Volume : 5 | Issue : 7 | July 2016 • ISSN No 2277 - 8179 | IF : 3.508 | IC Value : 69.48

Clinical Significance of Serum Dikkopf-1 (Dkk-1) As A Diagnostic Marker for Hepatocellular Carcinoma

Medical Science

KEYWORDS : Dickkopf -1, Hepatocellular carcinoma, alpha fetoprotein.

Hoda G.Bakr — Department of Internal Medicine, Faculty of Medicine, Zagazig University

Ola M.Elfarargy — Department of Medical Oncology &Haematology, Faculty of Medicine, Zagazig University

Amina M. Elnaggar — Department of Clinical Pathology, Faculty of Medicine, Zagazig University

ABSTRACT

Background: Hepatocellular carcinoma (HCC) is one of the commonest cancers worldwide. It is a major health problem and its incidence is increasing. Dickkopf-1 (DKK1) is a negative regulator of the Wnt signaling pathway, which plays an important role in proliferation, differentiation, survival, apoptosis and cell mortality. DKK1 has a potential oncogenic role in carcinogenesis. Aim of the study: Was to assess the expression of DKK1 in patients with hepatocellular carcinoma and its clinical significance. Subjects and Methods: This study comprised 50 hepatocellular carcinoma (HCC) patients, 40 males and 10 females, their ages ranged from 46-72 years, and control group: included 25 healthy individual, 16 males and 9 females, their ages ranged from 44-67. All participants were subjected to clinical evaluation and laboratory investigations; complete blood picture, liver & kidney function tests, alpha fetoprotein (AFP) and DKK1. Results: There was increased level of serum DKK1 in all HCC patient, with increased level in advanced than early stages. Increased AFP level was found in only 66% of patients while 34% of patients were AFP negative. Conclusion: Combination of serum α-fetoprotein with serum DKK1 could further improve the diagnostic accuracy in HCC. DKK-1 might be a key regulator in HCC progression and a potential therapeutic target in HCC

Introduction

Hepatocellular carcinoma (HCC) is one of the commonest cancers worldwide. HCC is a major health problem in Egypt and its incidence is increasing. HCC has unique risk factors, such as hepatitis C or B viral infection, dietary aflatoxin or vinyl chloride, cirrhosis, obesity and primary hemochromatosis[1].The high prevalence of HCV infection makes screening programs a very important tool for early detection of cases of HCC [2]. Cirrhosis is an important risk factor, account for 70% to 90% of HCC [3]. Hepatitis B virus (HBV) infection is a significant predisposing factor for the development of HCC with 100-fold increased risk and accounts for more than 50% of all cases [4]. Long duration of infection and high viremic load are also risk factors of HCC. Males are at greater risk of HCC than females, especially old male [5].

African males commonly exposed to HBV infection at a younger age, and so increases their risk for HCC. HBV genotypes A and D are most prevalent in African populations [6]. Family history of liver cancer, particularly among first degree relatives, in HBV-infected individuals has been shown to increase the incidence of HCC among siblings [7].

The Wnt signaling pathways are a group of signal transduction protein pathways, pass signals into cells by cell surface receptors.

Canonical Wnt pathway regulates gene transcription, noncanonical planar cell polarity pathway regulates cytoskeleton, and noncanonical Wnt/calcium pathway regulates cell calcium. Wnt signaling was first identified for its role in carcinogenesis[8]. Wnt/β catenin signaling pathway plays an important role in a variety of cellular processes, including proliferation, differentiation, survival, apoptosis and cell mortality. It have been proved to have a critical role in formation of HCC [9]. Wnt antagonists are important regulators of Wnt/β catenin signaling, DKK-1 is a known potent antagonist of Wnt/β catenin signaling pathway [10]. DKK-1 acts as inhibitory ligand of the low-density lipoprotein receptor-related protein 5/6 co-receptors resulting in blockage of their interaction with Wnt and β-catenin degradation[11]. DKK-1 was frequently found to be over expressed in patients with Wilms tumour, hepatoblastoma, multiple myeloma and breast cancer, indicating a potential oncogenic role of DKK1 in carcinogenesis[12].

Yu et al. (2009)[13] found that DKK-1 overexpression correlated with β catenin accumulation in clinical HCC samples and that high DKK-1 expression predicted unfavorable prognosis in HCC. These findings suggest that DKK1 is a novel prognostic predictor for HCC. Serum α-fetoprotein (AFP) level is the gold standard for HCC diagnosis, also high serum DKK-1 level was found to have higher diagnostic value for HCC. DKK family members have a diagnostic and/or prognostic markers for HCC especially for AFP-negative HCC, and can distinguish HCC from non-malignant chronic liver diseases.

The combination of serum DKK-1 and AFP levels enhance diagnostic accuracy for HCC compared to serum DKK-1 or AFP level alone [14]

This study aimed at assessment of the expression of Dickkopf-1(DKK-1) and its clinical significance as a diagnostic marker for early detection of patients with HCC

SUBJECTS&METHODS:

This study was performed in the departments of Internal Medicine, Medical Oncology & Hematology, and Clinical Pathology, Faculty of Medicine of Zagazig University Hospitals, from September 2014 to May 2015. Fifty adult patients with HCC in addition to 25 healthy control subjects were selected after obtaining approval from the Institutional Review Board of Zagazig University Hospitals.

Patients group: 50 newly diagnosed patients with HCC without induction therapy, they were 40 males &10 females, their mean age 57.1 ± 6.98 (46-72) years, 80 % were males and 20% were females. HCC patients were diagnosed clinically and by imaging studies including ultrasound (US), triphasic computed tomography (CT) and Magnetic Resonance Imaging (MRI). Liver cirrhosis was present in 33/50 (66%) of the patients. Diagnosis of liver cirrhosis was done by clinical, laboratory and radiological features of cirrhosis. Child-Pugh classification was also done. HCC stage was clinically defined according to the Barcelona Clinic Liver Cancer (BCLC) staging system [15]. Exclusion criteria were; patients with malignancies other than HCC,

diabetes mellitus, hypertension, cardiac, respiratory and renal diseases.

Control group: 25 healthy subjects were selected with normal liver function tests. No evidence of viral hepatitis (negative for HBsAg and HCV antibody) .Their mean age was 49.98 ± 5.4 (44-67) years, 16 (64 %) of them were males and 9 (36%) were females. In addition to full history taking, examination and routine laboratory investigations, assessment of alpha fetoprotein and serum Dickkopf-1 (DKK1) were performed.

Sample collection: Blood samples were obtained at the time of diagnosis prior to initial therapy. Samples were obtained after informed consent and in accordance with procedures approved by the human ethics committee. 5 ml venous blood were obtained from each patient & healthy control under complete aseptic condition.1 ml was delivered into EDTA vacutainer tube for complete blood count using an automated cell counter (sysmex KX-21N).2 ml was delivered into Na citrate vacutainer tube for prothrombin time (PT) by Sysmex 1500 (Dade Behring). 2ml were collected into anticoagulant- free tubes then centrifuged; liver function tests were done using automated analyzer (Dimention RXL MAX). AFP were done and serum were stored at -70°C till DKK1assay.

DKK1 assay:
Principle of test: Human Dickkopf-Related Protein 1 ELISA is a sandwich enzyme immunoassay for the quantitative measurement of human Dkk-1. It was performed according to the manufacturer's recommendations (Bio Vendor Research & Diagnostic). Standards and samples are incubated in microtitration wells pre-coated with polyclonal anti-human Dkk-1 antibody. After incubation and washing, biotin labelled polyclonal anti-human Dkk-1 antibody was added and incubated with the captured Dkk-1. Streptavidin-HRP conjugate was added. The remaining conjugate is allowed to react with the substrate solution (TMB). The reaction is stopped by addition of acidic solution and absorbance is measured. The absorbance is proportional to the concentration of Dkk-1. A standard curve is constructed by plotting absorbance values against Dkk-1 concentrations of standards. The optical density was measured at 450 nm and referenced to 570 nm. The DKK- 1 concentrations were obtained with a four-parameter logistic curve fitted against a standard curve and multiplied by the dilution factor.

Statistical Analysis: The data were tabulated and statistically analyzed using SPSS computer program (version 16.0; SPSS Inc., , Chicago, Illinois ,USA).Data were represented as Mean ± SD, median and range, and were analyzed statistically by using analysis of variance (ANOVA), Chi-squared test (χ^2), paired T test and correlation coefficient (r). Values were considered significant if $p < 0.05$.

RESULTS
Subjects' characteristics, the clinical and laboratory data were presented in tables 1 and 2, in which there was no significant difference between patients and control groups regarding age and sex. Serum DKK-1 & AFP were elevated significantly in patients compared to control**.** Assessment of DKK1 levels according to tumor size & multiplicity, showed that, there was significant elevation of DKK-1 among patient with HCC lesions <2 cm compared to healthy control, DKK-1 levels were increased significantly among patient with HCC lesions >5 cm compared to smaller one (<2cm). Patients with multiple HCC lesions had increased level of DKK-1 compared to solitary lesion but not reaching significant level **(Table3).** DKK-1 was tested according to Barcelona Clinic Liver Cancer (BCLC) staging system, the level of DKK-1 was significantly elevated in BCLC stage C-D than BCLC stage A-B P=0.000. **(Table3).** In **table (4)**, there was no significant difference in levels of DKK1 in patients with coma, GIT bleeding, but DKK-1 was significantly increased in patients with cirrhotic liver, ascites, vascular invasion, portal vein thrombosis and metastasis of lymph nodes when compared to patients without these findings.

Increased DKK1>3 ng/ml was observed in 17 patients (34%) with normal AFP (AFP≤10) i.e. there is increase in DKK1 in all patient ,while AFP was elevated in (33/50) (66%) of HCC patients. **(Table5).** There was no significant correlation between DKK-1 and AFP & liver function P >0.05.

Table (1): A summary of the patients' clinical data

Parameter	
Age: mean ±SD (range) (year)	57.1±6.98 (46-72)
Sex : Men: Number (%) Women: Number (%)	 40 (80) 10 (20)
Parameters	Number of patients (%)
Jaundice	28(56)
Disturbed level of Consciousness	22(44)
GIT bleeding	10 (20)
Ascites	27(54)
Oedema	15 (30)
Shrunken Liver	33 (66)
Vascular invasion	19 (38)
Splenomegaly	27(54)
LN metastasis	22(44)
Distant metastasis	6(12)

Table (2): Summary of the laboratory data of patient and control groups.

	Patients	Controls	P
T. Bilirubin(mg/dl) Median (range)	3.6 (1.2-16.9)	0.7 (0.5-1.2)	0.000
D. Bilirubin (mg/dl) Median (range)	1.5 (0.1-13.9)	0.21 (0.1-0.2)	0.000
ALT(U/L) Mean ±SD (range)	81.9±33.4 (21.9-151)	28.3±7.8 (18.9-40.1)	0.000
AST(U/L Mean ±SD (range)	115.3±60.6 (45-346)	31.5±7.4 (19-44.9)	0.000
T. protein (gm/dl) Mean ±SD (range)	6.2±0.6 (4.8-7.2)	7.2±0.20 (6.8-7.5)	0.000
S .albumin (gm/dl) Mean ±SD (range)	2.4±0.40 (1.8-3.7)	4.5±0.3 (4-5.1)	0.000
Hemoglobin (gm/dl) Mean ±SD (range)	10.0±2.1 (6.3-13.5)	12.5±1.5 (11.6-14.8)	0.001
WBCs ($\times 10^9$ /L) Mean ±SD (range)	9.0±4.5 (2.1-13.7)	7.2±2.3 (4.4-10.9)	0.148
Platelet ($\times 10^9$ /L) Mean ±SD (range)	126.2±78.5 (49-389)	271.5±50.4 (231-359)	0.000
PT(sec.) Median (range)	33 (21-43)	12 (9.5-13.5)	0.000
Serum DKK1(ng/ml) Mean ±SD (range)	9.6±2.9 (4.9-15.0)	2.04±0.91 (0.8-3.8)	0.000
AFP (ng/ml) Median (range)	50 (0.8-2949)	1.52 (0.5-4.5)	0.000

Volume : 5 | Issue : 7 | July 2016 • ISSN No 2277 - 8179 | IF : 3.508 | IC Value : 69.48

Table (3): Serum DKK1 level regarding size, multiplicity and BCLC staging of HCC lesions.

	DKK-1	Control	F	P
<2cm (No.16) 2-5cm (No .15) >5cm (No.19)	7.21±1.15 9.65±2.88 10.0±2.81**	2.04±0.91*	37.386	0.000
Single lesion (No.21) Multiple lesions (No.29)	9.21±2.65 10.03±2.81	2.04±0.91*	55.1	0.000
BCLC (A-B) (No.16) BCLC (C-D) (No.34)	7.61±1.23 10.1±2.65***	2.04±0.91*	37.12	0.000

*denotes that all patients significantly different than control

**denotes that lesion>5cm significantly different than lesion<2cm

*** denotes that BCLC C-D significantly different than BCLC A-B

Table (4): Serum DKK-1 level in different clinical and radiological presentation

	Dkk1 (Mean ± SD)	T	P
Disturbed conscious level (22 cases) Undisturbed conscious level (28 cases)	9.76±2.50 9.63±3.00	0.138	0.889
GIT bleeding (10cases) No GIT bleeding (40 cases)	9.34±2.80 9.72±2.80	0.287	0.775
Ascites (27 cases) No ascites (23cases)	12.43±2.20 6.23±0.52	3.891	0.000
Liver cirrhosis (33 cases) No liver cirrhosis (17 cases)	10.24±2.62 7.45±2.18	2.774	0.008
Vascular invasion (19 cases) No vascular invasion (31 cases)	10.62±2.58 8.74±2.66	2.223	0.032
LN metastasis (22 cases) No LN metastasis (28cases)	10.80±2.38 7.62±2.38	4.144	0.000

Table (5): Study of DKK-1 in HCC patients in relation to level of AFP

	HCC patients with AFP >10(ng/ml)	HCC patients with AFP≤10(ng/ml)
Dkk1>3(ng/ml)	33/50 cases (66%)	17/50 cases (34%)

Discussion

Hepatocellular carcinoma (HCC) is the most common primary malignancy of liver, forming about 85-90% of all primary liver cancers [3]. 80%-90% of patients with cirrhosis will progress to HCC, HCV infection was present in 27% - 73% and HBV infection in 12%-55% of cases [16]. Early HCC detection gives the opportunity for curative treatments which can prolong survival. Despite vigorous attempts to screen for early HCC by using serum α-fetoprotein (AFP) and ultrasound, early HCC is asymptomatic and most cases are presented late in which surgical treatments are not valuable [17]. 30–40% of HCC patients are AFP-negative and ultrasound detection accuracy for HCC is suboptimal [18]. As a result, the widespread use of serum AFP and ultrasound for HCC surveillance has been continuously questioned.

The Wnt/β-catenin signaling pathway plays a pivotal role in development of both normal liver and hepatic carcinogenesis [13]. The DKK family are secreted glycoproteins and have the ability in regulating Wnt/β-catenin. DKK-1 is the most studied member of the DKK family, it is hardly expressed in normal tissues except in placenta and embryonic tissues [19]. **Shen et al., 2012** [20] suggest that, DKK-1 may be a potential new marker in diagnosis of many types of cancers, and elevated expression of DKK-1 is associated with the development of HCC. Also they reported the diagnostic accuracy of DKK-1 as a serum marker for HCC in a large-scale, multicenter study. The study documented that, serum DKK1 has high sensitivity and specificity in diagnosing HCC, especially early stages and HCC cases with non-elevated α-fetoprotein (AFP). They also demonstrated that combination of serum α-fetoprotein with serum DKK-1 could further improve the diagnostic accuracy.

In this study, we tried to assess of the expression of Dickkopf-1 in HCC and its clinical significance as a diagnostic marker for early detection of HCC.

Our study revealed a higher incidence of HCC in males than females (80%and 20% respectively), male: female ratio was 4:1, which was also published by **El-Serag and Rudolph, 2007**[3]. Serum DKK-1 levels in our cases were significantly higher in HCC patients than healthy controls. **Yu et al., 2009** [13], **Patil et al., 2005** [21] **and Yamashita et al., 2008** [22] reported high expression of DKK-1 in HCC. Also **Seung UP et al 2015**[23] documented that, DKK-1 concentrations were significantly higher in all HCC patients than in the control group. That elevation of DKK-1 of our patients was significant in patients with liver cirrhosis, ascites, vascular invasion, thrombosed portal vein and LN metastasis when compared to patients without these features.

In this study, we found that, DKK-1 can be used as a diagnostic marker for early detection of HCC, as we assessed DKK-1 levels in relation to the diameter and multiplicity of the tumor. We found a significant elevation of serum DKK-1 levels in cases with HCC less than 2 cm in diameter (single lesion) when compared to healthy control, and the level was increased significantly to be higher among lesions >5 cm compared to smaller one (<2cm). Patients with multiple HCC lesions showed non-significant higher DKK-1 levels compared to single one .These results were in line with that of **Yu et al., 2009** [13]who reported that there is no correlation between DKK1-positivity and tumor size. On the other hand, it was noted that there is correlation between serum DKK1 level and a larger tumor size (≥5 cm) and the correlation disappeared when tumor size was less than 5 cm [20], [24].

Serum DKK-1was assessed in our cases according to BCLC staging of HCC, we noticed, significantly increased serum DKK-1 levels in patients with BCLC (C-D) than BCLC (A-B) stage, the same results was reported by **Seung UP et al., 2015**[23], they found that, DKK-1 levels in BCLC (A-B) and TNM I–II patients tended to be lower than BCLC (C-D) and TNM III–IV patients. Another study by **Yang LY et al., 2004**[25] found that Dkk-1 was significantly elevated in nodular HCC (multiple lesions) with high metastatic potential compared to solitaryHCC. We observed that, DKK1 noticed to be a good marker in diagnosing early-stage HCC in patients with negative AFP, as we found that, AFP was increased in 33/50 of patients (66%), while17/50(34%) of patients had normal AFP whereas DKK-1 was increased in all our patients (100%), and so 34% of studied patients with HCC are AFP negative. These results were also noted by **Shen et al., 2012** [20] who reported that serum DKK-1 had a high diagnostic accuracy for early-stage HCCs and single HCCs ≤2 cm. Furthermore, DKK1 can distinguish HCC patients from non-malignant chronic liver diseases, as DKK1 level in HCC patients with cirrhosis was significantly high-

er than that in patients with cirrhosis alone. **Gomceli et al., 2012**[26] reported that DKK-1 may have an important role in patients where AFP levels are negative or equivocal as that in chronic liver disease.

Tao et al., 2013[27] found that in comparison to serum AFP level, which remains the gold standard for HCC diagnosis, high serum DKK-1 levels have higher diagnostic value for HCC, especially for AFP-negative HCC, and can distinguish HCC from non-malignant chronic liver diseases.

Conclusion

DKK-1 has diagnostic value for HCC better than that of AFP, especially for patients with AFP-negative status and early-stage HCC. Combination of serum α-fetoprotein with serum DKK-1 could further improve the diagnostic accuracy in HCC diagnosis. DKK-1 may be a key regulator in HCC progression and a promising potential therapeutic target for HCC. DKK-1 is one of the trickiest biomarkers studied in malignancies, and its exact role in oncogenesis and mechanisms underneath remain ambiguous.

References

1- Schütte K, Bornschein J, Malfertheiner P. (2009): Hepatocellular carcinoma, epidemiological trends and risk factors. Dig Dis; 27:80-92.

2- Shaker MK, Abdella HM, Khalifa MO, El Dorry AK (2013) : Epidemiological characteristics of hepatocellular carcinoma in Egypt: retrospective analysis of 1313 cases. Liver Int.Nov; 33(10):1601-6.

3- El-Serag HB and Rudolph KL. (2007): Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. Gastroenterology; 132:2557-76

4- Sherman M, Llovet JM. (2011): Smoking, hepatitis B virus infection, and development of hepatocellular carcinoma. J Natl Cancer Inst; 103:1642–1643

5- Ferenci P, Fried M, Labrecque D, Bruix J, Sherman M, Omata M, et al. (2010): World Gastroenterology Organization guideline. Hepatocellular carcinoma (HCC): a global perspective. J Gastrointestinal Liver Dis; 19:311–317

6- Kew MC. (2010): Hepatocellular carcin-oma in African blacks: recent progress in etiology and pathogenesis. World J Hepato; 2:65–73.

7- McClune AC, Tong MJ. (2010): Chronic hepatitis B and hepatocellular carcinoma. Clin Liver Dis; 14:461–476.

8- Nusse R (Jan 2005). "Wnt signaling in disease and in development". Cell Research 15 (1): 28–32.

9- Thompson MD and Monga SP. (2007): WNT/beta-catenin signaling in liver health and disease. Hepatology; 45:1298-305.

10- Niida A, Hiroko T, Kasai M, Furukawa Y, Nakamura Y, Suzuki Y, et al. (2004):DKK1, a negative regulator of Wnt signaling, is a target of the beta-catenin/TCF pathway. Oncogene; 23:8520–8526.

11- Mao B, Wu W, Davidson G, Marhold J, Li M, Mechler BM, et al. (2002): Kremen proteins are Dickkopf receptors that regulate Wnt/betacatenin signalling. Nature; 417:664-7.

12- Gavriatopoulou M, Dimopoulos MA, Christoulas D, et al. (2009): Dickkopf-1: a suitable target for the management of myeloma bone disease. Expert Opinion on Therapeutic Targets; 13:839–48.

13- Yu B, Yang X, Xu Y, et al. (2009): Elevated expression of DKK1 is associated with cytoplasmic/nuclear beta-catenin accumulation and poor prognosis in hepatocellular carcinomas. J Hepatol; 50:948-57.

14- Sarwat Fatima, John M Luk, Ronnie TP Poon and Nikki P Lee (2014): Deregulated expression of dickkopfs for potential detection of hepatocellular carcinoma June, Vol. 14, No. 5, Pages 535-548

15- Forner A, Reig ME, de Lope CR, Bruix J. (2010): Current strategy for staging and treatment: the BCLC update and future prospects. Semin Liver Dis; 30:61-74.

16- Kanwal F, Hoang T, Kramer JR, Asch SM, Goetz MB, Zeringue A, et al. (2011): Increasing prevalence of HCC and cirrhosis in patients with chronic hepatitis C virus infection. Gastroenterology; 140: 1182-1188.

17- Kudo M, Han KH, Kokudo N, Cheng AL, Choi BI, Furuse J,et al (2010): Liver Cancer Working Group report. Jpn J Clin Oncol; 40 Suppl 1.

18- Wu W, Yao DF, Yuan YM, Fan JW, Lu XF, Li XH, et al. (2006): Combined serum hepatoma-specific alpha-fetoprotein and circulating alpha-fetoprotein-mRNA in diagnosis of hepatocellular carcinoma. Hepatobiliary Pancreat Dis Int; 5:538-44.

19- Krupnik, V. E., Sharp, J. D., Jiang, C., Robison, K. et al. (1999): Functional and structural diversity of the human Dickkopf gene family. Gene 238, 301-313.

20- Shen Q, Fan J, Yang XR, et al. (2012): Serum DKK1 as a protein biomarker for the diagnosis of hepatocellular carcinoma: a large-scale, multicenter study. Lancet Oncol; 13:817-26.

21- Patil MA, Chua MS, Pan KH, Lin R, Lih CJ, Cheung ST, et al. (2005): An integrated data analysis approach to characterize genes highly expressed in hepatocellular carcinoma. Oncogene; 24:3737–3747.

22- Yamashita T, Forgues M, Wang W, Kim JW, Ye Q, Jia H, et al. (2008): EpCAM and alpha-fetoprotein expression defines novel prognostic subtypes of hepatocellular carcinoma. Cancer Res; 68:1451–1461.

23- Seung Up Kim, Jeon Han Park, Hyon-Suk Kim, Jae Myun Lee, Hyun Gyu Lee et al (2015): Serum Dickkopf-1 as a Biomarker for the Diagnosis of Hepatocellular Carcinoma Yonsei Med J 2015 Sep;56(5):1296-1306.

24- Tung EK, Mak CK, Fatima S, et al. (2011): Clinicopathological and prognostic significance of serum and tissue Dickkopf-1 levels in human hepatocellular carcinoma. Liver Int; 31:1494-504.

25- Yang LY, Wang W, Peng JX, et al. (2004): Differentially expressed genes between solitary large hepatocellular carcinoma and nodular hepatocellular carcinoma. World Journal of Gastroenterology; 10:3569–73.

26- Gomceli I, Bostanci EB, Ozer I, et al. (2012): A novel screening biomarker in gastric cancer: Serum Dickkopf-1. Hepatogastroenterology; 59:1661-4

27- Tao YM, Liu Z, Liu HL. (2013): Dickkopf-1 (DKK1) promotes invasion and metastasis of hepatocellular carcinoma. Dig Liver Dis; 45: 25