**Name of journal:** ***World Journal of*** ***Gastroenterology***

**Manuscript NO: 35675**

**Manuscript Type: ORIGINAL ARTICLE**

***Case Control Study***

**Multi-parameter genes expression profiling in peripheral blood for early detection of hepatocellular carcinoma**

Xie H *et al*. Early detection of HCC

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**Supported by** National Key R&D Program of China, No. 2016YFC0106604; and National Natural Science Foundation of China, No. 81471761 and 81501568.

**Institutional review board statement:** The study was reviewed and approved by the 302 Hospital of People's Liberation Army and Institutional Review Board.

**Informed consent statement:** All study participants or their legal guardians provided written informed consent prior to study enrollment.

**Conflict-of-interest statement**: We declare that we have no financial or personal relationships with other individuals or organizations that can inappropriately influence our work and that there is no professional or other personal interest of any nature in any product, service and/or company that could be construed as influencing the position presented in or the review of the manuscript.

**Data sharing statement:** The study participants provided informed consent for data sharing. No additional data are available.

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**Manuscript source:** Unsolicited manuscript

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**Received:** August 9, 2017

**Peer-review started:** August 9, 2017

**First decision:** August 29, 2017

**Revised:** October 16, 2017

**Accepted:** November 22, 2017

**Article in press:**

**Published online:**

**Abstract**

***AIM***

In our previous study, we have built a 9 genes (*GPC3*, *HGF*, *ANXA1*, *FOS*, *SPAG9*, *HSPA1B*, *CXCR4*, *PFN1* and *CALR*) expression detection system based on the GeXP system. Based on peripheral blood and GeXP, we aim to analyze the results of genes expression by different multi-parameter analysis methods and build a diagnosis model to classify the hepatocellular carcinoma (HCC) patients and healthy people.

***METHODS***

Logistic regression analysis, discriminant analysis, classification tree analysis and artificial neural network were used for the multi-parameter genes expression analysis method. 103 early stage of HCC patients and 54 age matched health normal control were were used to build the diagnosis model. 52 early stage of HCC patients and 34 healthy people were used for validation. The area under the curve, sensitivity and specificity were used as the diagnosis indicators.

***RESULTS***

Artificial neural network of the total 9 genes had the best diagnosis value, the AUC, sensitivity and specificity were separately 0.943, 98% and 85%. At last, 52 HCC patients and 34 healthy normal controls were used for validation. The sensitivity and specificity were separately 96% and 86%.

***CONCLUSION***

Multi-parameter analysis methods may increase the diagnosis value compared to the single factor and it may be a trend of the clinical diagnosis in the future.

**Key words:** Hepatocellular carcinoma; Peripheral blood; Early detection; Multi-parameter; Diagnostic value

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**Core tip:** We aim to analyze the results of our previous 9 genes expression by different multi-parameter analysis methods and build a diagnosis model to classify the hepatocellular carcinoma patients and healthy people. Logistic regression analysis, discriminant analysis, classification tree analysis and artificial neural network were used for the multi-parameter genes expression analysis method.

Xie H, Xue YQ, Liu P, Zhang PJ, Tian ST, Yang Z, Guo Z, Wang HM. Multi-parameter genes expression profiling in peripheral blood for early detection of hepatocellular carcinoma. *World J Gastroenterol* 2017; In press

**INTRODUCTION**

Hepatocellular carcinoma (HCC) is one of the most common cancers in the world[1]. Chronic infection with hepatitis B or C virus, dietary aflatoxin B1 intake and alcohol abuse clearly show a significant correlation with the incidence of HCC. In China, > 90% of HCC patients are reported to experience chronic HBV infection[2]. Currently, HCC is often diagnosed at an advanced stage and has a poor prognosis. Clinical practice has demonstrated that early diagnosis of HCC can significantly increase the survival time. Many biomarkers have been proposed, and some are currently used in clinical diagnosis[3, 4]; however, even AFP, the most widely used biomarker for HCC diagnosis, has a poor diagnostic value[5]. Although pathology is used as a gold standard for diagnosis of HCC, it is invasive, and tissue samples are not easily obtained. Therefore, a non-invasive, accurate, and fast method for early detection of HCC is urgently needed.

Peripheral blood samples, which are easily and repeatedly obtained in the clinical setting, have been demonstrated to be valuable for disease prediction and classification, drug response and toxicity classification[6-8]. These features make peripheral blood samples attractive to aid in the early detection of HCC[9]. As we know, HCC is a complex multi-gene and multi-factorial disease, and a single biomarker is not adequate to reflect the HCC status. A panel of biomarkers is a promising method for early detection of HCC, and now some panels have been used for cancer prediction[10, 11]. A single gene analysis method is not sufficient when gene expression is used for diagnosis. Multi-parameter analysis methods, such as logistic regression analysis (LRA), discriminant analysis (DA), classification tree analysis (CTA) and artificial neural networks (ANN), which can analyze multiple factors, have been shown to increase the sensitivity and specificity of diagnosis and may be promising analysis methods for multi-parameter analysis[12-14].

In our previous study, we used Affymetrix to screen differential gene expression and built a 9-gene (*GPC3*, *HGF*, *ANXA1*, *FOS*, *SPAG9*, *HSPA1B*, *CXCR4*, *PFN1* and *CALR*) expression detection system based on the GenomeLab GeXP Genetic Analysis system[15], known as GeXP, which can detect up to 35 genes in one reaction[16]. Based on peripheral blood and GeXP, we compared multi-parameter gene expression using various multi-parameter analysis methods and built a diagnostic model to classify early-stage HCC patients and healthy people.

**MATERIALS AND METHODS**

***Patients and blood collection***

The study was reviewed and approved by the 302 Hospital of People's Liberation Army & Institutional Review Board. After obtaining patient consent, blood samples from 103 early-stage HCC patients with chronic HBV infection were collected in our hospital. Fifty-four age-matched healthy normal control samples were collected from the people who underwent a health examination. Both samples were used to build the diagnostic model. Fifty-two early-stage HCC patients and 34 healthy people were used for validation. The disease status of early-stage HCC patients was confirmed by histopathological analysis, and tumors were staged according to the Barcelona Clinic Liver Cancer (BCLC) staging classification as either T1 (single lesion < 2 cm in diameter) or T2 (single lesion between 2 and 5 cm in diameter or < 3 lesions each < 3 cm in diameter)[17]. In addition, peripheral blood from HCC patients was collected before any therapy. The clinical characteristics of all the samples used for this study are shown in Table 1.

Peripheral blood (2.5 mL) was collected and added into the PAXGene blood RNA tubes (Qiagen, Valencia, CA); After inverting, the tubes were stored at -80 °C until the total RNA extraction.

***RNA isolation and GeXP gene expression detection***

Total RNA was isolated according to the PAXGene Blood RNA Kit (Qiagen, Valencia, CA) instructions. The quality and quantity of RNA was measured by agarose gel electrophoresis and DU 800 spectrophotometry (Beckman Coulter, Fullerton, CA). The primers for the 9 genes were shown in our previous study. Reverse and forward primers were diluted 1 : 200 and 1 : 500 in nuclease-free water to a final concentration of 500 nmol/L and 200 nmol/L, respectively. Total RNA (50 ng) was used for reverse transcription (RT) with chimeric reverse primers in a single reaction. The concentrations of the primers were diluted at a ratio of 1 : 8. The RT reactions were 48 °C for 1 min, 42 °C for 60 min, 95 °C for 5 min and then held at 4 °C.

The polymerase chain reaction (PCR) reaction was performed in the 96-well PCR Detection Plate. The PCR reaction (10 μL) was 95 °C for 10 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 seconds, and 68 °C for 1 minute. The PCR products were diluted using nuclease-free water and added to the detection plate, which contained sample loading solution and DNA Size Standard 400. The GeXP system was used to match each gene fragment size and to measure the fluorescent dye signal strength in arbitrary units, and then the dataset was normalized to the housekeeping gene B2M. Finally, the dataset was log transformed.

***Multi-parameter gene expression analysis***

LRA[18, 19], DA[20, 21], CTA[22, 23] and artificial neural network analysis[24, 25] were used as the multi-parameter gene expression analysis methods. In LRA, the “Forward: Conditional” method was used to select variables. The stepwise probability was “Entry 0.05” and “Removal 0.10”, the “Probabilities” was saved, and then “CI for exp (B) 95%” was shown. The “Probabilities” of the HCC group were used for receiver operating characteristic (ROC) analysis, and the cutoff value was based on the Youden Index. In DA, “Use stepwise method” was used to select variables. The stepwise criterion was the “F value”. The “Entry” was 3.84 and the “Removal” was 2.71. The “Probabilities of group membership” was saved as a new variable, “Dis\_n,” which represented the probability of HCC, and then the “Probabilities of group membership” was used for ROC analysis. In the CTA, “Exhaustive CHAID” was used as a growth method, and 20% of the samples were selected as the validation samples. The “Predicted value” was saved as a new variable for the ROC analysis. In the artificial ANN, the ROC curve was saved, and 80% of the samples were used as the training group and 20% as the test group. After comparison, the best diagnosis model was chosen, and 52 HCC samples and 34 healthy normal controls were used to validate the model. The ROC curve, area under the curve (AUC), sensitivity and specificity were used as diagnostic indicators.

**RESULTS**

***Single gene used for diagnostic evaluation***

A total of 9 genes (*GPC3*, *HGF*, *ANXA1*, *FOS*, *SPAG9*, *HSPA1B*, *CXCR4*, *PFN1* and *CALR*) were used for HCC detection. We used the ROC curve of the 9 genes to evaluate the diagnostic value and then analyzed the *P* value and 95% confidence interval (CI). The diagnostic value of the AUC, the *P* value, 95% confidence interval, cutoff value, sensitivity and specificity are shown in Table 2. The ROC curves from 4 genes (*HGF*, *ANXA1*, *SPAG9*, and *PFN1*) showed *P* values less than 0.05. According to the *P* values, 4 genes (*HGF*, *ANXA1*, *SPAG9*, and *PFN1*) were chosen to have diagnostic value. In the multi-parameter analysis, both the 9-gene and the 4-gene sets were used.

***Multi-parameter logistic regression analysis***

In the LRA for HCC detection, we compared the diagnostic value of the full 9-gene set (*GPC3*, *HGF*, *ANXA1*, *FOS*, *SPAG9*, *HSPA1B*, *CXCR4*, *PFN1* and *CALR*) and the 4-gene set (*HGF*, *ANXA1*, *SPAG9* and *PFN1*). From the full 9-gene set, 5 genes were selected using a “Forward: Conditional” method. The diagnosis formula was as follows:

Y = -4.089 + 21.269 XANXA1 - 5.339 XFOS + 32.543 XSPAG9 - 2.743 XCXCR4 + 9.524 XPFN1 (Y = logit P)

In the 4-gene set, 3 genes were selected, and the diagnosis formula was as follows:

Y = -4.826 + 13.172 XANXA1 + 15.353 XSPAG9 + 8.755 XPFN1 (Y = logit P)

Then, the probability was used for evaluating the diagnostic value. The ROC curves are shown in Figure 1. The AUC of the 5 selected genes was 0.933, and that of the 3 selected genes was 0.878. This indicated that the 5 selected genes had a better diagnostic value, and when the cutoff of logit P was 0.548, the sensitivity and specificity were 94% and 80%, respectively. According to the diagnostic formula, we can know the risk of the detected sample. In addition, the odds ratio (OR) of the 5 selected genes was also analyzed and is shown in Table 3. An OR greater than 1 indicated that the factor was a risk factor for the disease, and an OR less than 1 meant it was a protective factor. Thus, the *ANXA1*, *SPAG9* and *PFN1* genes were risk factors. When the gene expression is higher, it may increase the incidence of HCC. The *FOS* and *CXCR4* genes were found to be protective factors. When their gene expression is higher, it may decrease the incidence of HCC.

***Multi-parameter discriminant analysis***

In the DA, the full 9-gene set was analyzed by Bayes DA. Six genes were selected by “Use stepwise method”. The following formulas were used, separately: Y1 = -7.306 + 8.078 XGPC3 + 20.770 XANXA1 - 3.414 XFOS + 12.652 XSPAG9 + 1.842 XCXCR4 + 18.248 XPFN1. Y2 = -5.612 + 15.760 XGPC3 + 4.432 XANXA1 + 1.585 XFOS - 10.298 XSPAG9 + 4.320 XCXCR4 + 12.370 XPFN1

In the 4-gene set, 3 genes entered the diagnosis formula. The formula was as follows:

 Y1 = -6.665 + 21.814 XANXA1 + 18.524 XSPAG9 + 16.663 XPFN1

Y2 = -2.806 + 13.784 XANXA1 + 8.081 XSPAG9 + 10.393 XPFN1

The probability of the healthy normal group and the HCC group analyzed by the 3 selected genes was saved as a new variable named Dis 1. The probability of the 6 selected genes was saved as Dis 2. The new variables Dis 1 and Dis 2 were used for the ROC analysis, as shown in Figure 2. The AUC of Dis 1 was 0.877 and of Dis 2 was 0.926. This result meant that the 6 selected genes had a better diagnostic value for HCC, and when the cutoff of Y was 0.628, the sensitivity and specificity were 82% and 89%, respectively.

***Multi-parameter classification trees analysis***

We compared the diagnostic value of the full 9-gene set and the 4-gene set by CTA; however, only ANXA1 entered the classification tree. The classification tree of the full 9-gene set was the same as the 4-gene set, as shown in Figure 3. When the cutoff was 0.629, the sensitivity and specificity were 70% and 60%, respectively. The total accuracy was only 66.20%.

***Multi-parameter artificial neural network analysis***

The full 9-gene set and the 4-gene set were then analyzed by the ANN method; their network had 1 hidden layer which contained 5 units. In the training group of the full 9-gene set, the percent incorrect prediction was 12.3%, and for the testing group, it was 9.1%. In the 4-gene set, the percentages were 20.9% and 8.7%. In addition, the full 9-gene set had better predictive probabilities than the 4 genes. The AUC of the full 9-gene set was 0.943, greater than the 4-gene set, which was 0.877.

***Comparison of multiple multi-parameter analysis methods and validation***

Multiple multi-parameter analysis methods were used to build models, and the AUC was used to evaluate the diagnostic value. When a single gene was used, the AUC of SPAG9 was greater than the other genes. The AUC of LRA, DA and ANN were greater than SPAG9, and the CTA was less than SPAG9. Among the methods, the ANN of the full 9-gene set had the best diagnostic value; the AUC, sensitivity and specificity were 0.943, 98% and 85%, respectively. Finally, 52 HCC patients and 34 healthy normal controls were used for validation. The sensitivity and specificity were 96% and 86%, respectively. Above all, multi-parameter analysis methods may increase the diagnostic value compared to single-factor analysis and this approach may be a trend for future clinical diagnostic methods.

**DISCUSSION**

Clinical peripheral blood samples can be obtained easily and in a minimally invasive way. Studies have shown that mRNA in peripheral blood has the potential to be used for the early detection of cancers. There are many mRNA detection methods; however, the most commonly used method, Real time PCR, is limited by the number of genes and the amplification efficiency. The Beckman Coulter (Fullerton, CA, United States) GenomeLab GeXP Genetic Analysis system was designed ideally for up to 35 genes per reaction and can be used to detect 192 samples simultaneously in one single detection[26]. In addition, the GeXP system uses the universal priming strategy to decrease the variations in amplification efficiency across multiple genes[16, 27]. Both strengths make GeXP an ideal multiple-gene expression detection method as well as a useful validation tool that is more similar to large-scale gene analysis methods, such as microarrays, than Real-time PCR. We combined gene-chip analysis, peripheral blood, the GeXP detection system and bioinformatics by using gene screening, model building, and bioinformatics analysis to build a gene expression profiling standard operating procedure for the early detection of cancer.

Studies have demonstrated that multi-parameter analysis can increase the sensitivity and specificity and is considered promising for future diagnostic methods. Lots of multi-parameter methods have been used for cancer early detection. In our study, logistic analysis increased the AUC to 0.933. Out of the 9 genes, 5 genes (*ANXA1*, *FOS*, *SPAG9*, *CXCR4*, *PFN1*) entered the diagnosis formula; however, *FOS* and *CXCR4*, which had poor diagnostic value, also entered the formula. This demonstrated that the genes showed significant differences between groups, and even if they had AUC values of less than 0.5, they may contribute to the logistic analysis. In the 4-gene set, 3 genes (*ANXA1*, *SPAG9* and *PFN1*) entered the formula, but the AUC was less than for the full 9-gene set. This demonstrated that genes with more significant differences may result in better diagnostic values. The logistic had a formula which can get a continuous value: logit (P). With a gene expression panel, we can predict the *P* value to differentiate the healthy people and the HCC patients, which may provide a clinical indication for both physicians and patients.

After OR analysis, the *ANXA1*, *SPAG9* and *PFN1* genes were detected as risk factors. The *FOS* and *CXCR4* genes were protective factors. Annexin1 (ANXA1) is a member of the annexin family of phospholipid-binding and calcium-binding proteins with a well demonstrated role in early delayed inhibitory feedback of glucocorticoids in the hypothalamus and pituitary gland[28]. Studies have demonstrated ANXA1 involved in tumorigenesis progress, and can increase the incidence of HCC[29]. Sperm associated antigen 9 (SPAG9) is a gene encoding C-jun-amino-terminal kinase-interacting protein 4. This enzyme is a scaffolding protein that connects the mitogen-activated protein kinases to related transcription factor targets for the activation of JNK signaling pathways [30]. SPAG9 was also demonstrated to be the biomarker for breast cancer and cervical carcinoma[31, 32]. Profilin-1 (PFN1) has been regarded as a tumor-suppressor molecule for breast cancer, and it can enhance ADP-to-ATP exchange on G-actin. In addition, it can also act as a shuttle to deliver ATP-bound G-actin to facilitate actin polymerization[33]. Studies have demonstrated that PFN1 is overexpressed in cancer cells by up-regulating PTEN and down-regulating AKT, and it is also inhibitor of mammary carcinoma aggressiveness[34]. If PFN1 is silenced, it can inhibit endothelial cell proliferation, migration, and morphogenesis[35]. All 3 genes may contribute to the development of HCC.

In the DA, the AUC was similar to the logistic analysis. This demonstrated that it may be a valuable analysis method; however, because it had strict demand on the data distribution, its application was greatly limited. In our study, although we got the discriminant formula, it may have high bias because the dataset was not normally distributed. In the CTA, only one gene had diagnostic value. This finding demonstrated that they were not suitable for our study. Artificial neural network analysis has been demonstrated to provide better diagnostic value in disease prediction and cancer early detection. In our study, ANN had the best diagnosis value compared to the other analysis methods, and the predicted probabilities of the groups were also shown. In addition, in the training group for the full 9-gene set, the percent incorrect prediction was 12.3%, and for the testing group, it was 9.1%. All of this demonstrates that the ANN model we built was successful.

In our previous study, we screened the mRNA in peripheral blood samples by Affymetrix GeneChip analysis, and 9 genes (*GPC3*, *HGF*, *ANXA1*, *FOS*, *SPAG9*, *HSPA1B*, *CXCR4*, *PFN1* and *CALR*) were used for differentiating the healthy normal control and HCC groups. We have now built an ANN detection system. The sensitivity and specificity were 96% and 86%, respectively, which was greater than the single gene analysis.

**ARTICLE HIGHLIGHTS**

***Research background***

We have built a 9 genes (*GPC3*, *HGF*, *ANXA1*, *FOS*, *SPAG9*, *HSPA1B*, *CXCR4*, *PFN1* and *CALR*) expression detection system based on the GeXP system in our previous study, we aim to analyze the results of genes expression by different multi-parameter analysis methods and build a diagnosis model to classify the hepatocellular carcinoma (HCC) patients and healthy people.

***Research motivation***

Although pathology is used as golden standard for diagnosis of HCC, it is invasive and tissue sample is not easily obtained. Therefor, a non-invasive, accurate, and fast method for early detection of HCC is pressing.

***Research objectives***

A non-invasive, accurate, and fast method for early detection of HCC may be provided by our research based on the mRNA of peripheral blood.

***Research methods***

We have successfully built an artificial neural networks detection system combining detection system and bioinformatics together for differentiating the healthy normal group and HCC group. The sensitivity and specificity were separately 96% and 86% which was greater than the single gene.

***Research results***

Artificial neural network of the total 9 genes had the best diagnostic value, the AUC, sensitivity and specificity were separately 0.943, 98% and 85%. At last, 52 HCC patients and 34 healthy normal controls were used for validation. The sensitivity and specificity were separately 96% and 86%.

***Research conclusions***

Based on the mRNA of peripheral blood, a multi-parameter analysis method was used to analyze the multi-genes which may increase the diagnosis value compared to the single factor for the early detection of HCC, and it may be a trend of the clinical diagnosis in the future. It may provide a non-invasive, accurate, and fast method for early detection of HCC.

***Research perspectives***

The GeXP system uses the universal priming strategy to decrease the variations in amplification efficiency across multiple genes, and it was an ideal multiple-gene expression detection method as well as a useful validation tool that is more similar to large-scale gene analysis methods. Combination of the peripheral blood, GeXP detection system and bioinformatics together may be the future strategy to build an assistant detection method for cancer.

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**P-Reviewer:** Boeckxstaens GE, Lee MW **S-Editor:** Chen K  **L-Editor: E-Editor:**

**Specialty type:** Gastroenterology and hepatology

**Country of origin:** China

**Peer-review report classification**

Grade A (Excellent): 0

Grade B (Very good): B, B

Grade C (Good): 0

Grade D (Fair): 0

Grade E (Poor): 0

**Table 1 Characteristics of early stage of hepatocellular carcinoma and healthy control samples**

|  |  |  |
| --- | --- | --- |
| **Variable** | **Building model** | **Model validation** |
| **HCC** | **Control** | **HCC** | **Control** |
| Number of patients | 103 | 54 | 52 | 34 |
| Male | 54 | 29 | 29 | 19 |
| Female | 49 | 25 | 23 | 15 |
| Age (mean ± SD) | 54 ± 12 | 49 ± 11 | 51 ± 14 | 52 ± 9 |
| Tumor size (> 3 cm) | 29 | 0 | 13 | 0 |
| Tumor size (< 3 cm) | 74 | 0 | 39 | 0 |
| Number of nodules (Unilocular) | 36 | 0 | 21 | 0 |
| Number of nodules (Multilocular) | 67 | 0 | 31 | 0 |
| Cirrhosis | 89 | 0 | 36 | 0 |
| BCLC staging (T1) | 27 | 0 | 19 | 0 |
| BCLC staging (T2) | 76 | 0 | 32 | 0 |

HCC: Hepatocellular carcinoma.

**Table 2 Diagnosis value of the 5 genes showed *P* value less than 0.05**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Gene**  | **AUC** | ***P* value**  |  **95% CI for AUC** | **Cutoff** | **Sen** | **Spe** |
| **Lower**  | **Upper**  |
| *HGF* | 0.620  | 0.014  | 0.532  | 0.708  | 0.102  | 0.408  | 0.926  |
| *ANXA1* | 0.697  | < 0.001 | 0.614  | 0.780  | 0.919  | 0.728  | 0.593  |
| *SPAG9* | 0.761  | < 0.001  | 0.681  | 0.842  | 0.477  | 0.942  | 0.481  |
| *PFN1* | 0.700  | < 0.001  | 0.620  | 0.781  | 0.383  | 0.437  | 0.907  |

*P* < 0.05 means significant difference. AUC: Area under curve; Sen: Sensitiviey; Spe: Specificity.

**Table 3 Odds ratio of the 5 selected genes after logistic regression analysis**

|  |  |  |  |
| --- | --- | --- | --- |
| **Gene** | ***P* value** | **OR** | **95% CI for OR** |
| **Lower** | **Upper** |
| *ANXA1* | < 0.001 | 1.73E + 09 | 1.67E + 05 | 1.79E + 13 |
| *FOS* | 0.012  | 4.80E - 03 | 7.30E - 05 | 3.16E - 01 |
| *SPAG9* | < 0.001 | 1.36E + 14 | 3.19E + 07 | 5.80E + 20 |
| *CXCR4* | < 0.001 | 6.44E - 02 | 1.47E - 02 | 2.81E - 01 |
| *PFN1* | < 0.001 | 1.37E + 04 | 9.82E + 01 | 1.91E + 06 |

*P* < 0.05 means significant difference. OR: Odds ratio.



**Figure 1** **Receiver operating characteristic analysis of probability after logistic regression analysis.** In the total 9 genes, 5 genes (*ANXA1*, *FOS*, *SPAG9*, *CXCR4* and *PFN1*) entered the diagnosis formula. In the 4 genes, 3 genes (*ANXA1*, *SPAG9* and *PFN1*) entered the formula. The probability was used for ROC analysis. The AUC of the 5 selected genes was 0.933 and the 3 selected genes was 0.878. It indicated that the 5 selected genes had a better diagnosis value, and the sensitivity and specificity were separately 94% and 80%.



**Figure 2** **Receiver operating characteristic analysis of probability after discriminant analysis.** In the total 9 genes,*GPC3*, *ANXA1*, *FOS*, *SPAG9*, *CXCR4* and *PFN1* genes entered the diagnosis formula. In the 4 genes, *ANXA1*, *SPAG9* and *PFN1* genes entered the formula. The probability was used for HCC detection. The AUC of the 6 selected genes was 0.926 and the 3 selected genes was 0.877. When the cutoff of the 5 selected genes was 0.628, the sensitivity and specificity were separately 82% and 89%.



**Figure 3 Classification tree of the total 9 genes and the 4 genes.** The tree of the total 9 genes was the same to the 4 genes. Only *ANXA1* genes entered the tree, and according to the node, it was divided into two parts.