Expression of IER3 in Primary Hepatocarcinoma: Correlation with Clinicopathological Parameters

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Abstract

Background: Studies indicate the immediate early response gene 3 (IER3) is involved in many biological processes. Recently, it was discovered that IER3 plays an important role in tumorigenesis and tumor progression. Thus it may be a valuable biomarker in tumor. This study was designed to investigate the expression status of IER3 in primary hepatocarcinoma (PHC) and correlation with clinicopathological parameters.

Materials and Methods: Real-time PCR was performed to evaluate the expression levels of IER3 in 62 pathologically diagnosed human PHC specimens.

Results: A statistically significant association was disclosed between the expression of IER3 and P53 mutant protein (short for P53), Ki-67, EGFR and the biggest diameter, differentiation grade of tumor.

Conclusions: This work is the first to shed light on the potential clinical usefulness of IER3, as an efficient tumor biomarker in PHC.

Keywords: Immediate early response gene 3 - primary hepatocarcinoma - expression - tumor progression
The columns were incubated in Dnase and washed with alcohol before RNA was eluted in 50 μl of water. RNA was quantified spectrophotometrically and only samples with sufficient quantity and appropriate optical (OD260/280 ratio=1.8-2.0) were used for subsequent analysis. Samples that did not meet the RNA quality and quantity requirements were excluded from the study. For cDNA synthesis, 4 μg total RNA and random 6 primers (100 μm) 50 pmol, Oligo dT primer (50 μm) 25 pmol were incubated at 65°C for 10 min. Reverse transcription was performed using the M-MLV (promega M1705) and followed the specifications of the manufacturer and were incubated at 37°C for 1 h followed by 75°C for 10 min.

Real-time quantitative PCR (R-T PCR)
R-T PCR were performed with the SYBR green real-time PCR (MJ research, PTC200) and using 2 μl of the cDNA mixture and 10 μl 2x RT mix in a 20 μl reaction. The BROAD light cycler real-time PCR system (Linegene K FQ D-48A) was used. The PCR parameters were 3 min for 95 °C and 40 cycles of 95 °C for 10 s and 60 °C for 1 min. Gene expression levels were calculated during the logarithmic amplification phase by determining the initial mRNA copy number using the standard curve. Amplification of each gene specific fragment was confirmed by examination of peaks of melting curves. To further control for equal amount of total RNA loading, parallel examination of the house keeping genes Actin was quantified in each sample.

Immunohistochemical evaluation
The expression of EGFR, P53, Ki-67, AFP in the tumor tissue specimens was assessed by immunohistochemistry. 4-μm-thick sections were cut from formalin-fixed, paraffin-embedded (FFPE) tumor tissue specimens. The sections were deparaffinized in xylene and rehydrated in a graded ethanol series and then endogenous peroxidase activity was blocked with H2O2 in methanol. After the sections were pretreated with heat for antigen unmasking, immunohistochemistry was performed using the Ventana automated immunostainer (Ventana medical systems, Tucson, AZ, USA) with antibody (1:100 dilution) and secondary antibody (Beijing Zhongshan Company, China). The slides were then counterstained with hematoxylin. The expression of EGFR, P53, Ki-67, AFP was scored as negative or positive, according to the intensity of stained tumor cells compared with that of the surrounding connective tissues. When at least 20% of tumor cells show staining intensity stronger than that of the surrounding connective tissues, the tumor was regarded as positive; otherwise it was scored as negative. All specimens were evaluated independently by two investigators without any knowledge of the patient characteristics.

Statistical analysis
Student’s t-test was used to determine statistical significance between two sample means. And the chi-squared test was used to analyze significant associations between the expression of IER3 and P53, Ki-67, EGFR and other clinicopathological features. The SPSS statistical package version 13.0 (SPSS, Inc., Chicago, USA) was used for the statistical analyses. A p-value less than 0.05 was chosen to indicate statistical significance between two samples.

Results
The expression of IER3 was assessed by real time PCR in tissue specimens from 62 patients with PHC. The patients included 51 males and 11 females of age ranging from 33 to 83 years. Among the 62 patients, IER3 expression was positive in 38 tissue specimens (61.3%) and negative in 24 (38.7%). Table 1 shows the relationship between the IER3 expression and other clinicopathological factors. Using the chi-square test of independence, IER3 and P53, Ki-67, EGFR expression were found to be significantly associated (Table 1). Approximately 74% of IER3-positive cases also expressed ki-67, while fewer than 17% of IER3 negative cases stained positive for ki-67. On the contrary, no such correlation was observed between IER3 and AFP, with a p-value of 0.06. The tumor biggest diameter, differentiation grade was also found to be associated with IER3 expression. While the age, gender of patient and tumor thrombus was not observed of such association.

Discussion
Recent studies have characterized the functional interaction of IER3 with several signaling pathways, such as NF-κB, PI-3K/Akt, ERK. NF-κB/rel proteins, p53 and oncogene c-Myc are critical transcription factors involved in coordinating cellular decision-making events in response to external stimuli. Consensus sequences for binding these three transcription factors are found in the

Table 1. Correlation Between the IER3 Expression and Clinicopathological Factors in 62 PHC Patients

| Factor                        | No. of patients | P       |
|-------------------------------|----------------|---------|
|                               | IER3 Positive (n=38) | IER3 Negative (n=24) |
| **Age(years)**                 |                |         |
| High(≥65)                     | 18             | 11      | 7       | 0.99   |
| Low(<65)                      | 44             | 27      | 17      |        |
| Gender                        |                |         |
| Male                          | 51             | 29      | 22      |        |
| Female                        | 11             | 9       | 2       | 0.12   |
| P53 Positive                  | 27             | 21      | 6       |        |
| Negative                      | 35             | 17      | 18      | 0.02*  |
| Ki-67 Positive                | 32             | 28      | 4       |        |
| Negative                      | 30             | 10      | 20      | 0.00*  |
| EGFR Positive                 | 24             | 19      | 5       |        |
| Negative                      | 38             | 19      | 19      | 0.02*  |
| AFP Positive                  | 19             | 15      | 4       |        |
| Negative                      | 43             | 23      | 20      | 0.06   |
| Differentiation Grade         |                |         |
| Low                           | 34             | 25      | 9       |        |
| High-media                    | 28             | 13      | 15      | 0.03*  |
| Biggest diameter              |                |         |
| ≥5cm                          | 25             | 11      | 14      |        |
| <5cm                          | 37             | 27      | 10      | 0.02*  |
| Tumor thrombus                |                |         |
| Yes                           | 22             | 13      | 9       |        |
| No                            | 40             | 25      | 15      | 0.79   |

*P<0.05
promoter region of IER3 gene that can either suppress or induce apoptosis in a cell and stimulus-dependent manner. Interestingly, NF-κB/rel-mediated activation of IEX-1 expression was synergized by p53, but strongly inhibited by c-Myc in a dose-dependent fashion (Huang et al., 2002). It may explain the varying effects of p53 on IEX-1 expression in different cells.

Interestingly, it has been proposed that IER3 may enhance cell proliferation under favorable growth condition in the absence of cellular stresses, but may facilitate apoptosis under stressed conditions, such as serum starvation. Because the tumor microenvironment often involves stressed conditions unfavorable for tumor cell growth, such as a lack of growth-promoting factors (cytokines), nutrition and oxygen. IER3 may function as a pro-apoptotic factor in tumor cells and thereby limit tumor progression (Sasada et al., 2008; Han et al., 2011). But, necrosis and inflammation, which is a stimuli, is common in tumor centre. And the cells in the margin of the tumor may have enough oxygen and nutrition. These may be the reasons for the high expression of IER3 in this study.

Several studies about IER3 expression in variable human tumors was controversy. Yang et al. reported an enhanced expression of IER3 in atypical ductal hyperplasia or ductal carcinomas in situ and invasive carcinomas of the breast (Yang et al., 2006). Zhang et al. reported that IER3 may prevent the activation induced cell death of T-cells (Zhang et al., 2002; Zhang et al., 2003). Ishimoto et al. found that IEX-1 suppressed apoptotic damage in human intestinal epithelial Caco-2 cells induced by coculturing with macrophage-like THP-1 cells secreting TNF-α (Ishimoto et al., 2011). In contrast, IER3 also plays a role of pro-apoptotic effect in other types (Xiao et al., 2008; Hamidi et al., 2012). Distinct reduction of IER3 expression was found in human colorectal cancers (Nambiar et al., 2004), breast cancers (Hu et al., 2004) and pancreatic cancers (Sasada et al., 2008).

Additionally, it is found that IER3 may be a tumor therapy target for it can be affected by several drugs. For example, IER3 expression was stimulated by hydroxyl-tamoxifen, that the degree of increase was greater in resistant cells (Semplali et al., 2004). Bruheim S et al. reported that small interfering RNA-mediated gene silencing of IER3 sensitized the human osteosarcoma cell line OHS to treatment with 4-hydroperoxysamide (Bruheim et al., 2009). The increased P53 protein bind the promoter region of IER3 gene, which repress the transcription and result in down-regulation of IER3 gene expression. Transcriptional factor P53 was up-regulated after the addition of As₂O₃ in human osteosarcoma cell MG-63 (Xiao et al., 2008). It is reported that IER3 upregulation is correlated with a decrease level of intracellular ROS and low TNFR1 expression on malignant lymphocytes. Targeting IER3 could be of interest for the development of future therapeutic strategies for patients with Sezary syndrome (Akilov et al., 2012).

Positive of P53 mutant protein, ki-67and EGFR means tumor cell grow faster. Consistent with the studies mentioned above, The present study showed that positive expression of IER3 in tissues from PHC patients was well correlated with P53, ki-67, EGFR, tumor biggest diameter and differentiation grade. These results suggested that IER3 is an valuable independent biomarker for PHC. To our knowledge, this is the first report to analyze the IER3 expression in PHC tissues and to demonstrate the relationship between the IER3 expression and other clinicopathological parameters. These results suggest that IER3 may be involved in promotion of the progression of PHC.

Acknowledgements

This work was supported by grants from the national nature science foundations of China (Grant No. 81372632 and 81402579), the special funds of post doctoral innovation projects of Shandong province of China (Grant No.201303063) and the application of post doctoral funds of Qingdao City, Shandong province of China (Grant No.20130118).

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