ORIGINAL ARTICLE

Potential prognostic value of leptin receptor in hepatocellular carcinoma

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Background: Obesity is associated with several human malignancies, including hepatocellular carcinoma (HCC). This association may result from the deregulated expression of adipokines.

Aims: To explore the potential role and the prognostic value of leptin receptor (Ob-R) in HCC.

Methods: 66 patients with pathologically confirmed HCC were included in this study. Immunohistochemistry was used to evaluate the expression of Ob-R, microvessel density (MVD) and Ki-67 index in these patients. Eventually, the profiles of Ob-R expression, obtained by a semiquantitative scoring system, were further correlated with Ki-67 expression, intratumour MVD, clinicopathological characteristics and overall survival.

Results: High Ob-R expression was seen in 53% of patients with HCC and was significantly correlated with intratumour MVD (high v low; 59.4 $\pm$ 3.2 v 44.7 $\pm$ 3.7; $p=0.004$), but not with Ki-67 expression. In addition, Ob-R expression was inversely correlated with vascular invasion ($p=0.037$), but not with other known clinicopathological characteristics. The Kaplan–Meier survival curve showed that high Ob-R expression was associated with a better overall survival ($p=0.027$). Meanwhile, multivariate analysis showed that Ob-R expression was a significant determinant for HCC (odds ratio 0.02, 95% confidence interval 0.01 to 0.85; $p=0.041$).

Conclusion: Ob-R expression may have a potential role in the carcinogenesis of HCC. The positive association of Ob-R expression in the cancerous lesions of HCC with the survival outcome can be explained by its inverse correlation with vascular invasion, and may have prognostic value in HCC.

The association of obesity with the risk of gastrointestinal cancers, including hepatocellular carcinoma (HCC), may result from the deregulated expression of adipokines.^{1–8} Prior studies have shown that aberrant serum leptin levels are correlated with many features of hepatocarcinogenesis,^{9–10} and imply that the deregulated expression of adipokines, such as leptin, may contribute to the development of HCC, directly or indirectly. It should be noted, however, that the effects of circulating leptin, which is secreted in either an autocrine or a paracrine manner, depend on its binding to the leptin receptor (Ob-R).¹¹ To date, the potential role of Ob-R in HCC is mostly undetermined.

Ob-R is originally identified in the brain, which explains the negative feedback loop of leptin.¹² It is also found in other tissues, including the colon, pancreas, liver and stomach.^{13–16} In addition, enhanced Ob-R expression is increasingly identified in the malignant cells of breast, lung and gastric carcinomas, and in leukaemic cells.^{17–20} Enhanced Ob-R expression is presumed to have an oncogenic role in human cancers. A recent report, however, shows that Ob-R expression is unexpectedly reduced in the cancer lesions of HCC.²¹ Although the authors do not provide any clinical information, the report implies that the potential role of Ob-R in HCC may differ from that in other human cancers.²¹ Furthermore, the role of Ob-R in the angiogenesis, proliferation and invasion behaviour of human cancer cells²² may also differ from tissue types *in vitro* and *in vivo*.²³

In this study, we characterised the expression patterns of Ob-R in the paraffin-wax-embedded specimens of 66 patients with HCC by immunohistochemistry, and delineated the possible role and prognostic value of Ob-R in HCC by analysing its association with Ki-67 expression, microvessel density (MVD), clinicopathological characteristics and overall survival.

MATERIALS AND METHODS

Subjects

In all, 66 patients (50 men and 16 women; mean age 57.2 (1.5), range 30–82 years) with HCC, who had undergone curative hepatectomy between January 1995 and December 2003, at the Department of Surgery, Kaohsiung Medical University Hospital, were enrolled in the study. Patients who had a previous hepatectomy or a history of trans-arterial chemoembolisation were excluded. None had any preoperative treatment. The study was conducted with approval from the ethics committee of our institute. Informed consent was obtained from each patient.

The patients were strictly followed up for a mean period of 32.1 months (SD 2.79, range 0.5–111.6 months). The pathological diagnosis and classification of variables were based on the criteria recommended in the General Rules for Clinical and Pathological Study of Primary Liver Cancer.²⁴ Clinicopathological parameters analysed included sex, age, liver pathology (hepatitis and cirrhosis), tumour size (<5 cm v ≥ 5 cm), tumour differentiation (well differentiated v moderately or poorly differentiated), tumour stage, vascular invasion (including vascular invasion or tumour thrombi in the portal or hepatic vein), intrahepatic metastasis (negative v positive) and serum α fetoprotein levels.

Immunohistochemistry

Sections 3 μ m thick were cut from representative tissue blocks, deparaffinised by rinsing with xylene, and rehydrated in distilled water through graded alcohol. Antigen retrieval was enhanced by autoclaving slides in sodium citrate buffer (pH 6.0) for 30 min. Endogenous peroxidase activity was

Abbreviations: HCC, hepatocellular carcinoma; Ob-R, leptin receptor; MVD, microvessel density

Figure 1 Scoring systems for leptin receptor (Ob-R) (A–D) and Ki-67 (F–I) immunostaining. The immunostaining results for Ob-R were evaluated by dividing the staining reactions into 4 scores based on the intensity: score 4, strong intensity of cytoplasmic Ob-R staining (A); score 3, moderate intensity of cytoplasmic Ob-R staining (B); score 2, mild intensity of cytoplasmic Ob-R staining (C); and score 1, absent or minimal intensity of cytoplasmic Ob-R staining (D). On the other hand, the Ki-67 immunostaining results were also divided into 4 scores based on the percentage of positive cells: score 4, $\geq 76\%$ positive cells (F); score 3, 51–75% positive cells (G); score 2, 26–50% positive cells (H); and score 1, $\leq 25\%$ positive cells (I). Negative controls that substituted the primary antibodies with the immunoglobulin fraction of non-immune rabbit (for Ob-R) and non-immune mouse serum (for Ki-67) in hepatocellular carcinoma are shown in panels (E) and (J), respectively (original magnification $\times 400$).

quenched by 30 min of incubation in a 0.3% hydrogen peroxide–methanol buffer. The slides were then incubated overnight at 4°C in humid chambers with primary rabbit polyclonal anti-Ob-R antibody at a dilution of 1:100 (M-18; Santa Cruz Biotechnology, Santa Cruz, California, USA).^{16–18, 25}

The slides were washed thrice in phosphate buffer solution and further incubated with a biotinylated secondary antibody for 30 min at room temperature. Antigen–antibody complexes were detected by the avidin–biotin–peroxidase method with diaminobenzidine as a chromogenic substrate (Dako, California, USA). Finally, the slides were counterstained with haematoxylin and then examined by light microscopy. Ki-67 expression was assessed using a purified antihuman monoclonal antibody (1:75, Dako).

Evaluation of Ob-R, CD31 and Ki-67 immunohistochemical staining

Positive staining for Ob-R was observed predominantly in the membrane and cytoplasm of both cancerous and

non-cancerous liver cells. The intensity of immunoreactive cancer cells for Ob-R was graded by a four-tiered semiquantitative system: score 1, absent or minimal immunostaining; score 2, mild immunostaining; score 3, moderate immunostaining; and score 4, strong immunostaining (fig 1A–D). The percentage of immunoreactive cancer cells for Ki-67 was also graded by a four-tiered semiquantitative system: score 1, $\leq 25\%$ positive cells; score 2, 26–50% positive cells; score 3, 51–75% positive cells; and score 4, $\geq 76\%$ positive cells (fig 1F–I). Notably, more than 1000 cells expressed in 3–4 different high-power field ($\times 200$) areas were analysed for each section. Sections of breast cancer tissue were used as positive controls for Ob-R immunostaining,¹⁶ whereas sections of tonsil tissues that were known to be positive for Ki-67 expression were included as positive controls for Ki-67. Negative controls were obtained by substituting the primary antibody with the immunoglobulin fraction of non-immune rabbit serum (for Ob-R) and non-immune mouse serum (for Ki-67; fig 1E,J).

MVD, assessed by immunoreactive CD31, is determined according to the method of Weidner.²⁶ Briefly, the

Figure 2 Leptin receptor (Ob-R; A and C) and CD31 (B and D) immunostaining. The cancer cells with high Ob-R expression (A) showed an increased density of microvessels (B), whereas the cancer cells with low Ob-R expression (C) showed a decreased density of microvessels (D).

Figure 3 Overall survival of cases of hepatocellular carcinoma between high and low leptin receptor (Ob-R) expression subgroups.

Table 1 Expression of leptin receptor (Ob-R) and Ki-67 in cases of hepatocellular carcinoma

Variable	S1 (0–25%)	S2 (26–50%)	S3 (51–75%)	S4 (76–100%)
Ob-R	16/66	15/66	19/66	16/66
Ki-67	35/66	16/66	11/66	4/66

S1, score 1; S2, score 2; S3, score 3; S4, score 4.

immunostained sections are scanned at low magnification ($\times 40$) and the tumour area with the highest density of distinctly highlighted microvessels ("hot spot") is selected. MVD is then determined in the hot spot by counting all vessels at a total magnification of $\times 200$. All the values were presented as mean $\pm$ SEM. The immunostaining for Ob-R and Ki-67, as well as MVD, was determined separately for each specimen and estimated by two independent pathologists. The rare cases with discordant scores were re-evaluated and scored on the basis of a consensus opinion.

Statistical analysis

Contingency table methods were used to analyse the correlation between Ob-R and other variables. For Ob-R and Ki-67 expression, score 1 plus score 2 and score 3 plus score 4 were categorised as high and low expressions, respectively. An MVD of 52.5 Vessels/mm² (overall mean value) was used as baseline to categorise the total cases of HCC into high (≥ 52.5) and low (< 52.5) MVD subgroups. For staging and dimensions, stage I–II and tumour size > 5 cm were used as baseline, respectively. Significance was evaluated by analysis of variance and χ^2 test or Fisher's exact test. Survival curves were calculated by the Kaplan–Meier method, and significance was evaluated by a log rank test. Multivariate odds ratio was calculated using Cox proportional hazards regression. All the calculations were carried out using the SPSS V.10.0 statistical software package. Results were considered significant at $p < 0.05$.

RESULTS

Expression profiles of Ob-R, Ki-67 and MVD in HCC

Table 1 summarises the expressions of Ob-R and Ki-67 evaluated in 66 cases of HCC. The expression of Ob-R showed a heterogeneous pattern between normal and cancer cells in terms of intensity of positive cells. A strong membranous or cytoplasmic immunostaining of Ob-R was seen in 24.2% of the cancerous lesions of HCC (fig 1A). On the other hand, the localisation of the Ki-67 protein was predominantly stained in the nucleus in HCC lesions. Intriguingly, a strong nuclear immunostaining was seen only in 6.1% cases of HCC (fig 1F). Most cases of HCC (35/66, 53%) showed very low Ki-67 expression (fig 1F). The mean value of MVD, assessed by immunoreactive CD31, of the total cases of HCC was 52.50 Vessels/mm² (2.57; range 15.0–104.67).

Correlation of Ob-R with Ki-67, MVD and clinicopathological characteristics

To explore the potential role of Ob-R in HCC, Ob-R expression was further correlated with Ki-67 expression and MVD (table 2) and with clinicopathological characteristics (table 3). Notably, the mean values of high and low MVD subgroups were 36.03 (1.92) and 70.01 (2.31), respectively. Ob-R expression was positively correlated with the value of MVD (table 2; $p = 0.015$; fig 2), but not with Ki-67 expression (table 2). In addition, the mean value of MVD of high Ob-R expression was significantly higher than that of low Ob-R expression (59.4 (3.2) ν 44.7 (3.7); $p = 0.004$), and Ob-R expression was correlated with the MVD subgroup (table 3; $p = 0.027$) but not with the Ki-67 subgroup (table 3).

Ob-R expression was inversely correlated with vascular invasion (table 3; $p = 0.037$), but not with liver cirrhosis, serum α -fetoprotein level, tumour stage, tumour grade, tumour size, intrahepatic metastasis or recurrence (table 3). Besides, no significant correlation of Ob-R expression was found among sex, age, viral hepatitis, capsular invasion, liver fibrosis and fatty liver (data not shown).

Ob-R as a prognostic parameter for HCC

The Kaplan–Meier survival curves showed a significant relationship between Ob-R expression and the overall survival of HCC (fig 3; $p = 0.027$). On the other hand, we found that Ob-R expression was also a predictor for the overall survival of HCC (OR 0.02, 95% CI 0.01 to 0.85; $p = 0.041$; table 4).

DISCUSSION

Recently, there have been several reports on the association of circulating leptin with HCC.^{9–10} The expression of Ob-R in HCC has, however, not been fully investigated. In accordance with Wang *et al.*,²¹ we found that $> 80\%$ of the adjacent non-cancerous parts of HCC presented enhanced membranous or cytoplasmic immunostaining of Ob-R (data not shown). In addition, the strong expression of Ob-R was seen in only 24.2% of HCC in our study (table 1). These results imply that reduced Ob-R expression in liver tissues may be associated with the carcinogenesis of human HCC. Results from our study that different Ob-R expression in the lesions of HCC was markedly associated with overall survival seemingly continued to support this hypothesis (fig 3).

Furthermore, we noticed that enhanced Ob-R expression was significantly associated with negative vascular invasion (table 3; $p = 0.037$). Little is known about the exact role of Ob-R in the vascular invasion of HCC, but this finding may provide the possible explanation as to how enhanced Ob-R expression could have better overall survival of HCC in our study. Vascular invasion is regarded as the most important risk factor of recurrence after resection of HCC. In fact, we also observed that patients with high Ob-R expression

Table 2 Correlation of leptin receptor expression with microvessel density and Ki-67 expression in cases of hepatocellular carcinoma

Variable	MVD, mean (SEM)	p Value*	KS1	KS2	KS3	KS4	p Value†
Ob-R		0.015					0.194
OS1	39.77 (4.09)		11	1	2	2	
OS2	49.96 (6.17)		10	3	2	0	
OS3	59.40 (4.10)		7	5	5	2	
OS4	59.44 (5.13)		7	7	2	0	

KS1, score 1 ($\leq 25\%$ positive cells); KS2, score 2 (26–50% positive cells); KS3, score 3 (51–75% positive cells); KS4, score 4 ($\geq 76\%$ positive cells); MVD, microvessel density; Ob-R, leptin receptor; OS1, score 1 (absent or minimal immunostaining); OS2, score 2 (mild immunostaining); OS3, score 3 (moderate immunostaining); OS4, score 4 (strong immunostaining).

*Analysis of variance; † χ^2 test.

Table 3 Correlation of leptin receptor expression with clinicopathological characteristics of cases of hepatocellular carcinoma (n=66)

Variables*	n	Ob-R expression		p Value†
		Low	High	
Liver cirrhosis				0.396
Negative	34	18	16	0.396
Positive	25	16	9	
Vascular invasion				0.037
Negative	45	22	23	0.037
Positive	21	16	5	
Intrahepatic metastasis				0.186
Negative	48	30	18	0.186
Positive	18	8	10	
Recurrence				0.149
Negative	33	16	17	0.149
Positive	22	15	7	
αFetoprotein (ng/ml)				0.314
<200	29	24	15	0.314
≥200	27	8	19	
Tumour stage				0.748
I/II	47	27	20	0.748
III/IV	17	9	8	
Tumour grade				0.824
High	11	6	5	0.824
Moderate/poor	55	32	23	
Tumour size (cm)				0.181
<5	37	19	18	0.181
≥5	28	19	9	
MVD				0.027
Low (<52.5)	34	24	10	0.027
High (≥52.5)	42	14	28	
Ki-67				0.160
Low (KS1+KS2)	51	27	24	0.160
High (KS3+KS4)	15	11	4	

KS1, score 1 (<25% positive cells); KS2 (score 2, 26–50% positive cells); KS3, score 3 (51–75% positive cells); KS4, score 4 (≥76% positive cells); MVD, microvessel density; Ob-R, leptin receptor.

*Undetermined in small cases; † χ^2 test.

showed a lower percentage of recurrence (7/22) than those with low Ob-R expression (table 3).

In addition, Ob-R expression was positively and significantly correlated with MVD, but not with Ki-67 expression in our study (tables 2 and 3). This result points to the possibility that Ob-R, in which leptin exerts its effects, may have a role in the angiogenesis, but not in the proliferation, of HCC. The above findings are in accordance with an in vitro study, which shows that leptin has no effect on the growth of liver cancer cell line expressing Ob-R.²¹

Recent studies suggest that increased MVD is positively associated with HCC progression.^{27–30} However, we did not observe the significant correlation of MVD with the overall survival of HCC in this study ($p = 0.754$). This may have

resulted from the use of antibody against CD31 in this study to evaluate intratumour MVD, because we found that the MVD values of cancer lesions and adjacent normal liver tissues were positively correlated ($p < 0.001$). Thus, this phenomenon hints that the prognostic value of CD31 may be used merely as a marker of vascular changes in the liver. On the other hand, the findings from our study that Ob-R expression was positively associated with MVD but was inversely correlated with vascular invasion (table 3) seemed confusing. Nevertheless, it should be noted that no available data have established the correlation of MVD with vascular invasion. Also, when we set Ob-R expression as a factor in the Kaplan–Meier curves of the low and high MVD subgroups, high Ob-R expression remained significantly

Table 4 Cox regression analysis of cases of hepatocellular carcinoma

Characteristics	OR (95% CI)	p Value
Ob-R expression (high/low)*	0.02 (0.01 to 0.85)	0.041
MVD (<52.5)	4.57 (0.69 to 30.16)	0.114
Ki-67 expression (high/low)	1.64 (0.49 to 5.49)	0.426
Sex (male/female)	0.85 (0.06 to 12.36)	0.903
Age (<65 years)	2.12 (0.28 to 17.98)	0.496
Liver cirrhosis*	34.14 (2.75 to 423.31)	0.006
Virus hepatitis	0.43 (0.06 to 2.94)	0.387
αFetoprotein (<200 ng/ml)	1.57 (0.02 to 112.80)	0.836
Tumour stage*	9.27 (1.44 to 59.57)	0.019
Tumour grade*	11.26 (1.05 to 120.35)	0.045
Tumour size (<5 cm)	0.25 (0.5 to 1.21)	0.084
Vascular invasion	0.53 (0.05 to 5.72)	0.603
Capsular invasion	0.78 (0.12 to 4.89)	0.788
Intrahepatic metastasis	1.67 (0.05 to 54.956)	0.775
Recurrence	6.97 (0.32 to 153.81)	0.219

MVD, microvessel density; Ob-R, leptin receptor.

* $p < 0.05$.

associated with a better overall survival in the high MVD subgroup ($p = 0.017$). Further investigations are still needed to explore the possible effects of Ob-R in angiogenesis and metastasis of HCC, such as VEGF expression, which is a biomarker for angiogenesis and metastasis.

In conclusion, we show for the first time that Ob-R expression may have a potential role in the carcinogenesis of HCC. Also, the positive association of Ob-R expression in the cancerous lesions of HCC with the survival outcome may result from its inverse correlation with vascular invasion. Further investigations are required to explore the possible mechanisms of Ob-R associated with the development of HCC.

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