Prognostic Role of Apelin Receptor Expression in Hepatocellular Carcinoma Treated With Curative Surgical Resection

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Abstract. *Background/Aim:* apelin and apelin receptor (APJR) are involved in the regulation of angiogenesis, and their high expression is related to poor outcomes in several cancer types. Recently, several positive results on APJR antagonists in cancer treatment have been reported at the preclinical level. The aim of this study was to evaluate the prognostic effect of APJR expression on hepatocellular carcinoma (HCC) survival. *Materials and Methods:* We evaluated APJR expression in 288 curatively resected HCCs using immunohistochemistry and investigated the correlation with clinicopathological features. *Results:* High APJR expression was significantly associated with the presence of microvascular invasion ($p<0.001$), intrahepatic metastasis ($p=0.004$), and early recurrence ($p=0.029$). The high-expression group showed shorter recurrence-free survival ($p<0.001$) and overall survival ($p=0.001$) than the low-expression group. In multivariate analysis, high APJR expression was an independent predictor of shorter recurrence-free survival (Hazard Ratio 1.49; 95% confidence interval 1.08-2.05, $p=0.016$). *Conclusion:* We described-high APJR expression and its prognostic effect in HCC. Emerging target agents may be applicable in patients with HCC and high APJR expression.

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Hepatocellular carcinoma (HCC) is the most common primary liver malignancy, the sixth most common cancer, and the fourth leading cause of cancer death worldwide (1). Approximately 800,000 patients are newly diagnosed and die each year globally. Although hepatic resection is the treatment of choice in HCC, it is not curative for the most patients due to the high recurrence rate of up to 70% (2). Recurrence is the main cause of death. Thus, prediction of recurrence is exceedingly important and prevention with appropriate therapy will improve patient outcome (3). Applications of target therapy are still limited despite many clinical trials. The multi-targeted kinase inhibitors sorafenib, regorafenib, and lenvatinib are approved systemic treatments in advanced HCC (4-6). Identification of new therapeutic targets and reliable biomarkers is needed to ensure more effective clinical treatment after curative resection (7).

Apelin, a ubiquitous peptide, is involved in the regulation of cardiovascular control, homeostasis, and angiogenesis (8-12). The significance of apelin and apelin receptor (APJR) has been studied in many tumors, including non-small cell lung cancer, colon cancer, and brain tumors (13-15). Relatively consistent results show that a high apelin level is related to vascular proliferation and poor outcome. Recently, apelin was highlighted as a promising target in the treatment of metabolic disease (16, 17). Several positive results using APJR antagonists in cancer treatment have been reported at the preclinical level (14, 15). Recently, Muto *et al.* reported an increased APJR expression in arterial smooth muscle cells in HCC and apelin mRNA expression in HCC. They also showed inhibition of tumor growth with APJR antagonist (18). However, APJR expression and its relation to clinical features and outcomes in HCC has not been fully elucidated.

In this study, we investigated APJR expression by immunohistochemistry (IHC) in 288 curatively resected HCC samples and analyzed the prognostic effect of APJR expression in patients with HCC and a median 119-month follow-up period.

Materials and Methods

Patients. Initially, 291 surgically resected HCC cases were collected from the surgical pathology database at Samsung Medical Center (Seoul, Korea) between 2000 and 2006. Three cases with no residual tissue were excluded, and finally 288 cases were included in this study. All hematoxylin and eosin-stained slides were reviewed by two pathologists (CKP and SYH). Clinical and laboratory data including age, sex, date of surgery, serum albumin level and alpha-feto protein (AFP) level, presence of viral hepatitis, recurrence-free survival (RFS), overall survival (OS), and follow-up were extracted from electronic medical records. The institutional review board of Samsung Medical Center approved this study and waived informed consent.

Curative resection was defined as complete resection of all tumor nodules without microscopic involvement of resection margins and no detected residual tumor on computed tomography (CT) scans at 1 month after surgery. Histopathologic features of HCCs, including tumor differentiation, microvascular invasion, major portal vein invasion, intrahepatic metastasis, multicentric occurrence, and non-tumor liver pathology, were reviewed by two liver pathologists (SYH and CKP). Tumor differentiation was determined according to the criteria of Edmondson and Steiner (19). Intrahepatic metastasis and multicentric occurrence were distinguished according to the criteria of the Liver Cancer Study Group of Japan (20). Patients were staged according to the American Joint Committee on Cancer (AJCC) staging system (21) and Barcelona Clinic Liver Cancer (BCLC) staging classification (22).

During the follow-up period, patients underwent CT with serum AFP measurement every 2 or 3 months postoperatively. Patients with suspicious imaging findings and/or continuously elevated AFP levels were further evaluated with positron emission tomography (PET)-CT and/or magnetic resonance imaging (MRI). The median follow-up period was 119.8 months (range=14.0-151.4). Recurrence was generally diagnosed using radiologic examinations without histologic confirmation. Intrahepatic HCC recurrence within the first two years after curative resection is mainly due to intrahepatic metastasis, whereas late recurrence usually results from multicentric disease (23). Using 2 years as a cut-off, intrahepatic tumor recurrence was classified as either early or late (24).

Tissue microarray construction. Representative tumor areas free from necrosis or hemorrhage were marked in formalin-fixed paraffin-embedded blocks. Two tissue cores of 2.0 mm in diameter were obtained from donor blocks and arranged in recipient paraffin blocks. Two cores of normal liver tissue from 12 patients with metastatic colonic carcinoma to the liver were included in each array block. Each tissue microarray (TMA) block contained up to 60 tissue cores.

Immunohistochemical staining and evaluation. IHC was performed in 4- μ m-thick tissue sections from TMA blocks, using a Bondmax automated immunostainer (Leica Biosystems, Melbourne, Australia) and a Bond™ Polymer refine detection system, DS9800 (Vision Biosystems, Melbourne, Australia). The primary antibody was a rabbit polyclonal antibody against APJ Receptor antibody - cytoplasmic domain (ab140508, 1:50, Abcam, Cambridge, MA, USA). Briefly, antigen retrieval was performed at 97°C for 20 min in ER2 buffer (Leica Biosystems, Melbourne, Australia). After blocking endogenous peroxidase activity with 3% hydrogen

peroxide for 5 min, primary antibody incubation was performed for 15 min at room temperature, and antigen-antibody chromogenic reactions were detected for 10 min.

Brain tissue was used as positive control according to manufacturer's recommendations, and cytoplasmic staining of tumor cells was interpreted as positive. APJR staining was analyzed by a semiquantitative method using H-score on a continuous scale of 0 to 300 by considering both percentage of stained cells and 4 intensity categories: 0 for negative, 1+ for weak, 2+ for moderate, and 3+ for strong positive (Figure 1). The IHC slides were independently interpreted by two pathologists (TL and SYH). In cases of disagreement, the final interpretation was determined by consensus using a multi-head microscope.

Statistical analysis. Calculated H-score was analyzed using X-tile statistical software program to determine the optimal cut-off point with the most significant prognostic effect in terms of RFS (25). Correlations between clinicopathologic data and APJR expression were analyzed by chi-square test or Fisher's exact test. Survival curves were plotted according to Kaplan-Meier analysis, and Cox proportional hazard model was used to predict RFS. Statistical analyses were performed using R and SPSS statistical package (IBM, NY, USA) and *p*-value <0.05 (two sided) was considered statistically significant.

Results

Clinicopathological features of the HCC patients. The clinicopathologic features are summarized in Table I. Among the 288 HCC cases, 237 (82.3%) patients were male and 51 (17.7%) were female, with ages ranging from 17 to 76 years old (median=53). In preoperative laboratory tests, 30 (10.4%) patients were hypoalbuminemic (≤ 3.5 g/dl) and 103 (37.1%) patients showed increased AFP level (>200 ng/ml). Forty-nine (17.0%) patients had non-viral associated HCC, and the other cases were viral associated. A total of 209 (72.6%) patients had hepatitis B virus (HBV), 26 (9.0%) patients had hepatitis C virus (HCV), and 4 (1.4%) patients had both HBV and HCV.

By histological examination, the majority of cases (233; 80.9%) were Edmonson grade II, which was followed by grade I (31; 10.8%) and grade III (24; 8.3%). One-hundred fifty-six (54.2%) cases showed peritumoral microvascular invasion, and 12 (4.2%) cases had major portal vein invasion. Sixty-six (22.9%) cases had intrahepatic metastasis, while 19 (6.6%) were multicentric. In the background liver (at least 2 cm away from the tumor), 145 (50.3%) cases showed cirrhosis.

One-hundred forty-two patients recurred within 2 years after surgery (early recurrence). Of the remaining 146 patients, 50 patients recurred over 2 years after surgery (late recurrence).

APJR expression and recurrence free survival. The median H-score value of APJR IHC was 115 (range=0-260) and the mean was 116.66 (standard deviation 56.58). Using X-tile

Figure 1. Representative images of apelin receptor immunohistochemistry according to intensity score: Score 0 (A), Score 1 (B), Score 2 (C), Score 3 (D).

Figure 2. Kaplan–Meier survival curves for apelin receptor expression (high expression: $H\text{-score} \geq 155$; low expression: $H\text{-score} < 155$) in 288 patients with hepatocellular carcinoma.

software, the optimal cut-off value with the most significant prognostic effect was 155. Seventy-two cases (25%) were considered as high expression. Normal hepatocytes, including control blocks and peritumoral liver, variably expressed negative to focal weak staining intensity.

The relationship between high APJR expression and clinicopathologic findings is shown in Table I. High APJR expression was significantly associated with younger age (≤ 55 , $p=0.033$), higher Edmondson grade ($p<0.001$), advanced AJCC T-stage ($p<0.001$), high AFP level ($p=0.023$), microvascular

Table I. Clinicopathologic features and correlation with apelin receptor IHC expression in 288 hepatocellular carcinomas.

	Variable	n	Low apelin expression, n (%)	High apelin expression, n (%)	p-Value
Age	≤55 years	167	117 (54.2%)	50 (69.4%)	0.033
	>55 years	121	99 (45.8%)	22 (30.6%)	
Gender	Female	51	37 (17.1%)	14 (19.4%)	0.789
	Male	237	179 (82.9%)	58 (80.6%)	
Tumor size	≤5.0 cm	192	151 (69.9%)	41 (56.9%)	0.061
	>5.0 cm	96	65 (30.1%)	31 (43.1%)	
Edmondson grade	I	31	28 (13.0%)	3 (4.2%)	<0.001
	II	233	179 (82.9%)	54 (75.0%)	
	III	24	9 (4.2%)	15 (20.8%)	
Microvascular invasion	(-)	132	113 (52.3%)	19 (26.4%)	<0.001
	(+)	156	103 (47.7%)	53 (73.6%)	
Major portal vein invasion	(-)	276	210 (97.2%)	66 (91.7%)	0.080
	(+)	12	6 (2.8%)	6 (8.3%)	
Intrahepatic metastasis	(-)	222	176 (81.5%)	46 (63.9%)	0.004
	(+)	66	40 (18.5%)	26 (36.1%)	
Multicentric occurrence	(-)	269	202 (93.5%)	67 (93.1%)	1.000
	(+)	19	14 (6.5%)	5 (6.9%)	
AJCC T-stage	1	124	107 (49.5%)	17 (23.6%)	<0.001
	2	115	80 (37.0%)	35 (48.6%)	
	3a	32	18 (8.3%)	14 (19.4%)	
	3b	11	5 (2.3%)	6 (8.3%)	
	4	6	6 (2.8%)	0	
BCLC stage	0-A	167	132 (61.1%)	35 (48.6%)	0.092
	B	107	76 (35.2%)	31 (43.1%)	
	C	14	8 (3.7%)	6 (8.3%)	
Albumin level	>3.5 g/dl	258	195 (90.3%)	63 (87.5%)	0.656
	≤3.5 g/dl	30	21 (9.7%)	9 (12.5%)	
AFP level ^a	≤200 ng/ml	175	140 (67.0%)	35 (50.7%)	0.023
	>200	103	69 (33.0%)	34 (49.3%)	
Etiology	Non-viral	49	37 (17.1%)	12 (16.7%)	1.000
	Viral (HBV or HCV)	239	179 (82.9%)	60 (83.3%)	
Liver cirrhosis	(-)	143	99 (45.8%)	44 (61.1%)	0.035
	(+)	145	117 (54.2%)	28 (38.9%)	
Early recurrence (≤2 years)	(-)	146	118 (54.6%)	28 (38.9%)	0.029
	(+)	142	98 (45.4%)	44 (61.1%)	
Late recurrence (>2years)	(-) ^b	96	82 (69.5%)	14 (50.0%)	0.083
	(+)	50	36 (30.5%)	14 (50.0%)	

AJCC, American Joint Committee on Cancer 7th edition; BCLC, Barcelona Clinic Liver Cancer; AFP, α-fetoprotein; HBV, hepatitis B virus; HCV, hepatitis C virus. Statistically significant p-values are shown in bold. ^aPartial data were not available; ^bNo early or late recurrence.

invasion ($p<0.001$), intrahepatic metastasis ($p=0.004$), and early recurrence ($p=0.029$). Absence of liver cirrhosis is also significantly associated with high APJR expression ($p=0.035$).

The RFS and OS rates of the 288 patients with HCC were 60.1% and 88.5% at 1 year, 45.1% and 80.2% at 2 years, 38.5% and 74.0% at 3 years, 28.5% and 64.2% at 5 years and 15.6% and 36.5% at 9 years, respectively. Both the RFS and OS of patients with high APJR expression were significantly shorter than in patients with low APJR expression ($p<0.001$ and $p=0.001$, respectively) (Figure 2A and 2B). The median RFS of patients with high and low APJR expression was 9.8 and 27.5 months, respectively.

On univariate analysis for prediction of RFS, larger size (>5.0 cm), Edmondson grade III, presence of microvascular

invasion, major portal invasion, intrahepatic metastasis, higher AJCC T-stage and BCLC stage, hypoalbuminemia, higher AFP level, HBV or HCV infection, and high APJR expression showed unfavorable effects (Table II). In multivariate analysis, high APJR expression was an independent predictor of shorter RFS [Hazard Ratio (HR)=1.486, 95% confidence interval (CI)=1.075-2.053; $p=0.016$], in addition to well-known prognostic factors such as intrahepatic metastasis and hepatitis virus infection (Table II).

Discussion

Apelin, a peptide encoded by the *APLN* gene, is an endogenous ligand for the seven-transmembrane G-protein-

Table II. Cox proportional hazard models for prediction of recurrence free survival in 288 hepatocellular carcinoma patients.

Variable	Univariate model		Multivariate model	
	HR (95% CI)	p-Value	HR (95% CI)	p-Value
Age, year (≤ 55 vs. > 55)	0.957 (0.716-1.278)	0.765		
Gender (Male vs. Female)	0.974 (0.678-1.400)	0.887		
Tumor size, cm (≤ 5.0 vs. > 5.0)	1.843 (1.377-2.465)	<0.001	1.129 (0.784-1.625)	0.515
Edmondson grade (I+II vs. III)	2.199 (1.395-3.468)	<0.001	1.111 (0.658-1.877)	0.693
Microvascular invasion (No vs. Yes)	2.255 (1.681-3.023)	<0.001	1.200 (0.826-1.745)	0.339
Major portal vein invasion (No vs. Yes)	6.533 (3.573-11.95)	<0.001	1.899 (0.988-3.652)	0.055
Intrahepatic metastasis (No vs. Yes)	5.588 (4.033-7.743)	<0.001	4.242 (2.948-6.104)	<0.001
Multicentric occurrence (No vs. Yes)	1.343 (0.747-2.414)	0.325		
AJCC T-stage (1 vs. 2+3+4)	2.298 (1.707-3.093)	<0.001		
BCLC stage (A vs. B+C)	2.216 (1.666-2.947)	<0.001		
Albumin level, g/dl (> 3.5 vs. ≤ 3.5)	1.913 (1.245-2.94)	0.003	1.382 (0.863-2.214)	0.178
AFP level, ng/ml ^a (≤ 200 vs. > 200)	1.700 (1.271-2.273)	<0.001	1.336 (0.986-1.810)	0.061
Etiology (Nonviral vs. Viral)	2.479 (1.309-4.694)	0.005	1.756 (1.082-2.848)	0.023
Liver cirrhosis (No vs. Yes)	1.319 (0.992-1.752)	0.057	1.233 (0.910-1.669)	0.176
Apelin expression (Low vs. High)	1.807 (1.326-2.461)	<0.001	1.486 (1.075-2.053)	0.016

AJCC, American Joint Committee on Cancer; BCLC, Barcelona Clinic Liver Cancer; AFP, α -fetoprotein; HR, hazard ratio; CI, confidence interval. Statistically significant *p*-values are shown in bold. ^aPartial data were not available.

coupled APJR (26). Since it was first introduced from bovine stomach extracts in 1998, wide expression of apelin and APJR in many human organs has been identified (27), supporting the multitudinous functions of apelin and APJR. A variety of physiologic functions has been described, such as blood pressure control, cardiac contractility, and fluid and glucose homeostasis (8-10). Above all, angiogenesis is one of the most impressive functions of apelin and APJR. Apelin and APJR expression is essential for embryonic vascular development and has been identified in adult vessels (11, 12).

Hypoxia plays an important role in angiogenesis (28). It upregulates the expression of apelin derived from adipose tissue, and apelin enhances the migration and proliferation of endothelial cells (29). Angiogenesis is essential for growth and metastasis of solid tumors; thus, it is closely related to clinical outcome (30). Recent studies showed that apelin induced vascular maturation and tumor growth. Apelin upregulation is also associated with poor clinical outcome in several cancer types (13-15). In non-small cell lung cancer, apelin mRNA level was increased and high apelin protein level was related to increased microvascular density and poor OS (13). Colon cancer, brain tumor, and HCC showed elevated expression of apelin and/or APJR and accompanying microvascular proliferation (14, 15, 31). Stimulation of tumor-induced angiogenesis raises the possibility of lymphovascular invasion and metastasis (32). A recent study on lymphangiogenesis revealed an association of apelin overexpression and lymph node metastasis (33).

HCC shows typical vascular abnormalities compared to non-tumorous liver. Unlike the dual arterial and portal blood

flow supply of non-tumor liver, HCC only receives arterial supply. Arteriogenesis of HCC originates from pre-existing arteries with proliferation of smooth muscle cells. A recent study by Muto *et al.* showed APJR expression in arterial smooth muscle cells of HCC, and induction of smooth muscle cell proliferation by apelin treatment (18). These results suggest the impact of apelin and APJR system on typical arteriogenesis in HCC.

In our study, high expression of APJR in HCC was significantly associated with microvascular invasion and intrahepatic metastasis. High expression of APJR in HCC was also an independent factor for shorter RFS. This finding supports the evidence that apelin is involved in arteriogenesis and vascular invasion in HCC. Apelin and APJR may play important roles in early recurrence. Intrahepatic metastasis is a major contributor to early recurrence, which is the most important factor in predicting clinical outcome with HCC. APJR expression can be considered an excellent predictive marker of RFS.

Due to its function in energy metabolism and insulin sensitivity, apelin has recently become a promising target for treating a broad range of diseases, including type 2 diabetes, obesity, dyslipidemia, cardiovascular disease, kidney disease, and liver disease (16, 17, 34, 35). On the basis of angiogenesis in tumor progression, the development of a potential targeting therapy has been suggested (13). In colon adenocarcinoma cell line, APJR antagonist significantly reduced the proliferation rate of tumor cells (14). Selective competitive antagonists of APJ receptor suppressed glioblastoma growth *in vivo* and prolonged survival of

intracranially xenografted mice (15). APJR antagonist also inhibited *in vivo* tumor growth in cholangiocarcinoma and HCC (18, 36). Based on the function of apelin and APJR in HCC progression, targeted antagonist may reduce tumor growth and angiogenesis while lengthening RFS.

Conclusion

High expression of APJR in HCC tissue specimens was demonstrated to be an excellent marker related to microvascular invasion, intrahepatic metastasis, and shorter RFS. Moreover, APJR expression was suggested as an independent predictor of RFS. To the best of our knowledge this is the first study to assess APJR expression on clinical outcomes of patients with HCC. Emerging target agents against apelin or APJR may be applicable in patients with HCC with high APJR expression.

Authors' Contributions

Conception and design: TL, SYH; Acquisition of data: CKP; Analysis and interpretation of data: TL, CKP, SYH; Drafting the article: TL, SYH; Revising and final approval of the article to be published: TL, SYH; All authors read and approved the final manuscript.

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