Circulating CD95-ligand as a Potential Prognostic Marker for Recurrence in Patients with Synchronous Colorectal Liver Metastases

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Abstract. *Aim: To assess whether circulating soluble CD95 ligand (sCD95L) levels are associated with recurrence-free survival (RFS) in patients with synchronous colorectal liver metastases. Patients and Methods: Blood samples were obtained from 62 patients with synchronous colorectal liver metastases before and after liver surgery. Serum sCD95L levels were determined using enzyme-linked immunosorbent assay (ELISA). Cox regression analysis was performed to determine the correlation between sCD95L levels and RFS and overall survival (OS). Results: Median follow-up was 33 months. High pre-operative sCD95L levels were associated with poor RFS and OS in univariable (p=0.019 and p=0.020) and multivariable analyses (p=0.020 and p=0.003). Conclusion: Preoperatives CD95L is a potential prognostic factor for RFS and OS of patients undergoing surgery for synchronous colorectal liver metastases. Low preoperatives CD95L levels may help identify a subgroup of patients with synchronous liver metastases that are likely to benefit from liver surgery.*

The presence of liver metastases is the major determinant of survival in patients with colorectal cancer. Approximately 25% of the patients with colorectal cancer already have liver metastases at diagnosis (1). The presence of synchronous liver metastases may indicate a more aggressive and unpredictable disease course when compared to metachronous metastasis (2). The optimal strategy for treatment of patients with synchronous liver metastases is currently debated (3).

CD95 ligand (CD95L/FS7 associated surface antigen ligand) and its receptor CD95 (apoptosis inducing protein 1/FS7 associated surface antigen) are transmembrane proteins that play an essential role in lymphocyte cytotoxicity and the maintenance of immunological homeostasis (4). CD95L is known to induce tumour cell apoptosis. However, CD95L can also act in a pro-tumourigenic fashion by stimulating tumour cell proliferation, survival and invasion (5, 6). In colorectal cancer, the expression of CD95L is higher in liver metastases than in matched primary tumours, and high expression is related to poor prognosis (7, 8). Furthermore, we have recently shown that CD95L stimulates migration and invasion of colorectal cancer cells, rather than apoptosis (5). Membrane-bound CD95L can be cleaved by metalloproteases, which results in a soluble form of CD95L (sCD95L) which can be detected in the circulation (9). Preclinical studies showed that tumour progression is selectively promoted by sCD95L (10).

In this study, we investigated whether high levels of sCD95L in patients undergoing surgery for synchronous liver metastases are associated with a more aggressive tumour phenotype and can be used to predict recurrence-free survival (RFS).

Patients and Methods

Blood samples were obtained from all consecutive patients with synchronous colorectal liver metastases before and 24 h after liver surgery between March 2004 and September 2008 at the University Medical Centre Utrecht in the Netherlands. The study protocol was approved by the Ethical Committee on Human Research. Written informed consent was obtained from all patients. Patients 18 years or older who underwent resection with curative intent for synchronous colorectal metastases confined to the liver were included in the study. Resection of the primary tumour was followed by liver resection as soon as patients had recovered from surgery of the primary tumour. Patients were excluded in cases of extrahepatic disease, treatment

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Key Words: Colorectal cancer, surgery, liver metastases, biomarker, CD95-ligand.

with local ablative therapies, or macroscopic residual disease (R2) after surgery. Patient and tumour characteristics, as well as surgical characteristics, were retrospectively drafted from our prospectively collected liver database.

sCD95L assay. Venous blood samples were drawn into sterile vacuum tubes before surgery and 24 h after surgery. Blood samples were centrifuged at 1450 ×*g* for 15 min and immediately frozen at –80˚C until assayed. The levels of sCD95L in the sera were determined using a commercially available solid-phase sandwich enzyme-linked immunosorbent assay (ELISA) kit for the quantitative detection of human sCD95L, recognizing both natural and recombinant human CD95L (ab45907; Abcam, Cambridge, UK). ELISAs were performed according to the manufacturers' protocol.

Follow-up. All patients were subjected to routine follow-up. Computerized tomography scans were acquired every 3 months to monitor recurrences. The follow-up data were updated by letters and telephone calls to referring physicians and general practitioners. The duration of the follow-up and the time between surgery and the detection of recurrence were obtained, as well as overall survival (OS) data.

Statistical analyses. RFA and OS were calculated from the day of surgery to the day of the first recurrence, or the day of death, respectively. Median RFS and OS were estimated by the Kaplan-Meier method. To determine the influence of possible risk factors on recurrence-free and overall survival a univariable COX regression analysis was performed. A multivariable COX proportional hazards model was used to determine the independent prognostic impact of all variables on RFS and OS. Statistical significance was assumed for *p*-values less than 0.05. Statistical analyses were performed using SPSS for Windows version 15.0 (SPSS, Chicago, IL, USA).

Results

Sixty-two patients undergoing partial hepatectomy for synchronous colorectal liver metastases with curative intent fulfilled the inclusion criteria and were enrolled in this study (40 male and 22 female patients, with a median age of 61.23 years, ranging from 33 to 81 years). Baseline characteristics are shown in Table I.

Among 62 patients, 24 died during follow-up. The remaining 38 patients had a median follow-up time of 33 months. None of the patients were lost to follow-up. Median RFS as calculated by the Kaplan-Meier method was 11.27 months and median OS was 50.46 months. The median preoperatives CD95L level was 0.1762 ng/ml (95% confidence interval, CI=0.12-0.41). The median postoperative level was 0.1643 ng/ml (95% CI=0.11-0.26).

Clinical factors that were significantly associated with poor RFS in univariable COX regression analysis included a high Memorial Sloan-Kettering Cancer Centre Clinical Risk Score (MSKCC-CRS) as defined by Fong *et al.* (11), (*p*=0.040, HR=1.395, 95% CI=0.151-0.979), the administration of neoadjuvant chemotherapy (*p*=0.028, HR=2.345, 95% CI=1.096-5.017), increasing tumour size (*p*=0.045, HR=1.096, 95%CI=1.001-1.205), omission of adjuvant chemotherapy (*p*=0.045, HR=0.395, 95%CI=0.151-0.979) and high pre-operative sCD95L levels (*p*=0.019. HR=2.322, 95%CI=1.272-3.590) (Table II). For Kaplan-Meier survival curves, patients were divided into high (above median preoperative levels) and low (below median preoperative levels) sCD95L groups. Patients in the high preoperative sCD95L group (n=31) had a median RFS of 8.08 months (95% C=4.371-11.79), whereas patients in the low pre-operative sCD95L group (n=31) had a median RFS of 15.13 months (95%CI=10.63-21.63) (Figure 1). Postoperative levels of sCD95L were not significantly

Table I. *Patient and tumour characteristics*.

Total number of patients	62
Male	40 (64.5%)
Female	22 (35.5%)
Age	
(Mean; Median; SD)	60.23; 61.23; 10.68
Location of primary tumour	
Rectum	19 (30.6%)
Colon	43 (69.4%)
Differentiation of primary tumour	
Good	5 (11.3%)
Moderate	50 (80.6%)
Poor	7 (11.3%)
Nodal status	
N+	36 (58.1%)
N–	26 (41.9)
Neoadjuvant chemotherapy	
Yes	11 (17.74%)
No	47 (75.81%)
Missing	4 (6.45%)
Type of resection	
Minor	34 (54.8%)
Major (3 segments resected or more)	28 (44.2%)
R0/R1 Resection	
R0	58 (93.5%)
R1	4 (6.5%)
Bloodtransfusion required	
No	52 (83.9%)
Yes	10 (16.1%)
Mean number of liver metastases/patient	2.32
Ischaemia (due to vascular clamping)	
None	25 (40.3%)
Minor	19 (30.6%)
Severe	18 (29.0%)
Adjuvant chemotherapy	
Yes	13 (21%)
No	49 (79%)
Preoperative CEA (ng/ml)	
(Mean; median; 95% CI)	44.51; 30.43; (32.33-57.73)
Preoperative sCD95L level (ng/ml)	
(Mean; median; 95% CI)	0.2132; 0.1762; (0.12-0.41)
Postoperative sCD95L level (ng/ml)	
(Mean; median; 95% CI)	0.1858; 0.1643; (0.11-0.26)

Table II. *Risk factors for recurrence-free survival and overall survival identified by univariable COX regression analysis.*

	Recurrence-free survival			Overall survival		
	Hazard ratio	95% CI	*p*-Value	Hazard ratio	95% CI	*p*-Value
Age	0.990	0.958-1.023	0.532	0.992	0.952-1.033	0.690
Gender	0.600	0.315-1.144	0.121	0.367	0.125-1.083	0.070
Location primary tumour (rectum/colon)	0.883	0.467-1.668	0.701	0.740	0.298-1.837	0.516
Differentiation (good/moderate/poor)	1.050	0.655-1.685	0.838	1.331	0.638-2.774	0.446
Nodal status (N+/N-)	1.761	0.945-3.281	0.075	0.859	0.375-1.965	0.719
Neoadjuvant chemotherapy	2.345	1.096-5.017	0.028	1.606	0.582-4.432	0.360
Blood transfusion	1.541	0.734-32.35	0.253	1.092	0.424-2.813	0.856
Major/Minor resection	1.289	0.717-2.317	0.397	1.331	0.581-3.051	0.449
R1/R0	0.901	0.278-2.919	0.862	3.022	0.865-10.55	0.083
No. of liver metastases	0.971	0.861-1.094	0.624	0.950	0.769-1.174	0.637
Ischaemia (none/minor/severe)	0.996	0.702-1.414	0.982	0.539	0.316-0.919	0.023
Size of biggest liver tumour	1.096	1.001-1.205	0.045	0.996	0.882-1.124	0.948
Preoperative CEA	1.006	0.998-1.014	0.146	1.003	0.993-1.012	0.548
Bilobar distribution	1.208	0.640-2.777	0.560	0.924	0.354-2.412	0.872
Adjuvant chemotherapy	0.395	0.151-0.979	0.045	0.368	0.048-2.799	0.334
MSKCC-CRS (11)	1.408	1.016-1.953	0.040	0.934	0.589-1.482	0.773
Iwatsuki score (17)	1.041	0.736-1.473	0.819	0.859	0.514-1.435	0.562
Preoperative sCD95L (above/below median)	2.322	1.272-3.590	0.019	2.692	1.168-6.206	0.020
Postoperative sCD95L (above/below median)	0.625	0.339-1.152	0.132	1.203	0.533-2.717	0.656

CI: Confidence interval; CEA: carcinoembryonic antigen; MSKCC-CRS: Memorial Sloan_Kettering Cancer Center-Clinical risk score.

associated with RFS (Table II). Next we employed a multivariable COX regression model containing the factors that displayed p-values less than 0.1 in univariable analysis. The preoperatives CD95L levels (p=0.009, HR=3.911, 95% CI=1.414-10.817) was found to be the only independent risk factor for poor RFS. Factors significantly correlating with OS in univariable COX regression analysis were the amount of ischaemia induced during surgery (12) and high pre-operative sCD95L (p=0.020, HR=2.69, 95%CI=1.168-6.206). Patients in the high preoperatives CD95L group (n=31) had a median OS of 31.57 months (95% CI=20.30-42.84), whereas patients in the low preoperativesCD95L group (n=31) had a median OS of 58.38 months (95%CI=44.45-72.31) (Figure 2).

Multivariable analysis showed a significant correlation of severe ischaemia (p=0.06, HR=0.457 95% CI=0.261-0.800) and high preoperative level of sCD95L (p=0.003, HR=3.674, 95%CI=1.536-8.786) with poor prognosis. To assess whether sCD95L was not merely a reflection of the overall tumour load or influenced by cell death induced by chemotherapy, we performed an additional binary logistic regression analysis comparing sCD95L expression levels with clinicopathological features (Table III) None of these variables were significantly associated with sCD95L.

Discussion

The findings presented point towards a potentially prognostic role of sCD95L regarding the biological behaviour of colorectal cancer presented with synchronous liver metastasis. Our results show that high preoperative levels of sCD95L upon presentation are associated with an unfavourable outcome, reflected by a median RFS of less than 11 months. sCD95L levels were not simply a reflection of overall tumour burden. sCD95L may therefore identify a subgroup of patients that is unlikely to benefit from liver surgery and that should be referred for up-front chemotherapy and/or biological therapies (13). In contrast, patients presented with low sCD95L levels might be considered for surgery, including a 'liver first' or 'simultaneous' approach. Poor OS was observed in the high sCD95L group.

Although synchronous liver metastasis is considered as a poor prognostic factor by itself, it does not preclude the possibility of long-term survival, and 5-year survival rates of up to 40% can still be achieved (11, 14). Various studies have defined factors predicting RFS in colorectal cancer patients with synchronous liver metastasis such as sex, tumour differentiation, postoperative carcinoembryonic antigen (CEA) levels, infiltration in other

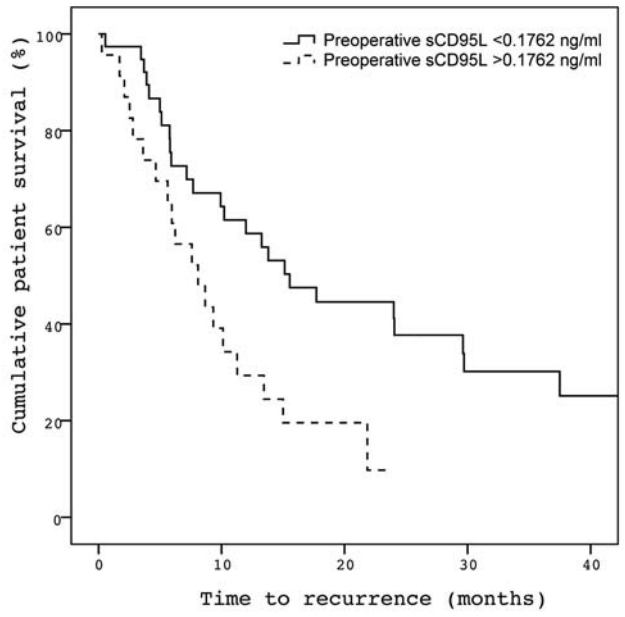

Figure 1. *Kaplan-Meier curves illustrating the effects of high preoperativesCD95L (above median preoperativevalue) on recurrence-free survival (p=0.019, log-rank test).*

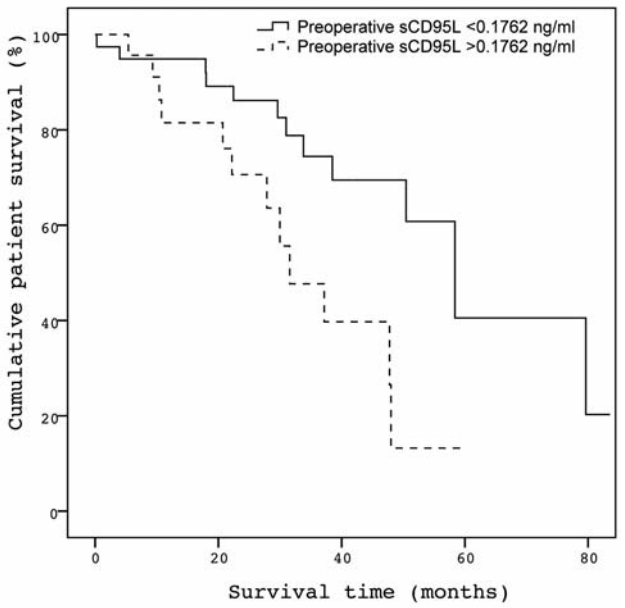

Figure 2. *Kaplan-Meier curves illustrating the effects of high preoperativesCD95L (above median preoperativevalue) on overall survival (p=0.020, log-rank test).*

organs, number of metastases and metastatic lymph nodes (15, 16). Different combinations of these factors have been proposed as clinical prediction models for selecting patients who could benefit from surgery (11, 17-19). Nonetheless, these models are still far from optimal, as they predict outcome with a considerable degree of variation (20, 21). Progression during preoperative chemotherapy is also suggested as a biological marker for poor prognosis. No substantial evidence exist that this approach is beneficial to RFS (22). Therefore the use of neoadjuvant chemotherapy should be preserved for large clinical trials, as chemotherapy induces liver toxicity, such as steatosis, steatohepatitis and sinusoidal changes. Preoperative chemotherapy increases the risk of postoperative complications (25% *vs*. 16% p=0.04) (22). This makes it essential to search for new molecular prognostic factors that inplicate tumour status and accurately reflect its biological behaviour (15, 23).

Accumulating evidence suggests a tumour-propagating role of (s)CD95L in malignancies, including colorectal cancer (5, 7, 10). Our results are in line with several other studies in which elevated serum sCD95L concentrations are correlated with poor prognosis in large granular lymphocytic leukaemia, NK lymphoma, bladder carcinoma, gastric carcinoma, hepatocellular carcinoma and breast carcinoma (24, 25). To our knowledge, this is the first study presenting a correlation between elevated serum sCD95L and poor RFS in colorectal cancer. Conversely, in squamous cell carcinoma of the oesophagus, sCD95L levels had no significant prognostic effect on RFS, suggesting that the merit of sCD95L measurements may be tumor type-specific (26). Whether sCD95L is merely associated with decreased RFS or whether it is causally involved in accelerating tumour progression will be the subject of further studies.

Although our study is retrospective in nature and based on a relatively small number of patients, the differences in outcome that are associated with different sCD95L levels are such that further validation studies are justified. Moreover, analysis of sCD95L levels in clinical practice is appealing as it can simply be measured in patient blood samples preoperatively by using thoroughly validated ELISAs (9, 27).

In conclusion, our data suggest that high pre-operative levels of sCD95L are associated with poor RFS and OS in patients scheduled for surgery for synchronous colorectal liver metastases. Obviously, these findings should be regarded as hypothesis-generating and require substantiation in larger patient cohorts. A low preoperatives CD95L level may help identify a subgroup of patients with synchronous liver metastases that are likely to benefit from liver surgery.

Acknowledgements

This study was supported by a grant from the Dutch Cancer Foundation (KWF #2009-4417). FJHH was supported by a grant from the PON foundation. MWN was supported by a grant from ZON-Mw (#920-03-534). The Authors thank all the patients who participated in this study.

Table III. *Factors associated with levels of preoperative sCD95L identified by univariable logistic regression analysis, according to whether values are below (low) or above (high) the median preoperative level (0.1762 ng/ml).*

	Low sCD95L (n=31)	High sCD95L (n=31)	*p*-value	HR	95%CI
Age (years)	61.65 (SD 9.63)	58.81 (SD 11.6)	0.296	0.975	0.928-1.023
Gender, n (%)					
Male	21 (67.7%)	19 (61.3%)	0.596	1.326	0.467-3.766
Female	10 (32.3%)	12 (38.7%)			
Location primary tumour, n (%)					
Rectum	8 (25.8%)	11 (35.5%)	0.410	0.632	0.213-1.881
Colon	23 (74.4%)	20 (64.5%)			
Differentiation, n (%)					
Good	4 (12.9%)	1 (3.2%)	0.380	0.724	0.351-1.491
Moderate	24 (77.4%)	26 (83.9%)			
Poor	3 (9.7%)	4 (12.9%)			
Nodal status, n (%)					
N+	16 (51.6%)	20 (64.5%)	0.305	1.705	0.616-4.720
N–	15 (48.4%)	11 (35.5%)			
Neoadjuvant chemotherapy					
Yes	4 (12.9%)	7 (22.6%)	0.445	1.677	0.433-6.502
No	23 (74.2%)	24 (77.4%)			
Blood transfusion					
Yes	5 (16.1%)	5 (16.1%)	1.000	1.000	0.258-3.871
No	26 (83.9%)	26 (83.9%)			
Type of resection					
Minor	18 (58.1%)	15 (48.4%)	0.610	1.298	0.476-3.538
Major	13 (41.9%)	16 (51.6%)			
Bilobar distribution					
Yes	7 (22.6%)	11 (35.5%)	0.266	1.886	0.616-5.768
No	24 (77.4%)	20 (64.5%)			
No. of liver metastases					
(mean, SD)	2.55 (SD 3.30)	2.10 (SD 1.83)	0.510	0.934	0.761-1.146
Size of biggest tumour (cm)					
(median, SD)	3.75 (SD 3.86)	4.42 (SD 2.51)	0.228	2.261	0.601-8.505
Ischemia					
None	11 (35.5%)	14 (45.2%)	0.645	0.867	0.474-1.588
Minor	11 (35.5%)	8 (25.8%)			
Severe	9 (29%)	9 (29%)			
Preoperative CEA (ng/ml)	46.81 (SD 46.7)	43.25 (SD 53.8)	0.771	0.999	0.989-1.009
Adjuvant chemotherapy, n (%)					
Yes	4 (12.9%)	8 (25.5%)	0.228	2.261	0.601-8.505
No	27 (87.1%)	23 (74.2%)			
Iwatsu score, n (%)					
Grade 1	0	0	0.436	1.279	0.689-2.375
Grade 2	21 (67.7%)	17 (54.8%)			
Grade 3	4 (12.9%)	7 (22.6%)			
Grade 4	6 (19.4%)	7 (22.6%)			
Fong score					
0	0	0	0.471	1.234	0.697-2.186
1	6 (19.4%)	6 (19.4%)			
2	13 (41.9%)	9 (29%)			
3	10 (32.3%)	13 (41.9%)			
4	2 (6.5%)	3 (9.7%)			

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Received August 28, 2011
Revised November 10, 2011
Accepted November 11, 2011