

Histology and Immunohistochemical Analysis in Patients with Liver Metastases for Colorectal Cancer Based on Tissue Microarrays

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ABSTRACT

Many studies have analyzed preoperative poor prognostic factors in patients undergoing liver resection for colorectal liver metastases in order to select patients for surgery. However, intraoperative and postoperative factors have been poorly analyzed. The purpose of this study was to establish postoperative prognostic factors by histological and immunohistochemical study (p53, Ki-67, and stem cell markers, CD44, CD133, and CD166) based on the tissue microarray technique (TMA). For this, data from 100 consecutive patients undergoing any liver resection (127 liver resections) for liver metastases from colorectal cancer from January 2004 to August 2008 were retrospectively analyzed (minimum follow-up, 12 months). Patients were managed by a multidisciplinary team. Overall survival rates (OS) 1, 3, and 5 years after hepatectomy were 92%, 48%, and 30% respectively, and disease-free survival rates (DFS) 1, 3, and 5 years after the first hepatectomy were 85%, 42%, and 24% respectively. The conventional histological type was most common (92%), and a good or middle tumor differentiation grade tumor was seen in 91%. Infiltrating growth was seen in 57% of our patients (expansive in 43%), and microsatellites in 17%. A resection margin less than 10 mm, presence of microsatellites more than 5 mm away from the resection margins, and tumor differentiation grade (poorly differentiated/undifferentiated) were found to be significant independent prognostic factors by univariate analysis. Presence of microsatellites more than 5 mm away from the leading edge of tumor was a poor prognostic factor in multivariate analysis. Univariate and multivariate analyses found no immunohistochemical factors associated to recurrence and/or mortality.

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Key words: Colorectal cancer; Liver metastases; Microsatellites; Prognostic factors; Stem cells markers; Tissue microarrays

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INTRODUCTION

The purpose of surgery for colorectal liver metastases (CLM) is liver resection (LR) of all liver lesions with a tumor-free margin, provided R0 resection may be achieved with low morbidity and mortality^[1,2] without endangering the life of the patient due to either liver insufficiency or postoperative complications^[3]. According to most authors, it should be noted that surgery, however extensive it is, does not prolong survival if residual microscopic or gross tumor is left^[4,5].

Since Woodington and Waugh^[6] reported the first favorable results of surgical treatment for CLM, a disease previously considered incurable, to date, 5- and 10-year survival rates of 30%-40% and 20%-25% respectively^[7], while survival without treatment is less than 2%^[8].

The most adequate treatment for CLM is the study conducted by a multidisciplinary team^[9,10].

The difficulty for assessing the indication stems from the fact that the presence of extrahepatic tumor, the possibility of achieving a tumor-free margin, and the actual number of liver metastases (LM) are known during the laparotomy. As regards surgery, the best predictor for survival is the resection margin, which is also the only factor on which the surgeon may act. A distance longer than 10 mm from the tumor to the excision border is considered to be optimum^[11].

Different studies^[8,12] have analyzed the traditional preoperative factors predicting for survival in order to select patients in whom unnecessary surgery could be avoided, factors related to the patient, the primary tumor and LM. However, some authors^[2] do not contraindicate surgery in these patients with poor prognostic criteria provided R0 resection may be obtained, as there are prognostic factors which are only known after LM resection and may guide us as to prognosis and efficacy of surgery performed. These factors include histological study (number, resection margin size, microsatellites, type of growth, presence of tumor pseudocapsule, tumor differentiation grade, histological type, nuclear grade, and number of mitoses/mm²) and immunohistochemical study of the resected specimen. The latter may combine the immunohistochemical markers of cell proliferation and cell cycle control, p53 and Ki 67.

In this regard, there is increasing evidence supporting the concept that in human cancer, a minority of cells, tumor stem cells^[13-15], have acquired in the tumor setting characteristics of uncontrolled growth and the ability to form metastases. This hypothesis is supported by different experimental observations made initially in acute myeloid leukemia^[16] and subsequently in human solid tumors such as breast^[17], brain^[18,19], colorectal^[20,21], head and neck^[22], and pancreatic cancer^[23]. However, this concept continues to be highly controversial^[24], and data reported in colorectal cancer are not conclusive yet^[21].

It is therefore interesting to know both the qualitative and quantitative stem cell population in the tumor using markers such as CD44, CD133, and CD166.

The TMA technique^[25,26] allows for monitoring and simultaneous evaluation of a great number of samples or tumor series in a single experiment, ensuring homogeneity of the techniques between specimens, and for validation of the results obtained with various histological, immunohistochemical, and in situ hybridization (FISH) techniques^[26,27].

METHODS

Design

Data from 100 consecutive patients undergoing any liver resection (127 liver resections) for liver metastases from colorectal cancer from January 2004 to August 2008 (minimum follow-up, 12 months) were retrospectively analyzed.

The study was conducted at the Miguel Servet General University Hospital in Saragossa, Spain, a third level reference hospital covering a population of 800 000 inhabitants.

Patients and Data acquisition

All patients were individually managed by a multidisciplinary team.

Both study variables and potential answers to them were given consecutive numbers. All variables were independent or isolated, with no coincident answers within a single variable.

Data related to surgery and subsequent follow-up were collected from patients. No patient died for reasons other than the tumor. Five patients in whom peritoneal carcinomatosis was found after laparotomy was completed and no LR was performed were excluded from the study.

The study was approved by the bioethics committee of our hospital, and all patients were given comprehensive information about the study nature and objectives and signed an informed consent.

Histology and immunohistochemistry

Samples from our patients were subject to hematoxylin-eosin staining and subsequently to immunohistochemical staining techniques for each of the markers defined in our study (p-53, Ki-67, CD133, CD44, and CD166).

p53 Protein: code IS616, Clone DO-7, Ready-to-Use, FLEX, for Dako® Autostainer Instruments, Dako®.

Ki-67 Antigen: code IS626, Clone MIB-1, Ready-to-Use, FLEX, for Dako® Autostainer Instruments, Dako®.

CD133: SC-130127, CD133 (32AT1672), Santa Cruz Biotechnology®, Inc.

CD44: EpCAMhigh-CD44⁺, Santa Cruz Biotechnology®, Inc.

CD166: 35264, CD166 LYO 1 ml (mililitre) EpCAMhigh-CD166+, A. Menarini Diagnostics®.

Data from Histological analysis

The basic histological study (Table 1) using hematoxylin-eosin staining techniques tested the following variables: Tumor necrosis (%); Presence of steatosis in non-neoplastic parenchyma (%); Number of resected metastases; Maximum size of metastases in millimeters (mm); Minimum tumor resection margin (mm); Tumor margin characteristics (infiltrating, expansive) (Figure 1, 2); Presence of pseudocapsule; Presence of tumor microsatellites in adjacent parenchyma; Histological type (conventional, mucinous) (Figure 3); Histological tumor differentiation grade (well differentiated, moderately differentiated, poorly differentiated, undifferentiated); Nuclear grade and Number of mitoses/mm².

According to different authors^[28-30], necrosis is related to response of LM to neoadjuvant chemotherapy.

For those same authors, steatosis refers to toxicity or damage to non-neoplastic healthy parenchyma affected by neoadjuvant chemotherapy^[28-30]. This toxicity may occur as non-alcoholic fatty liver disease (NAFLD), usually induced by 5-FU^[29], and sinusoidal obstruction syndrome (SOS), usually induced by oxaliplatin^[30].

Nuclear grade [based on the axiom that "... the greater the nuclear diameter, the higher the number of chromosomal aberrations ..."] refers to the number of times the nucleus of the neoplastic cell is greater than the lymphocyte nucleus, the cell arbitrarily taken as

Table 1 Univariate analysis (histological prognostic factors).

| | Cases, <i>n</i> (%) | 1-year OS,% | 3-year OS,% | 5-year OS,% | <i>p</i> | 1-year DFS,% | 3-year DFS,% | 5-year DFS,% | <i>p</i> |
|---|------------------------|----------------|----------------|----------------|----------|-----------------|-----------------|-----------------|----------|
| Histological factors | | | | | | | | | |
| Resected LMs (<i>n</i>=100) | | | | | | | | | |
| <3 | 83 (83%) | 92% | 54% | 36% | NS | 83% | 29% | 8% | NS |
| ≥3 | 17 (17%) | 94% | 20% | | | 88% | 19% | | |
| Maximum LM size (mm) (<i>n</i>=100) | | | | | | | | | |
| <25 mm | 37 (37%) | 95% | 51% | 22% | NS | 89% | 29% | | NS |
| ≥25 mm | 63 (63%) | 90% | 46% | 32% | | 81% | 27% | 7% | |
| LR margin (mm) (<i>n</i>=100) | | | | | | | | | |
| <10 mm | 63 (63%) | 89% | 43% | 31% | 0.025 | 81% | 22% | 4% | 0.034 |
| ≥10 mm | 37 (37%) | 97% | 56% | 68% | | 89% | 35% | 12% | |
| Histological characteristics of LR margin (<i>n</i>=100) | | | | | | | | | |
| Infiltrating | 57 (57%) | 93% | 40% | 22% | NS | 81% | 21% | 6% | NS |
| Expansive | 43 (43%) | 91% | 62% | 46% | | 88% | 36% | 9% | |
| Pseudocapsule (<i>n</i>=100) | | | | | | | | | |
| No | 84 (83%) | 94% | 49% | 28% | NS | 84% | 27% | 7% | NS |
| Yes | 16 (16%) | 81% | 37% | 37% | | 81% | 22% | | |
| Microsatellitosis (<i>n</i>=100) | | | | | | | | | |
| No | 83 (83%) | 94% | 52% | 32% | 0.042 | 88% | 28% | 7% | 0.039 |
| Yes | 17 (17%) | 82% | 25% | 13% | | 65% | 24% | 1% | |
| Differentiation grade (<i>n</i>=100) | | | | | | | | | |
| Well differentiated/Moderately differentiated | 91 (91%) | 93% | 50% | 32% | 0.018 | 85% | 29% | 7% | 0.049 |
| Poorly differentiated/Undifferentiated | 9 (9%) | 78% | 60% | 8% | | 78% | 11% | 3% | |
| Nuclear grade | | | | | | | | | |
| <3 | 19 (19%) | 95% | 42% | | NS | 84% | 30% | | NS |
| ≥3 | 81 (81%) | 91% | 48% | 32% | | 84% | 27% | 7% | |
| No of mitoses/mm² | | | | | | | | | |
| <10 | 46 (46%) | 96% | 47% | 25% | NS | 87% | 33% | 0% | NS |
| ≥10 | 54 (54%) | 89% | 48% | 34% | | 81% | 23% | 9% | |
| Histological type (<i>n</i>=100) | | | | | | | | | |
| Conventional/ductal | 92 (92%) | 92% | 49% | 31% | NS | 85% | 26% | 6% | NS |
| Mucinous | 8 (8%) | 88% | 33% | | | 75% | 38% | | |

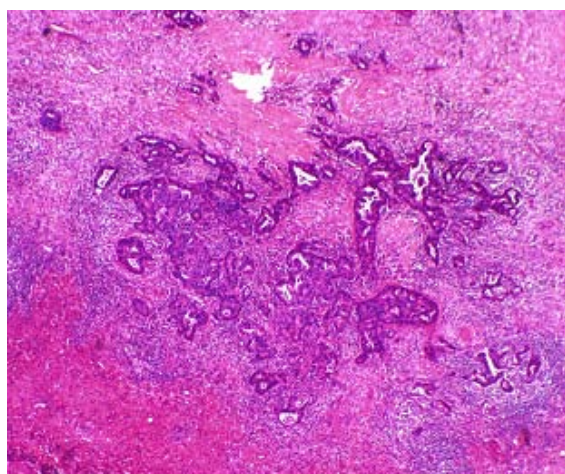


Figure 1 Adenocarcinoma infiltrating growth pattern.

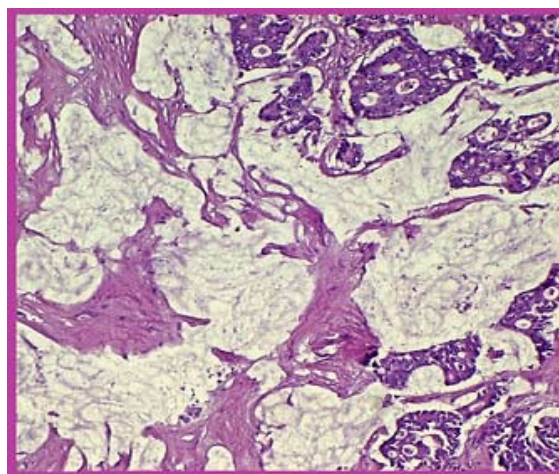


Figure 3 Adenocarcinoma mucinous histological type.

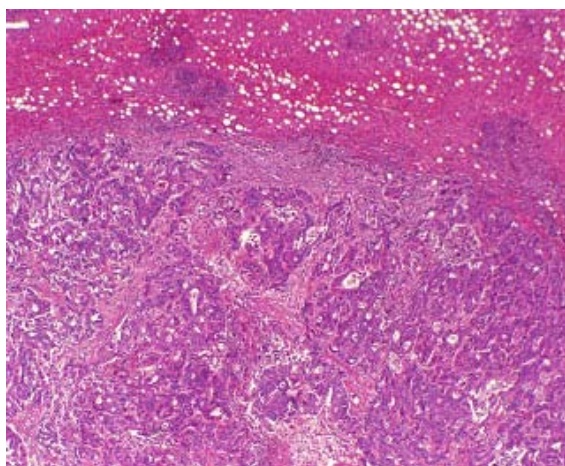


Figure 2 Adenocarcinoma expansive growth pattern.

reference because it is easy to measure (7 microns)].

For detection of microsatellites in our series, serial multiple sections were made in the periphery of the main lesion (sectioned thickness was 5 microns). Any neoplastic focus located more than 5 mm from the leading edge of tumor was considered as a microsatellite. Using a conventional optical microscope (×40, ×100, ×400 magnifications), the main lesion was required not to be continuous with the microsatellite.

The same pathologist examined the specimens with hematoxylin-eosin. The presence of gross satellite nodules in the main lesion is called satellitosis. When satellites are microscopically detected, some authors call them micrometastases, which may be located close to or distant from the main metastasis. When these micrometastases are close to the primary lesion, they could be called microsatellites, the term used by us and other authors^[2,31].

Data from Immunohistochemical analysis of TMA

This immunohistochemical study (Table 2) was conducted using the TMA technique based in Beecham Instruments Tissue Arrayer[®] [25,26]. Three tissue microarrays consisting of 60 to 90 tumor fragments previously selected from each patient were performed. These fragments were from the most infiltrating and representative tumor areas. At least two tumor fragments, all of them 1.5 mm in diameter, were included from each patient. TMA allowed for testing tumors in groups of 60, instead of one by one.

The indirect immunohistochemical staining method was used.

The standard indirect immunohistochemical method conducted consists of the following steps: Tissue or cell fixation with 4% paraformaldehyde (PFA) for a few hours; Tissue cryoprotection in 30% sucrose in 0.1M phosphate buffer (PB) at 4°C overnight (this step will only be performed for cryostat sections); 10-micron thick sections; Immunoreaction: Antigen recovery protocol (1 hour at 60°C to obtain crystals, which are incubated at pH 6/pH 9 for antigen recovery); Blockade for 56 min with 100 µL of Dako[®] endogenous peroxidase blocking agent or, failing this, 10% methanol; Wash in two baths (wash buffer) with 0.1 M phosphate buffered saline (PBS) at pH 7.4 containing 0.25% Triton (PBST) for 5 min each; Drain preparations, apply the specific primary antibody for each measurement (100 µL) and incubate for 30 min; Wash in two baths (wash buffer) with 0.1M PBS at pH 7.4 containing 0.25% PBST for 5 min each; Drain the slides and apply the secondary antibody (rabbit/mouse Envision). 100 µL visualization reagent for 30 min; Wash in two baths (wash buffer) with 0.1M phosphate buffered saline (PBS) at pH 7.4 containing 0.25% Triton (PBST) for 5 minutes each; Drain and apply 100 µL of the freshly prepared chromogen, diaminobenzidine tetrahydrochloride (DAB), for 10 min; Wash in a phosphate buffer (BP) bath with 0.1M Tris-HCl buffer at pH 7.2 (wash buffer) for 5 min; Transfer to Harris hematoxylin 200 µl for 5 min; Wash in distilled water; Dehydrate, rinse, and perform non-aqueous mounting.

Immunohistochemistry focused on p53; Ki-67; Membrane stem cell marker CD133, phenotype *AC133-CD133*^{+ [32]} (Figure 4); Membrane stem cell marker CD44, phenotype *EpCAM^{high} CD44*^{+ [33,34]} (Figure 5) and Membrane and cytoplasm stem cell marker CD166, phenotype *EpCAM^{high} CD166*^{+ [33,34]} (Figure 6).

An automated Dako[®] staining system was used to allow for objective control of the technique, avoiding random variations. The automated Dako staining system used was DakoCytomation Autostainer Plus[®]. A Carl Zeiss Axioskop 40[®] light microscope was used. The optimum entry concentration/dilution was searched for

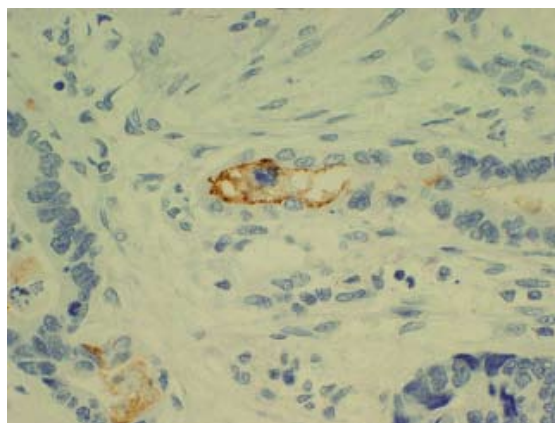


Figure 4 Immunohistochemical pathological study. Positive membrane staining for stem cell markers CD133, CS-130127, CD133 (32AT1672). Santa Cruz Biotechnology[®], Inc (×100).

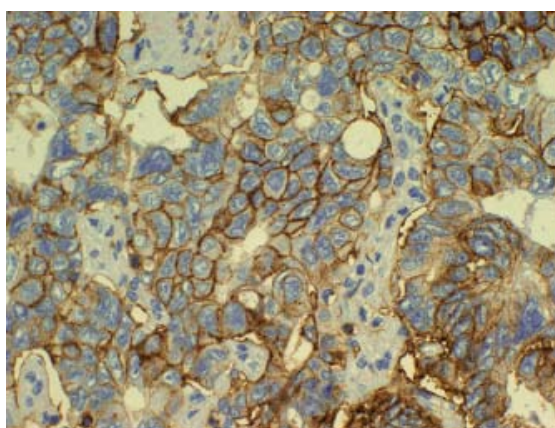


Figure 5 Immunohistochemical pathological study. Positive membrane staining for stem cell markers CD44, EpCAM^{high}-CD44+. Santa Cruz Biotechnology[®], Inc (×400).

each stem cell marker (antibody) in order to prevent overstaining or understaining, with a predefined pH to perform antigen “unmasking”. Dilutions used were 1:50 for p53, 1:100 for Ki 67, 1:10 at pH 9 for CD133, 1:60 at pH 6 for CD44, and 1:60 at pH 6 for CD166.

Surface antigen CD133 is a cell membrane glycoprotein which is considered as a cell surface marker expressed in stem cells of hematopoietic immature cells but not in mature blood cells.

CD133 has also been shown to be a marker of immature neuronal stem cells^[32,35,36]. It is recognized by two antibodies, CD133/1 or

Table 2 Univariate analysis (immunohistochemical prognostic factors).

| | Cases, n (%) | 1-year survival,% | 3-year survival,% | 5-year survival,% | p | 1-year DFS,% | 3-year DFS,% | 5-year DFS,% | p |
|-----------------------|--------------|-------------------|-------------------|-------------------|----|--------------|--------------|--------------|----|
| Histological factors | | | | | | | | | |
| p53 (n=87) | | | | | | | | | |
| Negative | 34 (39.08%) | 94% | 58% | 35% | NS | 82% | 37% | 11% | NS |
| Positive | 53 (60.92%) | 91% | 33% | 26% | | 83% | 11% | 0% | |
| Ki-67 (n=90) | | | | | | | | | |
| Negative | 15 (16.67%) | 73% | 52% | 17% | NS | 67% | 46% | 0% | NS |
| Positive | 75 (83.33%) | 96% | 42% | 34% | | 87% | 19% | 9% | |
| CD133 (n=91) | | | | | | | | | |
| Negative | 64 (70.33%) | 91% | 47% | 30% | NS | 81% | 28% | 7% | NS |
| Positive | 27 (29.67%) | 96% | 34% | | | 89% | 12% | | |
| CD44 (n=89) | | | | | | | | | |
| Negative | 39 (43.82%) | 82% | 35% | 20% | NS | 77% | 20% | 4% | NS |
| Positive | 50 (56.18%) | 98% | 50% | 35% | | 88% | 27% | 6% | |
| Membrane CD166 (n=86) | | | | | | | | | |
| Negative | 61 (70.93%) | 89% | 44% | 32% | NS | 82% | 22% | 5% | NS |
| Positive | 25 (29.07%) | 96% | 59% | 20% | | 84% | 37% | 16% | |
| Total CD166 (n=86) | | | | | | | | | |
| Negative | 46 (53.49%) | 85% | 41% | 30% | NS | 78% | 22% | 6% | NS |
| Positive | 40 (46.51%) | 98% | 56% | 26% | | 88% | 32% | 11% | |

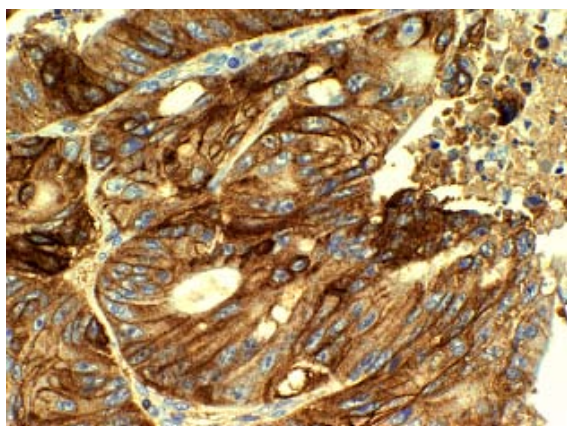


Figure 6 Immunohistochemical pathological study. Positive membrane staining for stem cell markers CD166, 35264, CD166 LYO 1 mL EpCAMhigh-CD166+. Menarini Diagnostics® (x40).

AC133 (which was the one used by us) and CD133/2 (AC141). CD133⁺ cells in colon cancer are helpful markers for detection of tumor initiating cells^[32,35,36] (Figure 4).

CD44 is considered as a cell membrane marker or epithelial cell adhesion molecule (EpCAM). Its phenotype *EpCAM^{high}-CD44⁺* is becoming established as a good marker for immature stem cells of human colon mucosa in certain series^[33,37,38] (Figure 5).

CD166 is considered as a marker for both cell membrane or EpCAM and cytoplasm (Figure 6). It is a marker of mesenchymal stem cells whose role in carcinogenesis is not fully clear^[33,37,38]. Its phenotype *EpCAMhigh-CD166⁺* added to *EpCAMhigh-CD44⁺* is starting to be considered as an additional marker of immature stem cells in human colon mucosa^[33].

Markers were identified in the nucleus (p-53 and Ki-67) with a threshold cut-off value >10%, in cytoplasm (CD166) and in cell membrane staining (CD44, CD133, and CD166), with significant staining of each of the stem cell markers with a threshold cut-off value >15%. Total CD166 stem cell marker was considered as the sum of CD166 expression in membrane and cytoplasm.

Statistical analyses

Data for quantitative variables were expressed as the mean±SEM, and data for qualitative variables as percentages. Kaplan-Meier survival curves were used. Univariate and multivariate analyses were also used to show the impact of study variables on overall survival (OS) and disease-free survival (DFS) with or without hepatic and/or extrahepatic recurrence (Tables 1, 2, 3). Statistical analysis was performed using a Student’s *t* test for non-paired samples, a Chi-square test, and an ANOVA test (Table 4). All analyses were done using SPSS™ version 15.0 statistical software (Statistical Package

Table 3 Multivariate analysis (Cox regression model). Poor prognostic factors.

| PROGNOSTIC FACTORS | value | OR | 95% CI | <i>p</i> |
|--------------------|-------|-------|----------------|----------|
| Microsatellitosis | Yes | 5.318 | 1.704 - 16.600 | 0.004 |

Table 4 Inferential statistics.

| VARIABLES COMPARED | TYPE OF STATISTICAL TEST | <i>p</i> |
|---|--------------------------|----------|
| TUMOR MARGIN CHARACTERISTICS (Infiltrating/Expansive) vs LM SIZE (>25 mm.) | <i>Chi-square</i> | 0.027 |
| NUCLEAR GRADE (>3) vs MARGIN (<10 mm.) | <i>Chi-square</i> | 0.035 |
| NUCLEAR GRADE (>3) vs No. of mitoses/mm ² (>15) | <i>Chi-square</i> | 0.007 |
| DIFFERENTIATION GRADE (Well-Moderately/Poorly-Undifferentiated) vs No. of mitoses/mm ² (>15) | <i>Chi-square</i> | 0.020 |
| CD133 (+/-) vs NUCLEAR GRADE (>3) | <i>Chi-square</i> | 0.001 |
| Ki 67 (+/-) vs No. of mitoses/mm ² (>10) | <i>Chi-square</i> | 0.023 |

for the Social Sciences v15, Chicago, IL). A value of *p*<0.05 was considered statistically significant.

RESULTS

Overall survival rates 1, 3, and 5 years after hepatectomy were 92%, 48%, and 30% respectively, while disease-free survival rates 1, 3, and 5 years after the first hepatectomy were 85%, 42%, and 24% respectively.

As regards data from the histological study (Table 1), a resection margin<10 mm was a poor prognostic factor for survival in the univariate analysis and was related to a greater number of local recurrences (OS, *p*=0.025; DFS, *p*=0.034). A liver resection margin<10 mm was related to a nuclear grade>3 (*p*=0.035) (Table 4). Among the histological markers tested in our patient series, both necrosis (40.1±26.5) and steatosis (11.4±17.6), had no statistically significant relation to neoadjuvant chemotherapy (results not shown).

A pseudocapsule was found in 16% of patients. Presence of microsatellites (17% of patients) was a poor prognostic factor for survival and recurrence in both the univariate (OS, *p*=0.042; DFS, *p*=0.039) and multivariate analyses (*p*=0.004) (Table 3). Infiltrating growth was seen in 57% of our patients, and expansive growth in 43%.

As regards LM size, this was not considered as a poor prognostic factor based on the size of the main LM. Patients in our series who showed infiltrating growth in pathological examination showed a significant relationship with a LM size>25 mm. (*p*=0.027) (Table 4). The conventional histological type was most common, as compared to the mucinous type (92% vs 8%), while all other types were non-existent. Well or moderately differentiated tumors were found in 91% of resected specimens.

A poorly differentiated or undifferentiated tumor was defined in our series as a poor prognostic factor for survival and was related to an increased number of local recurrences (OS, *p*=0.018; DFS, *p*=0.049) (Table 1). A comparison of the proportions of patients with>15 mitoses/mm² by tumor differentiation grade showed significant differences (*p*=0.020). More than 15 mitoses/mm² were found in 66.7% of patients with poorly differentiated-undifferentiated tumors, as compared to 26.4% of patients with well or moderately differentiated tumors (results not shown). Presence of a nuclear grade>3 was related to>15 mitoses/mm² (*p*=0.006) (Table 4).

As regards data from the immunohistochemical analysis using TMA (Table 2), 53 patients in our series had a statistical threshold cut-off value for p53>10%, and 34 patients a value<10%, while Ki-67 values were high in 75 patients and low in 15 patients. A relationship with a number of mitoses/mm² >10 was shown in 58.7% of patients with high Ki-67 values (*p*=0.023) (Table 4).

Twenty-seven patients in our series were considered CD133-positive, and 64 CD133-negative; 50 patients were CD44-positive and 39 CD44-negative; and 40 patients were CD166-positive and 46 CD166-negative. In our series, immunohistochemical markers tested (p53, Ki-67, CD133, CD44, and CD166) were not poor prognostic factors. A nuclear grade>3 was found in 92.2% of CD133⁺ patients, as compared to 59.3% of CD133⁻ patients (*p*=0.001) (Table 4).

DISCUSSION

Design, Patients and Data acquisition

A retrospective analysis was performed of data from 100 consecutive patients undergoing any liver resection (127 liver resections) due to the presence of CLM. This same retrospective data collection procedure from the clinical records has been used by several authors, but with some differences between them, particularly as regards the

sample size selected.

Our sample size was quite similar to that reported by European^[39], Japanese^[40], and American authors^[41]. However, our sample size was greater than that reported by other authors^[42,43].

Data from Histological analysis

Like us, many authors, including Nordlinger *et al.*^[12], Fong *et al.*^[8], and Figueras *et al.*^[44], considered resection margins <10 mm as positive. In our series, this was a poor prognostic factor for survival and was related to increased local recurrence rates (OS, $p=0.025$; DFS, $p=0.034$), in agreement with reports by other authors^[4,45,46] (Table 1). This is a controversial subject^[47,48], and quite a few authors advocate values lower than 10 mm. Thus, Elias *et al.*^[49] considered as positive margins those located within 2 mm of the leading edge of tumor. Kokudo-Makuuchi *et al.*^[47] reported recurrence rates of 13%, 2.8%, and 0% when the margin was <2 mm, 2-4 mm, and >5 mm, respectively.

A liver resection margin <10 mm was related to a nuclear grade >3 ($p=0.035$) (Table 4), probably because carcinomas with a nuclear grade >3 consist of more atypical cells with a high proliferation index, which results in shorter resection margins.

In our patients, presence of a pseudocapsule (found in 16% of patients) was not a statistically significant prognostic factor for survival or disease-free survival, unlike in the reports by Lunevicius *et al.*^[43], Weber *et al.*^[50], and Rajaganeshan *et al.*^[42]. However, very marked trends to a greater 5-year survival were found in patients with pseudocapsules (37% vs 28%) (Table 1).

Presence of microsatellites (found in 17% of patients) was related to recurrence in the section line, very common when the resection margin was <5 mm. In our study, microsatellites were defined as any neoplastic site located more than 5 mm away from the leading edge of tumor^[47], and hematoxylin-eosin was used in agreement with some authors^[2,31,51]. This leads to highly variable results as regards the percentage of microsatellites in the main lesion.

Thus, Marin *et al.*^[2] considered a microsatellite any neoplastic focus located within 1 cm of the leading edge of tumor and detected microsatellites in 15.5% of their 210 patients, who showed a significantly lower survival as compared to those with no microsatellites (33.6% versus 58% respectively). However, results reported by both Marin *et al.*^[2] and us are very different from those by Kokudo *et al.*^[47], who found microsatellites in 2% of their 194 patients.

Yokoyama *et al.*^[52] found microsatellites in 69% of their 46 patients (using multiple biopsies within and beyond 1 cm), with the resultant impact on survival. In this series, 10-year survival rates were 21% and 64% in patients with and without microsatellites respectively.

Wakai *et al.*^[51] studied microsatellites in the main lesion in 90 patients and detected 298 microsatellites in 52 patients (58%), of which 95% were located within 1 cm, and concluded that since most microsatellites are located within 1 cm, the resection margin in liver resections should be greater than 1 cm. Other authors, such as Kokudo *et al.*^[47], combined detection of microsatellites with genetic determination.

In our experience, microsatellites were detected with hematoxylin-eosin in 17% of cases (all those located within 1 cm of the main metastasis), which was a poor prognostic factor for survival and recurrence in both univariate (OS, $p=0.042$; DFS, $p=0.039$), and multivariate analyses ($p=0.004$). This contrasts with the results reported by many series^[2,8,12,44,47,53-57] (Table 1). Unlike these same authors, an analysis of tumor growth type showed very striking trends to a greater 5-year survival in patients with an expansive versus an infiltrating tumor margin (46% vs 22%) (Table 1).

As regards LM size, sizes of the main LM >2.5 cm, >4 cm^[8], >5 cm^[56], >8 cm^[58], or >10 cm^[54] have not been considered as poor prognostic factors (Table 1). Like us, other authors^[59] found no significant differences in tumor size.

As regards LM number, a number >1 was a poor prognostic factor for some authors such as Fong *et al.*^[8] and Nordlinger *et al.*^[12] in 1999. The number of LM associated to a poor prognosis was >3 for other authors such as Adam *et al.*^[54] and Pawlik *et al.*^[56] and ≥ 4 for Figueras *et al.*^[44,53]. In our patients, a number of resected LM >3 was not a poor prognostic factor, in agreement with in other series^[2,47,56,58]. However, a very marked trend was seen to a greater 5-year survival in patients resected ≤ 3 as compared to >3 LM (Table 1).

According to our results, the conventional histological type was most commonly found (92%) with a well or moderately differentiated tumor was found in 91% of the resected specimens. In our series, a poorly differentiated or undifferentiated tumor grade was found to be a poor prognostic factor for survival and was related to an increased local recurrence rate (OS, $p=0.018$; DFS, $p=0.049$), unlike reported in many other series^[2,8,12,44,47,53-57] (Table 1).

Nuclear grade and number of mitoses/mm² were analyzed in our series, unlike in other studies^[2,8,12,44,47,53-57]. However, these were not poor prognostic factors in our patients. A statistically significant relationship was found between a nuclear grade >3 and a number of mitoses/mm² >15 ($p=0.007$), and significant differences were also seen when tumor differentiation grade was compared to a number of mitoses/mm² >15 ($p=0.020$) (Table 4). In this regard, both nuclear grade and differentiation grade refer to cell anaplasia and undifferentiation, and it is natural to think that there was a correlation between these data and a higher tumor cell activity (mitoses/mm²). This may suggest that chromosomal aberrations are greater and, as a result, cells are more dysregulated and proliferate more.

A nuclear grade >3 was related to a liver resection margin <10 mm ($p=0.035$) (Table 4), suggesting that the surgical margin should be greater for tumors with a high nuclear grade. It would therefore be interesting to assess the nuclear grade of metastases during surgery in order to better define the surgical resection margin.

Data from Immunohistochemical analysis of TMAs

Like many authors, such as Kokudo *et al.*^[47], we analyzed p53 expression. The immunohistochemical markers tested in our study (p53, Ki-67) were not poor prognostic factors (Table 2). By contrast, authors such as Tanaka *et al.*^[40] reported that p53 or Ki-67 expression, for Weber *et al.*^[50] in LM, had a negative impact on survival.

It should be noted, however, that survival was longer than 5 years in patients with high Ki-67 levels and in those with a high mitotic index (>10 mitosis/mm²) (Tables 1 and 2), which is also another expression of the tumor proliferation index.

However, no significant relationship was found between cell proliferation as measured by Ki-67 and p53, whose changes express a loss of cell cycle control.

The immunohistochemical stem cell markers tested (CD133, CD44, and CD166), were not poor prognostic factors (Table 2). For O'Brien *et al.*^[35], most CD133⁺ stem cells had a 200-fold greater oncogenic potential than CD133⁻ cells for development of CRC. In addition, this subpopulation was able to maintain itself and to differentiate and become established again as a tumor when transplanted in certain solid organs of experimental animals. However, significant trends were found in our series with regard to membrane CD133 and CD166 markers. CD133⁺ patients had lower 5-year survival as compared to CD133⁻ patients (0% vs 30%) (Table 2). Similarly, patients with membrane-positive CD166 had a lower 5-year survival than those with no CD166 in their membranes (20% vs 32%) (Table 2).

In our series, positive CD133 and/or CD166 membrane markers were associated to a lower tumor activity index and did not correlate with a greater cell activity. Survival and DFS did not therefore change (Table 4).

According to O'Brien *et al.*^[20] and Ricci-Vitiani *et al.*^[21], in several colon tumors CD44 was more determinant than CD133, because CD44 was expressed in tumor lines not expressing CD133. In our study, patients with tumors expressing CD44⁺ had better survival and DFS rates at 1, 3, and 5 years as compared to patients with tumors low in CD44 (Table 2). Thus, our results for CD44 contradict the theories^[20,21,35] assuming that the greater the number of stem cells in the tumor, the poorer the survival.

Comparison with many of the groups is difficult because, unlike us, many authors^[2,8,12,44,47,53-57] performed no immunohistochemical studies. However, as regards study of immunohistochemical markers, more significant results were not achieved probably because immunohistochemistry arrays were only performed, but flow cytometry^[36] or other molecular biology techniques were not used.

CONCLUSIONS

With regard to postoperative prognostic factors, a resection margin less than 10 mm, presence of microsattellites more than 5 mm away from the resection margin, and a poorly differentiated/undifferentiated tumor were significant poor prognostic factors for survival and disease-free survival in the univariate analysis. Presence of microsattellites more than 5 mm away from the leading edge of tumor was a significant, independent poor prognostic factor in the multivariate analysis.

Univariate and multivariate analyses found no immunohistochemical factors associated to recurrence and/or mortality in the pathological study of TMA.

Postoperative factors should be considered in patients with CLM because they may inform us about tumor aggressiveness and the efficacy of surgery.

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