

Original Article

Assessment of different biomarkers provides valuable diagnostic standards in the evaluation of the risk of acute rejection

Jin Zheng[†], Xiaoming Ding[†], Xiaohui Tian, Zhankui Jin, Xiaoming Pan, Hang Yan, Xinshun Feng, Jun Hou, Heli Xiang, Li Ren, Puxun Tian, and Wujun Xue*

Hospital of Nephropathy, The First Affiliated Hospital of Medical College, Xi'an Jiaotong University, Xi'an 710061, China

[†]These authors contributed equally to this work.

*Correspondence address. Tel: +86-29-85323721; Fax: +86-29-85323718; E-mail: xwujun@126.com

Acute rejection (AR) is a strong risk factor for chronic rejection in renal transplant recipients. Accurate and timely diagnosis of AR episodes is very important for disease control and prognosis. Therefore, objectively evaluated the immune status of patients is essential in the field of post-transplantation treatment. This longitudinal study investigated the usefulness of five biomarkers, human leukocyte antigen (HLA)-G5 and sCD30 level in sera, intracellular adenosine triphosphate (iATP) release level of CD4⁺ T cells, and granzyme B/perforin expression in peripheral blood mononuclear cells (PBMCs) and biopsies, to detect AR and the resolution of biomarkers in a total of 84 cases of renal transplantation. The data demonstrated that recipients with clinical or biopsy proven rejection significantly increased iATP release level of CD4⁺ T cells, and elevated sCD30 but lowered HLA-G5 level in sera compared with individuals with stable graft function. Expression levels of granzyme B and perforin were also elevated in PBMCs and graft biopsies of AR patients. Taken together, we identified that upregulation of sCD30, iATP, granzyme B, perforin, and downregulation of HLA-G5 could provide valuable diagnostic standards to identify those recipients in the risk of AR. And iATP may be a better biomarker than others for predicting the graft rejection episode.

Keywords renal transplant; acute rejection; HLA-G5; sCD30; iATP

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Introduction

Renal transplantation is the most effective treatment for end-stage renal disease, but the effectiveness of this procedure is hampered by the rejection of graft kidney by the recipient's immune system. The assessment of patient immune status usually relies on monitoring the blood

concentration of immunosuppressive drug, the examination of renal function, and lymphocyte classification [1,2]. However, these strategies only help prevent drug toxicity, which is not adequate in avoiding rejection or infection [3].

Current practice for confirmation of renal graft rejection relies on a needle-based biopsy. But protocol routine renal transplant biopsies are not standard clinical practice in most transplant clinics. Based on the study of transplant immunology, a series of immune biomarkers that are associated with the acute rejection (AR) episodes were monitored to reflect the immune status from different aspects, and some of them have been proved to be valuable in predicting the risk of rejection.

Human leukocyte antigen (HLA)-G5 (the soluble full-length HLA-G1 counterpart), the leading subunit of HLA-G, plays an essential role in immune tolerance and has been negatively associated with acute and chronic rejection after organ transplantation [4–6]. The *in vitro* studies have shown that soluble HLA-G5 suppresses T-cell functions and induces regulatory T cells [7]. On the other hand, CD30 that belongs to the tumor necrosis factor- γ super family can serve as a T-cell signal transducing molecule and is expressed by a subset of activated T lymphocytes, CD45RO (+) memory T cells. Augmentation of soluble CD30 (sCD30) during kidney transplant rejection has been reported [8]. Soluble HLA-G5 and CD30 are the most important immune molecules for estimating the risk of AR in sera [6,9].

Intracellular adenosine triphosphate (iATP) levels in CD4⁺ T cells after stimulating with phytohemagglutinin (PHA) could reflect the degree of T-cell activity [10]. It was reported that application of ATP bioluminescence could assess cellular immune function by testing concentration of ATP in antigen-stimulated lymphocytes [11]. Granzymes and perforin are the main effector molecules of cytotoxic T lymphocytes (CTLs), and it is also suggested that, in kidney transplant recipients, serial measures of

peripheral blood perforin, and granzyme B gene expression levels have been a way to recognize graft rejection at sub-clinical stages [12]. Both iATP and granzyme B/perforin are valuable biomarkers of lymph cells for assessing the cellular immune function.

We used a cross-sectional design approach to show variations at expression levels of HLA-G5, sCD30, iATP, and granzyme B/perforin among patients with biopsy-proven renal graft rejection [6,11,13]. The purpose of the current study is to determine a more specific and sensitive rejection biomarkers that can be used to detect and monitor clinical rejection in renal transplant recipients. To achieve this purpose, different immune biomarkers of allograft renal transplant recipients were examined regularly over one year after transplant. For all participants, we collected clinical follow-up data since 2006.

Materials and Methods

Study design

A total of 84 cases of renal transplantation were recruited in the Hospital of Nephrology, the First Affiliated Hospital of Xi'an Jiaotong University. Blood samples were collected from 40 individuals with stable renal function as controls or from 44 other patients diagnosed as AR episodes by biopsy proven. Patients' samples were collected on the day when a biopsy was performed. Serum HLA-G5 and sCD30 level, iATP release level of CD4⁺ T cells, and perforin/granzyme B expression levels in peripheral blood mononuclear cells (PBMCs) and graft biopsy were examined. None of the patients included in this study had active Epstein–Barr virus, fungus, or cytomegalovirus infections at the time of rejection. All rejection episodes were biopsy proven and the severity of the rejection episode were classified according to the Banff classification [14,15]. The patients' general clinical characteristics were summarized in **Table 1**. All samples were stored at 4°C and analyzed within 24 h after collection. This study was approved by the ethics committee of the First Affiliated Hospital of Xi'an Jiaotong University and was carried out in compliance with the declaration of Helsinki. All participants provided written consent authorizing review of their medical records for research purposes.

Enzyme-linked immunosorbent assay analysis of HLA-G5 and sCD30 level in serum

Sera were harvested by centrifugation at 800 g for 10 min. Then HLA-G5 and sCD30 levels were measured with a commercial immunoassay kit (eBioscience, San Diego, USA) following the instruction manual.

Measurement of iATP release in CD4⁺ T cells

Whole blood of renal transplant patients were collected in tubes containing sodium heparin (Sigma, St Louis, USA) and processed ~2 h after blood collection by ImmuKnow test. Briefly, blood was diluted 1:4 with RPMI 1640 medium. For 100 µl of sample, 25 µl of a solution of phytohemagglutinin L (PHA; Sigma) was added and the mixture was cultured at 37°C in an incubator with 5% CO₂ for 15 h. Anti-CD4 monoclonal antibody coated magnetic particles (50 µl) were added to immuno-select CD4 cells from both the stimulated and non-stimulated cells and cultured for an additional 15 min at room temperature. Finally, the CD4⁺ cells were collected by using a magnet held in position for 2 min. After washing the selected CD4⁺ cells on a magnet tray, iATP was released with a lysis reagent and measured using the ImmuKnow test kit (Cylex Inc., Columbia, USA).

Analysis of perforin and granzyme B expression in PBMC and graft biopsy

To detect the expression level of intracellular perforin and granzyme B in PBMCs, cells were fixed by addition of 2% paraformaldehyde/phosphate-buffered saline. Anti-perforin-phycoerythrin and anti-granzyme B fluorescein isothiocyanate antibodies (BD Biosciences, San Jose, CA, USA) were added to the cells in the presence of saponin buffer [0.5% saponin (Sigma), 5% fetal calf serum, and phosphate-buffered saline]. The antibody-stained cells were analyzed on a Becton Dickinson FACS calibur flow cytometer (Franklin Lakes, USA), and the data were analyzed using Flow-Jo software (Tree Star, Inc., San Carlos, USA).

The graft samples of patients were fixed with 4% paraformaldehyde for over 24 h, and embedded in paraffin and sectioned. Sections (5 µm) were mounted onto poly-lysine coating slides. After blocking with 3% bovine serum albumin for 30 min, slides were then incubated with primary antibody anti-perforin (1:20) (Abcam, Cambridge, UK) and anti-granzyme B (1:100) (Abcam) at 4°C overnight. Quantification of immune-positive cells were determined by counting perforin/granzyme B-positive cells in a sequence of 10 consecutive computer images of ×400 high-power fields. Only immunoreactive cells with the clear identifiable nuclei were counted. Cells were scored positive when displayed a distinctly brown membrane or showed brown granular cytoplasmic pattern. The results were expressed as a mean number of immune-positive cells per square millimeter. In each specimen, staining intensity was recorded by two independent observers.

Data analysis

Data were processed using the SPSS17.0 software package for Windows (SPSS Inc., Chicago, IL). All results were expressed as the mean ± SD (standard deviation). One-way

analysis of variance (ANOVA) was used to determine the difference among groups. Results were considered statistically significant if $P < 0.05$.

Results

Patient demographics

A total of 84 cases of renal transplantation were recruited in this study. There were 44 renal transplantation recipients with biopsy proven AR episodes, and the other 40 cases were patients with stable renal function. No significant difference were found to associate with recipient age, gender, body weight, and primary disease between cases with AR episodes and cases with stable renal function (**Table 1**).

Banff'97 diagnostic classifications

The histopathological findings of the graft biopsy were analyzed according to Banff'97 criteria [14,15] by a pathologist who was blinded to the results. In AR patients, 40.4% cases were diagnosed as grade IA with significant interstitial infiltration ($>25\%$ of parenchyma affected) and foci of moderate tubulitis (>4 mononuclear cells/tubular cross section or group of 10 tubular cells); 36.2% cases were at the stage of grade IB with significant interstitial infiltration and foci of severe tubulitis (>10 mononuclear cells/tubular cross section or group of 10 tubular cells); 17% cases were grade IIA with mild-to-moderate intimal arteritis; and 6.4% cases were diagnosed as grade IIB with severe intimal arteritis comprising $>25\%$ of the luminal area (**Fig. 1**).

Serum HLA-G5 and sCD30 levels in AR patients before treatment

To investigate the association of HLA-G5 and sCD30 levels with acute renal allograft rejection, we used enzyme-linked immunosorbent assay (ELISA) to detect HLA-G5 and sCD30 in sera. As shown in **Fig. 2(A)**, HLA-G5 level of AR patients was significantly lower compared with those with normal renal function (stable group) at the same period. HLA-G5 level of AR patients is 72.13 ± 17.91 pg/ml (median, 78.39 pg/ml), which has a significantly difference compared with stable group (92.30 ± 26.27 pg/ml; median, 88.30; $P = 0.029$). In contrast to HLA-G5, sCD30 level of AR patients was markedly higher than those of stable group [**Fig. 2(B)**]. The sCD30 level of AR patients was 15.43 ± 7.39 ng/ml (median, 17.34 ng/ml), which is markedly higher than those of stable group (8.70 ± 4.92 ng/ml; median, 8.15; $P = 0.008$). Co-evaluation of HLA-G5 and sCD30 levels in AR patients and stable patients, we found the area under receiver operating characteristic (ROC) curve (AUC) was 0.879 [95% confidence interval (CI): 0.762–0.995], higher than their individual AUC [**Fig. 2(C)**]. The difference was statistically

Table 1 Patient demographics

Patient demographics	Acute rejection (patients $n = 44$)	Stable renal function (patients $n = 40$)	P value
Recipient average age (year)	36.9 ± 10.9	35.7 ± 10.3	0.86
Recipient gender (male/female)	27/17	24/16	0.94
Body weight (kg)	52.3 ± 10.6	53.1 ± 9.8	0.84
Panel reactive antibody (% positive)	$<10\%$	$<10\%$	
Primary disease			
Glomerulonephritis	25 (56.8%)	24 (60%)	0.63
Pyelonephritis	16 (36.4%)	13 (32.5%)	0.58
Polycystic kidney disease	1 (2.3%)	1 (2.5%)	0.99
Others	2 (4.7%)	2 (5%)	0.91

Values are shown as the mean \pm SD or N (%).

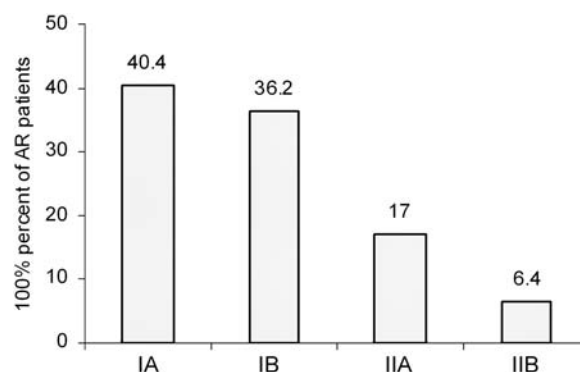


Figure 1 Banff '97 diagnostic classifications IA, cases with significant interstitial infiltration ($>25\%$ of parenchyma affected) and foci of moderate tubulitis (>4 mononuclear cells/tubular cross section or group of 10 tubular cells); IB, cases with significant interstitial infiltration ($>25\%$ of parenchyma affected) and foci of severe tubulitis (>10 mononuclear cells/tubular cross section or group of 10 tubular cells); IIA, cases with mild-to-moderate intimal arteritis; IIB, cases with severe intimal arteritis comprising $>25\%$ of the luminal area, $n = 44$.

significant ($P = 0.000$) compared with the reference curve AUC that was 0.5 (**Table 2**). These data indicated that co-evaluation of HLA-G5 and sCD30 was suitable as a biomarker for detecting renal transplant rejection.

iATP release of CD4⁺ T cell in AR patients before treatment

To evaluate the prognostic value of iATP level as a biomarker for AR, its level in CD4⁺ T cells of AR patients or stable patients was measured by Immuknow test. As shown in **Fig. 3(A)**, there was a statistical difference

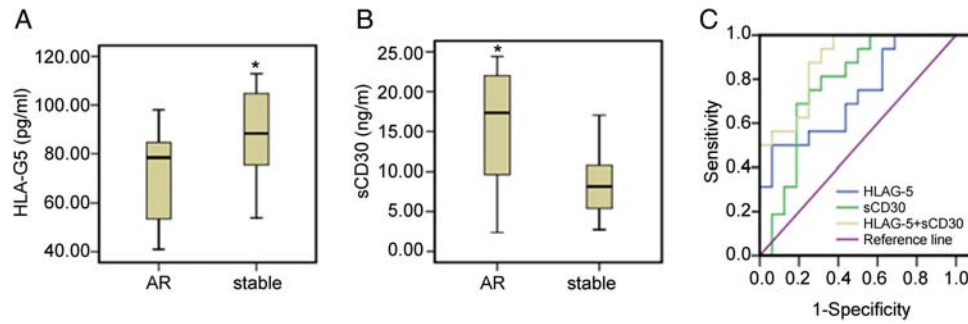


Figure 2 Serum HLA-G5 and sCD30 levels in AR patients before treatment HLA-G5 (A) and sCD30 (B) levels in serum between AR group ($n = 44$) and stable group ($n = 40$). $*P < 0.05$ vs. stable group. (C) Co-evaluation of HLA-G5 and sCD30 level in AR patients and stable patients by ROC curve. AUC is 0.879 ($P = 0.000$) vs. the reference curve AUC (AUC = 0.5). Data are representative of three separate experiments and each sample has four parallel samples.

Table 2 The ROC curve of HLA-G5 and sCD30

Test variables	AUC	SE	P value	95% CI	
				Lower	Upper
HLA-G5	0.727	0.090	0.029	0.551	0.902
sCD30	0.773	0.088	0.008	0.602	0.945
HLA-G5+sCD30	0.879	0.059	0.000	0.762	0.995

AUC, area under the curve; CI, confidence interval; SE, standard error.

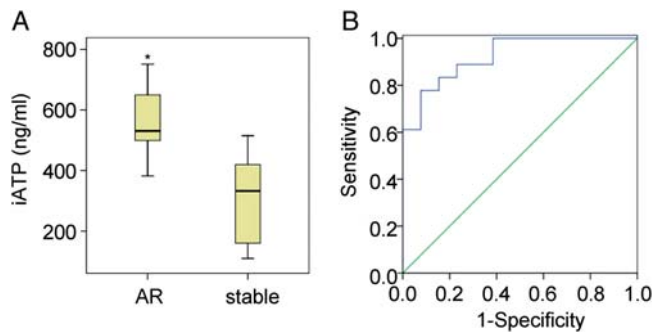


Figure 3 iATP release of $CD4^+$ T cell in AR patients before treatment (A) iATP release level between stable (renal function stable, $n = 40$) patients and AR patients ($n = 44$). $*P < 0.05$ vs. stable group. (B) Evaluation the iATP level in AR patients and stable patients by ROC curve. AUC is 0.923 ($P = 0.000$) vs. the reference curve AUC (AUC = 0.5).

between stable patients and AR patients (ANOVA, $P < 0.001$). The AR patients showed a mean iATP concentration of 560 ± 110 ng/ml (median, 531 ng/ml), significantly higher than those of stable patients (309 ± 140 ng/ml; median, 332 ng/ml). ROC analysis showed that iATP measurements could help to differentiate AR and stable patients apparently. The AUC was 0.923 (95% CI: 0.800–1.000; $P = 0.000$), indicating that iATP level of $CD4^+$ T cells may be a good biomarker for predicting graft rejection episodes [Fig. 3(B)].

Expression of perforin and granzyme B in PBMCs and biopsy in AR patients

To examine the association of perforin and granzyme B with acute renal allograft rejection, we used flow cytometry and immunohistochemistry to detect perforin and granzyme B expression levels in PBMCs and graft biopsy of transplant patients. Flow cytometry data showed the percentages of perforin and granzyme B positive PBMCs were significantly increased in AR patients compared with stable patients [Fig. 4(A,B)]. For graft biopsies, the expression levels of both perforin and granzyme B were increased in AR patients [Fig. 4(C,D)], and were significantly decreased after the anti-rejection treatment (data not shown). Graft biopsies were classified by three grades according to the expression degree of perforin and granzyme B: +++ indicated $>50\%$ of the renal tubular cells were found to express detected protein; ++ indicated 25%–50% tubular cells were stained; and + indicated $<25\%$ of the renal tubular cells were positive. For AR patients, grade ++ and +++ were 68% and 70.2%, respectively. On the contrary, the main expression grade of stable patients was + (57.9% for perforin expression and 63.2% for granzyme B). ROC analysis showed that expression of granzyme B and perforin could help differentiate AR and stable patients (Table 3). As shown in Fig. 5(A,B), the AUC of granzyme B and perforin were 0.790 (95% CI: 0.583–0.997; $P = 0.028$) and 0.770 (95% CI: 0.552–0.988; $P = 0.041$), respectively. It was also found that the AUC of co-evaluation of granzyme B and perforin was 0.780 (95% CI: 0.560–1.000), similar to their individual AUC [Fig. 5(C)].

Discussion

AR episodes are closely influencing the prognosis of kidney transplant [16]. Galante *et al.* [17] analyzed the follow-up information of 1544 cases and found that the 5-year survival rate of non-AR group was 82.2%, while that of AR group was 62.4% ($P < 0.01$). Therefore,

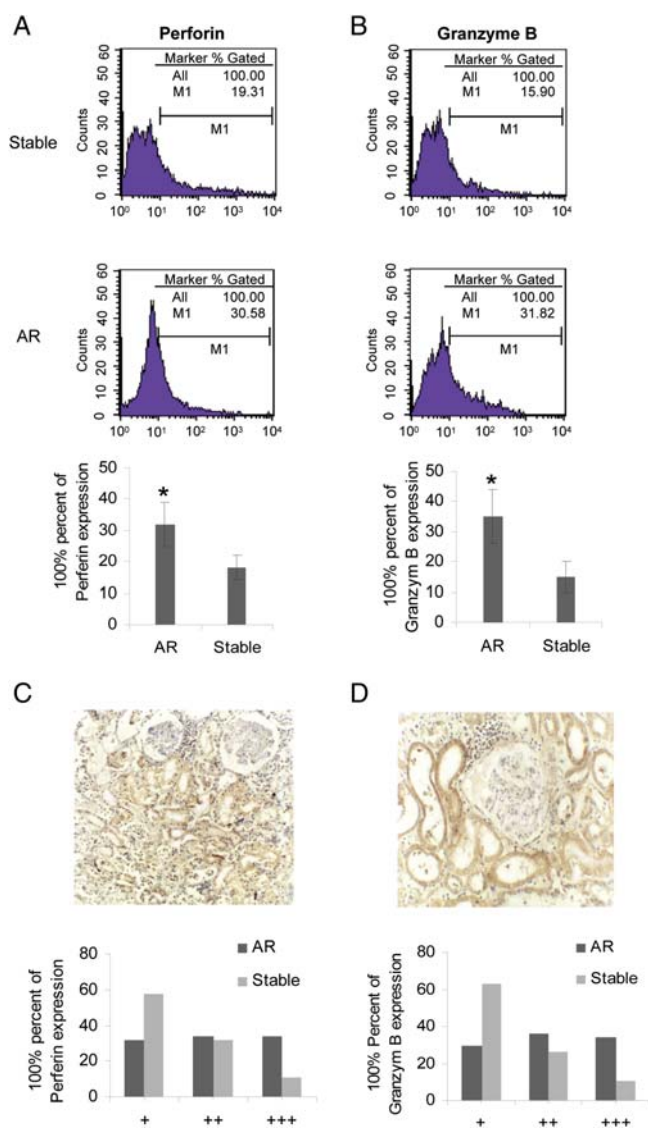


Figure 4 Expression of perforin and granzyme B in PBMCs and biopsy of AR patients before treatment Representative and group data showed perforin (A) and granzyme B (B) expressed in PBMC. Representative and group data of biopsies showed the grade of AR patients ($n = 44$) and stable patients ($n = 40$) according to the perforin (C) and granzyme B (D) expression: +++ indicated that >50% of the renal tubulars were found to expression detected protein; ++ indicated 25%–50% tubulars were positive; + indicated that <25% of the renal tubulars were positive.

Table 3 The ROC curve of granzyme B and perforin

Test variables	AUC	SE	P value	95% CI	
				Lower	Upper
Granzyme B	0.790	0.106	0.028	0.583	0.997
Perferin	0.770	0.111	0.041	0.552	0.988
Granzyme B+Perferin	0.780	0.112	0.034	0.560	1.000

AUC, area under the curve; CI, confidence interval; SE, standard error.

accurate and timely diagnosis of AR episodes is very important for disease control and prognosis. Currently, many non-invasive measurements of immune-associated molecules present in serum and lymph cells are possible alternatives to renal biopsy and serum creatinine for the detection of renal transplant rejection. These biomarkers may aid in differentiating acute allograft rejection from other etiologies of acute renal graft dysfunction.

HLA-G5 is a member of HLA-G family and associated with immune tolerance [4–6]. In this study, HLA-G5 was found to be decreased in the sera of AR patients and could be measured non-invasively by ELISA. Our data revealed that the level of HLA-G5 in serum of AR patients was significantly lower than those of stable patients. In contrast, sCD30 was found to be elevated markedly in serum of patients with an acute transplant rejection. Furthermore, co-evaluation of HLA-G5 and sCD30 level produced higher AUC than their individual AUC, indicating that co-evaluation of HLA-G5 and sCD30 was suitable as a biomarker for detecting renal transplant rejection.

Almost the entire renal transplant patients in clinical are using the immunosuppressant cyclosporine A and FK506, which both potently suppress T-cell activation by reducing transcription of interleukin-2. Both compounds inhibit mitochondrial respiration in lymphocytes, and then affect energy metabolism such as ATP production [18–20]. Hence, detecting internal ATP concentration of $CD4^+$ lymphocytes could reflect the immunosuppression degree of $CD4^+$ lymphocytes, and then predict the immune status of renal transplant recipients. In a multicenter study involving 162 kidney transplant recipients, iATP measurement was shown to be the first post-transplant test related to the risk of AR [21].

In our previous report, we found that iATP level of recipient pre-transplant was significantly lower than that of healthy human, increased in patients with acute transplant rejection episodes, but decreased to 132 ± 113 ng/ml in patients with infections [11]. In this study, we found that the iATP level of $CD4^+$ T cells also showed a statistical difference between the transplant patients with stable graft function and patients with graft rejection episodes ($P < 0.05$). ROC analysis of the iATP release level indicated that the release level of iATP in $CD4^+$ T cells was sensitive and specific in predicting AR.

It has been found that granzymes and perforin are the main effector molecules of CTLs [22–25]. Granzyme B is the most extensively studied granzyme that induces cell death through the activation of caspase-dependent and -independent pathways [26]. Perforin facilitates the entry of granzymes into the target cell cytoplasm where these serine proteases induce cell death through a number of pathways [27]. Histological studies have shown the abundance of granzyme B and perforin in many types of acutely rejecting

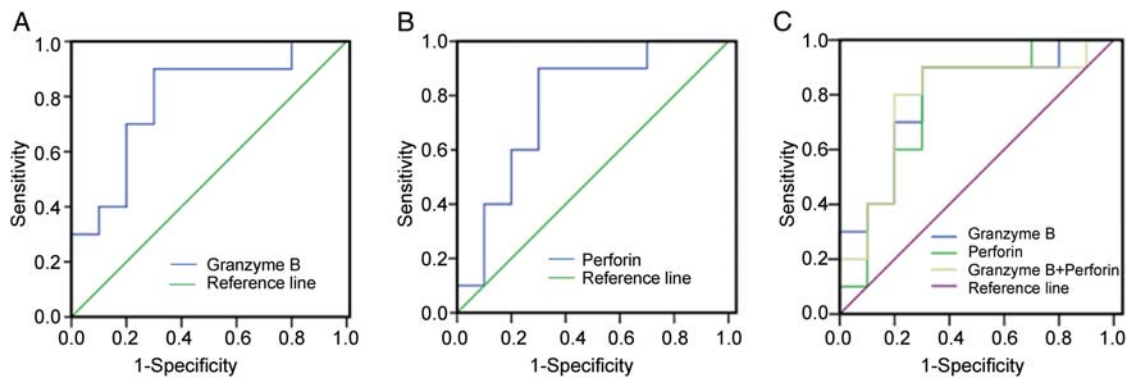


Figure 5 ROC curve analysis of perforin and granzyme B expression levels in AR patients before treatment (A) Evaluation granzyme B expression level in AR patients and stable patients by ROC curve. AUC = 0.79, $P = 0.028$. (B) Evaluation perforin expression level in AR patients and stable patients by ROC curve. AUC = 0.77, $P = 0.041$. (C) Co-evaluation granzyme B and perforin expression level in AR patients and stable patients by ROC curve. AUC = 0.78 ($P = 0.034$) vs. the reference curve AUC (AUC = 0.5).

allograft [28–32]. In addition, Griffiths *et al.* [33] and Legros-Maida *et al.* [34] investigated that increased expression of granzymes and perforin was predictive of the development of AR episodes. These studies showed that granzymes and perforin were main mediators involved in cellular rejection of solid organ allograft.

In our study, it was found that perforin/granzyme B expression levels in either PBMC or graft tissue were significantly increased in the period of AR episodes, and were significantly decreased after the anti-rejection treatment, suggesting that monitoring of perforin and granzyme B levels can be used in the assessment of post-transplant patient's immune status. Co-evaluation of perforin and granzyme B expression in biopsies showed that the AUC of perforin and granzyme B was similar to their individual one, indicating that either perforin or granzyme B could evaluate immune status of post-transplant patient, which provides valuable information for clinical treatment.

Taken together, we analyzed HLA-G5, sCD30, iATP, granzyme B, and perforin by AUC. The AUC of iATP, HLA-G5/sCD30 and granzyme B/perforin were 0.923 ($P = 0.000$), 0.879 ($P = 0.000$), and 0.78 ($P = 0.034$), respectively. The values of AUC are between 1.0 and 0.5, and the value is more close to 1, indicating the better the diagnosis is. AUC in 0.5–0.7 has lower accuracy, 0.7–0.9 has certain accuracy, and above 0.9 has a higher accuracy. All of these results lead to a speculation that iATP may be not only a better biomarker to assess the immune status of renal transplant patients but also a non-invasive measurement for the detection of renal transplant rejection.

In summary, we showed that upregulation of sCD30, iATP, granzyme B, perforin, and downregulation of HLA-G5 could provide valuable diagnostic standards to identify those recipients in the risk of AR. iATP may be a better biomarker than others for predicting graft rejection episodes. It is foreseeable that the ability to predict and prevent AR could markedly reduce the risk of post-

transplant patients' long-term survival. Future studies could utilize this information to devise new valuable biomarkers for predicting the risk of AR in post-transplant patients.

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