Establishment and clinical application of a highly sensitive TAT-2 time-resolved fluorescence immunoassay detection

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Research Article

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Abstract

Background: A rapid and highly sensitive assay for tumor-associated trypsinogen-2 (TAT-2) based on the time-resolved fluorescence immunoassay (TRFIA) detection technique was developed for the determination of serum TAT-2 levels in cancers.

Results: The measurement range of TAT-2-TRFIA was 1.53-300 ng/mL. The within-run and between-run coefficients of variation of TAT-2-TRFIA were 4.38% and 7.82%, respectively. The recovery rate of TAT-2-TRFIA was 103.0%. The cross-reaction rates of trypsin and T-cell immunoglobulin mucin 3 were 0.02% and 0.82%, respectively. The TAT-2-positive rates in lung cancer, liver cancer, nasopharyngeal cancer, cholangiocarcinoma, brain cancer, and pancreatic cancer were 45.9%, 50.0%, 45.0%, 64.3%, 50.0%, and 41.7%, respectively, with the areas under ROC curves of 0.788, 0.734, 0.862, 0.720, 0.887, and 0.585, respectively. In patients with lung cancer, the positive rate of the single indicator CEA was 28.4%, which increased to 60.6% after combined use with TAT-2. In patients with cholangiocarcinoma, the positive rate of CA-199 was 35.7%, which increased to 71.4% after combined use with TAT-2.

Conclusions: TAT-2 is expected to be used as an auxiliary diagnostic indicator for the combined use of tumor markers to improve the positive rate and accuracy of detection.

1 Introduction

Trypsinogen is the precursor of trypsin. Its molecular weight is approximately 25 kDa. Human pancreatic secreta (pancreatic juice) contains two main trypsinogen isozymes: trypsinogen-1 and trypsinogen-2. Previous studies have found that all the serum samples of patients with excised pancreas still contain trypsinogen-2, suggesting that the expression of trypsinogen-2 is not limited to the pancreas(1). Trypsinogen-2 is mainly distributed in the skin, esophagus, stomach, small intestine, lung, kidney, liver, bile duct, prostate and other tissues or organs(2). In addition, some scholars have found that trypsinogen-2 has a high expression level in the tumor cells of patients with gastric cancer, lung cancer, cholangiocarcinoma, ovarian cancer and other cancers(3–6). Therefore, trypsinogen-2 with high expression in tumor cells is called tumor-associated trypsinogen-2 (TAT-2). TAT-2 promotes tumor invasion by inducing the hydrolyzation of surrounding stromal proteins(7–9).

Previous studies have shown that TAT-2 is closely related to many kinds of tumor diseases. Koivunen et al.(10) found that TAT-2 increases the expression of proteolytic enzymes in malignant tumor cells. This result suggests that TAT-2 is closely related to ovarian and colon malignancies. Hedström et al.(11) measured the concentrations of TAT-2 and trypsin-2-α1-antitrypsin (trypsin-2-AAT) in 145 patients with malignant gastrointestinal disease and 61 patients with benign gastrointestinal disease. They discovered that the levels of TAT-2 and trypsin-2-AAT have significantly increased by 46% and 42%, respectively, in patients with malignant tumors compared with those in patients with benign tumors ($P < 0.05$). Subsequently, they measured the concentrations of TAT-1, TAT-2, and tumor-associated trypsin inhibitor (TATI) in the bile of 23 patients with benign biliary disease, 2 patients with biliary cancer, and 15 patients with pancreatic cancer and found high levels of TAT-1, TAT-2, and TATI in the majority of the patients(12). Under normal conditions, only a small amount of TAT-2 is expressed in human blood. When some tissues are damaged by tumors, a large amount of TAT-2 enters the blood, resulting in significantly increased blood concentrations of TAT-2(13). This situation suggests that sera from patients with cancer may contain large amounts of TAT-2.

At present, the level of TAT-2 in sera is detected via enzyme-linked immunoassay (ELISA). However, ELISA has low sensitivity and may lead to false negatives. Therefore, we need a highly sensitive detection method to complete our research. Time-resolved fluorescence immunoassay (TRFIA) is a newly developed detection technology that is mainly used to label antibodies or antigens with trivalent lanthanide elements that possess unique fluorescence characteristics. After immune reaction between antigens and antibodies, a TRFIA analyzer is used to detect fluorescence counts and the level of target antigens is determined by the standard curve established from the fluorescence counts and the antigen concentrations. This assay has the advantages of high sensitivity, convenient manipulation, wide detection ranges, high stability, and nonradioactive labeling over other detection methods(14–16) and can thus be used as an important noninvasive tool for elucidating the possible diagnostic role of serum TAT-2 in vitro.

2 Materials And Reagents

2.1 reagents and instruments

TAT-2 antigen, coating antibody and labelled antibody were provided by Zhejiang Boshi Bio Co., Ltd. Diethylenetriaminepentaacetate (DTPA), bovine serum albumin (BSA), Tris, and Triton X-100 were purchased from Guangzhou Saiguo Biotech Co., Ltd. A europium-labeling kit including Eu^{3+}-N1-(p-isothiocyanatobenzyl)-diethylenetriamine-N^1,N^2,N^3,N^4-tetraacetic acid (Eu^{3+}-DTTA) was purchased from Perkin-Elmer (USA). Ultracel-50K ultrafiltration tubes were purchased from Millipore, Inc. A Sephadex G50 column was bought from Shanghai Cell Biological Technology Co., Ltd. Ninety-six-well plates were purchased from Xiamen Yunpeng Technology Development Co., Ltd. A micro-oscillator was purchased from Jiangsu Kangjian Medical Products Co., Ltd. A peristaltic pump and an automatic partial collector were sourced from Shanghai Qingpu Luxi Instrument Factory. A time-resolved immunofluorescence analyzer was procured from Foshan Daan Medical Equipment Co., Ltd.

2.2 Serum samples

We collected sera from 221 patients with cancer and 91 heathy subjects from Zhejiang Xiaoshan Hospital. All patients provided informed consent prior to study participation. The overall design of this research was approved by Zhejiang Xiaoshan Hospital, and this research was approved by the Ethics Committee of Zhejiang Xiaoshan Hospital and the Ethic number is 2020-011.

3 Methods

3.1 Solid antibody coating

Ninety-six-well plates were coated with 1.5μg/mL captured coating TAT-2 antibodies diluted in coating solution (50 mmol/L Na_2CO_3-$NaHCO_3$, pH 9.6) through overnight incubation at 4°C and then blocked with the blocking buffer (50 mmol/L Tris-HCl, 0.9% NaCl, 1% BSA and 0.05% NaN_3, pH 7.8) for 2 h. After the blocking solution was removed, the wells were dried under high vacuum. The plates were sealed and stored at -20°C.

3.2 Antibody labeling

A total of 300 μL of labelled TAT-2 antibody was added to an ultracel-50K ultrafiltration tube and centrifuged at 7100 g/min for 8 mins. Then, 200 μL of labeling buffer (50 mmol/L Na_2CO_3-$NaHCO_3$, pH 9.0) was added and centrifuged at 7100 g/min for 8 mins. This procedure was repeated 7 times. Subsequently, 50 μL of labeling buffer was added to the ultrafiltration tube and centrifuged at 1000 g/min for 1 min. This step was repeated twice. The labelled antibody was mixed with 60 μL of Eu^{3+}-DTTA and incubated at 30°C overnight. The next day, the labelled antibody was

purified with Sephadex G50 and then diluted with elution buffer containing 0.2% BSA as a stabilizer. Finally, the Eu^{3+}-labelled antibody was stored at -20°C.

3.3 Experimental procedures

The TRFIA of TAT-2 were performed via a one-step noncompetitive sandwich-type technique. In brief, 25 μL of samples and 75 μL of 100-fold diluted Eu^{3+}-labelled antibody solution in assay buffer (0.05 mol/L Tris-HCl, pH 7.8, 0.9% NaCl, 0.2% BSA, 0.01% Tween-20, 20 μmol/L DTPA, and 0.05% sodium azide) were pipetted into the coated microtiter wells. The plates were incubated at 37°C with shaking for 60 mins. After washing the wells for 6 times, 100 μL of enhancement solution (15 μmol/L β-NTA, 50 μmol/L tri-noctylphosphine oxide, and 0.1% Triton X-100, pH 3.2) was dispensed into each well. The plates were shaken for 5 mins before fluorescence readings were taken. The construction of the calibration curve and the calculation of concentrations in the unknown samples were performed automatically, wherein a spline algorithm was used for logarithmically transformed data.

3.4 Assessment of the TAT-2-TRFIA method and statistical analysis

We evaluated the linearity, sensitivity, precision, specificity, and recovery rate of the TAT-2-TRFIA method. SPSS 26.0 was used for statistical analysis, and GraphPad Prism 8 was used for drawing. $P < 0.05$ was considered as statistically significant. We confirm that all methods were carried out in accordance with relevant guidelines and regulations.

4 Result

4.1 Assessment of the TAT-2-TRFIA method

The calibration graph of logarithms is shown in Fig. 1. Here, $R^2 = 0.9918$, indicating a good linear relationship between the concentrations of the standard substance dilutions and the corresponding fluorescence value (F value). The sensitivity of this method was 1.53 ng/mL, and the measurement range was 1.53–300 ng/mL. The within-run and between-run coefficients of variation of TAT-2-TRFIA were 4.38% and 7.82%, respectively. The recovery rate of TAT-2-TRFIA was 103.0%. The cross-reaction rates of trypsin and T-cell immunoglobulin mucin 3 were 0.02% and 0.82%, respectively.

4.2 Clinical applications

We collected serum samples from 221 patients with cancer and 91 healthy subjects to investigate the clinical application value and significance of TAT-2 in sera. The serum TAT-2 concentrations of healthy subjects = mean ± SD (standard deviation) = 7.51 ± 3.80 ng/mL and the cut-off value was 15.11 ng/mL when it was set to be equal to mean + 2SD. The samples of the patients were divided into 6 groups, namely, the lung cancer (109), liver cancer (30), nasopharyngeal cancer (20), brain cancer (14), cholangiocarcinoma (12), and pancreatic cancer (36) groups. The levels of other tumor markers were determined at Zhejiang Xiaoshan Hospital.

Table 1 and Fig. 2 show Serum TAT-2 levels in patients with lung cancer, liver cancer, nasopharyngeal carcinoma, brain cancer, cholangiocarcinoma, and pancreatic cancer were all signifcantly higher compared to the levels in the healthy subjects ($P < 0.001$). As can be seen from Table 2, when the cut-off value was set as 15.11 ng/mL, the TAT-2 positive rates of patients with pancreatic cancer, nasopharyngeal cancer, lung cancer, brain cancer, liver cancer and cholangiocarcinoma were 41.7%, 45.0%, 45.9%, 50.0%, 50.0% and 64.3%, respectively. Moreover, some tumor markers with high positive rates, such as serum AFP in liver cancer (43.3%) and serum CA-199 in pancreatic cancer (83.3%), were observed. And we combined these tumor markers with TAT-2. In patients with lung cancer, the positive rate of the single indicator CEA was 28.4%, which increased to 60.6% after combined use with TAT-2. In patients with

cholangiocarcinoma, the positive rate of CA-199 was 35.7%, which increased to 71.4% after combined use with TAT-2.

Table 1
Comparison between healthy subjects and patients

	Healthy subjects (n = 91)	Lung cancer (n = 109)	Liver cancer (n = 30)	Nasopharyngeal carcinoma (n = 20)	Cholangiocarcinoma (n = 14)	Brain cancer (n = 12)	Pancreatic cancer (n = 36)
Age	49.2 ± 17.4	60.8 ± 13.0	60.7 ± 12.9	60.7 ± 12.9	60.9 ± 12.9	60.9 ± 12.8	60.8 ± 12.9
Male/female	45/46	62/47	23/7	13/7	8/6	8/4	24/12
M (ng/mL)	6.2	14.0	29.0	15.0	25.3	17.4	9.4
QL-QU (ng/mL)	4.3–10.6	7.4–24.2	15.8–26.0	10.2–29.2	3.3–37.0	10.9–38.1	3.2–68.0
M: median; QL-QU: quarter spacing							

Table 2
Comparison of the positive rates between single tumor marker and combined use

	Lung cancer (n = 109)	Liver cancer (n = 30)	Nasopharyngeal carcinoma (n = 20)	Cholangiocarcinoma (n = 14)	Brain cancer (n = 12)	Pancreatic cancer (n = 36)
TAT-2	45.9%	50.0%	45.0%	64.3%	50.0%	41.7%
AFP	0.9%	43.3%	0	14.3%	8.3%	0
CEA	28.4%	3.3%	0	35.7%	0	22.2%
CA-125	31.2%	33.3%	5.0%	71.4%	0	55.6%
CA-153	12.8%	10.0%	0	14.3%	0	11.1%
CA-199	13.8%	33.3%	5.0%	35.7%	8.3%	83.3%
TAT-2 + AFP	46.8%	73.3%	45.0%	71.4%	50.0%	41.7%
TAT-2 + CEA	56.0%	56.7%	45.0%	78.6%	50.0%	55.6%
TAT-2 + CA-125	56.0%	63.3%	45.0%	78.6%	50.0%	72.2%
TAT-2 + CA-153	48.6%	60.0%	45.0%	71.4%	50.0%	44.4%
TAT-2 + CA-199	49.5%	66.7%	45.0%	71.4%	58.3%	86.1%

We used the ROC curve analysis of serum TAT-2 to distinguish lung cancer, liver cancer, nasopharyngeal carcinoma, brain cancer, cholangiocarcinoma, and pancreatic cancer (Fig. 3). Table 3 shows that the area under ROC curve of TAT-2 was higher in nasopharyngeal cancer and brain cancer (0.862 and 0.887, respectively) than in other cancers.

Table 3
Parameters of the ROC curve of nasopharyngeal carcinoma and brain cancer

	Lung cancer	Liver cancer	Nasopharyngeal carcinoma	Cholangiocarcinoma	Brain cancer	Pancreatic cancer
AUC	0.788	0.734	0.862	0.720	0.887	0.585
P values	< 0.0001	0.0002	< 0.0001	0.0082	< 0.0001	0.137
Sensitivity	47.7%	53.3%	85.0%	64.3%	75.0%	44.4%
Specificity	100%	98.9%	72.5%	100.0%	85.7%	97.8%

5 Discussion

TAT-2 can act as a growth stimulator by binding to cell receptors to activate receptors and promote cell proliferation. Consequently, TAT-2 is believed to be an important factor in promoting tumor invasion. Chemical modifications by tetracycline, doxycycline, and TATI can inhibit the migration of cancer cells by down-regulating the expression or activity of TAT-2, thus suggesting that TAT-2 may be a potential therapeutic target for tumors and that reducing the expression of TAT-2 may be useful for tumor treatment(17). In addition, previous studies have shown that TAT-2 can be used for the preliminary diagnosis and prognosis of certain types of cancers. Paju et al.(18) found that the preoperative elevation of trypsin-2-API in serum is a strong prognostic factor for advanced epithelial ovarian cancer and demonstrated that the expression of TAT-2 plays a certain role in the development of ovarian cancer. Furthermore, immunohistochemically detected trypsin is associated with the recurrence and poor prognosis of human colorectal cancer(19). Lempinen et al.(5) found that TAT-2 in serum is highly accurate in differentiating between cholangiocarcinoma and primary sclerosing cholangitis.

In the past, scholars usually used ELISA and radioimmunoassay to detect TAT-2 levels in sera from patients(1, 20, 21). In ELISA, enzymes are used to tag antigens or antibodies. However, high-activity enzymes are strictly required in this assay. In addition, ELISA needs 2–3 h to detect serum samples, has low sensitivity, can only perform semiquantitative detection, and is unsuitable for application in clinical detection(22, 23). The lack of stability and the short ranges of measurement are two other reasons why ELISA cannot be used in clinical testing. Therefore, developing a serum TAT-2 detection method with increased sensitivity and accuracy is crucial. In this research, we used TRFIA for detection. TRFIA has the advantages of high sensitivity and rapid detection and is thus suitable for clinical detection.

In this work, a method for the detection of TAT-2 in sera by TRFIA was established for the first time and preliminarily applied to detect the level of TAT-2 in sera from patients with cancer. The diagnostic value and clinical application of TAT-2 were evaluated by comparing the levels of TAT-2 in healthy subjects and patients with cancer. The positive rate of TAT-2 was found to be more than 40% in patients with lung cancer, liver cancer, nasopharyngeal cancer, cholangiocarcinoma, brain cancer, and pancreatic cancer, and the positive rates of this tumor marker in patients with related cancers were 20%-50%, indicating that the diagnostic value of TAT-2 in lung cancer, liver cancer,

nasopharyngeal cancer, cholangiocarcinoma, brain cancer, and pancreatic cancer was close to that of general tumor markers. TAT-2 concentrations are associated with MMP-9 activation in ovarian cancer(24), and MMP-2 and − 9 are type IV collagenases that participate in tumor invasion; the expression of the inhibitor of MMP-2 is associated with lung cancer(25) and may be one of the reasons for the elevated TAT-2 levels in patients with lung cancer. Hedström et al.(11) showed that elevated TAT-2 and TAT-2-AAT levels in serum are the most common feature in patients with hepatobiliary and pancreatic tumors. Our experimental results were consistent with previous results. the involvement of TAT-2 in the pathogenesis of nasopharyngeal cancer and brain cancer has not been revealed. In addition, through the literature, we found that nasopharyngeal carcinoma and brain cancer lack an appropriate clinical serological marker. Clinical imaging and immunohistochemical methods are usually used for the diagnosis of these malignancies(26, 27). However, due to small pathological changes or low resolution, clinical imaging methods may cause misdiagnosis. Immunohistochemistry requires pathology detection through biopsy, which is a complicated process and may destroy tumor tissues, thus leading to the metastasis of tumor cells into the blood. Through TAT-2-TRFIA detection, we found that the TAT-2 positive rates of patients with nasopharyngeal cancer and brain cancer were approximately 50% and there were few other serological indicators that can reach this level. This result indicated that TAT-2 might be helpful in the diagnosis of nasopharyngeal cancer and brain cancer and might become a new diagnostic index with a simple detection process that could be used in combination with clinical imaging methods to improve the accuracy of diagnosis. In addition, we found that in lung cancer, the positive rates of CEA and CA-125 were higher than other tumor markers, and the positive rate could be improved significantly after combined use with TAT-2. In liver cancer, AFP is a tumor marker of liver cancer with a high positive rate, and the positive rate can be increased by 30% after combined use with TAT-2. In cholangiocarcinoma, the positive rates of CEA, CA-125 and CA-199 were all high, which was consistent with the experimental results of predecessors(28). The positive rates of CEA and CA-199 could improve obviously after combined use with TAT-2, while the positive rate of CA-125 was less improved. In pancreatic cancer, and the positive rate of CA-199 is high, up to 83.3%, but when combined with TAT-2, the positive rate hardly increases.

In conclusion, we successfully established a serological detection method for TAT-2 and found that this detection method can be used for cancer diagnosis. The combined use of TAT-2 and other tumor markers could further improve the positive rate of detection and help to screen patients with cancer.

Declarations

Ethical approval and consent to participate

We collected sera from 221 patients with cancer and 91 heathy subjects from Zhejiang Xiaoshan Hospital. All patients provided informed consent prior to study participation. The overall design of this research was approved by Zhejiang Xiaoshan Hospital, and this research was approved by the Ethics Committee of Zhejiang Xiaoshan Hospital. Ethic number is 2020-011.

Consent for publication

Not Applicable.

Availability of data and material

All data generated or analysed during this study are included in this published article [and its supplementary information files].

Competing interests

Authors state no conflict of interest.

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Author contributions

Xindong Chen wrote the main manuscript text and prepared all of the figures and tables. Jianfeng Hong and Hongming Fang provided the sera. Han Zhao did the data processing. Zhongyi Xiang, Yuan Qin, Xiumei Zhou, Yigang Wang, Liping Zheng and Biao Huang offered their opinions on the paper. Pengguo Xia, Hongming Fang, Yingwei Zhu and Biao Huang provided the funds. Hongming Fang, Yingwei Zhu and Biao Huang supervised the project.

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Figures

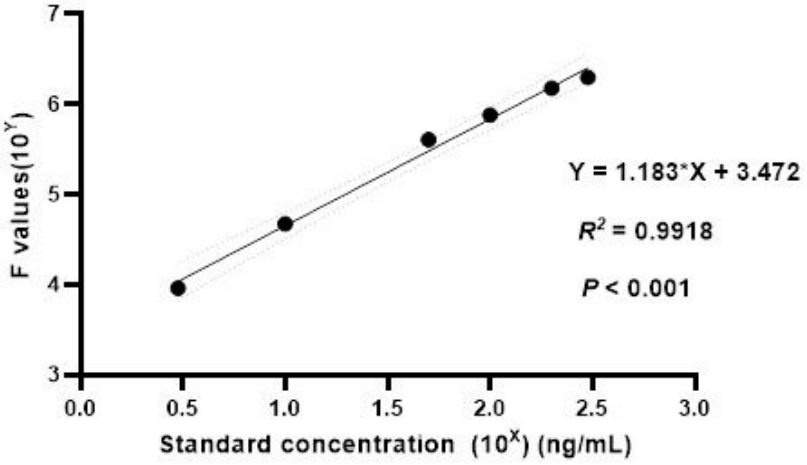

Figure 1

The standard curve of TAT-2 antigen, florescent (F)

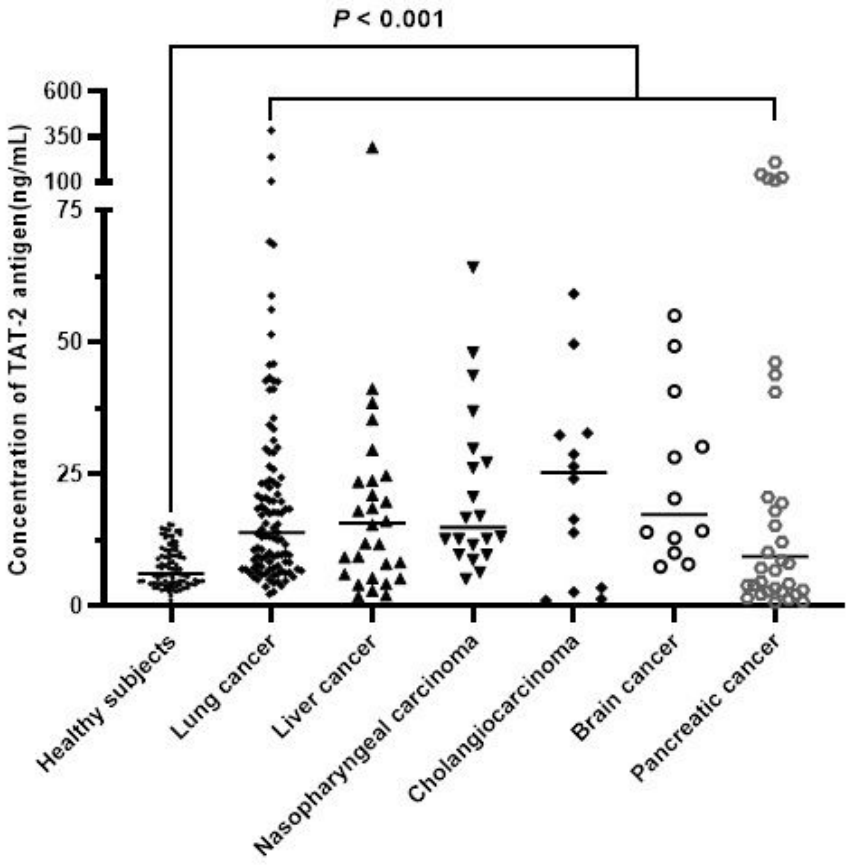

Figure 2

The concertration of TAT-2 in the cancer patients serum

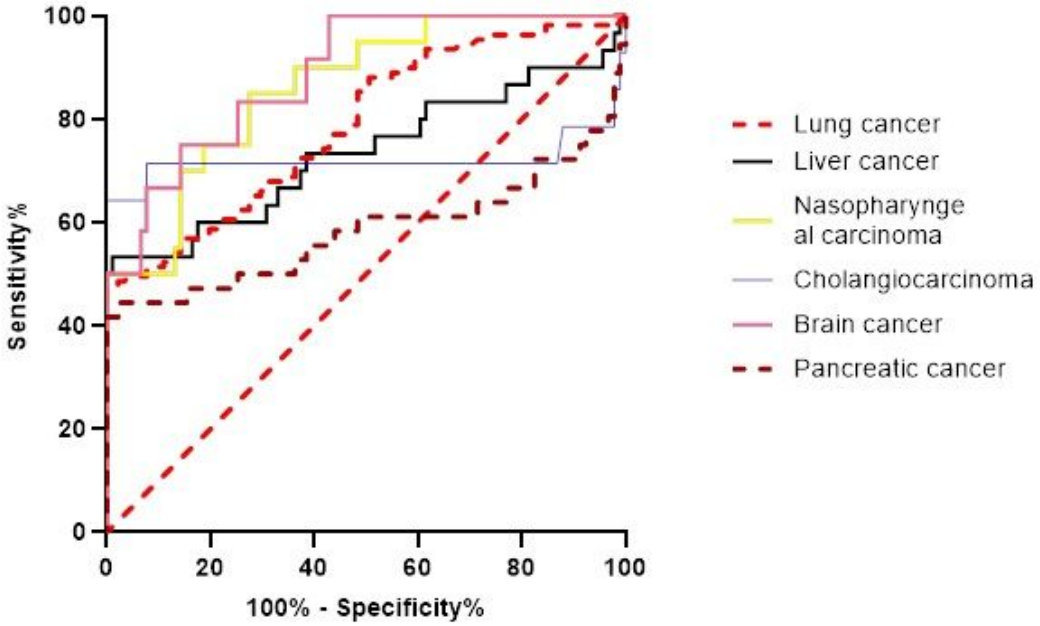

Figure 3

ROC of TAT-2 which distinguished healthy subjects and patients with cancer.

Supplementary Files

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