Intrahepatic lymphatic invasion but not vascular invasion is a major prognostic factor after resection of colorectal cancer liver metastases

short title: intrahepatic lymphatic invasion in CRCLM

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ABSTRACT

Background: Despite the advances on diagnosis and surgical strategies, up to 70% of patients will develop recurrence of the disease after resection of colorectal cancer liver metastases (CRCLM). The purpose of our study was to determine the frequency of 4 different mechanisms of intrahepatic dissemination, and to evaluate the impact of each mechanism on patient's outcomes. **Methods:** The medical records of 118 patients who underwent a first resection of CRCLM during the period between 2000 and 2010 were reviewed. Clinicopathologic variables and outcome parameters were examined. Resected specimens were submitted to routine histological evaluation, and immunohistochemical staining with D2-40 (lymphatic vessels), CD34 (blood vessels), CK-7 (biliary epithelium), and CK-20 (CRC cells). **Results:** The mean follow-up after resection was 38 months. Tumor recurrence was observed in 76 patients, with a median interval of 13 months after resection. Overall survival and disease-free survival (DFS) rates after hepatectomy were 62%, 56%, and 26%, 24% at 3-, and 5-years, respectively. Intrahepatic microscopic invasion included portal venous in 49 patients, sinusoidal in 43 patients, biliary in 20 patients, and lymphatic in 33 patients. Intra-hepatic lymphatic invasion was the only mechanism of dissemination independently associated with the risk of hepatic recurrence (OR=2.75), and shorter DFS (p=0.006). **Conclusion:** Intrahepatic lymphatic invasion is a significant prognostic factor. Other mechanisms of invasion, although frequently observed, are not related to recurrence or survival, suggesting that the lymphatic system is the main route for dissemination of CRCLM. Furthermore, immunohistochemical detection of intrahepatic lymphatic invasion might be of value in clinical practice.

Background

The liver is the most common site of distant metastases in patients with colorectal cancer. Surgery represents the basis for curative treatment of colorectal cancer liver metastases (CRCLM) with long-term survival rates ranging from 36% to 58% and from 23% to 36% at 5 and 10 years, respectively [1-6]. Despite the advances on diagnosis, staging and surgical strategies, 60 % to 70% of patients will develop recurrence of the disease even after R0 resection of CRCLM [6,7].

In the last few years, the aim of many studies has been to identify biomarkers capable of stratifying CRCLM into prognostic groups. Hereupon, reassess histomorphological features related to different mechanisms of intrahepatic tumor spread can be of potential clinical value.

The presence of lymphovascular invasion has been shown to be an independent adverse prognostic factor for early recurrence, disease-free survival (DFS), and overall survival (OS) after resection of colorectal cancer [8,9]. Moreover, in stage II patients it is taken into account when determining if adjuvant therapy should be started.

For CRCLM, the presence of intrahepatic lymphatic, blood vascular, and sinusoidal dissemination have been reported to be associated with an increased risk of intrahepatic recurrence and worse survival after liver resection [10-13].
The aims of the present study were to (a) distinguish between intrahepatic lymphatic vascular invasion (LVI), blood vascular invasion (BVI), biliary invasion (Bili), and sinusoidal invasion (SI) in order to determine which type of intrahepatic spread is more frequent, (b) compare the accuracy of the assessment of the different intrahepatic mechanisms of dissemination using routine H&E-stained sections with

that obtained from immunohistochemistry (IHC) stained analysis, and (c) to investigate the prognostic significance of each mechanism in a well-characterized group of CRCLM patients.

Patients and methods

After the approval by the Institutional Ethics Committee, the medical records of all consecutive patients who underwent a first resection of CRCLM during the period between January 2000 and January 2010 were reviewed. Patients with repeated hepatectomies were excluded from the study. A total of 118 patients underwent a first hepatic resection for CRCLM. Patients with incomplete macroscopic resection (R2) (n=2) and early post-operative death (within 90 days; n=3) were excluded. The remaining 113 patients were included in this retrospective study. Intraoperative ultrasonography was employed in all patients. Liver resections were performed as previously published [14] and included nonanatomic partial hepatectomy in 16 patients, segmentectomy in 11, bisegmentectomy in 14, central hepatectomy (removal of Couinaud´s segments IV, V, and VIII) in 4, right hepatectomy in 48, left hepatectomy in 14, and right trisectionectomy in 8 patients.

Pathologic evaluation

Resected specimens were submitted to conventional histological evaluation. The number of CRCLM was determined in each patient by both preoperative imaging and macroscopic examination of multiple slices from each resected specimen, and did not include satellite lesions according to the criteria of Taylor et al [15]. In patients with multiple tumors, the largest tumor was chosen as representative and used for IHC staining [10,12,13]. Surgical margin status were histologically classified as either R0 (no residual tumor) or R1 (presence of tumor cells on the resection margin). Size of

liver lesions was measured by the pathologist, in centimeters, before fixation of the specimen.

Immunohistochemistry

One to three paraffin-embedded blocks (median, 1.2 blocks) from each patient were used for immunohistochemistry. After routine histopathological examination, five serial 4µm-thick sections of each previously chosen formalin-fixed paraffin-embedded tissue bloc were cut and immunohistochemically stained in a fixed order : the first section of each block was used for routine histological examination using H&E staining, the second section was used for immunohistochemical staining with D2-40 monoclonal antibody specific for lymphatic vessels (730-16, SIGNET, Cambridge, UK; dilution 1:100), the third section used for immunohistochemical staining with CD34 polyclonal antibody for blood vessels (ab64480, Abcam, Cambridge, MA; dilution 1:200 dilution), the fourth section used for immunohistochemical staining with CK-20 monoclonal antibody [16] (monoclonal, M7019, Dako, Denmark; dilution 1:100), and the fifth section used for immunohistochemical staining with CK-7 (monoclonal, OV-TL 12/30, Dako, Denmark; dilution 1:100).

Assessment of intrahepatic mechanisms of invasion

First, all H&E slides were screened for the presence of the 4 mechanisms (BVI, LVI, Bili, and SI) of intrahepatic invasion. Then, all IHC slides were screened using strict criteria to confirm or modify the findings obtained from H&E slides. LVI or BVI were considered positive when either single tumor cells or cell clusters were clearly visible within an endothelium-lined vessel-like structure that showed immunoreactivity for D2-40 or CD 34 antibody respectively (Figures 1A and 1B) beyond the border of the studied metastasis. Perineural invasion in the resected liver was included in the category of intrahepatic lymphatic invasion as described elsewhere [10]. Bili was

considered positive when either single tumor cells or cell clusters were clearly visible within an epithelium-lined structure that showed immunoreactivity for CK-7. SI was considered if discrete microscopic cancerous lesions, ranging from a single cell to clusters of cells showing immunoreactivity for CK-20 antibody, were identified within the hepatic parenchyma and separated from the invasive front of the metastasis by a rim of hepatic sinusoids. An experienced pathologist (ESM) blinded to clinical details assessed each section.

Prognostic factors

Blood transfusion during or 24 hours following surgery was defined as perioperative. Neoadjuvant or adjuvant chemotherapy was administered in selected cases after a multidisciplinary staff meeting. Major liver resection was defined as any resection of 3 or more liver segments [17]. Clinicopathologic variables studied included: gender, age >60 years, node positive primary cancer, liver metastases confirmed within 12 months after primary cancer resection, CEA level ≥200 ng/mL, size of metastases greater than 5 cm, bilateral hepatic metastases, and multiple (>1) metastases.

Follow-up

Patients were regularly followed with physical examination, assessment of tumor markers (CEA and CA 19.9), and computed tomography or abdominal ultrasound with chest X-ray every 4 to 6 months. Hepatic recurrence was considered when liver metastases were present (whether solely or associated with other sites) at the first sign of disease-recurrence. DFS was defined as the time interval between the date of hepatic resection and the date of the first documented recurrence at any site. OS was defined as the time interval between the date of hepatic resection and the date of death or the most recent date of follow-up if the patient was alive. The criteria for establishing recurrent disease were histologic confirmation, radiologic evidence of

progression with subsequent clinical progression, and supportive biochemical data (eg, rising serum level of CEA or CA-19.9).

Statistical analysis

The univariate associations between clinicopathologic variables and hepatic recurrence were examined using *chi*-square test and/or Fisher´s exact test when appropriate. Factors independently associated with hepatic recurrence were identified by multiple logistic regression analysis, which included all variables with $P<0.1$ at univariate analysis. The risk prediction was reported as p-value, odds ratio (OR) and 95% confidence intervals (95% CI).

Survival curves were constructed using the Kaplan–Meier method, and the differences in survival were compared using the two-tailed log rank test. The Cox proportional hazards regression model was used to identify factors that were independently associated with overall survival (OS) and disease-free survival (DFS). A 2-sided *P* value was always computed and a difference was considered statistically significant at $P<0.05$. Assessment of κ value was used as a measure of agreement between results of H&E and IHC. Statistical analysis was performed with SPSS for Windows, version 20.0 (SPSS Inc, Chicago, Illinois).

Figure 1

Results

There were 64 men (57%) and 49 women with a mean age of 59 years (range: 28 to 81 years). All patients had undergone curative resection of their primary tumor previous to the hepatic resection. Primary tumor location was the colon and rectum for 55 and 58 patients, respectively. Thirty-eight patients received neoadjuvant chemotherapy prior to surgery. Seven patients had concomitant extrahepatic metastases at the time of hepatectomy: 6 pulmonary metastases and one localized

peritoneal metastasis. In all patients with pulmonary metastases, resection was performed within 1 to 3 months after liver resection. Clinical and pathologic characteristics of the entire cohort are shown on Table 1.

The mean postoperative hospital stay was 10.7 days (range: 2 to 70). The mean follow-up after resection was 38 months (range: 3 to 122 months). After multidisciplinary discussion indication for adjuvant chemotherapy was retained for 60 patients. Tumor recurrence was observed in 76 patients (68%), with a median interval of 13 months after resection. The overall cumulative survival rates after hepatectomy were 87%, 62%, and 56% at 1-, 3-, and 5-years. The overall cumulative disease-free survival rates were 49%, 26%, and 24% at 1-, 3-, and 5-years.

Table 1

Pathological results

The mean number of metastatic liver tumors was 2.2 per patient (range: 1 to 10 lesions), with 23 patients (20%) having more than 3 nodules, and 48 patients (42%) with solitary lesions. Mean size of the largest lesion was 5.3 cm (range: 0.5 to 23.5 cm). Resection was considered R0 in 88% of patients.

After IHC analysis, intrahepatic microscopic invasion included BVI in 49 patients, SI in 43 patients, Bili in 20 patients, and LVI in 33 patients. All intrahepatic lymphatic invasion foci were evident outside the tumor's border. Intrahepatic lymphatic invasion foci were evident within 2mm from the metastatic liver tumor, whereas portal venous invasion were evident within 8mm from the tumor edge. The level of agreement between H&E and IHC was excellent for BVI, SI, and Bili, but markedly lower for LVI, as shown in Table 2.

Table 2

Factors influencing hepatic recurrence, disease-free survival, and overall survival for the whole series

Univariate analysis showed CEA levels > 200ng/mL (p=0.012), multiple lesions (p=0.013), and bilateral lesions (p=0.016) to be associated with hepatic recurrence. Multivariate logistic regression (which also included factors with p<0.1 in univariate analysis: R0 resections, BVI, and LVI) showed CEA levels > 200ng/mL (p=0.029), multiple lesions (p=0.041), and LVI to be independently associated with the risk of hepatic recurrence, with LVI associated with an almost 3-fold increased risk (OR=2.75; 95% CI=1.01-7.45, p=0.04).

DFS was significantly associated with the presence of multiple lesions, positive surgical margins, bilateral tumors, and LVI. Multivariate Cox regression analysis confirmed positive surgical margins, and LVI to be independently associated with DFS (Table 3). Actuarial DFS rates according to the presence of intra-hepatic lymphatic invasion for 1-, 3-, and 5-years were 30% *vs*. 56%, 6% *vs*. 34%, and 6% *vs*. 31%, respectively. (Figure 2)

CEA levels > 200ng/mL was the only variable associated with OS (p=0.003). Noteworthy, the actuarial OS of patients presenting LVI was considerably shorter than patients without LVI (mean, 85 months vs. 35 months, p=0.15) with OS rates for 3-, and 5-years of 52% *vs.* 66%, and 42% *vs.* 61%, respectively. (Figure 2)

Table 3

Factors influencing Recurrence risk, Disease-free survival, and Overall survival in R0 patients

In the R0 group (n=99 patients), univariate analysis showed bilateral lesions (p=0.028) and multiple lesions (p=0.001) to be related to hepatic recurrence, but after multivariate logistic regression only the presence of multiple lesions was

independently associated with a higher risk of hepatic recurrence (OR 3.97; p=0.007).

DFS was associated with node positive primary cancer (p=0.04), multiple metastases (p=0.007), and LVI (p=0.008). After Cox proportional hazards regression model, multiple metastases (HR 1.89; 95% CI=1.12-3.2, p=0.016) and LVI (HR 1.96; 95% CI=1.18-3.24, p=0.009) remained independently associated with a shorter DFS in R0 patients.

Univariate analysis failed to demonstrate any significant effect of the studied variables on OS in the R0 group. However, CEA levels > 200ng/mL (p=0.065) and LVI (p=0.092) showed a trend towards shorter OS. Actuarial DFS and OS rates for 3-, and 5-years in R0 patients according to the presence of LVI were 7% *vs*. 36%, 7% *vs*. 33%, and 52% *vs*. 67%, 41% *vs*. 61%, respectively.

Effect of chemotherapy in patients with LVI

The effect of preoperative chemotherapy in DFS and OS of patients presenting LVI should also be investigated, however, due to the limited number of patients included in this series that received preoperative chemotherapy (34%) we were unable to provide any reliable data about this important topic. Meanwhile, the frequency of LVI in patients with (11 out of 38; 28.9%) or without (22 out of 75; 29.3%) preoperative chemotherapy was not statistically different (p=0.827).

Among the 33 patients with confirmed LVI, 18 patients (55%) had postoperative chemotherapy. Postoperative chemotherapy didn't show any effect on DFS but was related to better OS (mean OS, 85.3 months *vs.* 31.2 months; p=0.04) in this subgroup of patients with poorer outcome. On the other hand, among the 80 patients without LVI, 42 patients (52%) had postoperative chemotherapy, which did not show any effect on DFS (p=0.918) or OS (p=0.520).

Discussion

This study has shown that LVI is a significant prognostic factor after surgical resection for CRCLM. It has also demonstrated that microscopic invasion of other structures (blood vessels, biliary ducts or sinusoids), although frequently observed is not related to recurrence or survival. Recent studies support the hypothesis that lymphatic metastasis from a primary tumor site is the major pathway to progression into the systemic circulation and to distant organ sites [18-20].

The hypothesis that blood and lymph vessels invasions are not only two different routes that cancer cells use to metastasize, but are characterized by a different biological behavior is supported in our study by the fact that some patients exclusively show BVI or LVI.

The frequency and the prognostic implication of hepatic pedicle lymph nodes invasion as though as the indication, extent, and therapeutic role of hilar lymphadenectomy are presently the objects of ongoing interest [21-24]. Unfortunately, the retrospective character of our study did not allow us to evaluate the relationship between the presence of hilar lymph node invasion and LVI.

Tumor budding is a morphologic phenomenon observed at the advancing edge of neoplasms characterized by isolated or small clusters of tumor cells that detach from the main lesion and migrate into the neoplastic stroma [25]. In colorectal cancer it is considered as a factor of poor prognosis and has, recently, been the subject of increasingly interest [25,26]. Sinusoidal invasion (SI) can be considered the morphological representation of tumor budding for liver tumors, although an universally accepted definition is warranted. In our study, the presence of sinusoidal

invasion was a common phenomenon (38%) but unrelated to recurrence and/or survival.

The role of perioperative chemotherapy in resectable CRCLM is still a matter of debate [27]. Although postoperative chemotherapy after resection of CRCLM has become an accepted standard of care, data on its benefit are limited [27,28]. In the present study, postoperative chemotherapy was related to a better OS in the poor prognostic group of LVI+ patients whereas it did not prolong DFS or OS in the LVI- group, thus suggesting that it might be use as a criterion in future trials dealing with adjuvant chemotherapy for CRCLM.

This study is limited by its retrospective nature, the small number of patients, and the range of different anticancer agents administered to some patients. However, it raises an important subject for discussion and future investigation.

In conclusion, the current study is, to our knowledge, the largest series to date to evaluate the presence of intrahepatic lymphatic invasion using D2-40 staining [29]. Also, it is the first study to use specific IHC staining to assess the four commonest mechanisms of intrahepatic micrometastatic dissemination (biliary, sinusoidal, lymphatic, and blood vascular). It shows that lymphatic invasion rather than blood vessel, biliary duct or sinusoidal invasion is the key prognostic marker of aggressiveness and spread in CRCLM. Furthermore, we have demonstrated that the use of immunohistochemical staining with a lymphendothelial specific marker (D2-40) increases the accuracy of the assessment of tumor associated lymphatic spread and reinforce its use as part of an optimal histopathology report.

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Figures' legends

Figure 1 (A) Immunohistochemical staining with D2-40 monoclonal antibody reveals tumor cell clusters clearly outlined by endothelial cells (arrow) showing D2-40 immunoreactivity, indicating LVI outside the tumor borders (arrow heads) (B) Immunohistochemical staining for CD-34 polyclonal antibody reveals tumor cell clusters clearly outlined by CD-34 immunopositive endothelial cells (arrow), indicating portal venous invasion

Figure 2 Kaplan-Meier actuarial survival curves (A) Overall survival (B) Disease-free survival

TABLE 1 Clinicopathologic criteria of patients

Characteristics	**N°. of patients (n, %)**
Age	
>60 yr	61 (54%)
≤60 yr	52
Gender	
Male	64 (57%)
Female	49
Primary tumor site	
Colon	55 (49%)
Rectum	58
Primary tumor lymph nodes	
Negative	47 (42%)
Positive	66
Interval (months)	
<12	50 (44%)
>12	63
CEA levels (ng/mL)	
<200	85 (75%)
>200	18
Extra-hepatic disease	
Yes	7 (6%)
No	106
Preoperative chemotherapy	
Yes	38 (34%)
No	75
Distribution	

Unilobar	73 (65%)
Bilobar	40
Tumor size (cm)	
<5	68 (60%)
≥5	45
No. of hepatic lesions	
single	48 (42%)
> 1	65
Transfusion	
Yes	33 (29%)
No	80
Adjuvant chemotherapy	
Yes	60 (53%)
No	53
Type of hepatectomy	
Major (≥3 segments)	74 (65%)
Minor	39
Surgical margin	
R0	99 (88%)
R1	14
Intrahepatic sinusoidal invasion (SI)	
Yes	43 (38%)
No	70
Intrahepatic blood invasion (BVI)	
Yes	49 (43%)
No	64

Intrahepatic lymphatic invasion (LVI)	
Yes	33 (29%)
No	80
Intrahepatic biliary invasion (Bili)	
Yes	20 (18%)
No	93

TABLE 2 Intrahepatic invasion by H&E and correlation with IHC.

Type of invasion	**H&E (n° of patients)**	**Correlation**
Sinusoidal (SI)		κ = 0.926
false +	1	
false -	3	
Blood (BVI)		κ = 0.928
false +	1	
false -	2	
Biliary (Bili)		κ = 0.862
false +	0	
false -	5	
Lymphatic		κ = 0.601
false +	1	
false -	15	

TABLE 3 Correlation of clinical and pathologic factors to Disease Free Survival: univariate and multivariate analysis

Characteristics	**Univariate analysis**	**Multivariate analysis**		
		OR	95% confidential interval	p
Age	p=0.317			
>60 yr				
≤60 yr				
Gender	p=0.488			
Male				
Female				
Primary tumor site	p=0.755			
Colon				
Rectum				
Primary tumor lymph nodes	p=0.170			
Negative				
Positive				
Interval (months)	p=0.551			
<12				
>12				
CEA levels (ng/mL)	p=0.042	_____	_____	NS
<200				
>200				
Extra-hepatic disease	p=0.278			
Yes				
No				

Preoperative chemotherapy	p=0.192			
Yes				
No				
Distribution	p=0.005	_____	_____	NS
Unilobar				
Bilobar				
Tumor size (cm)	p=0.088			
<5				
≥5				
No. of hepatic lesions	p=0.015	_____	_____	NS
single				
> 1				
Transfusion	p=0.671			
Yes				
No				
Adjuvant chemotherapy	p=0.951			
Yes				
No				
Type of hepatectomy	p=0.241			
Major (≥3 segments)				
Minor				
Surgical margin	p=0.003	0.357	0.181 – 0.703	p=0.003
R0				
R1				
Intrahepatic sinusoidal invasion (SI)	p=0.398			
Yes				

No				
Intrahepatic blood invasion (BVI)	p=0.201			
Yes				
No				
Intrahepatic lymphatic invasion (LVI)	p=0.006	2.126	1.12 – 4.036	p=0.021
Yes				
No				
Intrahepatic biliary invasion (Bili)	p=0.906			
Yes				
No