ID: ZJCH-CK19A

Initial version (1.0) date: Nov.11, 2016

Final version (2.0) date: Feb.2, 2017

NCT02992067

CK19 Combined With Contrast-enhanced Ultrasound: a Prediction System on Axillary Lymph Node Metastasis in Breast Cancer

Study Protocol with SAP

OBJECTIVE:

Based on our previous research, we developed a model predicting the risk of lymph node involvement in early breast cancer patients by combining CK19 mRNA in peripheral blood and Contrast-enhanced Ultrasound.

DESIGN:

Recruiting patients diagnosed cI, cII or cT3N1M0 (operable) invasive breast cancer as training set. In these patients, we tested the CK19 mRNA in peripheral blood and contrast-enhanced ultrasound (CEUS). With these two parameters and other clinical pathological characteristics, we can build a model calculating the risk of lymph node involvement by Logistic method. The predicting accurate, false negative rate will be evaluated firstly in the training set.

METHODS:

We identified cases diagnosed as operable invasive breast cancer from Dec 2016 to Aug 2018 in Zhejiang Cancer Hospital.

- Sampling Method: Probability Sample

Minimum Age: 18 Years
Maximum Age: 70 Years
Sex:Female

Gender Based:
Accepts Healthy Volunteers: No

- Criteria:

Inclusion Criteria:
female, age 18~70 years old
the test group should be in accordance with the pathological diagnosis of breast cancer, the clinical stage of breast cancer (stage cTis, cI, cII and cT3N1M0), the initial treatment to receive surgical treatment. Complete preoperative clinical diagnosis data, including the ipsilateral axillary lymph node CEUS data and peripheral blood sample; The negative-control group should comply with the pathological diagnosis, breast adenosis, fibrocystic lesions, breast cysts and other benign lesions, with complete preoperative clinical data, also including ipsilateral axillary lymph node CEUS data and peripheral blood sample
Exclusion criteria:
metastatic breast cancer, inflammatory breast disease, surgery without lymph node staging
pregnancy or lactation

patients with hematopoietic system disease or cancer, autoimmune diseases preservation of substandard peripheral blood samples.

- CK19 mRNA testing

Peripheral blood sample collection

Patient peripheral blood samples were collected one day before operation. A total of 10 ml anticoagulated peripheral blood was taken from each patient. The first 2 ml of blood was discarded to avoid potential contamination by normal epithelial cells, and the remaining 8 ml was collected into 20-ml sterilized tubes containing EDTA.

Specimen processing

One ml of saline and 2 ml of lymphocyte separation medium were added to 1 ml of anticoagulated blood. Samples were centrifuged at 2500 rpm for 20 min after

standing, and the white cell layer was drawn out and centrifuged at 12000 rpm for 5 min. Trizol (1 ml) was added to the precipitate, and was fully mixed. We then added 0.2 ml chloroform to the precipitate, which was mixed on a shaker at 4°C for 5 min and then centrifuged at 12000 rpm for 10 min. We collected 500 ul of the upper aqueous phase into a sterilized centrifuge tube and added an equal volume of isopropanol. The tube was gently mixed at 4°C for 10 min and then centrifuged at 12000 rpm for 15 min.

RNA preparation

The RNA precipitate was washed with 1 ml of 75% ethanol and centrifuged at 7500 rpm for 5 min at 4°C. Total cellular RNA was extracted by the acid guanidium thiocyanate-phenol-chloroform extraction procedure [20]. The RNA was resuspended in 25 ul of diethy pyrocarbonate (DEPC)-treated water and spectrophotometrically quantitated at 260 nm and 280 nm to assess purity. Extracted RNA was treated with RNase- free Dnase I (TaKaRa Biotechnology, Da Lian, China) according to the manufacturer's instructions to remove contaminating DNA.

Reverse transcription reaction

CK19 reverse transcription was performed according to manufacture's instructions. Briefly, 1 ug of RNA was reverse-transcribed for 15 min at 37°C in a 25 ul reaction mix containing 0.5 ul PrimeScript RT enzyme mix I, 0.5 ul oligo dT primer, 2 ul random 6-mers and 10 ul RNase free dH_2O. The reaction

was terminated by heating at 85°C for 5 sec.

RT-PCR

CK19 primers were designed from previously published sequences [21]. The sense primer, 5'-AAGCTA ACCATGCAGAACCTCAACGACCGC-3', and antisense primer, 5'-TTATTGGCAGGTCAGGAGAAGAGCC-3', were used for the first round of amplification. The second PCR amplification was carried out using the CK19 sense primer, 5'-TCCCGCGACTACAGCCACTACTAC ACGACC-3', and antisense primer, 5'-CGCGACTTGATG TCCATGAGCCGCTGGTAC-3'. PCR reaction conditions were as follows: 94°C for 3 min and 94°C for 30 sec for 40 cycles, followed by a final extension at 60°C for 35 sec. All RT-PCR products were separated by electrophoresis in a 1.5% agarose gel and were analyzed automatically via computer. CK19 levels were considered positive when copies ≥ 1000.

- CEUS

GE Logic E9 ultrasonic diagnostic instrument was adopted. During angiography with 9L probe, the transmitting frequency of the probe was 8.4-9.0mhz, and the mechanical index was 0.11. Parameters: 2 harmonics, gain 125, and time gain compensation remained unchanged during each angiography. SonoVue lyophilized powder (containing 59mg sulfur hexafluoride microbubbles, Bracco, Italy) was used for contrast. During the angiography, 1 SonoVue was taken, 5ml of normal saline was used to prepare the suspension, and the suspension was oscillated for 30s for later use. According to the results of general ultrasound examination, the axillary lymph nodes were scanned and the target suspected lesions were selected for display. After shaking the standby suspension well, 2.4ml was extracted and injected through the anterior cubital vein group, and then 5ml normal saline was injected to flush the tube, and the movement and distribution of contrast agent microbubbles in the lesions were immediately observed. The dynamic observation time was 3 minutes, and the image was saved for analysis by ITC software. The results of each report were independently determined and recorded by two tumor ultrasound doctors with secondary or higher titles. According to the references and the European federation of ultrasound medicine and biology, ceus guidelines for non-liver clinical application, the following criteria were used to evaluate the scores :(1) lymph node morphology, short diameter >1cm or long/short diameter <2(1 point), otherwise 0 point; (2) lymph node morphology, short diameter >1cm or long/short diameter <2(1 point); (2) cortex ≥3mm 1, cortex < 3mm 0; (3) mixed, non-uniform enhancement or no enhancement 2 points, 0 points of uniform enhancement; Calculate the total score and record it. Doctors in the two groups reported consistency test, random sampling of 30 cases (sampling rate of 10%), only one item in the three aspects of morphology, cortex and enhancement was inconsistent in the two cases, scoring coincidence rate reached 93.33%.

STATISTICAL ANALYSIS PLAN (SAP)

SPSS (v22.ibm ® Statistics) was used for data collection and statistical analysis. The general data hypothesis test level was set as a =0.05 (double-tailed), and H1

hypothesis was accepted when $P < 0.05$, i.e. the statistical difference between the two samples was statistically significant. Calculation of sample size required for modeling (diagnostic experiment) : n= (U alpha/delta) 2× (1-p) ×P. U alpha: alpha =0.05, U alpha =Z alpha /2=1.960. In the modeling standard study, delta =0.06, P sensitivity =0.9, P specificity =0.8, plus 10% of the additional sample size, the result is 310.06. In SLNS and detected nSLN pathological diagnosis of lymph nodes (gold standard: positive/negative) as dependent variable, incorporated into the general clinical data, CK19 were a grade and CEUS, application of binary linear regression model, the input method (Enter) to screen effective modeling variables, Forward the method to estimate the maximum likelihood ratio (Forward LR, higher reliability, the parameter estimation and Wald statistics) to establish the forecast model of combined method of formula of calculating the SLNS and nSLN detected probability of each case goal. With the pathological diagnosis of lymph nodes as the gold standard, the four grid table method of diagnostic test was used to calculate the prediction effect indexes of the prediction model formula: sensitivity (SEN), specificity (SPE), accuracy (AR) and Youden index. The prediction efficiency test of the prediction model formula is: area under the ROC curve (AUC); Hosmer and Lemeshow test was used to test the goodness of fit of the prediction model formula.