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Original Article

Evaluation of serum LINE-1 hypomethylation as a prognostic marker for hepatocellular carcinoma

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ABSTRACT

Background and study aims: Global hypomethylation is one of the most consistent epigenetic changes in cancer. Development of hepatocellular carcinoma (HCC) must be understood as a multistep process with accumulation of genetic and epigenetic alterations. In the last decades, in addition to genetic alterations, epigenetic changes have been recognized as an important and alternative mechanism in tumourigenesis. We investigated the clinical implications of global hypomethylation in the sera of patients with hepatocellular carcinoma (HCC).

Patients and methods: PCR was used to assess the methylation status of long interspersed nuclear element type 1 (LINE-1) repetitive sequences in genomic DNA derived from sera of 50 patients with HCC, 20 patients with cirrhosis, 20 patients with chronic hepatitis C and 10 healthy subjects.

Results: Serum genome hypomethylation was significantly increased in patients with HCC ($p < 0.001$). The levels of serum LINE-1 hypomethylation at initial presentation correlated significantly with tumour size, tumour number and alpha-foetoprotein level. Moreover high serum LINE-1 hypomethylation correlates significantly with poor survival.

Conclusion: Serum LINE-1 hypomethylation may serve as a prognostic marker for patients with HCC.

Introduction

Hepatocellular carcinoma (HCC) represents one of the most common cancers worldwide, accounting for 500,000 new cases annually [1].

In the last three decades, cancer has been understood as a summary of altered genetic and epigenetic events. In contrast to genetic events, the epigenetic pathway is a reversible alteration and is characterized by three main mechanisms: (1) DNA hypermethylation leading to inactivation, (2) DNA hypomethylation causing genomic instability, (3) histone modifications affecting chromatin conformation.

These processes, especially aberrant DNA methylation and histone modifications are closely linked with each other by a protein complex of transcript activators and repressors and alter mRNA transcript expression of affected genes [2].

Characteristically, DNA methylation does not change the genetic information. It just alters the readability of the DNA and results in inactivation of genes by subsequent mRNA transcript repression [3].

Aberrant DNA methylation is involved in the initiation and progression of carcinogenesis and includes both hypermethylation of CpG islands at gene promoters and global hypomethylation. While a small portion of hypomethylation occurs at gene promoters, resulting in overexpression of certain oncogenes [4], the majority occurs at repetitive elements, such as long interspersed nuclear elements (LINE-1s or L1s) [5].

Despite remarkable improvements in surgical and ablative therapies, the overall prognosis of patients with HCC remains unsatisfactory because of its aggressiveness and high recurrence rates [6]. As a result, a reliable serum marker is needed for monitoring tumour progression, treatment responsiveness, and predicting prognosis. Although several molecular and biological factors related to HCC have been studied in recent years, a prognostic marker for this cancer in routine clinical practice is not yet available.

Our aim was to examine the methylation status of long interspersed nuclear element type 1 (LINE-1) in serum samples of patients with HCC, and compare it with that of healthy individuals, patients with cirrhosis and chronic hepatitis C. Clinicopathological correlations and prognostic significance of this epigenetic alteration in sera of patients with HCC were also evaluated.

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Patients and methods

This study was conducted in the period between April 2008 and May2010. It was performed on ninety patients and 10 healthy subjects; fifty patients had hepatocellular carcinoma, twenty patients had liver cirrhosis and twenty patients had chronic hepatitis C (based on the presence of persistently elevated liver enzymes for at least 6 months and detection of HCV RNA by PCR technique).

HCC was diagnosed by histopathology and/or two imaging modalities (ultrasound [US], triphasic computed tomography [CT]) showing vascular enhancement. Diagnosis of cirrhosis was based on clinical, laboratory and imaging evidence of hepatic decompensation or portal hypertension. Informed consent was obtained according to the regulations of the local ethical committee.

Overall survival time of HCC patients was defined as the period from initial presentation to the time of last follow-up (May 2010) or till death.

The selected patients were set into four groups: Group A consisted of 50 patients with HCC, group B consisted of 20 patients with liver cirrhosis, group C consisted of 20 patients with chronic hepatitis C and group D consisted of 10 healthy subjects. Patients with other malignancies were excluded.

All patients were subjected to history taking, clinical examination, lab. investigations including liver biochemical profile and AFP, evaluation of serum LINE1 hypomethylation using PCR. Serum samples were collected from each subject at the time of their clinical evaluation and stored at -70 until further tested.

DNA was extracted from serum samples using QIA amplification blood kit (Qiagen, Hilden, Germany).

One μg DNA (concentration 50 ng/μL) of the extracted DNA was denatured by sodium hydroxide (0.22 mol/L) at 37 °C for 10 min. Thirty μL of hydroquinone(10 mmol/L) and sodium bisulfite (520 μL) of concentration 3 mol/L were added. The mixture was incubated for 20 h at 50 °C. DNA purification was done by adding sodium hydroxide (0.33 mol/L) at 25 °C for 3 min. DNA was precipitated and washed with ethanol. Bisulfite-treated DNA was resuspended in 20 μL of RNase free water and stored at −20 °C until use. After bisulfite treatment, the conversion of C into T was expected to be 100%. It was possible to insert a C/T single-nucleotide polymorphism into the sequence to be analyzed that will result in a 100% T if conversion was efficient.

The treated DNA with bisulfite was amplified using 5′ UTR of LINE-1.2 sequence from NCBI Accession Number M80343. The sequence of the primers was as follows:

5′-CCGTAAGGGGTTAGGGAGTTTTT-3

5′-TAAAACCCTCCAAACCAAATATAAA-3. The amplified product was 160 bp.

The PCR mixture (total volume 50 μl) was formed of 2 μL of the treated DNA, 5 μl of 10× reaction buffer with MgCl2 (Promega, Madison, WI, USA), 50 Pmol of each primer (sense and antisense), 100 μmol/μl dNTPs each (Perkin–Elmer Corporation, Foster City, CA, USA) and 2 units Taq DNA polymerase (Amersham Pharmacia Biotech, USA).

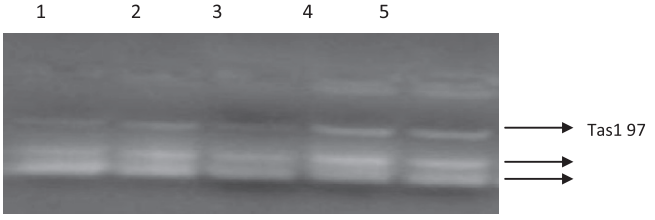

Fig. 1. Combined bisulfite restriction analysis of LINE-1 (COBRA LINE-1) in sera (Lanes 1 and 2: healthy control; Lanes 3–5: cirrhotic patients).

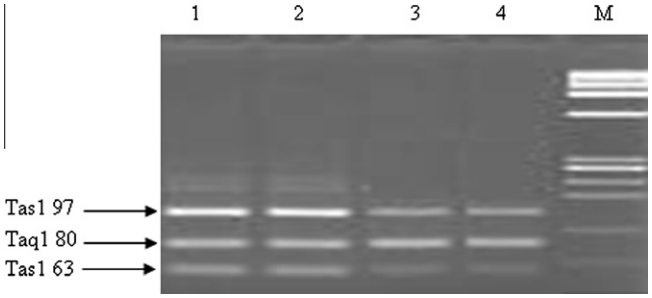

Fig. 2. Combined bisulfite restriction analysis of LINE-1 (COBRA LINE-1) in sera (M: marker; Lanes 1 and 2: chronic hepatitis patient; Lanes 3 and 4: HCC patients).

The Cycling condition was as follows: denaturation at 95 °C for 5 min. followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 50 °C for 45 s and extension at 72 °C for 1 min. Final extension cycle of 72 °C for 7 min. was done.

For measurement of hypomethylation 10 μl of PCR products were digested using 2U of Taq1 or 8U of Tas1 restriction enzymes (MBI Fermentas) at 65 °C overnight and the resultant fragments resolved on a 12% polyacrylamide gel. The LINE-1 amplicon size is 160 bp. After digestion, the methylated amplicons, Taq1 positive yielded two fragment length of 80 bp DNA. The unmethylated amplicons, Tas1 positive yielded two fragments of length of 63 and 97 bp DNA (Figs. 1 and 2).

The percentage of LINE-1 hypomethylation was expressed as percentage (%):

$$\frac{\text{The intensity of unmethylated LINE-1digested with Tas1}}{\text{methylated LINE-1 digested with Taq1} + \text{methylated LINE-1 with Tas1}}$$

Statistical methods

Analysis of the data was done using Software Package for Social Science (SPSS). The quantitative variables were described in the form of mean and standard deviation, median and range when appropriate. The qualitative variables were described in the form of frequency and percentages. Unpaired *t*-test (*t*-value) was used to compare a quantitative variable between two independent groups in parametric data. AFP values were transformed into their log values as they were not of normal distribution. Chi square test (χ^2 value) was used to compare a qualitative variable between two independent groups. Pearson correlation was used to correlate quantitative variables fulfilling normal distribution and Spearman correlation test (rho value) was used to rank different nonparametric variables against each other, either positively or inversely. *p*-value (which is either non significant (NS) if >0.05, significant (S) if <0.05, or highly significant (HS) if <0.01) was calculated. The analysis of overall survival was calculated by the Kaplan–Meier method.

Results

The ages of the studied groups ranged from 21 to 83 years with a mean of 50.24 ± 13.40 and a median of 54 years. The age and gender of patients of different groups were presented in Table 1. HCC incidence was more in middle and old ages. It was about two times more in males than in females.

All HCC patients had cirrhosis as underlying liver disease.

Twenty-three patients (46%) had a single focal lesion, and twenty-seven patients (54%) had multiple focal lesions. Twenty lesions (40%) were <5 cm while 30 lesions (60%) were >5 cm in diameter. Portal vein was patent in 45 patients (90%) and thrombosed in five patients (10%).