ORIGINAL ARTICLE

Potential role of leptin expression in hepatocellular carcinoma

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Background: Obesity is associated with hepatocellular carcinoma (HCC). The association may result from the aberrant expression of adipokines.

Aim: To explore the potential biological effect and prognostic value of leptin, one of the adipokines, in HCC.

Methods: Immunohistochemistry was used to evaluate the expression of leptin in 68 patients with HCC. The expression of Ki-67 and microvessel density (MVD) of tumorous lesions in HCC were also analysed. The result of leptin expression was further correlated with Ki-67 expression, intratumour MVD, clinicopathological characteristics, overall survival and the postoperative use of medroxyprogesterone acetate (MPA).

Results: High leptin expression was seen in 60.3% of patients with HCC and was significantly correlated with intratumour MVD (high v low; 59.2 (standard deviation 3.2) v 44.2 (19.5), $p=0.004$), but not with Ki-67 expression. No marked correlation was seen between leptin expression and clinicopathological characteristics. However, using a multivariate Cox's proportional hazards model, leptin expression was a predictor for improved overall survival of patients with HCC (odds ratio 0.16; 95% confidence interval 0.03 to 0.87; $p=0.033$). In addition, the Kaplan–Meier survival curve showed that high leptin expression was associated with a better survival in patients with HCC, treated postoperatively with MPA ($p=0.008$, log rank test).

Conclusion: High leptin expression was associated with an increased intratumour MVD and thus may be associated with HCC development. In addition, high leptin expression was a predictor for improved survival of patients with HCC, treated postoperatively with MPA.

Hepatocellular carcinoma (HCC) is the fifth most common malignancy in the world and causes roughly half a million deaths annually.¹ In addition to established risk factors (eg, hepatitis B and C), accumulated epidemiological studies have found an increased risk (1.5–4-fold) of HCC among obese people.^{2–4} However, the underlying mechanisms that link obesity to various types of human cancers, including HCC, are poorly understood. One possible hypothesis is deregulated adipokines (eg, leptin), which may contribute to tumorigenesis and to the metastatic potential of human cancers.^{5–7}

Leptin, a product of the obese gene, is mainly produced by adipose tissue. Leptin binds to the leptin receptor and exerts its effects in an autocrine or paracrine manner.⁸ In addition to its potential association with haematopoiesis, thermogenesis, obesity-related diseases and polycystic ovary syndrome,⁹ some authors have suggested that leptin is also associated with the initiation and progression of human cancers.^{10–15} In addition, it has been shown that leptin has a potential role in the development of HCC.¹⁶ However, the biological and clinical roles that leptin has in HCC are still undetermined.

Although advances in early diagnosis and surgical techniques have improved survival in patients with HCC, long-term survival after surgical resection is still unsatisfactory.¹⁷ Adjuvant therapies such as radiotherapy, chemotherapy and immunotherapy have been attempted, but have not effectively prolonged survival in most patients with HCC.¹⁸ Some clinical studies reported that the use of medroxyprogesterone acetate (MPA) can improve patient survival,^{19–20} but this conclusion was refuted by other studies.²¹ These differences may result from the different patient groups studied. Thus, to select patients who may benefit from postoperative MPA after HCC resection, it is important to define the biological and clinical markers that predict a good response to treatment with MPA.

In this study, we evaluated the expression patterns of leptin in 68 HCC specimens by immunohistochemistry. We also investigated the possible effects of leptin in the progression of HCC by analysing its association with microvessel density (MVD) and Ki-67, and verified its potential prognostic value by analysing its association with clinicopathological characteristics (including the postoperative use of MPA) and overall survival.

PARTICIPANTS AND METHODS

Patients

Between December 1994 and December 2003, 68 naive patients (51 men and 17 women; mean age 57 (standard deviation (SD) 1.5, range 30–82) years) with HCC who underwent hepatectomy at the Department of Surgery, Kaohsiung Medical University Hospital, Kaohsiung, Taiwan, were enrolled in the study. Patients who had undergone previous hepatectomy or transarterial chemoembolisation were excluded. None of them had undergone any preoperative treatment.

The patients were strictly followed up for a mean of 31.7 (SD 2.75, range 0.4–111.6) 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.²² Clinicopathological parameters analysed included sex, age, liver cirrhosis, tumour size (<5 v ≥ 5 cm), serum α -fetoprotein levels (<200 v ≥ 200 ng/ml), tumour grade, tumour stage, vascular invasion (including vascular invasion or tumour thrombi in the portal or hepatic vein), capsular invasion and postoperative MPA usage. Notably, from the

Abbreviations: HCC, hepatocellular carcinoma; MPA, medroxyprogesterone acetate; MVD, microvessel density; SEM, standard error of mean

Figure 1 Scoring systems for leptin (A–D) and Ki-67 (F–I) immunostaining in hepatocellular carcinoma (HCC). The immunostaining results for leptin were evaluated with four scores based on the staining intensity: (A) +++, strong cytoplasmic staining; (B) ++, moderate cytoplasmic staining; (C) +, weak cytoplasmic staining; and (D) –, negative cytoplasmic staining. Nuclear Ki-67 immunostaining was also divided into four scores based on the percentage of positive cells: (F) Score 4, $\geq 76\%$ positive cells; (G) Score 3, 51–75% positive cells; (H) Score 2, 26–50% positive cells; and (I) Score 1, $\leq 25\%$ positive cells. Negative controls that substituted the primary antibodies for the immunoglobulin fraction of non-immune rabbit (for leptin) and non-immune mouse serum (for Ki-67) in HCC are shown in panels (E) and (J) ($\times 400$).

available medical records (n = 51), 22 patients with HCC received postoperative treatment with MPA at a daily dose of 500 mg orally for 6 months, whereas 29 patients did not.

Immunohistochemistry

Sections, 3 μ m thick, from representative tissue blocks were cut, deparaffinised with xylene rinse and rehydrated with distilled water through graded alcohol. Antigen retrieval was enhanced by autoclaving slides in 10 mM sodium citrate buffer (pH 6.0) for 30 min. Endogenous peroxidase activity was quenched by incubation in 0.3% hydrogen peroxide–methanol buffer for 30 min. The slides were then incubated overnight at 4°C in humidified chambers with primary rabbit polyclonal anti-leptin antibody at a dilution of 1:100 (A-20; Santa Cruz Biotechnology, Santa Cruz, California, USA).^{13 23 24}

The slides were washed three times in a phosphate-buffered 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, using diaminobenzidine as a chromogenic substrate (DAKO, Carpinteria, California, USA). Finally, the slides were counterstained with haematoxylin and then examined by light microscopy. Ki-67 expression was assessed using purified anti-human

monoclonal antibody to Ki-67 (1:75; DAKO), and intratumour MVD was assessed using purified anti-human monoclonal antibody to CD31 (1:30; DAKO).

Evaluation of leptin, CD31 and Ki-67 immunohistochemical staining

Leptin was predominantly located in the cytoplasmic portions of both tumorous and non-tumorous liver tissue. The intensity of leptin immunostaining in cancer tissue was graded by a semiquantitative system: –, negative; +, weak; ++, moderate; +++, strong (fig 1A–D). The percentage of cancer cells immunoreactive for Ki-67 was also scored with a semiquantitative system: S1, $\leq 25\%$ positive cells; S2, 26–50% positive cells; S3, 51–75% positive cells; S4, $\geq 76\%$ positive cells (fig 1F–I). More than 1000 cells expressed in 3–4 different high-power fields ($\times 200$) were analysed for each section. Sections of breast cancer tissue were used as positive controls for leptin immunostaining,¹³ whereas sections of tonsil tissue 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 leptin) and non-immune mouse serum (for Ki-67; fig 1E,J).

Table 1 Expression profiles of leptin and Ki-67 in hepatocellular carcinoma cases (n = 68)

Variable	– or S1	+ or S2	++ or S3	+++ or S4
Leptin (N)	4/62	10/62	25/62	23/62
Leptin (T)	6/68	21/68	24/68	17/68
Ki-67 (T)	37/68	16/68	11/68	4/68

N, the adjacent non-tumorous liver tissue; T, tumorous lesion.
Immunostaining for leptin: –, negative; +, weak; ++, moderate; +++, strong.
Percentage of cancer cells immunoreactive for Ki-67: S1, $\leq 25\%$ positive cells; S2, 26–50% positive cells; S3, 51–75% positive cells; S4, $\geq 76\%$ positive cells.

Table 2 Correlation of leptin expression with MVD and Ki-67 expression in patients with hepatocellular carcinoma (n = 68)

Variable	MVD (Mean (SEM))	p Value*	Ki-67 expression				p Value†
			S1	S2	S3	S4	
Leptin		0.040					0.790
–	45.06 (21.16)		5	0	1	0	
+	44.00 (19.61)		10	6	3	2	
++	59.63 (18.45)		11	7	5	1	
+++	58.65 (23.68)		11	3	2	1	

MVD, microvessel density; S1, ≤25% positive cells; S2, 26–50% positive cells; S3, 51–75% positive cells; S4, ≥75% positive cells; –, negative; +, weak; ++, moderate; +++, strong.

*By analysis of variance.

†By χ^2 test.

Intratumour MVD assessed by CD31 staining was determined using the methods proposed by Weidner.²³ Briefly, the immunostained sections were scanned at low magnification ($\times 40$), and the tumour area with the highest density of distinctly highlighted microvessels (hot spot) was selected. MVD was then determined in the hot spot by counting all vessels at a total magnification of $\times 200$. All values were presented as mean (standard error of mean (SEM)). The immunostaining for leptin and Ki-67, as well as for MVD, was determined separately for each specimen, 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 leptin and other variables. To assess leptin expression, negative (–) and weak (+) were categorised as low expression, and moderate (++) and strong (+++) as high. To assess Ki-67 expression, S1 and S2 were categorised as high expression, and S3 and S4 as low. The overall mean value of MVD = 53.3 was used as the cut-off value to divide HCC cases into subgroups with high (≥ 53.3) and low (< 53.3) MVD. Thus, the mean values for the high and low MVD groups were 72.2 (SEM 2.3) and 37.4 (SEM 1.9), respectively. For stage and tumour dimensions, stages I and II and tumour size < 5 cm were used as baselines. Significance was evaluated by analysis of variance, χ^2 test and Fisher's exact test. Survival curves were calculated using the Kaplan–Meier method, and significance was analysed by the log rank test. Multivariate odds ratio (OR) was calculated using Cox's proportional hazards regression. Data were analysed using the SPSS V.10.0 statistical software package. Results were considered significant at $p < 0.05$.

RESULTS

Expression profiles of leptin, Ki-67 and MVD in HCC

Table 1 summarises the expressions of leptin and Ki-67 in 68 patients with HCC. Of the 68 tumorous lesions, 17 showed strong (+++; fig 1A), 24 moderate (++; fig 1B) and 21 weak (++; fig 1C) cytoplasmic immunostaining for leptin, whereas 6 showed negative immunostaining (–; fig 1D). On the other hand, in the non-tumorous liver tissues from 62 available cases of HCC, 23 showed strong, 25 moderate, 10 weak immunostaining, whereas 4 showed negative immunostaining for leptin (table 1).

Ki-67 protein was localised predominantly in the nucleus in HCC cells. Most HCC tissues showed very low Ki-67 expression (S1; fig 1I). Of the 68 HCC cases, 16 showed low Ki-67 expression (S2; fig 1H), 11 showed moderate (S3; fig 1G) and 4 showed strong Ki-67 expression (S4; fig 1F). The mean value of MVD in HCC tissues was 53.3 (SEM 2.6, range 15–104.67).

Table 3 Correlation of leptin expression with clinicopathological characteristics of patients with hepatocellular carcinoma (n = 68)

Variables	n	Leptin		p Value*
		Low	High	
Liver cirrhosis				0.085
Negative	39	12	27	
Positive	27	14	13	
Vascular invasion				0.230
Negative	46	16	30	
Positive	22	11	11	
Capsular invasion				0.110
Negative	48	22	26	
Positive	20	5	15	
α -fetoprotein (ng/ml)				0.273
< 200	42	17	15	
≥ 200	16	4	12	
Tumour size (cm)				0.457
< 5	39	14	25	
≥ 5	29	13	16	
Tumour grade				0.361
I	12	4	8	
II	48	18	30	
III	8	5	3	
Tumour stage				0.298
I or II	50	18	32	
III or IV	18	9	9	
MVD				0.032
Low	37	19	18	
High	31	8	23	
Ki-67				0.979
Low (S1 or S2)	53	21	32	
High (S3 or S4)	15	6	9	

MVD, microvessel density.

S1, ≤25% positive cells; S2, 26–50% positive cells; S3, 51–75% positive cells; S4, ≥76% positive cells.

*By χ^2 test.

Correlation of leptin with Ki-67, MVD and clinicopathological characteristics in patients with HCC

To explore the potential role of leptin in HCC, the expression profile of tumorous leptin was correlated with Ki-67 expression and MVD (table 2) and with clinicopathological characteristics (table 3).

Leptin expression was significantly and positively correlated with MVD ($p = 0.04$; table 2), and a significant difference in MVD was seen between the groups with high and low leptin expression (59.2 (3.2) v 44.2 (19.5); table 3). However, we found no marked correlation between leptin and Ki-67 expression in HCC tissue (tables 2, 3). Moreover, leptin expression was not correlated with liver cirrhosis, vascular invasion, capsular invasion, serum levels of α -fetoprotein, tumour size, stage and grade (table 3). We also found no marked correlation between leptin expression in

Table 4 Cox's regression analysis of patients with hepatocellular carcinoma

Characteristics	OR (95% CI)	p Value
Expresses leptin (high or low)*	0.16 (0.03 to 0.87)	0.033
MVD (<53.3)	0.74 (0.14 to 3.97)	0.727
Expresses Ki-67 (high or low)	0.45 (0.11 to 1.82)	0.266
Sex	1.35 (0.30 to 6.08)	0.694
Age (<65 years)	1.80 (1.40 to 8.03)	0.443
Liver cirrhosis	0.34 (0.03 to 3.39)	0.357
Tumour stage*	12.38 (1.28 to 119.88)	0.030
Tumour grade	5.37 (0.72 to 40.25)	0.102
Tumour size (<5 cm)	2.81 (0.44 to 17.95)	0.275
Vascular invasion*	40.1 (3.52 to 551.26)	0.003
Capsular invasion	1.02 (0.14 to 7.24)	0.985
Treatment with MPA*	0.02 (0.002 to 0.31)	0.004

MPA, medroxyprogesterone acetate; MVD, microvessel density.

*p<0.05.

patients with HCC and sex, age, viral hepatitis type and fatty change (data not shown).

Leptin expression as a prognostic parameter in patients with HCC

When a multivariate Cox's proportional hazards model was used, we found leptin expression to be a significant predictor of overall survival of patients with HCC (OR 0.16; 95% CI 0.03 to 0.87; $p = 0.033$; table 4). Notably, tumour stage, vascular invasion and treatment with MPA were also found to be robust predictors of overall survival of patients with HCC (table 4).

Neither expression of leptin nor postoperative treatment with MPA led to a significant difference in overall cumulative survival of patients with HCC ($p = 0.383$ and 0.171 , log rank test; fig 2A,B). In addition, we also found no considerable

correlation between MVD and Ki-67 expression (data not shown). However, we found that in patients with HCC treated with postoperative MPA, overall cumulative survival was better in the high leptin expression group than in the low leptin expression group ($p = 0.008$, log rank test; fig 2C,D).

DISCUSSION

Accumulated studies have suggested that leptin may be associated with the initiation and progression of human cancers, but the controversy about its role as an inhibitor or promoter remains unresolved. Recent data show that circulating levels of leptin are higher in cases of breast cancer²⁶ and colorectal cancer,²⁷ and are lower in cases of uterine leiomyomas.²⁸ Even in the same cancer type (eg, prostate cancer), discordance exists among studies from different groups.²⁹⁻³⁰ Thus, leptin may have distinct effects on human cancer cell development depending on tissue type.

Recent studies show that circulating leptin levels are considerably higher in patients with HCC than in healthy controls.³¹ However, immunohistochemical studies show that leptin expression is increased in adjacent non-cancerous liver tissues, but is markedly decreased in the cancerous lesions.¹⁶ Consistent with the observations of Wang *et al*,¹⁶ we also found that most adjacent non-tumorous liver tissues showed an increased leptin expression in patients with HCC (77.4%, with moderate to strong staining; table 1). In addition, the expression of leptin in non-tumorous liver tissue was significantly correlated with portal inflammation ($p = 0.026$), but not with MVD, Ki-67 and clinicopathological characteristics. This result may be consistent with the role of leptin as a proinflammatory cytokine.³² On the other hand, although no noticeable correlation was found between tumorous leptin expression and known clinicopathological characteristics, tumorous leptin expression was associated with intratumour MVD and overall survival of patients with

Figure 2 Actuarial analysis of overall survival was used (A) in 68 patients with hepatocellular carcinoma (HCC) with high ($n = 41$) and low ($n = 27$) leptin expression ($p = 0.383$) and (B) in 51 patients with ($n = 22$) and without ($n = 29$) postoperative treatment with medroxyprogesterone acetate (MPA; $p = 0.171$). Actuarial analysis of overall survival in 51 patients with HCC with high and low leptin expression showed a significant difference in the subgroup (C) without postoperative treatment with MPA ($n = 29$; $p = 0.319$) (D) postoperatively treated with MPA ($n = 22$; $p = 0.008$), but not in the subgroup.

HCC in our study (tables 2–4). Wang *et al*¹⁶ have also shown that leptin expression is higher in the vascular endothelial cells of HCC, suggesting the association of leptin in angiogenesis of cancer tissues. Taken together, we believed that aberrant leptin expression in HCC affected the development of HCC, possibly through its angiogenic effects. However, the Kaplan–Meier survival curve implied that leptin expression alone may not have an important role in HCC (fig 2A).

Tumour progression entails the combined ability of cancer cells to proliferate and to sustain angiogenesis uncontrollably. In our study, leptin expression was not markedly correlated with Ki-67 expression or HCC tumour size (tables 2, 3). Thus, it may be assumed that leptin may not have a role in the proliferation of HCC. This finding is also consistent with an *in vitro* study showing that leptin has little effect on the growth of HCC cells.¹⁶ On the other hand, our results showed that leptin expression positively and significantly correlated with the MVD of the tumorous lesions in HCC (tables 2, 3). Therefore, our results suggested that leptin may be associated with angiogenesis, but not with the proliferation of HCC cells. Further investigation is needed to explore whether the considerable correlation between leptin and angiogenesis in HCC contributes to its potential participation in HCC development.

Several adjuvant treatments (eg, chemotherapy and radiotherapy) have been attempted to improve the survival of patients with HCC, but the results are generally frustrating. Although some clinical studies^{19–20} show that megestrol acetate, a synthetic progesterone analogue, has an inhibitory effect on advanced HCC and the growth of HepG2 cells grown *in vitro* and *in vivo*,³³ these findings are not supported by other studies.²¹ In our study, we found that the use of MPA after hepatectomy was a strong predictor of overall survival of patients with HCC (table 4, $p = 0.004$), although the use of MPA was imperfectly associated with a better overall survival of patients with HCC (fig 2B; $p = 0.171$). In addition, we noticed that in patients treated postoperatively with MPA, those with high leptin expression had a remarkably better survival than those with low leptin expression (fig 2C; $p = 0.008$). Thus, our finding indicated that leptin expression may intensify the curative effect of MPA in patients with HCC and may serve as a predictor for response to treatment with MPA. Nevertheless, this finding requires further investigation.

In conclusion, we suggest that leptin may be associated with the development of HCC. In addition, the versatile roles of leptin in the sequential events of HCC progression (including angiogenesis) and the response of HCC to postoperative hormone treatment deserve further investigation.

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