

Analysis of *N-ras* gene mutation and p53 gene expression in human hepatocellular carcinomas*

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Abstract

AIM To study the relationship between *N-ras* gene mutation and p53 gene expression in the carcinogenesis and the development of human hepatocellular carcinomas (HCC).

METHODS The *N-ras* gene mutation and the p53 gene expression were analyzed in 29 cases of HCC by polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) and immunohistochemistry.

RESULTS Thirteen cases of HCCs were p53 positive (44.8%), which showed a rather high percentage of p53 gene mutation in Guangxi. The aberrations at *N-ras* codon 2-37 were found in 79.31% of HCCs and 80.77% of adjacent non-tumorous liver tissues. More than 2 point mutations of *N-ras* gene were observed in 22 cases (75.86%). Twelve cases (41.37%) of HCCs showed both *N-ras* gene mutation and p53 gene expression.

CONCLUSIONS *N-ras* gene and p53 gene may be involved in the carcinogenesis and the development of HCC. That 38% of HCCs with *N-ras* gene mutation did not express p53 protein indicates that some other genes or factors may participate in the carcinogenesis and the development of HCC.

INTRODUCTION

The hepatocellular carcinogenesis is presumably a multiple-step process and is influenced by many factors. The accumulation of genetic changes is necessary for emergence of hepatocellular carcinoma (HCC). Activation of pro-oncogenes and inactivation of tumor suppressor genes might be related to the carcinogenesis and the development of HCC. It has been reported that there was mutation of tumor suppressor gene p53 and overexpression of *N-ras* oncogene in human HCC^[1-3], but there have been few reports on the relationship between *N-ras* gene mutation and p53 gene expression. We analyzed the *N-ras* gene mutation by polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) and p53 protein by immunohistochemistry in 29 cases of human HCC in order to explore the relationship between these two kinds of genetic changes and human HCC.

MATERIALS AND METHODS

Clinical samples

Twenty-nine HCC surgical resected specimens were collected in Guangxi Cancer Institute during the period of 1987-1992, 28 of them had HCC adjacent liver tissues. All samples were fixed with 10% formalin, embedded in paraffin and stained with haematoxylin and eosin (HE).

Immunohistochemistry

Immunostaining was performed using a streptavidin-biotin immunoperoxidase method. p53 protein was detected with monoclonal anti p53 antibody (Oncogene Science, PAb DO1) and Strept ABC kit (DAKO A/S Denmark). The tissues were firstly microwaved 5min, 4 times and then used for immunohistochemistry.

DNA extraction

Genomic DNA was prepared by the proteinase K-Phenol-Chloroform extraction method.

PCR-SSCP

Oligomers that flank codon 2-37 of *N-ras* genes was synthesized as a primer by the Department of Molecular Medicine in King's College Hospital, UK. One of them was 5'-end labelled with r-³²P ATP by T4 Polynucleotide Kinase reaction. The

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primersets were: 5'-GACTGAGTACAACTGGTGG-3', 5'-GGGCCTCACCTCTATGGTG-3'.

The amplified product obtained by the PCR is 118bp. 0.1µg DNA taken from tissues, which was added with 9µl PCR mixture (containing 1pmol/L primer, 0.2mmol/L dATP, dGTP, dCTP, dTTP, 0.25U Taq DNA polymerase, 50mmol/L KCl, 10 mmol/L Tris, 2.5mmol/L MgCl₂ and 0.45% Tween 20), covered with mineral oil. PCR reaction underwent 5min denature at 94°C, then went to 35 cycles. One cycle included 30sec denature at 95°C, 1min annealing at 55°C, 1min 30sec extension at 72°C, and finally 10min extension at 72°C.

Two µl PCR product was added to 2µl dilution containing 95% formamide, 20mmol/L EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol dye. The mixture was denatured 5min at 95°C, then applied to a 6% polyacrylamide gel (21cm×40cm×0.4cm) with 1×TBE buffer at 45mA current and 45 Watt in cold room (4°C) for 4-5 hours. The gel was dried at 80°C and autoradiographed at -70°C.

RESULTS

Pathologic histology

The tumor specimens were all diagnosed as hepatocellular carcinomas. The histological diagnosis of tumor adjacent liver tissues were: 14 (50%) cases of chronic hepatitis, including chronic active type (CAH) and chronic persistent type (CPH); 6 of them had liver cirrhosis with CAH, 6 had liver cirrhosis and 2 cases acute hepatitis.

Point mutation of N-ras gene

The N-ras PCR-SSCP showed only 3 bands in normal control samples including human liver tissues (2 cases) and human placenta DNA (1 case). These 3 normal bands were also clearly discerned in all HCC and their adjacent tissues. Some of detected specimens presented mobility shift bands, which was the point mutation in N-ras gene. The aberrations at N-ras codon 2-37 were found in 79.31% of HCCs and 80.77% of adjacent non-tumor liver tissues, respectively. There was no significant difference between them (χ^2 test, $P>0.05$). Twenty-two of 29 HCC (75.86%) showed 2-5 mobility shifts.

p53 protein detection

p53 protein was mainly localized in HCC cell nucleus. Both nuclei and cytoplasmic staining were observed in 75% of p53 protein positive HCC. Non-tumor liver tissues showed only cytoplasmic type of p53 protein. p53 protein positive rate was 44.8% (13/29), 12 (41.37%) of them had N-ras mutation as well.

DISCUSSION

PCR-SSCP analysis is based on single strand DNAs

of the same nucleotide length in which the nucleotide sequences different at only one or some positions can be separated by polyacrylamide gel electrophoresis. DNA polymorphisms at a variety of positions in a fragment could cause a difference in its conformation and result in change in mobility of the single strands on gel electrophoresis.

In HCC, N-ras was first proved as one of the transforming genes^[4], which belongs to G protein family. When it is converted to active oncogene by point mutation, chromosome rearrangement or gene amplification, the signal transmission of cell membranes may change, which drives cell division, results in abnormal differentiation and finally forms neoplasm. Enhanced expression of N-ras gene in human HCC has been reported^[3]. The 'hot spot' mutation at codon 12, 13 or 61 in the N-ras gene has been described in many kinds of human cancers^[5]. But no point mutation around these 'hot spot' was found in HCC from South Africa^[6] and Japan^[5]. In China, in FANG Dian Chun's study^[7], 37.2% (16/43) of HCC showed N-ras gene point mutation at codon 12 by PCR-restriction fragment length polymorphisms (PCR-RFLP). In our study, the PCR product contained codon 12, 13 of N-ras. This gene point mutation was found in 79.31% (23/29) of HCC, indicating that the frequency is rather high in HCC from Guangxi region. In contrast to the reports about no N-ras gene point mutation or only one mutation at codon 12 in HCC, we found that 75.86% (22/29) of HCC had 2-5 point mutations around codon 2-37. This data showed that the mutation positions were not limited at codon 12 or 13. Therefore, if we only investigate the 'hot spot' mutation of N-ras gene, there is a possibility of missing mutations in other regions of the gene. The mobility shifts scattered at different positions. No clustering position could be considered as a hot spot in this study. We do not know the exact codon of mutation in this fragment by PCR-SSCP analysis. They could be confirmed by DNA sequencing.

SSCP bands can be clearly discerned in HCC (79.31%) and their adjacent liver tissues (80.77%). There was no significant difference between them, $P>0.05$. Most of these non-tumorous liver tissues presented as chronic hepatitis and/or liver cirrhosis, which was indicative of a close correlation with HCC. Our result indicates that N-ras gene mutations are involved in the carcinogenesis and development of HCC.

Many studies have shown that p53 abnormalities were involved in the genesis of HCC^[1-2]. One of the most common mechanisms leading to functional defect of this tumor suppressor gene in carcinogenesis is point mutations and expression of a conformationally altered protein that is immunohistochemically identifiable. Wild-type (normal) p53

protein has a very short half-life^[8] and can not be identified immunohistologically. In contrast, most mutant p53 protein becoming more stable with an extended half-life (4-5 hours) and being overexpressed in cells were readily detected by immunohistochemistry. We examined 29 HCC for p53 protein by ABC immunohistochemistry, 13 cases were found positive, accounting for 44.8% of HCC, which showed a high frequency of p53 gene mutation in HCC in Guangxi area.

It has been evidenced that mutation of the tumor suppressor gene p53 can convert *ras* gene into oncogene^[9]. The mutant p53 gene cooperation with mutant *ras* gene in cell transformation has been proved^[10]. We noticed that 12/29 (41.37%) cases of HCC had both mutations of N-*ras* gene and p53 gene. The result indicates that the mutations of N-*ras* gene and p53 gene may play an important role in the carcinogenesis and maintenance of HCC. It is interesting that 38% of HCC with N-*ras* mutations was p53 protein negative. The explanation might be that some other genes or factors except N-*ras* and

p53 participated in the formation and maintenance of HCC in Guangxi region.

REFERENCES

- 1 Bressac B, Kew M, Wands J, Ozturk M. Selective G to T mutations of p53 gene in hepatocellular carcinoma from Southern Africa. *Nature*, 1991;350(6317): 429-431
- 2 Hsu IC, Metcalf RA, Sun T, Welsh JA, Wang NJ, Harris CC. Mutational hotspot in the p53 gene in human hepatocellular carcinoma. *Nature*, 1991;350(6317): 427-428
- 3 Farshid M, Tabor E. Expression of oncogenes and tumor suppressor genes in human hepatocellular carcinoma and hepatoblastoma cell lines. *J Med Virol*, 1992;38(4):235-239
- 4 Gu JR, Hu LF, Wan DF, Tian PK, Guo C, Huang LH. Study on N-*ras* gene: the human primary liver cancer transformation gene. *Tumour*, 1985;5(2): 52-55
- 5 Tada M, Omata M, Ohto M. Analysis of *ras* gene mutations in human hepatic malignant tumors by polymerase chain reaction and direct sequencing. *Cancer Res*, 1990;50(4):1121-1124
- 6 Leon M, Kew MC. Analysis of *ras* gene mutations in hepatocellular carcinoma in Southern African blacks. *Anticancer Res*, 1995;15(3):859-861
- 7 Fang DC, Luo YH, Lu R, Xiao WH, Liu WW, Men RP *et al.* Studies of the point mutation of *ras* oncogene and suppressor gene p53 in 43 cases of hepatocellular carcinoma. *Acta Acad Med Militaris Tertiae*, 1994;16(5):340-343
- 8 Slagle BL, Zhou YZ, Butel JS. Hepatitis B virus integration event in human chromosome 17p near the p53 gene identifies the region of the chromosome commonly deleted in virus positive hepatocellular carcinomas. *Cancer Res*, 1991;51(1):49-54
- 9 Harris AL. Telling changes of base. *Nature*, 1991;350(6317):377-378
- 10 Hinds P, Finlay C, Levine AJ. Mutation is required to activate the p53 gene for cooperation with the *ras* oncogene and transformation. *J Virol*, 1989;63(2): 739-746