European Review for Medical and Pharmacological Sciences 2013; 17: 1095-1101

Significance of relaxin-2 expression in hepatocellular carcinoma: relation with clinicopathological parameters

H.-Z. PAN[1], A.-B. DONG[2], L. WANG[2], S.-S. TAN[2], Q. YANG[1], X.-Y. TONG[3], J. LIANG[3], J.-R. WANG[2]

Department of Clinical Laboratory[1], Thyroid Surgery[2] and Oncology[3], the Affiliated Hospital of Medical College, Qingdao University, Qingdao, Shangdong, R.P. China

Huazheng Pan and *Anbing Dong* contributed equally to this work

Abstract. – BACKGROUND: A number of putative roles, including the modulation of tumor growth, neovascularization, metastasis and oncogenic progression, have been correlated to relaxin-2 overexpression. However, the clinical significance of relaxin-2 expression in hepatocellular carcinoma (HCC) remains unclear. The aim of this study was to investigate the expression of relaxin-2 in HCC and determine its correlation with tumor progression and prognosis.

PATIENTS AND METHODS: 180 HCC patients who had undergone curative liver resection were selected and immunohistochemistry was performed to analyze relaxin-2 expression in the respective tumors.

RESULTS: Immunohistochemistry confirmed relaxin-2 overexpression in HCC tissues compared with their adjacent nonneoplastic tissues ($p < 0.01$). Additionally, immunostaining showed more relaxin-2 positive cells in the higher tumor grade (III) than in the lower tumor stage (I, II; $p = 0.026$). Moreover, HCC patients with high relaxin-2 expression were significantly associated with lower 5-year overall survival ($p < 0.01$) and lower 5-year disease-free survival ($p < 0.01$), respectively. Furthermore, immunostaining showed more relaxin-2 positive cells in the tumor recurrence (ETR) patients than non-ETR patients ($p = 0.001$). The Cox proportional hazards model further showed that relaxin-2 was an independent poor prognostic factor for both 5-year disease-free survival (hazards ratio [HR] = 1.872, 95% confidence interval [CI] = 1.18-5.146, $p = 0.023$) and 5-year overall survival (HR = 3.637, CI = 1.443-7.15, $p = 0.001$) in HCC.

CONCLUSIONS: Our data suggest for the first time that the overexpression of relaxin-2 protein in HCC tissues is of predictive value on tumor progression and poor prognosis.

Key Words:
Hepatocellular carcinoma, Relaxin-2, Prognosis.

Introduction

The current trend in cancer treatment is shifting from uniform protocols to personally customized methods. The prediction of prognosis or therapy response has become central to the efficiency of customized cancer therapies. Prognosis is an important factor used to decide the extent of surgery. Immunohistochemistry is an excellent technique to link basic research data to therapy and is critical for the identification of specific proteins overexpressed in malignant tissues. However, only after rigorous testing, can one determine if these proteins can be considered as suitable candidates as prognostic/predictive markers.

Hepatocellular carcinoma (HCC) is the fifth most common cancer of men and the eighth most cancer of women worldwide, which causes 250,000 deaths each year[1-2]. Early HCC is clinically silent and often well advanced at the first manifestation, only 10-20% of patients are suitable for surgical treatment[2]. Even having undergone surgical treatment, the prognosis of patients with HCC is still poor[3]. The poor prognosis is largely attributed to the invasion at early stage in HCC. Considering its high incidence of occurrence and the large range of therapeutic approaches provided to HCC patients, it has become clear that there exists a pressing need for additional prognostic/predictive markers.

Relaxin is a short circulating peptide hormone .Two highly homologous genes on human chromosome 9 encode relaxin-1 and relaxin-2 peptides with predicted 82% identity at amino acid level[4-5]. Despite having two peptide-coding genes, relaxin gene 1 and 2, the major stored and circulatory form of relaxin in humans is relaxin-

Corresponding Author: Jueru Wang, MD; e-mail: qyfywang@163.com

2. Relaxin-2 is produced in the prostate by males[6] and corpus lutea in females[7], and relaxin-1 is a pseudogene, which does not translate into a functional peptide in rodents, humans and other non-human species.

The effect of relaxin-2, particularly during pregnancy, is well established in rodents. Levels of relaxin change with the different stages of pregnancy and these patterns are dissimilar across species. Furthermore, relaxin-2 has been shown to increase oocytes fertility[8]. Besides exerting its effect in female reproduction, relaxin-2 is also involved in maintaining sperm motility in the male reproductive system[9]. Relaxin-2 has also been shown to be involved in nonreproductive functions. While well known for its reproductive and antifibrotic roles, most recently relaxin-2 has been associated with cancer biology. A number of putative roles, including the modulation of tumor growth, neovascularization, metastasis and oncogenic progression, have been correlated to relaxin-2 overexpression[10-12].

The clinical significance of relaxin-2 in HCC has not been reported. This paper investigates whether relaxin-2 could be useful as a prognostic marker in HCC using immunohistochemical staining. The staining results obtained for the tumor tissues were correlated with the clinical data of patients, including survival rates.

Patients and Methods

Patient and Tissue Samples

The study was approved by the Affiliated Hospital of Medical College, Qingdao University, Qingdao, China. Ethical approval for human subjects was obtained from the research Ethics Committee of the Affiliated Hospital of Medical College, and informed consent was obtained from each patient. A total of 180 patients with primary HCC who underwent a curative liver resection at the Affiliated Hospital of Medical College, Qingdao University, were included in this retrospective study. Tissues used in the study were retrieved from the tissue bank of the Department of Pathology in the Affiliated Hospital of Medical College, Qingdao University. These patients were diagnosed as HCC between 2002 and 2007. The preoperative studies for the diagnosis and staging of the hepatocellular carcinoma included biochemical parameters of liver function, alpha fetoprotein, viral serology, an abdominal ultrasound and a TC scan of the chest and abdomen; also, a splenic angiogram with splenoportography was performed in selected cases. All the patients under study were assessed with intraoperative ultrasound, and underwent liver resection. Surgical resection was defined as complete resection of all tumor nodules with the cut surface being free of cancer by histologic examination[13]. Of the 180 surgical operated HCC patients (110 men and 70 women, aged from 27 to 76 years old, mean age is 52 years old), 28 patients was serum HCV-positive, 124 patients was serum HBV-positive, 14 patients was both serum HBV-positive and HCV-positive and 42 patients was both serum HBV-negative and serum HCV-negative. 32 paired adjacent nontumor liver tissues were also selected from the same sample bank. Elevated alpha-fetoprotein (AFP; ≥ 200 ng/mL) was detected in 112 cases (62%). Liver cirrhosis was found in 74 patients (38%). Liver function was assessed by Child-Pugh classification. None of the patients recruited in this study had chemotherapy or radiotherapy before the surgery.

Histological Study and Tumor Staging

Tumor differentiation was defined according to the Edmondson grading system. Tumor grade was divided into 3 groups: well-differentiated (grade I, 37 cases), moderately differentiated (grade II, 80 cases), and poorly differentiated (grade III, 63 cases). Tumor staging was defined according to the Sixth Edition of Tumor-Node-Metastasis (TNM) Classification of International Union Against Cancer. Stage I HCC included tumors that were ≤ 2 cm and showed no evidence of liver and vascular invasion (7 cases). Stage II HCCs included tumors that were ≤ 2 cm for which vascular invasion was limited to small vessels in the tumor capsule, as well as encapsulated tumors > 2 cm with no evidence of liver or vascular invasion (73 cases). Stage IIIA HCCs included invasive tumors > 2 cm with invasion of small vessels in the tumor capsule and/or satellites near the tumor, but no portal vein invasion (22 cases). Stage IIIB HCCs included tumors with invasion of the portal vein branch near the tumor, but not of the distant portal vein in the liver parenchyma (28 cases). Stage IV included tumors with involvement of major portal vein branches, satellites extending deeply into the surrounding liver, tumor rupture, or invasion of the surrounding organs (50 cases). No evidence of distant metastasis and local metastasis was noted at the time of surgery in any of the cases. 34 cases had regional lymph node metastasis. Among the 180 patients studied, 165 were eligible for the

evaluation of early tumor recurrence (ETR; ≤ 12 months). 15 patients who died within 1 year after surgery without objective evidence of tumor recurrence were excluded from the evaluation of ETR. The clinicopathological features of 180 patients are summarized in Table I.

Histological Staining and Immunohistochemistry

5-μm paraffin sections were adhered to silanized slides, deparaffinized and hydrated by passing through xylene (3×5 min), a graded series of ethanol (1×100%, 1×180%, 1×70% and 1×50%) for 5 min and distilled water for 10 min. Endogenous peroxidase activity was blocked using 3% hydrogen peroxide in methanol (30 min). After each following step, sections were washed with 0.5 mol/l Trisbuffer, pH 7.6 (3×10 min). When required, permeabilization of the sections was achieved by incubation with 0.05% trypsin for 30 min. Then the tissue sections were covered with 2% normal rabbit serum in Tris

Table I. Clinicopathological features and the expression of relaxin-2 in 180 hepatocellular carcinoma.

			Relaxin-2 expression		
	Features	Case	Positive	Negative	*p*-value
Age (Years)					0.846
	< 50	102	53	49	
	> 50	78	40	38	
Gender					0.783
	Male	110	56	54	
	Female	70	37	33	
HBV					0.236
	Yes	124	64	58	
	No	56	29	27	
HCV					0.187
	Yes	28	15	13	
	No	152	78	74	
HBV+HCV					0.153
	Yes	14	6	8	
	No	166	87	79	
AFP					0.34
	> 200	112	63	49	
	< 200	68	30	38	
Child-Pugh					0.462
	A	146	79	67	
	B	34	14	20	
Cirrhosis					0.631
	Yes	74	35	39	
	No	106	58	48	
Growth pattern					0.72
	Trabecular	131	72	59	
	Nontrabecular	49	21	28	
Size (cm)					0.152
	< 5	82	36	46	
	> 5	98	57	41	
Grade					0.089
	Well	37	17	20	
	Moderately	80	39	41	
	Poorly	63	37		
Stage					0.026
	I + II	80	24	56	
	IIIA-IV	100	69	31	
ETR					0.001
	Yes	100	62	38	
	No	65	25	40	
5-year survival					0.004
	Yes	68	19	49	
	No	112	74	38	

Buffered Saline (TBS) for 20 min, and were incubated with the anti-relaxin-2 antibody (1:100 dilution). For each case, a corresponding section was incubated in TBS without the primary antibody as a control for non-specific staining. Biotinylated rabbit anti-mouse (DAKO, Hamburg, Germany) was added for 40 min, followed by the avidin-biotinylated peroxidase complex for an additional 40 min. After washing with distilled water for 10 min, staining was achieved using 3,3'-diaminobenzidine. Then the sections were counterstained with Mayer's haemalum and mounted for microscopy. All stainings were examined by three independent investigators.

Immunohistochemistry Evaluation

Positive cases were those that had clear-cut positive staining in at least 5% of the total cellular constituents. The amounts of immunopositive cells were estimated semiquantitatively: grade "–" corresponds to ≤ 5%; grade "+" corresponds to 5-10%, grade "++" to 10-50%, and grade "+++" to more than 50% positive cells. All series included positive and negative controls. The results of control staining were satisfactory.

Statistical Analysis

The χ^2 test was performed to analyze the correlation between relaxin-2 expression and clinicopathological parameters. Cumulative survival time was calculated by the Kaplan-Meier method and analyzed by the log-rank test. Univariate and multivariate analyses were based on the Cox proportional hazards regression model. Statistical analyses were done using SPSS 11.0 for windows (SPSS Inc., Chicago, IL, USA). $p < 0.05$ was considered statistically significant.

Results

Expression of Relaxin-2 Protein in HCC

Immunohistochemical analysis revealed that relaxin-2 staining was absent in adjacent nonneoplastic liver tissues (–) (Figure 1a). In HCC cells, relaxin-2 staining was localized in the cytoplasm (Figure 1b-e). Relaxin-2 expression was found in 93 (51.6%) of 180 HCC tissues. The grade –, +, ++ and +++ was 87, 17, 28 and 48 cases, respectively). Thus, the relaxin-2 immunostainings in HCC tissues were significantly higher than those in the adjacent nonneoplastic liver tissues ($p < 0.001$).

Clinicopathologic Significance of Relaxin-2 Expression in Hepatocellular Carcinoma

To evaluate whether relaxin-2 protein expression was associated with clinicopathological features of patients with HCC, we correlated immunohistochemical relaxin-2 staining results with major clinicopathologic features of HCC. As shown in Table I, relaxin-2 expression was associated with high T stage, ETR and 5-year

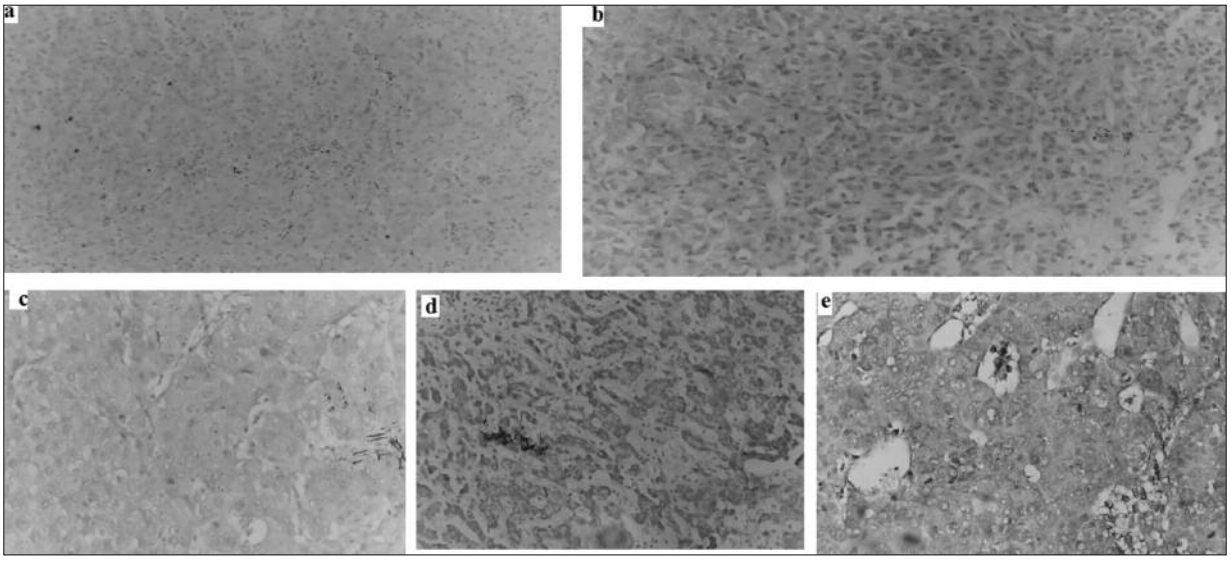

Figure 1. Relaxin-2 expression in hepatocellular carcinoma (HCC) and adjacent nonneoplastic liver tissues. ***A,*** Relaxin-2 negative staining was seen in adjacent nonneoplastic liver tissues. ***B,*** Relaxin-2 negative staining was seen in cytoplasm of HCC tissues. ***C-E,*** Relaxin-2 positive staining was seen in cytoplasm of HCC tissues (+, ++, +++). (Original magnification × 200). Bar, 50 um.

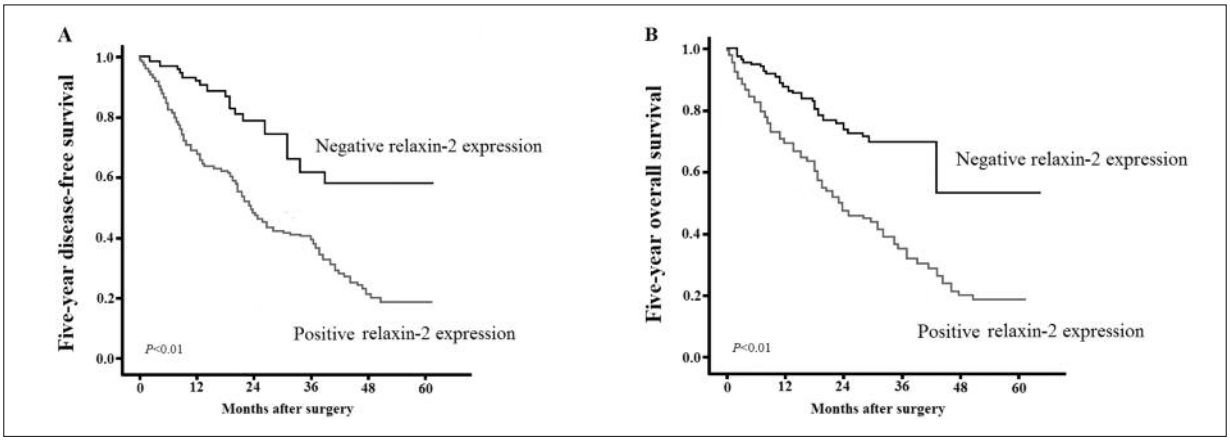

Figure 2. Kaplan-Meier survival curves for relaxin-2 expression in hepatocellular carcinoma (HCC) patients. The HCC patients with positive relaxin-2 expression showed significantly shorter disease-free survival ($p < 0.01$, A) and overall survival ($p < 0.01$, B) rates than those with negative relaxin-2 expression.

survival, but not with serum AFP level, age, gender, chronic hepatitis B/C virus infection, liver functional reserve (Child-Pugh class), presence of cirrhosis and tumor grade.

Relaxin-2 Expression Predicts Early Tumor Recurrence and Poor Prognosis

HCC with relaxin-2 expression were associated with worse 5-year survival than HCC without relaxin-2 expression ($p = 0.004$; Table I). Moreover, HCC with relaxin-2 overexpression showed more frequent ETR ($p = 0.001$; Table I). Five-year disease-free survival was observed in 68 (37.8%) patients, whereas in 100 (60.6%) patients, disease recurred, and 112 (62.2%) even died during a 5-year follow-up period. We observed a trend that 5-year disease-free survival in the group with relaxin-2 overexpression was significantly poorer than that in the group with low relaxin-2 expression ($p < 0.01$, log-rank test; Figure 2A). Additionally, the Kaplan-Meier plot of 5-year overall survival curves stratified by relaxin-2 expression was shown in Figure 2B. A significant relationship was found between relaxin-2 expression and 5-year overall survival ($p < 0.01$, log-rank test, Figure 2B). Importantly, relaxin-2 expression correlated with major clinicopathologic parameters related to tumor progression by univariate analyses, including large tumor size ($p = 0.036$), higher tumor grade ($p = 0.004$), and higher tumor stage ($p < 0.001$) (Table II). Relaxin-2 expression was significantly associated with overall survival ($p =$

Table II. Univariate analysis of different prognostic factors in 180 HCC patients.

Variable	Overall survival		Disease-free survival	
	HR (95% CI)	*p*-value	HR (95% CI)	*p*-value
Age (years)	1.253 (0.372-3.218)	0.374	1.421 (0.453-4.025)	0.287
Gender	0.774 (0.287-2.374)	0.536	0.823 (0.402-2.921)	0.520
HBV	1.18 (0.52-2.35)	0.874	1.24 (0.73-2.49)	0.852
HCV	0.617 (0.45-1.623)	0.558	0.83 (0.44-1.524)	0.536
HBV+HCV	0.475 (0.15-1.73)	0.26	0.403 (0.18-1.93)	0.247
AFP	1.84 (1.02-3.35)	0.082	1.92 (0.94-3.84)	0.073
Child-Pugh	0.68 (0.26-1.83)	0.352	0.48 (0.19-1.68)	0.287
Cirrhosis	0.83 (0.46-1.85)	0.592	0.74 (0.38-1.92)	0.626
Growth pattern	1.14 (1.53-2.86)	0.148	1.26 (1.37-2.74)	0.196
Size	1.89 (1.46-6.47)	0.032	2.15 (1.24-4.94)	0.027
Grade	1.96 (2.48-6.94)	0.004	1.83 (2.16-6.15)	0.016
Stage	5.43 (2.28-12.31)	< 0.001	5.6 (2.44-13.92)	< 0.001
ETR	0.58 (0.30-1.12)	0.082	0.60 (0.29-1.48)	0.079
Relaxin-2	3.56 (2.54-8.33)	0.004	4.26 (2.83-11.46)	0.001

Table III. Multivariate analysis of overall and disease-free survival rates of HCC patients

Variable	Overall survival		Disease-free survival	
	HR (95% CI)	*p*-value	HR (95% CI)	*p*-value
Stage	2.749 (1.780-6.14)	0.002	2.154 (1.420-5.452)	0.014
Relaxin-2	3.637 (1.443-7.15)	0.001	1.872 (1.18-5.146)	0.023

0.001) and disease-free survival ($p = 0.02$) of HCC patients (Table II). Futhermore, in a multivariate Cox model, we found that tumor stage and relaxin-2 expression was an independent poor prognostic factor (Table III).

Discussion

In the present study, we provide the first analysis of relaxin-2 expression in human HCC tissue and its association with patient clinical outcome. Relaxin-2 immunoreactivity was significantly increased in a substantial proportion of HCC cases compared with their adjacent nonneoplastic liver tissue.

Relaxin-2 were significantly higher in patients with lymph node metastasis and higher tumor stage than in those without lymph node involvement and lower tumor stage. Importantly, relaxin-2 expression correlated with major clinicopathologic parameters related to tumor progression by univariate analyses, including large tumor size ($p = 0.036$), higher tumor grade ($p = 0.004$), ETR ($p < 0.01$) and higher tumor stage ($p < 0.001$). By multivariate analyses, we showed that relaxin-2 expression was associated with high-stage (stages IIIA, IIIB, and IV) HCC, which exhibits vascular invasion and various extent of microscopic intrahepatic spread. This suggested that downregulation of relaxin-2 in HCC could be of clinical use for distinguishing a set of patients with poor prognosis.

Although the diagnosis and management of HCC have progressed significantly, the prognosis for patients receiving surgical treatment remains poor because of the high early tumor recurrence (ETR)[14]. Hence, the identification of molecular factors to predict ETR will help develop better strategies for patient management. Here, we showed that HCC with relaxin-2 overexpression had a greater than 2.4-fold higher chance of ETR than HCC without the overexpression. Consistent with its association with high-stage HCC and frequent ETR, high relaxin-2 expression is associated with a significant trend toward both poorer disease- free survival and poorer overall survival. Our study further confirms that high relaxin-2 expression independently predicts a higher risk of disease relapse or death after multivariate adjustment for other prognostic factors. These findings are consistent with the correlation of relaxin-2 overexpression with poor tumor differentiation and worse patient survival in thyroid cancers[15]. Taken together, our findings suggest that relaxin-2 overexpression serves as a useful marker predicting ETR and hence poor prognosis. Our results suggest that relaxin-2 selective inhibitors are potential drugs for HCC treatment. Nevertheless, whether targeting relaxin-2 alone is a better therapeutic strategy, will require further exploration.

Conclusions

Our study suggests that relaxin-2 is overexpressed in HCC tissues compared with their benign counterparts. To the best our knowledge, this is the first study evaluating the expression levels of relaxin-2 in HCC tissues and its association with clinicopathologic parameters. Especially, the most important finding of this study is that relaxin-2 is a novel and potential factor for predicting the poorer prognosis of HCC patients after surgery. Further studies are needed to investigate the precise function of relaxin-2 in the progression of HCC. Although the current results which are based on a cohort of Chinese patients should be further confirmed in other HCC cohorts, our findings suggest relaxin-2 as a new and promising prognostic biomarker for HCC progression and prognosis.

References

1) Trevisani F, Cantarini MC, Wands JR. Recent advances in the natural history of hepatocellular carcinoma. Carcinogenesis 2008; 29: 1299-1305.

2) Clark HP, Carson WF, Kavanagh PV. Staging and current treatment of hepatocellular carcinoma. Radiographics 2005; 25(Suppl 1): S3-S23.

3) Yeo W, Mok TS, Zee B. A randomized phase III study of doxorubicin versus cisplatin/interferon alpha-2b/doxorubicin/fluorouracil (PIAF) combination chemotherapy for unresectable hepatocellular carcinoma. J Natl Cancer Inst 2005; 97: 1532-1538.

4) Sherwood OD. Relaxin's physiological roles and other diverse actions. Endocr Rev 2004; 25: 205-234.

5) Bathgate RA, Ivell R, Sanborn BM, Sherwood OD, Summers RJ. International Union of Pharmacology LVII: recommendations for the nomenclature of receptors for relaxin family peptides. Pharmacol Rev 2006; 58: 7-31.

6) Feng S, Agoulnik IU, Bogatcheva NV, Kamat AA, Kwabi-Addo B, Li R, Ayala G, Ittmann MM, Agoulnik AI. Relaxin promotes prostate cancer progression. Clin Cancer Res 2007; 13: 1695-1702.

7) Shabanpoor F, Separovic F, Wade JD. The human insulin superfamily of polypeptide hormones. Vitam Horm 2009; 80: 1-31.

8) Brener SHL, Schoenfeld C, Amelar RD, Dubin L, Weiss G. Stimulation of human sperm cervical mucus penetration in vitro by relaxin. Fertil Steril 1984; 42: 92-96.

9) Weiss G. Relaxin in the male. Biol Reprod 1989; 40: 197.

10) Silvertown JD, Geddes BJ, Summerlee AJ. Adenovirusmediated expression of human prorelaxin promotes the invasive potential of canine mammary cancer cells. Endocrinology 2003; 144: 3683-3691.

11) Silvertown JD, Ng J, Sato T, Summerlee AJ, Medin JA. H2 relaxin overexpression increases in vivo prostate xenograft tumor growth and angiogenesis. Int J Cancer 2006; 118: 62-73.

12) Silvertown JD, Symes JC, Neschadim A, Nonaka T, Kao JCH, Summerlee AJ, Medin JA. Analog of H2 relaxin exhibits antagonistic properties and impairs prostate tumor growth. FASEB J 2007; 21: 754-765.

13) Yang XR, Xu Y, Yu B, Zhou J, Li JC, Qiu SJ, Shi YH, Wang XY, Dai Z, Shi GM, Wu B, Wu LM, Yang GH, Zhang BH, Qin WX, Fan J. CD24 is a novel predictor for poor prognosis of hepatocellular carcinoma after surgery. Clin Cancer Res 2009; 15: 5518-5527.

14) Carr BI. Hepatocellular carcinoma: current management and future trends. Gastroenterology 2004; 127(5 Suppl 1): S218-24.

15) Feng S, Agoulnik IU, Bogatcheva NV, Kamat AA, Kwabi-Addo B, Li R, Ayala G, Ittmann MM, Agoulnik AI. Relaxin promotes prostate cancer progression. Clin Cancer Res 2007; 13: 1695-1702.