Methylation status of c-fms oncogene in HCC and its relationship with clinical pathology

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INTRODUCTION
The mechanism that DNA hypomethylation leads to activation of oncogene and occurrence of malignant neoplasm is being increasingly recognized by researchers. Normal DNA methylation plays an important role in stabilizing the phenotype of cell. DNA methylation status reduction and/or pattern alteration are related to activation and abnormally high expression of some oncogenes and cellular malignancy [1-6]. c-fms oncogene encodes for colony stimulating factor 1 receptor (CSF-1R) [7], c-fms/CSF-1R was highly expressed in hepatocellular carcinoma (HCC) tissue, but the mechanism remained obscure [8,9]. In this study, restrictive endonucleases Hpa II/Msp I digestion and Southern blot were used to study methylation status alteration of c-fms oncogene in HCC tissue and matching circum-cancer liver tissue, meanwhile the relationship between the alteration and clinical pathology of HCC was investigated. The gist of this study was to clarify the mechanism leading to c-fms oncogene high expression in hepatocellular carcinogenesis.

MATERIALS AND METHODS

Subjects
Thirty HCC patients were verified with pathological examination (25 males and 5 females, age range 32-76 years, mean 55 years). Fresh hepatic tissue of HCC focus and circum-tissues 2 cm from HCC focus were cut off, normal control was from HBV negative hepatic tissue of renal transplantation donor. All the tissues were washed with physiological saline and put in freezing tube, and then they were put in liquid nitrogen for storage.

Main reagents
Restrictive endonucleases Hpa II/Msp I were purchased from Japan TaKaRa Bio-Company. DIG random labeling and detection kit were purchased from Boster Bio-Company. Nylon transfer membranes used for Southern blot were purchased from Shanghai Bio-Company. c-fms plasmid was kindly provided by academician Gu Jianren in the National Laboratory for Oncogene and Related Genes, Shanghai Cancer Institute.

METHODS

DNA extraction
About 0.5g hepatic tissue was triturated in mortar, and a dissolvent (10 mmol/L Tris-Cl pH 8.0, 10 mmol/L EDTA pH 8.0, 0.5% SDS) 500 µL was added. The preparation was digested with proteinase K and RNase, then it was extracted twice with saturated hydroxybenzene and once with chloroform:isoamyl alcohol (chloroform:isoamyl alcohol = 24:1), and at last 2.5 volumes of absolute alcohol and 0.1 volume of 3 mol/L sodium acetate (pH 5.2) were added to precipitate the DNA. The extracted DNA was dissolved in TE buffer (pH 8.0), and DNA concentration was determined with an ultraviolet spectrophotometer.

Probe labeling
Plasmid extraction, restrictive endonucleases digestion, reclamation and purification were performed as the methods recorded in Molecular Cloning. The probe was labeled with DIG random labeling and detection kit.

Southern blot
Genome DNA 10 µg was digested with restrictive endonucleases Hpa II and Msp I 50U each for 12 hours respectively. Digested DNA was examined with agarose electrophoresis. The buffer used for electrophoresis was 0.5 x TBE. Electrophoresis was ended when 2 kb marker shifted to the middle of the gel. Photo was taken under ultraviolet ray transmission. The gel was immersed in 0.25 mol/L
HCl for 10 minutes, washed with distilled water for 20-30 minutes, and then immersed in denaturalizing liquid twice for 15 minutes each time. The gel was again washed twice with distilled water, and then it was immersed into neutralizing liquid twice for 15 minutes each time. A piece of nylon membrane of the same size as the gel was cut, it was immersed into 2 × SSC for 20 minutes. Transfer was performed for 18 hours at room temperature by the capillary transfer method. Then the nylon membrane was taken out and washed several times with 2 × SSC. After this, it was fixed for 20 minutes under long wave ultraviolet radiation. The transferred nylon membrane was sealed in a plastic bag and then pre-hybridizing reagent was perfused into the bag. The bag was put in a 42°C water bath for 2 hours, and then pre-hybridizing reagent was decanted off. The bag was sealed, after the hybridizing reagent containing the DIG-labeled probe had been put in. The bag was put into a 42°C water bath and hybridized for 20 hours. After hybridization the nylon membrane was taken out, and showed coloration according to the instruction of the DIG random labeling and detection kit.

Pathological examination and classification

The tumors were variously classified into unifocal or multifocal, and massive or nodular (a main tumor mass with satellites). The size and inner-condition of the tumors were recorded. The histological grading was based on Edmondson standard I-IV scale.

RESULTS

Southern blot of HCC tumor tissue and circum-cancer liver tissue

Hpa II and Msp I are isoschizomers. Both can digest CCGG sequence, but when the sequence is methylated into C-mCGG, only Msp I can digest while Hpa II can not. Methylation status of the gene may thus be determined by relying on such a difference. The data in this study showed that after digestion with Hpa II, hybridizing bands of less than 6.5kb in size were observed in 36.7%(11/30) of HCC tissue and 13.3%(4/30) of circum-cancer liver tissue, which resembled those of the hybridizing bands digested with Msp I. The results indicated that the methylation status of c-fms oncogene in the tissue was decreased (Figures 1,2).

Methylation status comparison of c-fms oncogene in HCC tissue and circum-cancer liver tissue

Hypomethylation rate of c-fms oncogene in HCC tissue and circum-cancer liver tissue was compared, the difference was determined, the result is shown in Table 1.

| Item          | Total number | HCC tissue | Circum-cancer liver tissue |
|---------------|--------------|------------|----------------------------|
| Sex           |              | Number     | %  | Number     | %  |
| Male          | 25           | 10         | 41.0 | 3       | 12.0 |
| Female        | 5            | 1          | 20.0 | 1       | 20.0 |
| Age(yrs) <60  | 22           | 7          | 31.8 | 2       | 9.1  |
| Age(yrs) ≥60  | 8            | 4          | 50.0 | 2       | 25.0 |
| Edmondson I-II| 14           | 2          | 14.3 | 1       | 7.1  |
| Edmondson III-IV| 16       | 9          | 56.3 | 3       | 18.8 |

Note: \( \chi^2=4.36, P<0.05 \), hypomethylation rate of c-fms oncogene in HCC tissue was higher than that in circum-cancer liver tissue.

Relationship between hypomethylation of c-fms oncogene and clinical pathology

The relationship between hypomethylation of c-fms oncogene in hepatic tissue and the sex, age and pathological Edmondson scale of patients was analysed; the results are shown in Table 2.

Chi-square test showed that there was no significant relationship between hypomethylation of c-fms oncogene and sex and age of patients \((P>0.05)\). There was significant relationship between Edmondson scale and hypomethylation of c-fms oncogene in HCC tissue \((\chi^2=5.66, P<0.05)\), while there was no significant relationship between Edmondson scale and hypomethylation of c-fms oncogene in circum-cancer liver tissue \((P>0.05)\).

Figure 1 Southern blot of HCC tissue genome DNA digested with Hpa II/Msp I.
Lane 1, 3, 5, 7, 9, 11 Msp I digestion; Lane 2, 4, 6, 8, 10, 12 Hpa II digestion; Lane 13 λDNA/Hind III markers; Lane 1, 2 normal control hepatic tissue; Lane 3-12 HCC tissue; Lane 8, 10 hybridizing bands of less than 6.5kb in size appeared after Hpa II digestion.
alterations of DNA. DNA methylation is a kind of modificatory mode of cellular DNA at transcription level. In human genome, CpG islands in house keeping genes including a great number of oncogenes are in the form of methylation. Transcription and expression of the methylated genes especially oncogenes are limited. Decline of DNA methylation status at special sites of oncogene induces abnormal expression of the gene.[28,40-43]

c-fms oncogene locates at 5q 33.3 of human chromosome and encodes CSF 1R, which has tyrosine kinase activity[41]. c-fms/CSF-1R is highly expressed in hepatocellular carcinogenesis, and its expressing level in HCC tissue is higher than that in circum-cancer liver tissue. Through the mechanism of signal transduction, CSF-1R stimulates the growth and development of HCC [45-50]. The whole length of c-fms oncogene is 43kb, in which there are 49 CCGG sites. The data in this study indicated that the hypomethylation rate of c-fms oncogene in HCC tissue was higher than that in circum-cancer liver tissue, while no hypomethylation of c-fms oncogene in normal hepatic tissue was observed. So hypomethylation is an important activating mechanism of c-fms oncogene in hepatocellular carcinogenesis. The data also showed that the hypomethylation rate of c-fms oncogene in Edmondson III-IV HCC was higher than that in Edmondson I-II HCC. All the evidence indicated that hypomethylation of c-fms oncogene might be an important late genetic incident in hepatocellular carcinogenesis. HCC with hypomethylation of c-fms oncogene may show the characteristics of higher malignancy and rapid development, and provide a clinical guide in the selection of surgery, radiotherapy and chemotherapy, and also in judging the prognosis of patients.

c-fms/CSF-1R is abnormally high-expressed in hepatocellular carcinogenesis at a late stage, meanwhile hypomethylation of c-fms oncogene occurs, and the hypomethylation rate of c-fms oncogene in HCC tissue is higher than that in the circum-cancer liver tissue. These data indicate that hypomethylation of c-fms oncogene is a kind of molecular mechanism leading to abnormally high CSF-1R expression and promoting the occurrence and development of HCC. So blocking the process of hypomethylation of c-fms oncogene, consequently decreasing a high expression of CSF-1R may have great clinical significance in decreasing malignant phenotype of HCC and improving prognosis of patients.

REFERENCES

1. Yao D, Jiang D, Huang Z, Lu J, Tao Q, Yu Z, Meng X. Abnormal expression of hepatoma specific gamma-glutamyl transferase and alteration of gamma-glutamyl transferase gene methylation status in patients with hepatocellular carcinoma. Cancer. 2006;88:761-769
2. Nagai H, Kim YS, Yasuda T, Ohmachi Y, Yokouchi H, Monden M, Emi M, Konishi N, Nogami M, Okumura K, Matsubara K. A novel sperm-specific hypomethylation sequence is a demethylation hotspot in human hepatocellular carcinomas. Gene. 1999;237:15-20
3. Tsuchiuchi T, Tsurumaki M, Sasaki Y, Takahama M, Konishi Y. Hypomethylation of Cpg sites and c-myc gene overexpression in hepatocellular carcinomas, but not hyperplastic nodules, induced by a choline-deficient L-amino acid-defined diet in rats. Jpn J Cancer Res
