Correlation of adrenomedullin with the erythropoietin receptor and microvessel density in hepatocellular carcinoma

Zhen Qu[1], Yong Jiang[2], Ming Xu[3], Ming Zhu Lu[3], Bin Zhou[3], Yitao Ding[1]

[1]Department of Hepatobiliary Surgery, Drum Tower Hospital, Medicine School of Nanjing University, Nanjing, China
[2]Department of Hepatobiliary Surgery, Changzhou 1st People's Hospital, Jiangsu, China
[3]Department of Hepatobiliary Surgery, Affiliated Soochow University Changzhou Tumor Hospital, Changzhou, China

Corresponding author:
Assoc. Prof. Yong Jiang MD, PhD
Changzhou 1st People's Hospital
185 Juqian St
213003 Changzhou
Jiangsu, China
Phone: 86-519-68871341
E-mail: yjiang8888@hotmail.com

Abstract

Introduction: Uncontrolled angiogenesis plays an essential role in the occurrence, metastasis and malignant progression of hepatocellular carcinoma (HCC). This study aimed to investigate the expression of adrenomedullin (ADM) in human HCC and its correlation with the expression of erythropoietin receptor (EPOR), microvessel density (MVD) and the tumor pathological characteristics.
Material and methods: Fresh tumor tissues were obtained from 30 HCC patients after hepatectomy. Ten cirrhotic and 10 normal liver tissues were included as controls. Expression of ADM and EPOR was determined by real-time PCR. The MVD was determined by counting the number of microvessels.
Results: The MVD and the mRNA levels of ADM and EPOR in cancer tissues were significantly higher than those in the non-cancer tissues ($p < 0.05$). Expression of ADM was significantly correlated with the MVD and EPOR ($r = 0.68$ and 0.74, $p < 0.01$). Adrenomedullin and EPOR mRNA levels in HCC tissues were correlated with capsule invasion, pathological differentiation and tumor metastasis ($p < 0.05$).
Conclusions: Our findings suggest that ADM and EPOR may serve as new regulatory factors involved in angiogenesis of HCC and represent novel targets for the treatment of HCC.

Key words: hepatocellular carcinoma, adrenomedullin, erythropoietin receptor, microvessel density.

Introduction

Uncontrolled cell growth and angiogenesis are key features of occurrence, metastasis and malignant progression of hepatocellular carcinoma (HCC). It was recently found that adrenomedullin (ADM) plays an important role in tumorigenesis, invasion and metastasis. Nakata *et al.* demonstrated that different gene expression was observed in multicentric HCCs. Adrenomedullin has been identified as a lead gene in the gene expression signature; it was overexpressed in HCCs with progression of intrahepatic metastasis [1]. Park *et al.* studied the role of ADM gene in the growth of HCC cells under hypoxic conditions. They found that hypoxia-induced HepG2 and Hep3B cell proliferation is mediated by

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JMJD1A up-regulation and subsequent decrease in methylation in the ADM promoter region [2]. In this study, we investigated the expression of ADM in human HCC and its correlation with the expression of erythropoietin receptor (EPOR) and microvessel density (MVD). We found that ADM expression in tumor tissues was significantly higher than non-cancer tissues. We also found that ADM expression was positively correlated with the expression of EPOR and MVD in HCC, suggesting a potential role of ADM in tumor angiogenesis.

Material and methods

Materials

Fresh tumor tissues were obtained from 30 HCC patients after hepatectomy. Ten cirrhosis and 10 normal liver tissues were included as control groups. Samples were stored in liquid nitrogen. Hepatocellular carcinoma was verified by pathological examination. Written informed consent was obtained from all patients. Affiliated Soochow University Changzhou Tumor Hospital Human Research Ethics Committee approval was obtained for the use of all samples by using a protocol that conforms to the provisions of the Declaration of Helsinki (as revised in Seoul, 2008).

Patient information

The average age of the HCC subjects (n = 30; male: 23, female: 7) included in this study was 44.3 years (range: 35–81). Tumors in 24 HCC subjects were well differentiated, while 6 subjects had poor differentiation. Fifteen subjects had a tumor diameter of less than 5 cm and the other 15 subjects had a tumor diameter of more than 5 cm. Sixteen subjects had an intact capsule and 14 subjects had capsule involvement. Eight subjects had metastases (including extrahepatic metastases, hilar lymph node metastasis, and portal vein thrombosis). Eighteen subjects had cirrhosis. Preoperative α fetal protein (AFP) was negative in 9 subjects and positive in 21 subjects. Hbsag (+) occurred in 23 subjects and hbsag (–) occurred in 7 subjects.

Real time-polymerase chain reaction

Expression of ADM, EPOR and β-actin genes was measured by RT-PCR with the following primers: H-ADM-F:5'TTGTCCTCCCCTATTTTAAGACG3';H-ADM-R: 5' CTTCCACACAGGAGGTAATCAGTC3'; H-EPO-F1: 5' CAGAGCAGGAAGCATTCAGAG3'; H-EPO-R1: 5' TGGTAGGTGCGAAAACAGGTA3'.

The reaction conditions were as follows: 94°C for 25 s, then 40 cycles of 64°C for 25 s, 72°C for 30 s, and a final extension at 72°C for 5 min.

Relative ADM or EPOR expression was measured by the comparative CT method (ΔΔCT method; Applied Biosystems) using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA as an internal standard. In this method, the threshold cycle (CT) indicates the fractional cycle number at which the amount of amplified target reaches a fixed threshold. The ΔCT value was determined by subtracting the average GAPDH CT value from the average ADM or EPOR CT value. The calculation of ΔΔCT involves subtraction by the ΔCT baseline sample. The amount of target, normalized to GAPDH and relative to the baseline sample, is given by 2-ΔΔCT. The resulting ΔΔCT factor enables relative quantification of the RNA of interest.

Measurement of microvessel density

Microvessel density was measured as described previously [3]. Briefly, tissues were fixed with 10% formalin, embedded in paraffin and subjected to HE staining. Any vessels with brown staining in the tumor tissues were considered positive. Any brown-stained endothelial cell or cell cluster was regarded as one vessel. For special types of microvessel with a large vascular lumen and a relatively small number of vessels per unit area, a length of 40 µM was regarded as one MVD value, as described previously [4].

Statistical analysis

Data are presented as mean ± standard deviation. Significance of difference between two groups was determined by χ^2 and Student's t-test. Correlation between the parameters was determined by Spearman univariate correlation analysis. Value of $p < 0.05$ was considered as indicating statistical significance. All statistical analyses were conducted using SPSS version 12 (SPSS Inc., Chicago, IL, USA).

Results

ADM and EPOR gene expression and MVD were increased in HCC tissues

The mRNA level of ADM and EPOR genes and MVD in HCC tissues were significantly higher than those in cirrhotic tissues and normal tissues (Table I). In contrast, mRNA levels of ADM and EPOR genes and MVD were not significantly different between cirrhosis and normal liver groups (Table I). Furthermore, expression of ADM in HCC tissues with capsule invasion, poor differentiation and tumor metastasis was significantly higher than that in the control groups ($p < 0.05$). In contrast, expression of ADM was not correlated with the patient age, tumor size, AFP level, hepatitis B virus infection or presence of cirrhosis ($p > 0.05$) (Table II).

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Table I. ADM mRNA, EPOR mRNA and MVD ($x \pm s$) in each group

Variable	Cases	ADM mRNA	EPOR mRNA	MVD
HCC	30	2.10 ±1.28*	15.37 ±12.75*	48.51 ±10.36*
Cirrhosis	10	0.51 ±0.29	3.58 ±2.40	17.36 ±3.57
Normal	10	0.44 ±0.16	2.94 ±2.11	16.15 ±3.74

*P < 0.05, HCC vs. control.

Table II. Relationship between ADM mRNA, EPOR mRNA level and clinic-pathological features of HCC

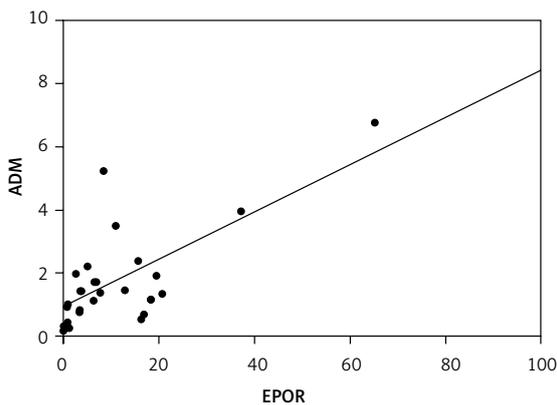| Clinicopathological features		Cases	ADM mRNA		EPOR mRNA	
			x ± s	Value of p	x ± s	Value of p
Age	> 55	18	2.25 ±1.38		15.77 ±11.85	
	≤ 55	12	2.34 ±1.41	> 0.05	16.22 ±12.34	> 0.05
HBsAg	+	23	2.41 ±1.50		16.13 ±13.04	
	−	7	2.20 ±1.15	> 0.05	15.62 ±12.50	> 0.05
AFP [ng/ml]	> 20	21	2.19 ±1.18		15.26 ±12.20	
	≤ 20	9	2.38 ±1.46	> 0.05	15.34 ±11.88	> 0.05
Cirrhosis	+	18	2.53 ±1.31		15.59 ±12.81	
	−	12	2.12 ±1.07	> 0.05	15.46 ±12.17	> 0.05
Tumor size [cm]	> 5	15	2.17 ±1.35		16.43 ±12.72	
	≤ 5	15	2.05 ±1.27	> 0.05	15.49 ±12.14	> 0.05
Capsule invasion	+	14	2.97 ±1.74*		19.60 ±4.71*	
	−	16	1.21 ±0.79	< 0.05	12.04 ±11.38	< 0.05
Metastasis	+	8	2.97 ±1.74^		11.44 ±10.07^	
	−	22	3.26 ±1.82	< 0.05	18.73 ±13.41	< 0.05
Pathological differentiation	L	6	3.66 ±1.61˚		19.35 ±14.40˚	
	H/M	24	0.87 ±1.02	< 0.05	12.21 ±11.36	< 0.05

*^P < 0.05, vs. control.

Figure 1. Scatter plot of ADM mRNA and EPOR mRNA level in HCC. A linear correlation between ADM and EPOR expression was observed (correlation coefficient: 0.74)

Correlation of ADM expression with EPOR expression and MVD

The mRNA level in HCC tissues was positively correlated with EPOR expression and MVD value ($p < 0.01$) (Figure 1).

Discussion

Recent studies have shown that ADM is overexpressed in many diseases including myocardial infarction [5], renal cell carcinoma [6], gliomas [7] and ovarian carcinoma [8]. Adrenomedullin influences the occurrence and development of malignant tumors by stimulating mitosis, suppressing the immune response, promoting angiogenesis and inhibiting apoptosis. In addition, hypoxia can induce the expression of ADM in hepatoma and pancreatic cancer cells [2, 9, 10]. Erythropoietin receptor is an erythropoietin receptor belonging to the type I cytokine receptor superfamily. In addition to the regulation of erythrocytopoiesis, EPO is involved in the development of a variety of human malignancies and promotes tumor angiogenesis [11, 12]. Nakamatsu *et al.* demonstrated that EPOR was highly expressed in the tumor tissues and tumor microvascular endothelial cells in the chemical reagent-induced HCC [13].

In this study, we showed that ADM expression in tumor tissues was higher than tumor adjacent

tissues and normal tissues, suggesting that tumor cells can produce and secrete ADM. Adrenomedullin may directly or indirectly promote and maintain tumor cell proliferation in an autocrine and paracrine manner. We also found that ADM expression in tissues with capsule invasion, poor differentiation and liver metastasis was higher than that in normal tissues. These results suggest that ADM is a new metastasis-related factors involved in the invasion and metastasis of liver cancer. Recent studies have suggested that the potential of angiogenesis is one of the key features of tumor cell growth. Currently, MVD is used for quantitative assessment of tumor angiogenesis. In our study, ADM expression in HCC is positively correlated with MVD. These results suggest that ADM plays an important role in the process of angiogenesis and the occurrence and progression of HCC as an angiogenic regulatory factor.

In conclusion, ADM is a new angiogenic regulatory factor involved in tumor angiogenesis, recurrence and metastasis of tumors. The selective interruption of ADM signaling may reduce the size of the tumor and the surface area of the vascular bed [14].

Acknowledgments

Conflict of interest

The authors declare no conflict of interest.

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