Supplementary Material

Analysis of Lamin B1, Vimentin and Anti-Ku86 as Prospective Biomarkers of Hepatocellular Carcinoma in Patients with Hepatitis C Virus Infection

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Supporting information

A. Routine Laboratory investigation:

- Complete blood picture was done on Sysmex KX-21(Wakinohama-kaigandori, Japan).
- Urea ,creatinine (by Hitachi 902)
- Prothrombin time and concentration (by sysmex)
- Liver function tests:alanine transaminase, aspartate transaminase, albumin, total protein, total bilirubin, direct bilirubin, alkaline phosphates, gamma-glutamyl transferase (GGT). Were conducted using Integra400 autoanalyzer (Roche-Diagnostics,Germany).
- HBsAg and hepatitis C virus antibody tests were conducted by the electrochemiluminescence immunoassay 'ECLIA' using Elecsys 2010 autoanalyzer (Roche-Diagnostics). Measurement of serum AFP was taken by Elecsys2010 autoanalyzer (Roche-Diagnostics). *AFP by chemiluminescence using immulite 1000.
- HCV anti-bodies by architect system in a two step immunoassay,using chemiluminescent microparticles immunoassay (CMIA) technology ,for the qualitative detection of anti-HCV in human serum

B. Chemical s and Reagents

The following items show the commercial sources and some characterizations of purchased chemicals used in

***1*. Biochemical assessment of Serum anti Ku86**: The level of the Human Anti 86 kDa subunit of Ku antibody was measured using a human Ku antibody (Ku86) ELISA kit according to the manufacturer's protocol [(Catalog #MBS749325,(MyBioSource's Products)]

Reactivity: human

Method type: sandwich ELISA detection

Quantity: 96 tests

Sample type: serum

Detection range: 15ng/ml-400ng/ml

Reagent preparation

1-wash buffer (1x)-if crystals have formed in the concentrates ,warm up to room temperature and mix gently until the crystals have completely dissolved .dilute 20 ml of buffer concentrate (30 x) into deionized or distilled water to prepare 600 ml of wash buffer (1 x)

2- Standard

Dilute the standard: pipette 50ul standard dilution in each tube . pipette 100ul standard (540ng/ml) in the fifth tube .and take out 100ul from the fifth tube into the fourth. pipette 50ul from the fourth tube to the third tube and produce dilution series .the undiluted standard serves as the high standard (540ng/ml).sample diluent serves as the zero standard (blank well)(0ng/ml)

Assay procedure:

Step 1: prepare all reagent, working standards, blank and samples as directed in the previous sections .

Step 2 : refer to the assay layout sheet to determine the number of wells to be used and put remaining wells and the desiccant back into the pouch and seal the Ziploc, store unused wells at 4 c

Step 3 : pipette standard 50ul to testing standard well ,pipette sample dilution 40ul to testing sample well ,then add testing sample 10ul (sample final dilution is 5-fold),pipette sample to wells to, do not touch the well wall as far as possible ,and mix gently

Step 4: incubate: cover with the adhesive strip provided ,incubate for 30 min at 37 c

Step 5: configurate liquid: dilute wash solution 30 fold with distilled water.

Step 6: washing: uncover the adhesive strip , discard liquid ,pipette washing buffer to every well ,still for 30s the drain ,repeat 5 times.

Step 7: add enzyme: pipette HRP-conjugate reagent 50ul to each well ,except blank well .

Step 8: incubate: operation with 4.

Step 9 : washing :operation with 6.

Step 10: color: pipette chromogen solution a 50ul and chromogen solution B to each well, avoid the light preservation for 15 min at 37 c

Step 11: stop the reaction: pipette stop the reaction (the blue change to yellow)

Step 12 : calculate :take blank well as zero .read absorbance at 540 nm after pipette stop solution within 15 min

Calculation of result:

Take the standard concentration as horizontal ,the OD value for the vertical, draw the standard curve on graph paper .find out the corresponding concentration according to the sample OD by the sample curve ,multiplied by the dilution multiple ,or calculate the straight line regression equation of the standard curve with the standard concentration and OD value , with the sample OD value in the equation ,calculate the sample concentration ,multiplied by dilution factor ,the result is the sample actual concentration.

2. RT-PCR of Lamin B1 (*LMNB1*), Vimentin(*Vim*) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)mRNA from HumanPlasma.

Aim of The PCR Protocol :The test is based on these major processes: sample preparation, RNA extraction, RT-PCR amplification of target DNA using LMNB1 specific complimentary primers, hybridization of the amplified products to SYBER green dye and detection of the SYBER green dye-bound amplified products by colorimetric determination.

(1) RNA extraction from whole blood: Total RNA was extracted from 0.5 ml up to 1.0 ml of whole blood using Quiagen Mini Kit 250 Cat No./ID: 74106.

(2) Kit is tested functionally for use in RT-PCR. The SensiFAST™ SYBR® Hi-ROX One-Step Kit .It has been optimized for fast, efficient, unbiased cDNA synthesis and subsequent highly-sensitive, reproducible real-time PCR detection in a single tube.

(3) PCR amplification was performed in a 20 μL reaction mixture containing 3 μL of cDNA template and 200 nM of each primer *LMNB1* . The primer sequences for ***LMNB1*** were 5′-TCGCAAAAGC ATGTATGAAGA 3′ (sense) and 5′-CTCTACCAAGCGCGTTTCA-3′ (antisense) and ***Vim*** 5'-GCTGCAGGCCCAGATTCA-3' , 5'-TTCATACTGCTGGCGCACAT-3'for ***GAPDH*** were 5′-AGCCACATCGCTCAGACAC-3′ (sense) and5′-GCCCAATACGACCAAATCC-3′ (antisense).glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)]. *GAPDH*was used as an internal control for PCR quality

(4) PCR amplification was done by the following steps: (a) Denaturation: generally, a 2 min initial denaturation step at 95°C is sufficient. (b) Annealing: optimize the annealing conditions by performing the reaction starting 5°C below the calculated melting temperature of the 95 primers (63°C) and increasing the temperature in increments of 1°C to the annealing temperature. The annealing step is typically 1 min at 65°C.(c) Extension: the extension reaction is typically performed at the optimal temperature forDNA polymerase, which is 2°C. A final extension of 2 min at 72°C is recommended.

(5) The step of reverse transcription is repeated using house keeping gene GAPDH.

(6) Hybridization of the amplified products to SYBRgreen dye: This step is done using 5′ Hot FirepolEva Green qPCR Mix Plus catalog number: (08-25-0001). Pack size: 1 ml (250 reactions).Purchased from (Solis BioDyne, Tartu, Estonia).

(7) Calculation of relative expression level: The mean expression levels of LMNB1 mRNA relative toGAPDH mRNA level were calculated using thequantitation-comparative *C*t ($\Delta\Delta C$t) method on theABI 7500 fluorescence quantitative PCR

analyzer. After correction with the corrector sample, the relative quantitation values of the expression levels were used for the statistical analysis. The RT-PCR assay was repeated twice and PCR products were size fractionated by and confirmed by DNA sequencing and visualized under UV light

(8) **Gel Electrophoresis: PCR** products were size fractionated on 2% agarose gels, after ethidium bromide staining PCR products and visualized using an ultraviolet illuminator. The PCR products were size fractionated by and confirmed by DNA sequencing and visualized under UV light.

Reagents and Materials: for preparation: ● tank, tray, comb • normal melting point agarose powder • 10 x TBE buffer solution, gel stain • microwave oven, Erlenmeyer flask, measuring cylinder, scales for loading: pipette, PCR tubes or , power supply for documentation: camera/ gel documentation system

1. Formulate sufficient electrophoresis buffer (1:10 dilution of TBE:distilled water)
2. Position the comb 0.5-1 mm overhead the plate so that a widespread well is formed when the agarose is added. 2. - Prepare agarose gel. For a 2% agarose gel:
3. After 30 minutes at room temperature carefully remove the comb. - Location the gel into the gel electrophoresis tank. Improve sufficient TBE buffer to security the gel to a complexity of about 5 mm.
4. Loading. Mix the DNA samples with gel-loading buffer with pipettes: 1/10 of PCR reaction volume Prepare marker DNA of known size: 6 µl from Promega Marker
5. Load the mixtures gradually into the slots. Evade making bubbles.
6. Attach the electrical leads so that DNA can move toward the anode (red lead).
7. Run the gel until the gel-loading buffer stain has migrated the appropriate distance
8. Documentation: Examine the gels: Carefully by placing it on an ultraviolet transilluminator and take a photo.