Expression of Augmenter of Liver Regeneration and Nuclear Faction-κB in Human Hepatocellular Carcinoma

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Abstract: To determine whether the levels of expression of the Augmenter of Liver Regeneration (ALR) and nuclear faction-κB(NF-κB) were assoiated with the clinicopathological features in human Hepatocellular Carcinoma (HCC). Gene expression was measured by using RT-PCR in the 30 human HCC tissues, matched adjacent tissues and 8 normal hepatic tissues. The clinicopathological data of all patients were also collected. Higher expression of ALR mRNA was observed in HCC tissues compared with normal liver tissue ($p<0.05$), it did not differ from the expression of matching non-neoplastic tissue in the same patient ($p>0.05$). Expression of both two genes were not found to be significantly associated with clinical/pathologic factors. ($p>0.05$). The expression of ALR mRNA in HCC tissues was positively correlated with NF-κB mRNA ($r = 0.382$, $p<0.05$). Up-regulation of ALR may play partial role of carcinogenesis through MAPK pathway in HCC.

Key words: Augmenter of liver regeneration, hepatocellular carcinoma, nuclear faction-κB, partial role, clinicopathological, China

INTRODUCTION

Augmenter of Liver Regeneration (ALR), a kind of polypeptide is mainly produced and stored in parenchymal cells. It has been proved that a large number of acceptors of ALR exist on the surface of hepatic cells. Little is known about the expression of ALR in Hepatocellular Carcinoma (HCC). Nuclear faction-κB (NF-κB) has the function of anti-apoptosis. The correlation of these two in HCC has not been reported. The aim of this study, conducted in a series of patients diagnosed with HCC was therefore to determine the relation between the clinical/pathologic factors and the levels of gene expression of ALR and NF-κB.

MATERIALS AND METHODS

Patients and tissue specimens: The present study was based on 30 patients of HCC for whom tumor tissue was available between 2007 and 2008. The fresh HCC sample and matched paracancerous tissues were obtained from several teaching hospitals in Wuhan.

The paracancerous tissue was 3 cm from tumor fringe. Eight cases of normal hepatic tissues were obtained from hepatorrhexis patients. All samples were qualified by histology. Samples were rapidly frozen in liquid nitrogen and stored at -80°C. Peripheral blood samples were also collected before operation. About 21 cases were males

and 9 cases were females, median age at diagnosis was 54 years (rang, 35-76) with no previous radiotherapy and chemotherapy.

Materials: The following reagents were used in this study: Trizol reagents (Invitrogen, Carlsbad, CA,USA); cDNA primer linkage synthetic kit (Fermentas, Lithuania); The primers, PCR kit (Sai Baisheng Gene Limited Company, Shanghai, Chian). The PCR amplification meter (Mannheim, Germany).

RNA isolation: About 100 mg of each tissue sample was homogenized on ice in 1mL Trizol reagent using a DEPC treated homogenizer. Total RNA was then extracted using a one-step RNA isolation technique. About 50 μL RNase-free solution was added to the collected RNA before storing at -70°C. After diluting 12 μL RNA sample in 3 mL DEPC-treated water, the concentration and purity of each sample was evaluated using a UV 240 spectrophotometer. The A260/A280 absorbance ratio of the RNA samples ranged from 1.9-2.0.

Primer design: The following primers were purchased from Sai Baisheng Gene Limited Company. ALR forward primer: 5'-AAGCGGGACACCAAGTTTAGG-3', reverse primer: 5'-CAGCTTGCGG TTCA CTTC-3' with 288 bp for amplification. NF-κB forward primer: 5'-AAAGGTTAGGGTCAAGAT-3', reverse primer:

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5'-TAGGAATTGCAGGTGTAT-3' with 405 bp for amplification. β-Actin forward primer: 5'-CTGGGACGACATGGAGAAAAT-3', reverse primer: 5'-AGGAAGGAAGGCTGGAAGAGT-3' with 567 bp for amplification.

Reverse transcription: RNA samples were reverse transcribed using a 20 µL reverse transcription reaction system. About 4 µL RNA, 1 µL of oligo (dT)$_{18}$ and 7 µL of DEPC-treated water were added into an EP tube and incubated at 70°C for 5 min. The EP tube was then transferred onto ice and the following reagents were added: 4 µL 5× buffer, 1 µL RNasin and 2 µL 10 mMdNTP mix. The reaction mix was centrifuged down and incubated at 37°C for 5 min after which 1 µL M-MuLV reverse transcriptase (200 µ µL^{-1}) was added. After centrifuging, the final reaction mix was incubated at 42°C for 1 h followed by 70°C for 10 min. The product was stored at -20°C.

PCR: The suitable PCR conditions and cycle number were determined during a preliminary experiment to amplify genes Tankyrase 1, TRF 1 and hTeRT. The β-Actin gene was used as an endogenous control gene. The reaction mix contained 2 µL cDNA, 2 µL each of forward and reverse primers, 4 µL of dNTP (10 mM), 5 µL of 10× PCRBuffer (within Mg^{2+}) and 2 µL of TaqDNA polymerase (5U µL^{-1}) in a total volume of 50 µL , added DEPC-treated water to total volume of 50 µL. The PCR conditions were as follows: initial denaturation of 94°C for 5 min followed by 30 cycles of 94°C for 30 sec, 55°C for 45 sec, 72°C for 60 sec (denaturation) and 72°C for 5 min (annealing and elongation) and finished with a 4°C hold for 5 min. The reaction terminated during the log phase of amplification.

Gel electrophoresis: About 10 µL product of PCR was separated through 1.5% agarose gel and the bands analyzed using the EDAS290 electrophoresis imaging system and Kodak 1D3.5 Image Analysis Software.

Statistical analysis: SPSS13.0 statistics software was used for t-test, paired t-test and Spearman correlationt analysis.

RESULTS AND DISCUSSION

Expression of ALR and NF-κB mRNA: Expression level of ALR mRNA was 1.32±0.41 in HCC tissue, 1.18±0.33 in paracancerous tissue and 0.27±0.13 in normal tissue. The expression of NF-κB mRNA was 0.96±0.21 in HCC, 0.77±0.27 in paracancerous tissue and 0.56±0.24 in normal tissue. Although, there were no significant difference in
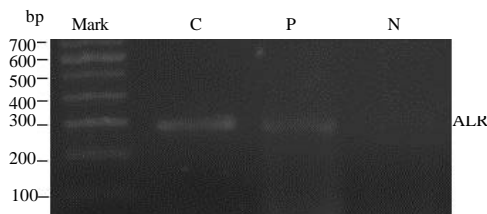

Fig. 1: The expression of ALRmRNA in HCC, peficancerous tissue and normal tissue (C: HCC; P: Peficancerous tissues; N: control group)

ALR and NF-κB mRNA expression between HCC and paracancerous tissues, the level of both of them in HCC tissues was much higher than normal tissue (p<0.01) (Fig. 1).

Relationship between the expression of ALR mRNA and clinicopathological features: No significant association of ALR mRNA expression was identified with hepatic cirrhosis, HbsAg (+), tumor size, number of tumors, Child-Pugh classification before operation, TNM staging, Edmondson classification and serum AFP level of the patients (p>0.05) (Table 1 and Fig. 2).

Correlation between the expression of ALR and NF-κB: The expression of ALR mRNA in HCC tissue was positively correlated with NF-κB mRNA (r = 0.382, p<0.05).

ALR which was first discovered in partial liver resections of rats is not normally expressed in human liver tissue but is present in cirrhotic liver tissue. It was recently found that ALR can stimulate the proliferation of hepatocytes directly (Liu *et al.*, 2004) and also functions as a disulphide oxidase using thioloxidase as a reductant to inhibit the proliferation of inflammatory cells. This study showed that ALR expression in HCC tissue was significantly elevated compared to normal liver tissue but its expression levels was not higher than the matching paracacinoma tissues in the same patients. These results coincided with the findings of Thasler *et al.* (2005). This suggests that some stimulator would enhance the expression of ALR in liver, advance activation of MAPK and phosphorylation of EGFR during the process of cell mitosis and thus accelerate the proliferation of cells (Polimeno *et al.*, 2000).

At the same time, it was discovered that injecting ALR into the integrated liver of normal rats can inhibit activity of NK cells (Lewis *et al.*, 2000). It can then be hypothesized that in addition to uncontrollable hepatocyte proliferation, high expressions of ALR may contribute to immune escape of cancerous cells.

Table 1: ALR mRNA, NF-κBmRNA expression and clinical/pathologic factors

| Clinical pathology	Case (n)	Strength of ALRmRNA HCC	P	Strength of NF-κBmRNA HCC	P
Hepatitis					
HBsAg+	23	1.42±0.33		1.10±0.31	
HBsAg-	7	1.27±0.41	0.3275	0.89±0.18	0.1013
Corrhosis					
Yes	21	1.37±0.15		0.93±0.15	
No	9	1.24±0.23	0.0752	1.07±0.22	0.0517
AFP					
≤400 μg L⁻¹	9	1.16±0.50		1.13±0.26	
>400 μg L⁻¹	21	1.38±0.57	0.3248	0.91±0.28	0.0539
Size					
≤5 cm	10	1.09±0.47		0.85±0.41	
>5 cm	20	1.42±0.58	0.1306	1.01±0.11	0.1089
Envelope					
+	17	1.57±0.63		1.18±0.53	
-	13	1.27±0.31	0.0564	0.92±0.13	0.0959
Tumor number					
Single	25	1.40±0.44		1.01±0.40	
Multi	5	1.32±0.61	0.5753	0.95±0.15	0.7426
TNM					
I--II	12	1.29±0.36		0.93±0.12	
III--IV	18	1.71±0.65	0.0607	1.08±0.2	0.0641
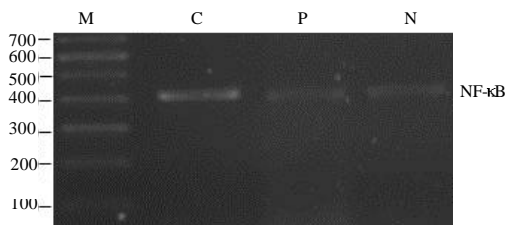
Fig. 2: The expression of NF-κBmRNA in HCC, peficancerous tissue and normal tissue (C: HCC; P: Peficancerous tissues; N: Control group)

Therefore, ALR may be involved in the pathogenesis of HCC. The results of this study indicated that there was no correlation between the expression of ALR and hepatic cirrhosis, HbsAg (+), tumor size, number of tumors, Child-Pugh classification before operation, TNM staging, Edmondson classification and serum AFP level in HCC (p>0.05). It not only suggested that HCC is a complex neoplasm regulated by multiple genes but also implied that ALR may play a promotive role in proliferation of HCC cell. ALR does not induce proliferation in normal hepatocytes directly but can promote proliferation via Kupffer cells (Wang *et al.*, 2006). This may suggest that ALR can indirectly promote regeneration of the liver via inflammatory factors. NF-κB is a ubiquitous inflammatory factor.

It has 5 isoforms and a diverse range of biological functions including playing a role in inflammatory reactions (Ogata *et al.*, 2004) blood vessels regeneration

and apoptosis. The expression of NF-κB in HCC is significantly higher than that of normal tissue. However, there was no significant difference between HCC and adjacent tissue. The adjacent tissue had more or less of inflammation or fibrosis and the histopathology was different from the normal tissue. This may partly explain that there was no significant difference in expression of NF-κB between HCC and adjacent tissue.

High levels of NF-κB promote the expression of TNF-α, IL-6, CRP and PAI-I (Zamara *et al.*, 2004). TNF-α and IL-6 are important mitotic factors in liver regeneration and may therefore advance malignant proliferation of tumor cells. At the same time, the another function of NF-κB is anti-apoptotic.

Therefore, it can be implied from above that NF-κB may also play an important role in cancer development and in particular, HCC. Zheng *et al.* (2008) found that interrupting the expression of NF-κB can inhibit proliferation and promote apoptosis of tumor cells (Zheng *et al.*, 2008), indicating that NF-κB can be used as a new target point in gene therapy. Up-regulation of ALR expression increases the activity of MAPK which increases the activity of NF-κB (Lu *et al.*, 2002).

CONCLUSION

The upregulation of ALR in HCC possibly allows, indirectly, the proliferation of cancerous cells unimpeded by an immune response. ALR also up-regulates the activity of NF-κB and thus allows cancerous cells to escape apoptosis.

This synergetic effect is a possible pathaphysiology behind the malignant proliferation of cancerous cells. However, whilst ALR has anti-inflammatory properties, NF-κB promotes an inflammatory response. The fact that the expression of both ALR and NF-κB are u p-regulated in HCC shows the complexity of HCC development. The interaction of ALR and NF-κB in HCC is still under discussion.

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