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Association between Osteopontin and EGFR Expression with Clinicopathological Parameters in Hepatocellular Carcinoma

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Abstract

Osteopontin (OPN) and epidermal growth factor receptor (EGFR) are important factors associated with tumor progression, invasion and metastasis in humans. The aim of this study was to assess the correlation of OPN and EGFR expression with hepatocellular carcinoma (HCC) progression. Expression of OPN and EGFR was assessed by immunohistochemistry in 100 HCC specimens. Immunostaining scores (0 to 400) were calculated from the percentage of cells (0 to 100) at each immunostaining intensity and the immunostaining intensity (0 to 4). The average immunostaining score for OPN was correlated with tumor grade (56.1 for grade I, 104.6 for grade II, and 141.2 for grade III; $P = 0.023$) and T stage (58.6 for stage T1, 85.9 for stage T2, 126.8 for stage T3, and 189.1 for stage T4; $P = 0.029$). Similarly, the average immunostaining score for EGFR was correlated with tumor grade (80.5 for grade I, 142.1 for grade II, 230.6 for grade III; $P = 0.011$) and T stage (96.4 for stage T1, 135.5 for stage T2, 221.3 for stage T3, and 261.4 for stage T4; $P = 0.026$). In addition, OPN and EGFR immunostaining scores were also correlated with M, N, and AJCC stages. In conclusion, higher expression of OPN and EGFR is significantly associated with advanced histological grades, advanced pathological stages and poorer survival rates in HCC. OPN and EGFR may be used as novel biomarkers for diagnosis or monitoring of progression of hepatocellular carcinoma.

Key Words: hepatocellular carcinoma, immunohistochemistry, osteopontin, EGFR

Introduction

Hepatocellular carcinoma (HCC) is the most common histological type of primary liver cancer, accounting for 7.4% and 3.2% of all malignancies in males and females, respectively (29). The incidence varies with geographic area and is more than 30/100,000 in Taiwan and southeast Asia (29). For most patients without obvious symptoms, HCCs are not easily detected before progression to metastatic disease (40). The presence of portal vein thrombosis and TP53 mutation is related to poor prognosis and therapeutic failure in HCC patients (1, 35). Several studies have demonstrated the influence of certain genetic factors, such as p16 protein (14), transforming growth factor-β (TGF-β) (13), vascular endothelial growth factor (VEGF) (24, 38) and hepatocyte growth factor (HGF) (42), on the progression and neovascularization of HCC. In recent research, HCC development has been attributed to signaling pathways

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such as receptor tyrosine kinase, Wnt/β-catenin, ubiquitin-proteasome, epigenetic promoter methylation and histone acetylation, PI3kinase/AKT/mTOR, angiogenesis and telomerase (18, 32). However, no evidence has linked these factors to the clinicopathological staging system and prognosis of HCC.

Osteopontin (OPN), a highly phosphorylated and glycosylated secretory protein, is expressed in different cell types including osteoclasts, arterial smooth muscle cells, macrophages, T lymphocytes and various types of epithelial cells (37). And OPN expression is associated with cell adhesion and migration, inflammatory processes, antiapoptosis, suppression of nitric oxide synthase and bone calcification (9, 15, 17, 31, 34, 45). Additionally, overexpression of OPN can enhance cancer progression, invasion and even metastasis in several human cancers, including hepatocellular (25, 31), breast (41), lung (4), prostatic (39), gastric (12), nasopharyngeal (43), laryngeal and hypopharyngeal (21) and clear-cell renal cell carcinomas (23) and melanoma (22). The ability of tumors to migrate depends on the GRDS domain of OPN which recognizes the cell adhesion sequence αvβ3 integrin (17, 45). In an earlier study, the expression of OPN was linked to poor prognosis, early recurrence and high risk for metastasis in HCC (19, 30). However, evidence showing a correlation between OPN immunostaining and clinicopathological parameters in HCC is lacking.

Epidermal growth factor receptor (EGFR) belongs to the ErbB family of receptor tyrosine kinases and is encoded by the *c-erbB-1* gene in humans (46). Activation of EGFR may play an important role in cell adhesion, proliferation, differentiation, apoptosis and tumor metastasis (8). A previous study showed that EGFR was upregulated in various human malignancies, including cancer of the head and neck, lung, colorectum and prostate (33). Schiffer *et al*. successfully used EGFR inhibitors to prevent the development of HCC in the cirrhotic livers of rats (36). However, the expression profiles of EGFR in human HCC are unclear, especially in the Chinese population.

In the present study, we evaluated OPN and EGFR expression in 100 HCC cases by immunostaining and correlating the immunostaining scores with pathological grade and clinical stage. To our knowledge, this is the first study to address the relationship between the expression of these two biomarkers and various clinicopathological parameters of HCC. Our results demonstrated the association of increased OPN and EGFR immunostaining scores with more advanced stages of HCC.

Materials and Methods

HCC Samples

Table 1. Clinicopathological characteristics of 100 patients with hepatocellular carcinomas

Variables	Numbers
Gender	
Male	69
Female	31
HBV carrier	
Yes	73
No	27
HCV carrier	
Yes	24
No	76
Liver cirrhosis	
Yes	68
No	32
Tumor necrosis	
Yes	57
No	43
Tumor number	
Single	45
Multiple	55
The largest tumor size (cm)	
≤ 5	22
> 5	78
Lymphovascular invasion	
Yes	21
No	79

Paraffin-embedded tumor tissues were collected from the Department of Pathology at Tri-Service General Hospital between 1998 and 2005. The patients were 22-85 years old and the median age was 61 (Table 1). Tissue microarray slides were constructed from 100 specimens of HCC (20 well differentiated [grade I], 47 moderately differentiated [grade II], and 33 poorly differentiated [grade III]) and eight specimens taken from non-tumorous parts of the liver (at least 4 cm from the tumor). The staining of all tissues on the microarray slides was as uniform as the staining of the original paraffin-embedded specimens. The pathological diagnosis of these cases was reviewed by at least two experienced pathologists. All HCC cases were divided into groups based on histological grading and AJCC pathological staging (11).

Immunohistochemistry

Tissue microarray sections were de-waxed in xylene, rehydrated in alcohol, immersed in 3% hydrogen peroxide for 5 min to suppress endogenous peroxidase activity, heated (100°C) for 30 min in 0.01 M sodium citrate buffer (pH 6.0) to retrieve the antigen, rinsed (3 times, each for 5 min) in phosphate buffered saline (PBS), incubated with a polyclonal mouse anti-rabbit

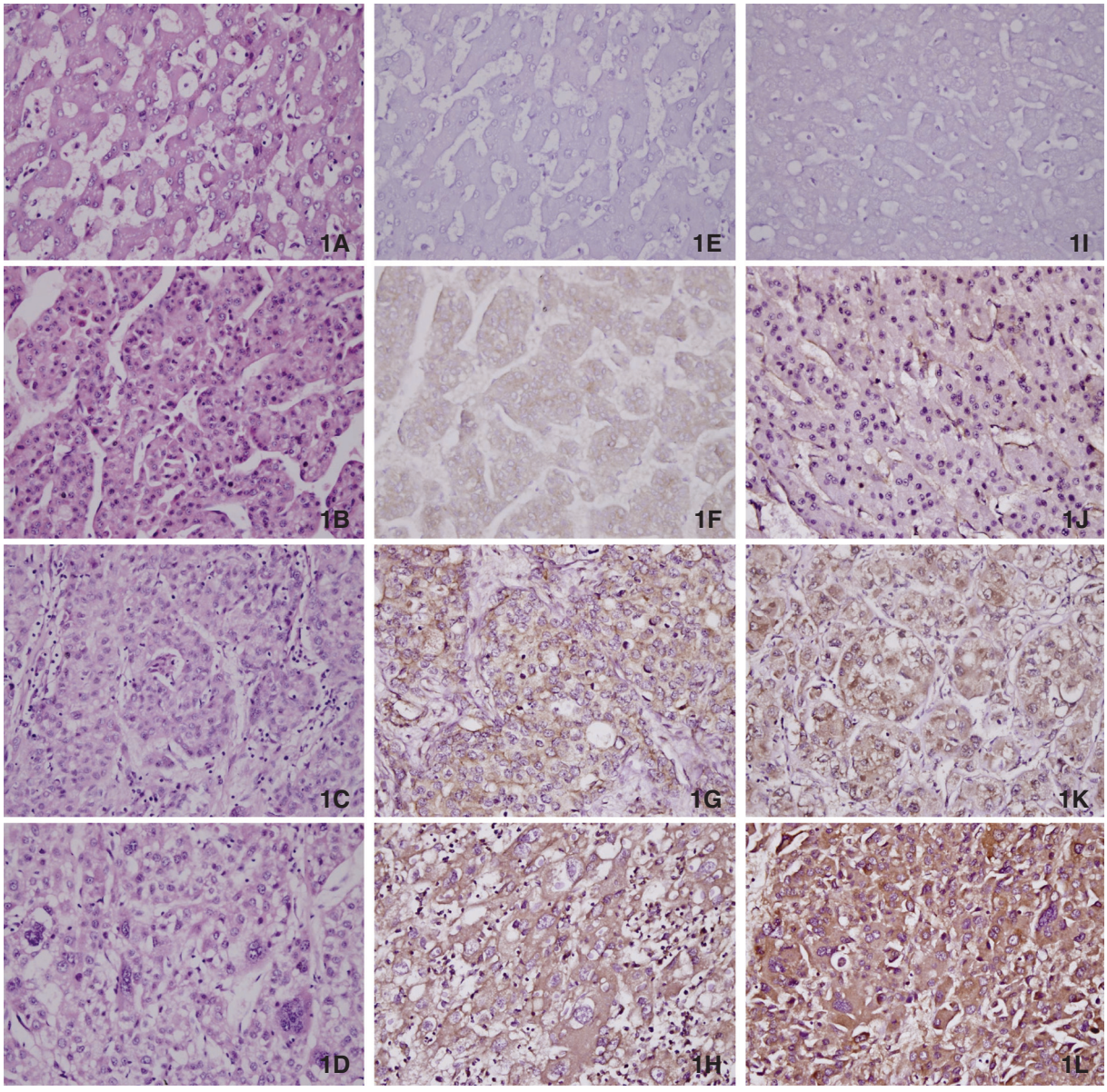

Fig. 1. Hematoxylin and eosin staining of non-neoplastic liver tissue (1A), grade I HCC (1B), grade II HCC (1C), and grade III HCC (1D); immunohistochemical analysis of OPN in non-neoplastic liver tissue (1E), grade I HCC (1F), grade II HCC (1G), and grade III HCC (1H); and immunohistochemical analysis of EGFR in non-neoplastic liver tissue (1I), grade I HCC (1J), grade II HCC (1K), and grade III HCC (1L). Original magnification × 400.

OPN antibody (1:100, Thermo Fisher Scientific, Waltham, MA, USA) or a polyclonal mouse anti-human EGFR antibody (1:25, Zymed, San Francisco, CA, USA) diluted in PBS for 2 h at room temperature, washed (3 times, each for 5 min) in PBS, incubated with horseradish peroxidase-labeled goat anti-mouse immunoglobulin (1:100, DAKO, Glostrup, Denmark) or mouse anti-rabbit immunoglobulin (DAKO) for 1 h at room temperature, washed 3 times, and treated with AEC+ substrate chromogen (DAKO) at room temperature to visualize the peroxidase reaction.

For assessment of OPN and EGFR expression, a non-tumorous part of the liver parenchyma was used as a negative internal control. Immunostaining scores were calculated from the percent and staining intensity of tumor cells with cytoplasmic and membrane staining. The immunoreactivity and histological appearance of all tissue specimens were evaluated twice and the slides were examined and scored by two authors concurrently. The intensity of cytoplasmic and membrane immunostaining of tumor cells was scored on a scale of 0 (no staining) to 4 (strongest intensity), and the percentage of tumor cells with cytoplasmic or membranous staining at each intensity was estimated.

Table 2. Immunostaining patterns of osteopontin and clinicopatholigcal parameters of hepatocellular carcinomas and non-neoplastic liver tissues

	No. of Cases	Average Intensity*	Average % Tumor*	Average Score*	Correlation
Normal liver tissue	8	0	0	0	
Histological grading					
Well differentiated	20	1.1	48.8	56.1	Positive correlation (P = 0.023)
Moderately differentiated	47	1.4	69.8	104.6	
Poorly differentiated	33	1.8	74.3	141.2	
TNM stage					
T stage					
T1	53	1.1	51.3	58.6	Positive correlation (P = 0.029)
T2	22	1.3	72.4	85.9	
T3	15	1.7	75.2	126.8	
T4	10	2.1	80.4	189.1	
N stage					
N0	77	1.3	70.2	73.8	Positive correlation (P = 0.032)
N1	23	1.9	78.1	154.5	
M stage					
M0	82	1.4	71.5	90.3	Positive correlation (P = 0.037)
M1	18	2.0	79.3	164.5	
Clinical stage					
Stage I	48	1.0	49.8	50.3	Positive correlation (P = 0.026)
Stage II	15	1.2	67.5	81.1	
Stage IIIA	5	1.5	73.9	108.7	
Stage IIIB	5	1.6	74.8	115.2	
Stage IIIC	3	1.8	75.7	138.9	
Stage IVA	6	1.8	76.2	150.1	
Stage IVB	18	2.0	79.3	164.5	

Asterisks (*) showed mean value.

The percentage of cells (from 0 to 100) at each intensity was multiplied by the corresponding immunostaining intensity (from 0 to 4) to obtain an immunostaining score ranging from 0 to 400.

Statistical Analysis

Statistical analysis was performed using the Mann-Whitney *U*-test. With *P* value less than 0.05, correlation of clinicopathological parameters with immunostaining scores was considered significant. In addition, overall survival was calculated as the time from the date of surgery to the date of death. In all, 84 of the 100 HCC patients included in the study were followed up for at least five years. These patients were divided into two groups based on mean OPN and EGFR scores to determine the relationship between survival time and OPN and EGFR immunostaining scores. Survival rates were analyzed using the Kaplan-Meier survival test. Additionally, multivariate analysis was performed using Cox's proportional hazard model.

Results

OPN Expression in HCC

The expression of OPN was undetectable in the 8 specimens of normal liver parenchyma (Figs. 1A and 1E) and varied in the 100 HCC specimens. The average OPN staining intensity, percentage of stained cells and immunostaining score were, respectively, 1.1, 48.8 and 56.1 in grade I specimens (Figs. 1B and 1F), 1.4, 69.8 and 104.6 in grade II specimens (Figs. 1C and 1G) and 1.8, 74.3 and 141.2 in grade III specimens (Figs. 1D and 1H). OPN staining score was positively correlated

Table 3. Immunostaining patterns of EGFR and clinicopatholigcal parameters of hepatocellular carcinomas and non-neoplastic liver tissues

	No. of Cases	Average Intensity*	Average % Tumor*	Average Score*	Correlation
Normal liver tissue	8	0	0	0	
Histological grading					
Well differentiated	20	1.1	66.5	80.5	Positive
Moderately differentiated	47	1.8	77.8	142.1	correlation
Poorly differentiated	33	2.7	83.8	230.6	(P = 0.011)
TNM stage					
T stage					
T1	53	1.4	70.5	96.4	Positive
T2	22	1.7	78.1	135.5	correlation
T3	15	2.7	81.8	221.3	(P = 0.026)
T4	10	3.0	85.7	261.4	
N stage					
N0	77	1.7	75.9	132.7	Positive correlation
N1	23	2.8	83.2	236.4	(P = 0.024)
M stage					
M0	82	1.8	77.8	142.1	Positive correlation
M1	18	2.9	83.6	244.6	(P = 0.027)
Clinical stage					
Stage I	48	1.3	70.1	92.3	
Stage II	15	1.5	76.3	115.4	
Stage IIIA	5	2.3	79.5	183.6	Positive
Stage IIIB	5	2.6	80.1	208.5	correlation
Stage IIIC	3	2.7	80.3	218.0	(P = 0.018)
Stage IVA	6	2.7	82.1	223.2	
Stage IVB	18	2.9	83.6	244.6	

Asterisks (*) showed mean value.

with histological grade (Table 2, P = 0.023).

The more advanced T stages of HCC were associated with higher OPN intensity and immunostaining score. The average OPN immunostaining score was 58.6, 85.9, 126.8 and 189.1 in specimens from patients with stage T1 (n = 53), T2 (n = 22), T3 (n = 15), and T4 (n = 10) HCC, respectively. OPN staining score was positively correlated with T stage (P < 0.05), and higher OPN expression was associated with more advanced M or N stage. Finally, HCC cases were divided on the basis of the clinical staging system into stage I, II, IIIA, IIIB, IIIC, IVA and IVB. The corresponding immunostaining scores were 50.3, 81.1, 108.7, 115.2, 138.9, 150.1 and 164.5, respectively. The OPN immunostaining score was positively correlated with clinical stage (P = 0.026, Table 2).

EGFR Expression in HCC

The scores for EGFR immunostaining are shown in Table 2. EGFR expression was absent in normal liver parenchyma (Fig. 1I) but was present on the cell membrane and cytoplasm of tumor cells in all HCC specimens. The average intensity, percentage of stained tumor cells and immunostaining score were 1.1, 66.5 and 80.5, respectively, in grade I specimens (Fig. 1J), 1.8, 77.8 and 142.1 in grade II specimens (Fig. 1K), 2.7, 83.8 and 230.6 in grade III specimens (Fig. 1L). EGFR immunostaining score was positively correlated with histological grade (Table 3, P = 0.011).

Additionally, the average EGFR immunostaining score was 96.4 in specimens of stage T1, 135.5 for stage T2, 221.3 for stage T3, and 261.4 for stage T4 tumors. Higher EGFR immunostaining scores were

Table 4. Multivariate analysis of factors associated with overall survival in hepatocellular carcinoma patients

	Hazard Ratio (95% CI)	*P* value
Overall survival		
Tumor number (multiple *vs*. single)	2.226 (1.128-3.538)	0.138
OPN (1+, 2+, 3+, 4+) *vs*. OPN (-)	2.353 (1.413-3.492)	0.002
EGFR (1+, 2+, 3+, 4+) *vs*. EGFR (-)	2.238 (1.420-3.312)	0.002
Lymphovascular invasion (yes *vs*. no)	2.043 (0.655-2.741)	0.113
TNM stage (III-IV *vs*. I-II)	2.324 (1.374-3.557)	0.003

significantly correlated with more advanced T stage ($P < 0.05$) and more advanced M or N stage ($P < 0.05$). The immunostaining scores were 92.3, 115.4, 183.6, 208.5, 218.0, 223.2 and 244.6 for specimens from stage I, II, IIIA, IIIB, IIIC, IVA and IVB tumors. The EGFR immunostaining score was also significantly correlated with the clinical stage ($P = 0.018$, Table 3).

Relationship of OPN and EGFR Expression with Survival Time in HCCs

In 84 HCC cases with 5 years or more follow-up, more than one-half had higher OPN expression (immunostaining score ≥ 100) and higher EGFR expression (score ≥ 150). Higher OPN and EGFR expression levels were significantly associated with shorter survival time (Fig. 2). In addition, multivariate analysis revealed that OPN and EGFR expression as well as TNM stage are independent poor prognostic factors for overall survival (Table 4).

Relationship between OPN and EGFR in HCC

The relationship between OPN and EGFR immunostaining scores is shown in Fig. 3. Significantly higher OPN immunoscores were positively correlated with higher EGFR immunoscores in HCC specimens.

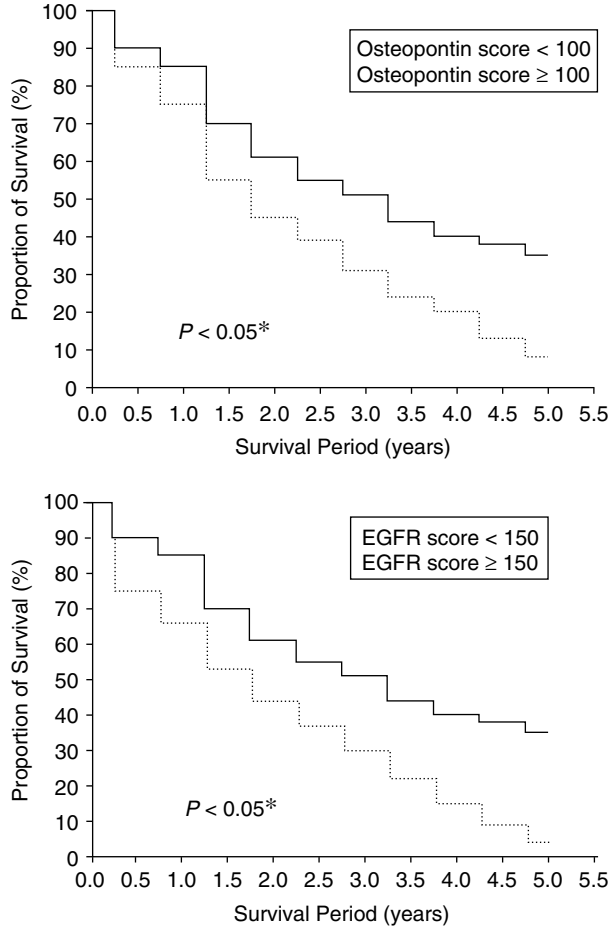

Fig. 2. Overall survival of 84 patients with HCC. Higher OPN and EGFR immunostaining scores were associated with short survival periods. Survival rates were analyzed using the Kaplan-Meier survival test ($P < 0.05$).

Discussion

HCC is the fifth most common malignant tumor in men and eighth most common in women (25). The main risk factors of HCC include hepatitis B virus (HBV), hepatitis C virus (HCV), alcohol consumption, metabolic disorders, drug abuse and exposure to toxins (25). Although several signal pathways of tumor progression have been identified in HCC, the overall survival rate remains disappointing. The overall 5-year survival rate in HCC is only 10%. The presence of cirrhosis, poor histological differentiation of tumor and male sex with higher age are related to worse outcome (5, 26-28). Short survival and therapeutic failure have been attributed to early vascular dissemination and lymph node metastasis. Recently, molecular biological evidence has revealed the mechanisms of VEGF-induced tumor metastasis and hepatocyte growth factor (HGF)-induced progression (44).

OPN is an acidic glycoprotein consisting of aspartate, glutamate and serine as well as about 30 monosaccharides (3). Although it is known that OPN overexpression can induce liver cancer invasion and progression, the mechanism is not fully understood

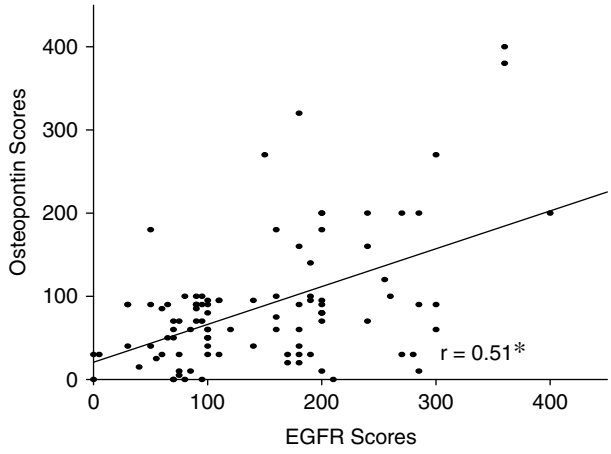

Fig. 3. Relationship between OPN and EGFR immunostaining scores in HCC. These results show higher immunostaining scores of OPN significantly correlating with higher EGFR immunostaining scores. Asterisks (*) indicates statistical significance of linear regression testing.

(17). Chen *et al*. discovered that the stimulation of HCC infiltration by OPN might depend on the interaction of OPN with CD44v6, and on upregulating MMP-2 and urokinase type plasminogen activator expression (7). Previous studies have shown that OPN is a sensitive biomarker of HCC development since immunostaining does not detect OPN in normal liver tissues (17, 36). However, the relationship between OPN expression and overall prognosis is limited to early stage HCC cases. In the present study, the OPN immunostaining score was positively correlated with histological grade and clinical stage. Our results support the hypothesis that OPN may be a crucial indicator of tumor metastasis and poor prognosis in HCC patients.

EGFR is a well-known and important feature of several malignancies in humans (21). Although HCC progression has recently been associated with genetic changes, the relevance of EGFR signaling genes in HCC is controversial (44). EGFR mutation in exons 18-21 has rarely been detected in HCC cases even in those with EGFR overexpression (44). No previous studies have established a relationship between EGFR immunostaining and tumor grade, pathological stage and overall survival. Our results show that the EGFR immunostaining scores indeed correlate with tumor progression and prognosis of HCC. In addition, our results indicate that EGFR is a potential biomarker of malignant transformation of hepatocytes.

The use of histological and immunohistochemical techniques on a tissue microarray is a powerful tool for simultaneous evaluation of tumors (16). Previous studies of individual cases were limited because variations in environmental conditions between tests had led to variations in immunohistochemical intensity between specimens (16). The reliability of immunohistochemistry studies conducted on tissue microarray slides has been established (16). In our study, there was a clear-cut difference in OPN or EGFR immunostaining between non-tumorous parts of the liver parenchyma and tumors, validating the use of tissue microarray slides in such studies. Therefore, the immunostaining scores used in our study could reflect the relative levels of OPN or EGFR protein expression in HCC.

Several signaling pathways are involved in HCC progression, including the transforming growth factor α/epidermal growth factor receptor (TGFα/EGFR) pathway (2). Likewise, OPN-induced tumor progression, invasiveness and metastasis in HCCs are dependent on the activation of the mitogen-activating protein kinase (MAPK), NF-κB pathway and on the overexpression of matrix metalloproteinase-2 (MMP-2) (6). Furthermore, increase in the activity of MMP-2 may cause the phosphorylation of EGFR (10). Our results not only demonstrate that OPN and EGFR can induce tumor invasiveness and metastasis, but also that EGFR upregulation (associated with OPN elevation) may synergistically enhance tumor progression in HCCs.

In conclusion, our study demonstrates that analysis of OPN and EGFR expression is effective in predicting tumor behavior, including progression, invasion and malignant transformation of HCC. Although the mechanisms involved in the progression of HCC remain unknown, our work suggests that OPN and EGFR play important roles in metastasis and poor prognosis of HCC. Immunostaining indicating wide distribution of both biomarkers in HCC may imply their importance in tumor progression. Therefore, these markers may help the pathologists to discriminate between benign liver nodules and malignant HCC, especially in small lesions with good differentiation.

Acknowledgments and Potential Conflicts of Interest

We declare no conflicts of interest relating to the work reported in this study. This study was supported by grants from the Tri-Service General Hospital, TSGH-C100-110 and TSGH-C100-059, Taiwan, R.O.C.

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