Changes in the Urine Proteome in Patients With Advanced Lung Cancer After Different Drug Treatments

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Research Article

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Changes in the urine proteome in patients with advanced lung cancer after different drug treatments

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Abstract

Lung cancer is one of the most threatening diseases to human life and health because of its mortality. How to choose more efficient drugs and the appropriate timing of treatment for patients with advanced lung cancer is still a problem. As the urine proteome can sensitively reflect the pathological or physiological changes of the body, it has the potential to reflect the dynamic changes of the body after drug treatment. To investigate the changes in the urine proteome of patients with advanced lung cancer after different drug treatments, urine samples were collected and analyzed at different time points. The changes in the urine proteome from the pretreatment state were different in each patient, although some of them were treated with the same drugs. The changes in the biological processes reflected by the differential urinary proteins

were consistent with the changes in the clinical manifestations of the patients. This study demonstrates that the pathophysiological changes of patients with advanced lung cancer can be reflected by changes in urinary protein after different drug treatments. In addition, the changes in urinary proteins can reflect the different biological processes in patients after the same drug treatment, and the patients' clinical condition assessment results are consistent with these changes. These findings may provide additional information for clinical treatment.
Keywords: Urine; Proteome; Advanced lung cancer; Pathophysiological changes

Introduction

In recent years, lung cancer has become a serious threat to human lives and health because of its rapidly increasing incidence and mortality. It is reported to be the most commonly diagnosed cancer (11.6%) and the leading cause of cancer deaths (18.4% of total cancer deaths)[1]. In addition, lung cancer is the second most frequent cancer in both sexes combined worldwide, and it is also the most frequent cancer in the Chinese population[2,3]. Among lung cancers, non-small-cell lung cancer (NSCLC) and small-cell lung cancer (SCLC) are the two main histologic classes. NSCLC is the most common subtype, accounting for approximately 83% of all lung cancers[4].

Treatment options for lung cancer include surgery, chemotherapy, radiation therapy, and immunotherapy. The choice of therapeutic modality depends on multiple factors, including the type and stage of the cancer[5]. Platinum-based regimens are the standard of treatment for advanced lung cancer in current clinical treatment, and they had better advantages in the survival rate and symptom control when combined with chemotherapy. However, their clinical effectiveness is affected by cumulative hematotoxicities and neurotoxicities, highlighting the requirement for alternative treatments[5]. In terms of adverse events, carboplatin mostly causes

thrombocytopenia, and cisplatin mostly causes nausea and vomiting. Therefore, the choice of platinum compounds should take into account the expected toxicity profile, patient comorbidities, and patient preferences[6]. Angiogenesis inhibitors are promising and effective ways to treat lung cancer. For example, bevacizumab, as a commonly used therapeutic drug for lung cancer, can promote tumor cell apoptosis. However, recent studies have found that it has certain toxic side effects in clinical applications, such as hypertension[7], bleeding[8], and proteinuria[9]. Among immunotherapies, pembrolizumab is an immune checkpoint inhibitor that is being rapidly developed and is approved as a first-line treatment for advanced NSCLC[10]. However, immune-related adverse effects caused by immunotherapy can affect various systems, such as abnormal thyroid function, pneumonia, and severe skin reactions[11]. Some patients will have different degrees of resistance after clinical targeted therapy, which further impacts the clinical prognosis and quality of life. Based on these problems, determining the most effective treatment for each patient, the appropriate treatment method and combined application of different methods, and the ideal timing of the treatment as well as providing the best treatment effect and reducing adverse effects are still challenges that cannot be ignored in the treatment of lung cancer. Clinicians should be mindful of these factors when considering therapeutic options for patients.

Urine proteome can sensitively reflect physiological changes in the body. With no regulation of homeostatic mechanisms, urine can accumulate changes throughout the body at a very early stage[12]. Many studies have shown that changes in the urine proteome can provide clues for the early diagnosis of many diseases, such as myocarditis model[13], Alzheimer model[14], liver fibrosis model[15], glioma model[16], lung fibrosis model[17], and chronic pancreatitis model[18]. In addition, the urine proteome can sensitively distinguish more subtle differences. It has been reported that urinary proteins have the potential to differentiate the same tumor cells grown in

different organs[19]. Urinary proteins can also sensitively reflect the changes caused by very few cells growing in the body[20]. We speculate that the potential of the urine proteome likely remains to be unleashed completely. Theoretically, if urine samples taken at different periods from one person are compared, and that the person had no significant lifestyle changes during that period, the changes in the urine should reflect the person's physiological or pathological changes during this period. The careful analysis of changes in urinary proteins caused by many different drugs with different side effects may provide clues about disease-related pathways and biological processes in the patient, which can aid in choosing the appropriate drugs to avoid adverse side effects[21].

In this study, we collected urine samples from eight patients with advanced lung cancer after different drug treatments and analyzed them by liquid chromatography-mass spectrometry (LC-MS/MS). Each patient chose their own controls to compare differences in their urine proteome at different stages. This study aims to discover dynamic changes in urinary proteins in patients with advanced lung cancer after different drug treatments and whether the urine proteome has the potential to reflect the pathophysiological changes that are consistent with clinical manifestations. It also further investigates the ability of the urine proteome to differentiate the changes in biological process induced by different drugs.

Results

Changes in the urine proteome in patients with advanced lung cancer. From October 2018 to June 2019, a total of 23 urine samples were collected and analyzed from 8 patients at different time points after drug treatment, and label-free LC-MS/MS quantification was used to characterize the differential urinary proteins. The number of urinary proteins identified in 8

patients is shown in Table 1. The collection time of urine samples, medications and clinical manifestations are shown in Tables 2 to 9. The screening conditions were set as follows: fold change ≥2 or ≤0.5; and *P*-value <0.05.

Table 1 The number of urinary proteins identified in 8 patients.

Patient ID	The number of total urinary proteins	The number of differential urinary proteins identified at different time points and the percentage in the total differential urinary protein		
		T1	T2	T3
P1	1,448	262, 55.5%	223, 47.2%	241, 51.1%
P2	1,534	161, 32.8%	302, 61.5%	232, 47.2%
P3	1,049	71, 100%	-	-
P4	1,107	315, 100%	-	-
P5	1,178	233, 100%	-	-
P6	1,838	276, 32.5%	330, 38.8%	628, 73.9%
P7	1,215	212, 100%	-	-
P8	1,248	194, 47.5%	329, 80.6%	-

- means no urine samples were collected. T1, T2, and T3 mean different collection times of urine samples.

Changes in the urine proteome and functional analysis in patient P1. The urine samples of patient P1 at four time points before and after drug treatment were collected and analyzed. More details about the urine samples and the clinical symptoms of the patient are shown in Table 2. Compared to those seen before drug treatment, a total of 1,448 urinary proteins were identified after treatment. After screening, 472 differential urinary proteins were identified, of which 262, 223, and 241 were identified at the three time points, T1, T2 and T3 (Table 1). A Venn diagram showed overlapping differential urinary proteins at the three time points (Fig. 1A). Seventy-four proteins changed continuously at three time points, and 130, 66, and 96 unique differential urinary proteins changed at the T1, T2 and T3 time points, respectively.

This study was evaluated by a single-blind study in which the medication and clinical manifestations of all patients were unknown when the functional analysis of urinary proteins was performed. The differential proteins identified at the T1, T2, and T3 time points were analyzed by the DAVID database and classified based on biological processes. At the three time points,

some biological processes related to immunity and tumor growth changed significantly (Table 2, Fig. 1B). At the T1 time point, complement activation, cellular response to growth factor stimulus, complement activation and classical pathway, cell response to tumor necrosis factor, regulation of immune, innate immune response, antigen processing and presentation, Fc-epsilon receptor signaling pathway, Fc-gamma receptor signaling pathway involved in phagocytosis, and chronic inflammatory response were altered. Compared with that before administration, the immune-related response in the patient changed significantly at the T1 time point. At the T2 time point, the significantly affected biological processes included complement activation, complement activation and classical pathway, Fc-gamma receptor signaling pathway involved in phagocytosis, complement activation and alternative pathway, and immune response. These immune-related response changes significantly in the overall biological process, indicating that after the activation of the immune system, a large number of tumor cells were continuously recognized and killed, which is consistent with the partial response of the evaluation in the patient at this time point. At the T3 time point, complement activation, complement activation and classical pathway, immune response, complement activation and alternative pathway, regulation of complement activation, inflammatory response, and gluconeogenesis were altered, and gluconeogenesis has been reported to be associated with tumor growth[22]. At this point, the evaluation of the patient was stable disease.

136 Table 2 The clinical information of patient P2 and the changed biological processes at different time points.

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Urine collection time	Target focus length (mm)	Non-target focus	Clinical manifestations	Treatment duration	Medication use	Biological processes change significantly (in part)
Control (2018.12.11)	35.2	Yes	Baseline	None	None	None
T1 (2019.01.23)	36.4	Yes	Stable disease	2019.01.08	Sintilimab: 200mg (first day); pemetrexed:991mg (first day); cisplatin:149mg (first day)	T1: proteolysis, cellular protein metabolic process, methionine biosynthetic process, fructose metabolic process, gluconeogenesis, cell-cell adhesion, cell recognition, serine family amino acid biosynthetic process, neutrophil aggregation, positive regulation of inflammatory response
T2 (2019.04.29)	-	-	Progressive disease	2019.02.21 2019.03.29	Sintilimab:200mg (first day); pemetrexed:991mg (first day); cisplatin:149mg (first day) Sintilimab:200mg; pemetrexed:991mg (first day)	T2: platelet degranulation, cell adhesion, carbohydrate metabolic process, proteolysis, gluconeogenesis, NIK/NF-kappaB signaling, proteolysis involved in cellular protein catabolic process, Fc-epsilon receptor signaling pathway, Fc-gamma receptor signaling pathway involved in phagocytosis, tumor necrosis factor-mediated signaling pathway, Wnt signaling pathway, planar cell polarity pathway, regulation of immune system process, regulation of immune response, methionine biosynthetic process, negative regulation of angiogenesis, cell recognition, cell-cell adhesion, fructose metabolic process, complement activation, positive regulation of canonical Wnt signaling pathway, glycolytic process, regulation of inflammatory response, complement activation, classical pathway, serine family amino acid biosynthetic process, L-methionine salvage
T3 (2019.06.04)	94.3	Yes	Progressive disease	2019.06.04	Sintilimab:200mg (first day); pemetrexed:991mg (first day)	T3: proteolysis, receptor-mediated endocytosis, cell adhesion, platelet degranulation, cellular protein metabolic process, Fc-gamma receptor signaling pathway involved in phagocytosis, regulation of immune system process, complement activation and classical pathway, Fc-epsilon receptor signaling pathway, proteolysis involved in cellular protein catabolic process, complement activation, Wnt signaling pathway, planar cell polarity pathway, acute inflammatory response, tumor necrosis factor-mediated signaling pathway, leukocyte migration, immune response, L-methionine salvage, carbohydrate metabolic process, negative regulation of angiogenesis

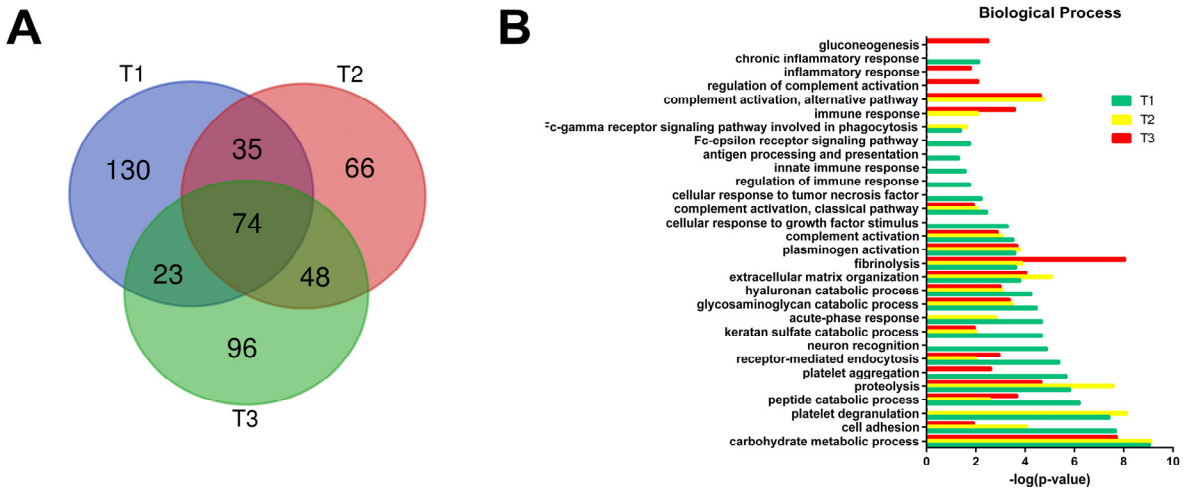

Fig. 1 Proteomic analysis of urine samples in patient P1. **A**, overlap evaluation of the differential proteins identified at three time points, T1, T2, and T3. **B**, biological processes of differential proteins at three time points, T1, T2 and T3.

Changes in the urine proteome and functional analysis in patient P2. The urine samples of patient P2 at four time points before and after drug treatment were collected and analyzed. More details about the urine samples and the clinical symptoms of the patients are shown in Table 1. Compared to those seen before drug treatment, a total of 1,534 urinary proteins were identified. After screening, 491 differential urinary proteins were identified, of which 161, 302 and 232 were identified at the three time points, T1, T2, and T3, respectively (Table 1). A Venn diagram showed overlapping differential urinary proteins at the three time points (Figure 2A). Twenty-seven proteins changed continuously at three time points, and 79, 146, and 89 unique differential urinary proteins changed at the T1, T2 and T3 time points, respectively.

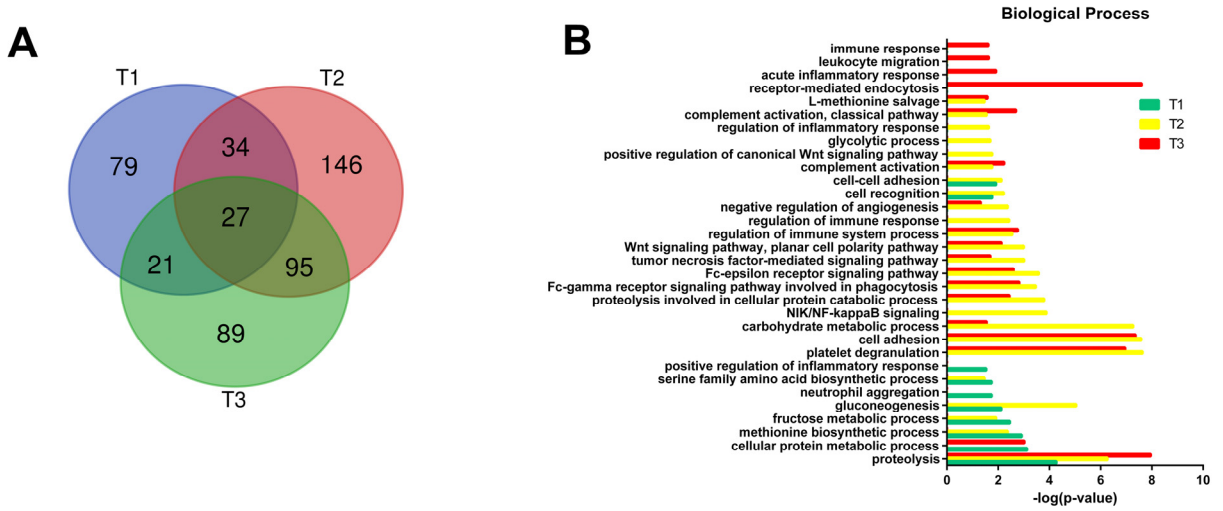

Fig. 2 Proteomic analysis of urine samples in patient P2. **A**, overlap evaluation of the differential proteins identified at three time points, T1, T2, and T3. **B**, biological processes of differential proteins at three time points T1, T2 and T3.

The differential proteins identified at the T1, T2, and T3 time points were analyzed by the DAVID database and classified based on biological processes. Changes in biological processes are shown in Table 3 and Figure 2B. At the T1 time point, processes related to proteolysis, cellular protein metabolic process, methionine biosynthetic process, fructose metabolic process, gluconeogenesis, serine family amino acid biosynthetic process, neutrophil aggregation and positive regulation of inflammatory response changed significantly, and proteolysis continuously changed significantly at each of the three time points. Compared with those before administration, few immune responses in patient P2 changed, and the biological processes associated with tumor growth changed more significantly. At this point, the evaluation of the patient was stable disease. Some of these changed biological processes have been reported in cancer, such as neutrophil aggregation, which is involved in many processes, including acute

injury and repair, chronic inflammatory processes, cancer, and autoimmunity[23]. It has been reported that methionine biosynthetic process is related to cancer, as many kinds of cancer cells require exogenous methionine to survive[24]. The abnormal dependence of cancer cells on methionine leads to methionine limitation as a potential treatment strategy[25]. Serine, as an important one-carbon donor of the folic acid cycle, can play an antioxidant role because many kinds of cancer cells are highly dependent on it[26].

At the T2 and T3 time points, the biological processes that commonly changed included platelet degranulation, cell adhesion, carbohydrate metabolism, proteolysis involved in cellular protein catabolic process, Fc-gamma receptor signaling pathway involved in phagocytosis, Fc-epsilon receptor signaling pathway, tumor necrosis factor-mediated signaling pathway, Wnt signaling pathway and planar cell polarity pathway, regulation of immune system process, negative regulation of angiogenesis, cell recognition, cell adhesion, complement activation, complement activation and classical pathway, and L-methionine salvage. Except for the common biological processes, the changed biological processes at the T2 time point included NIK/NF-kappaB signaling, regulation the immune response, positive regulation of canonical Wnt signaling pathway, glycolysis process, and regulation of inflammatory response. The changed biological processes at the T3 time point included receptor-mediated endocytosis, acute inflammatory response, leukocyte migration, and immune response. Some biological processes have been reported to be associated with tumors, for example, tumor cells usually show a reduction in cell-to-cell and/or cell-matrix adhesions, and there is increasing evidence that the reduction in cell adhesion is associated with tumor invasion and metastasis[27]. NF-κB has anti-inflammatory effects in vivo[28]; (3) Wnt signaling abnormalities are generally considered to be

key factors that trigger and maintain many cancers by affecting cancer stem cells (CSCs)[29]. Angiogenesis is considered a common feature of tumorigenesis[30].

Those changes in biological processes that are negatively related to tumor growth may be related to the use of chemotherapy drugs in patient P2. A large number of immune-related biological processes were increased at two time points, which may be related to the use of sintilimab. However, in the overall biological processes, the changes in biological processes negatively related to tumor growth changes were less significant, while those related to the immune system appeared later. At this point, the evaluation of the patient was progressive disease. It may be that the combination of these drugs used in patient P2 has no good therapeutically effective.

Changes in the urine proteome and functional analysis in patient P3. The urine samples of patient P3 at two time points before and after drug treatment were collected and analyzed. More details about the urine samples and the clinical symptoms of the patient are shown in Table 4. Compared to those seen before drug treatment, a total of 1,049 urinary proteins were identified. After the screening, 71 differential urinary proteins were identified (Table 1).
Functional enrichment analysis was performed on the identified differential urinary proteins by using the DAVID database. Changes in biological processes are shown in Table 4 and Fig. 3. Processes related to platelet degranulation, platelet aggregation, blood coagulation and fibrin clot formation, blood coagulation, wound healing and spreading of cells significantly changed, which may be related to the clinical manifestations in patient P3, such as cough and hemoptysis before treatment. After treatment, these symptoms were relieved. Changes in immune-related reactions include positive regulation of NF-kappaB transcription factor activity, positive regulation of

213 Table 3 The clinical information of patient P1 and the changed biological processes at different time points.

Urine collection time	Clinical manifest-ations	Treatment duration	Medication use	Biological processes change significantly (in part)
Control (2018.11.25)	Baseline	None	None	None
T1 (2019.01.08)	Stable disease	None	None	T1: carbohydrate metabolic process, cell adhesion, platelet degranulation, peptide catabolic process, proteolysis, platelet aggregation, receptor-mediated endocytosis, neuron recognition, keratan sulfate catabolic process, acute-phase response, glycosaminoglycan catabolic process, hyaluronan catabolic process, extracellular matrix organization, fibrinolysis, plasminogen activation, complement activation, cellular response to growth factor stimulus, complement activation, classical pathway, cellular response to tumor necrosis factor, chronic inflammatory response, regulation of immune response, Fc-epsilon receptor signaling pathway, innate immune response, Fc-gamma receptor signaling pathway involved in phagocytosis, antigen processing and presentation
T2 (2019.02.21)	Partial response	2019.01.23	Pemetrexed:930 mg(first day);cisplatin:40 mg(first day to third day);bevacizumab:500mg(first day)	T2: carbohydrate metabolic process, platelet degranulation, proteolysis, extracellular matrix organization, complement activation and alternative pathway, cell adhesion, fibrinolysis, plasminogen activation, glycosaminoglycan catabolic process, hyaluronan catabolic process, complement activation, acute-phase response, peptide catabolic process, immune response, complement activation and classical pathway, keratan sulfate catabolic process, receptor-mediated endocytosis, Fc-gamma receptor signaling pathway involved in phagocytosis
T3 (2019.04.10)	Stable disease	2019.04.09	Pemetrexed:930 mg(first day); bevacizumab:500mg(first day)	T3: fibrinolysis, carbohydrate metabolic process, proteolysis, complement activation and alternative pathway, extracellular matrix organization, plasminogen activation, peptide catabolic process, immune response, glycosaminoglycan catabolic process, hyaluronan catabolic process, receptor-mediated endocytosis, complement activation, platelet aggregation, gluconeogenesis, regulation of complement activation, keratan sulfate catabolic process, cell adhesion, complement activation and classical pathway, inflammatory response

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inflammatory response, neutrophil aggregation, and leukocyte migration involved in inflammatory response. Other biological processes, such as cell adhesion and the positive regulation of nitric oxide biosynthesis, may be related to tumors. It has been reported that nitric oxide has different effects on tumors, as it can either stimulate the growth of tumor cells or promote their death depending on its source[31].

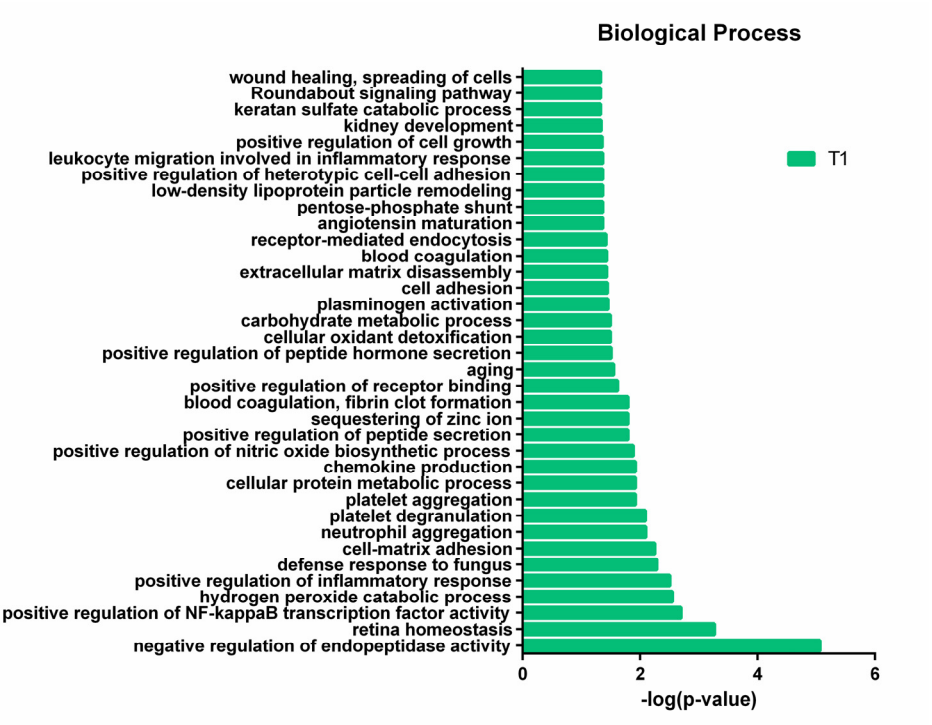


Fig. 3 Biological processes of differential proteins in patient P3.

During disease progression in patient P3, the biological processes related to vigorous tumor growth activities, such as glycolysis and gluconeogenesis, did not change significantly. The results indicate that pembrolizumab had a certain effect in patient P3. This is consistent with the evaluation as stable disease, and the efficacy of the drug may require follow-up observation.

Table 4. The clinical information of patient P3 and the changed biological processes.

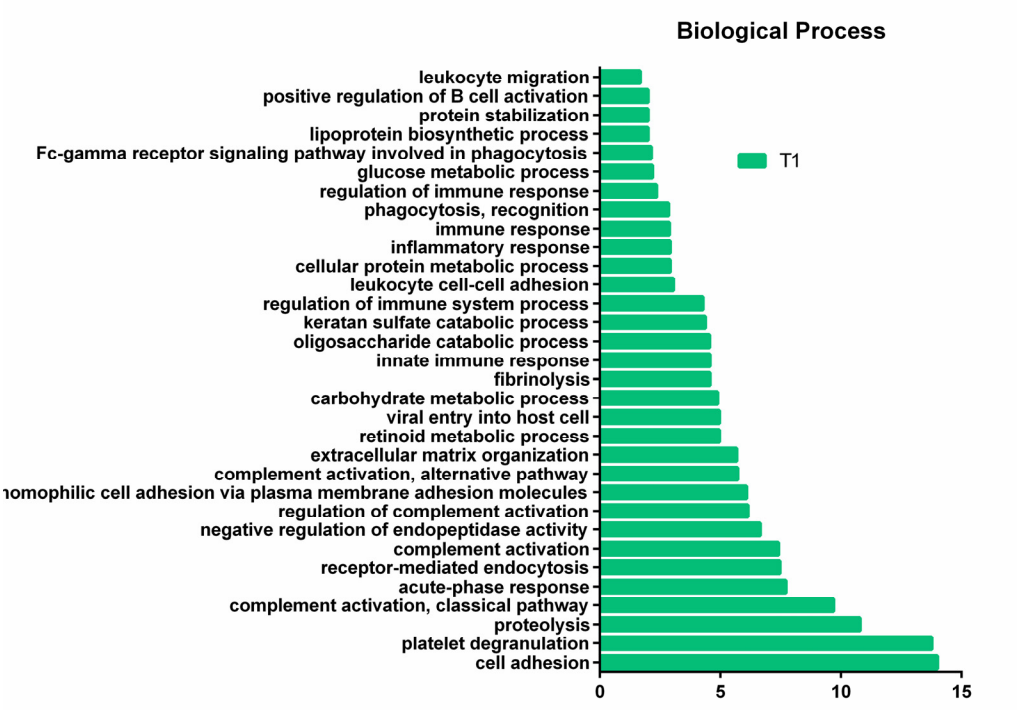
Urine collection time	Clinical manifestations	Treatment duration	Medication use	Biological processes change significantly (in part)
Control (2018.12.25)	Baseline	None	None	None
T1 (2019.02.22)	Stable disease	2019.02.02	Pembrolizumab:200mg(first day)	T1: negative regulation of endopeptidase activity, retina homeostasis, positive regulation of NF-kappaB transcription factor activity, hydrogen peroxide catabolic process, positive regulation of inflammatory response, defense response to fungus, cell-matrix adhesion, neutrophil aggregation, platelet degranulation, platelet aggregation, cellular protein metabolic process, chemokine production, positive regulation of nitric oxide biosynthetic process, positive regulation of peptide secretion, sequestering of zinc ion, blood coagulation and fibrin clot formation, cell adhesion, blood coagulation, leukocyte migration involved in inflammatory response, wound healing and spreading of cells

Changes in the urine proteome and functional analysis in patient P4. The urine samples of patient P4 at two time points before and after drug treatment were collected and analyzed. More details about the urine samples and the clinical symptoms of the patient are shown in Table 5. Compared to before drug treatment, a total of 1,107 urinary proteins were identified. After the screening, 315 differential urinary proteins were identified (Table 1).

The differential proteins were analyzed by the DAVID database and classified based on biological processes. Changes in biological processes are shown in Table 5 and Fig. 4. Compared with patient P3, who also had only one time point and was treated with the same medication, patient P4 had more differential proteins and more changes in biological processes. This indicates that patient P4 was more affected by the drug, pembrolizumab, than patient P3. The biological processes with significant changes included a large number of immune response

processes, such as complement activation and classical pathways, acute phase response, complement activation, regulation of complement activation, complement activation and alternative pathway, innate immune response, and regulation of immune system process. In addition, after treatment with pembrolizumab, the impact of the immune response occupies an important position in the change of the overall biological processes. Some biological processes have been reported to be related to tumors. In addition to those biological processes that were described in the previous patients above, such as cell adhesion, platelet degranulation, and proteolysis, vitamin A metabolic processes also play an important role because of the ability of retinoids and their synthetic derivatives to regulate cell growth, induce cell differentiation, and apoptosis. Vitamin A has become a recognized anticancer therapy[32]. Fibrin promotes cell migration by providing a matrix for tumor cell migration and interacting with adhesion molecules and integrins. Fibrinolytic components have different effects on tumors, including promoting tumor cell adhesion to the extracellular matrix and cell proliferation and increasing the survival of tumor cells[33]. On the other hand, the biological processes that are positively related to tumor growth changed to a lesser degree than those negatively related to tumor growth. Although the number of changed biological processes in patient P4 is greater than that in patient P3, the number of biological processes that are positively related to tumor growth was not been significantly increased. This indicates that the differential proteins are more affected by the immune response.

The changes in the biological processes indicate that the changes reflected by urinary protein are consistent with clinical manifestations, and the use of pembrolizumab is better for this patient.



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267 Fig. 4 Biological processes of differential proteins in patient P4.

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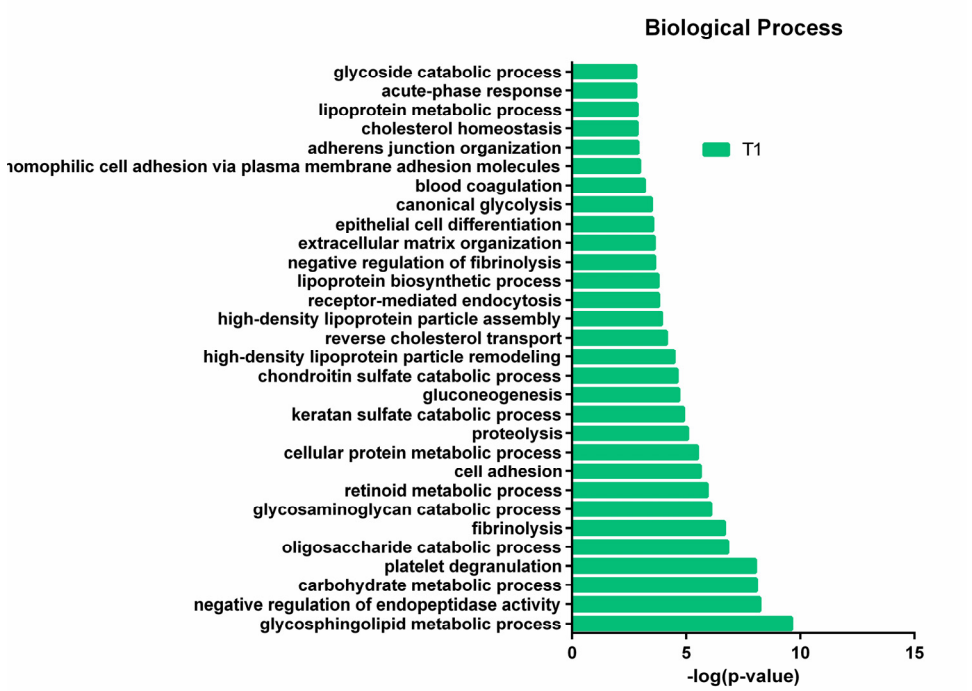
269 Table 5. The clinical information of patient P4 and the changed biological processes.

Urine collection time	Clinical manifestations	Treatment duration	Medication use	Biological processes change significantly (in part)
Control (2019.01.04)	Baseline	None	None	None
T1 (2019.06.28)	Partial response	2019.06.17	Pembrolizumab: 200mg (first day)	T1: cell adhesion, platelet degranulation, proteolysis, complement activation and classical pathway, acute-phase response, receptor-mediated endocytosis, complement activation, negative regulation of endopeptidase activity, regulation of complement activation, homophilic cell adhesion via plasma membrane adhesion molecules, complement activation and alternative pathway, extracellular matrix organization, retinoid metabolic process, viral entry into host cell, carbohydrate metabolic process, innate immune response, regulation of immune system process, leukocyte cell-cell adhesion, inflammatory response, immune response, phagocytosis and recognition, glucose metabolic process, Fc-gamma receptor signaling pathway involved in phagocytosis

270

Changes in the urine proteome and functional analysis in patient P5. The urine samples of patient P5 at two time points before and after drug treatment were collected and analyzed. More details about the urine samples and the clinical symptoms of the patient are shown in Table 6. Compared to those seen before drug treatment, a total of 1,178 urinary proteins were identified. After the screening, 233 differential urinary proteins were identified (Table 1).

Functional enrichment analysis was performed on the identified differential urinary proteins by using the DAVID database. Changes in biological processes are shown in Table 6 and Fig. 5. Patient P5 also had many differential proteins that were the same as those in patient P4. In terms of biological processes with significant changes, a large number of catabolic processes that may be related to tumors changed significantly, including glycosphingolipid metabolic process, chondroitin sulfate catabolic process, carbohydrate metabolic process, proteolysis, glycosphingolipid metabolic process, glycosaminoglycan catabolic process, retinoid metabolic process, cellular protein metabolic process, oligosaccharide catabolic process, and fibrinolysis. Although there are many changed biological processes, there are fewer biological processes related to immune response than to other signaling processes. The significance of changes in immune-related processes, such as acute phase response, complement activation and alternative pathway, regulation of complement activation, and innate immune response was lower. One possible reason is related to medication use.



289

290 Fig. 5 Biological processes of differential proteins in patient P5.

291

292 Table 6. The clinical information of patient P5 and the changed biological processes.

Urine collection time	Clinical manifestations	Treatment duration	Medication use	Biological processes change significantly (in part)
Control (2018.12.21)	None	None	None	None
T1 (2019.03.19)	Stable disease	2019.02.19	Etoposide:170mg (first day to third day); cisplatin:50mg (first day),40mg (second day to third day)	T1: glycosphingolipid metabolic process, negative regulation of endopeptidase activity, carbohydrate metabolic process, platelet degranulation, oligosaccharide catabolic process, fibrinolysis, glycosaminoglycan catabolic process, retinoid metabolic process, cell adhesion, cellular protein metabolic process, proteolysis, keratan sulfate catabolic process, gluconeogenesis, chondroitin sulfate catabolic process, canonical glycolysis, blood coagulation, homophilic cell adhesion via plasma membrane adhesion molecules, adherens junction organization, cholesterol homeostasis, lipoprotein metabolic process, acute-phase response, glycoside catabolic process

Based on the changes in biological processes, the combined treatment of etoposide and cisplatin used in this patient had an effect. At this time, the patient was evaluated as having stable disease, and the efficacy of the drugs may require follow-up observation.

Changes in the urine proteome and functional analysis in patient P6. The urine samples of patient P6 at four time points before and after drug treatment were collected and analyzed. More details about the urine samples and the clinical symptoms of the patient are shown in Table 7. Compared to those seen before drug treatment, a total of 1,838 urinary proteins were identified. After screening, 850 differential urinary proteins were identified, of which 276, 330 and 628 were identified at the three time points, T1, T2 and T3 (Table 1). A Venn diagram showed overlapping differential urinary proteins at the three time points (Fig. 6A). Eight-six proteins changed continuously at each of the three time points, and 84, 109, and 359 unique differential urinary proteins changed at the T1, T2 and T3 time points, respectively.

The differential proteins identified at the T1, T2, and T3 time points were analyzed by the DAVID database and classified based on biological processes. Changes in biological processes are shown in Table 7 and Fig. 6B. Some common biological processes changed at the three time points, including platelet degranulation, regulation of complement activation, complement activation and alternative pathway, proteolysis, negative regulation of endopeptidase activity, complement activation, cell adhesion, glycosaminoglycan catabolic process, blood coagulation, complement activation and classical pathway, negative regulation of cell adhesion, cellular amino acid metabolic process, acute phase response, leukocyte migration, cell-cell adhesion, metabolic processes, carbohydrate metabolic process, and oxidation-reduction process. These changed biological processes included many processes related to the immune response and tumor

316 growth. In addition to those biological processes affected in the other patients, this patient
317 showed some affected biological processes involving tumor growth; for example, tumors will
318 reprogram nutrient acquisition and metabolic pathways to meet the bioenergy, biosynthetic, and
319 redox demands of malignant cells[34]. As some metabolic characteristics are altered in many types
320 of cancer cells, altered metabolism is often considered a hallmark of cancer[35,36]. At the T1 time
321 point, immune-related biological processes, such as the regulation of complement activation,
322 complement activation and alternative pathway, complement activation, regulation of immune
323 response, and complement activation and classical pathway, changed significantly, indicating
324 that immune system function was activated. At the T2 time point, with the activation of the
325 immune system, biological processes such as the regulation of complement activation, leukocyte
326 migration, complement activation and alternative pathway, and tumor necrosis factor-mediated
327 signaling pathway changed significantly. Glycolysis and gluconeogenesis, which are related to
328 tumor growth, were also affected. At the T3 time point, the number of differential proteins
329 increased significantly compared with those seen at the other two time points, and some
330 metabolic decomposition processes, glycolysis and gluconeogenesis, were significantly affected.
331 The immune response-related processes continued to be identified at the same time.

Table 7. The clinical information of patient P6 and the changed biological processes at different time points.

Urine collection time	Target focus length (mm)	Non-target focus	Clinical manifestations	Treatment duration	Medication use	Biological processes change significantly (in part)
Control (2018.10.28)	66.6	Yes	Baseline	None	None	None
T1 (2018.12.12)	60.7	Yes	Stable disease	2018.11.22	Pemetrexed:881 mg (first day); cisplatin: 132mg (first day)	T1: platelet degranulation, regulation of complement activation, fibrinolysis, complement activation and alternative pathway, proteolysis, negative regulation of endopeptidase activity, cell adhesion, complement activation, glycosaminoglycan catabolic process, regulation of immune response, blood coagulation, complement activation and classical pathway, negative regulation of cell adhesion, cellular amino acid metabolic process, negative regulation of fibrinolysis, carbohydrate metabolic process, oxidation-reduction process, retinoid metabolic process, leukocyte migration, acute-phase response, metabolic process, immune response, positive regulation of T cell proliferation, cell-cell adhesion
T2 (2019.01.25)	54.6	Yes	Stable disease	2019.01.04	Pemetrexed:881 mg (first day); cisplatin: 132mg (first day)	T2:cell-cell adhesion, proteolysis, proteolysis involved in cellular protein catabolic process, platelet degranulation,, glycosaminoglycan catabolic process, regulation of complement activation, blood coagulation, metabolic process, leukocyte migration, cell adhesion, negative regulation of endopeptidase activity, oxidation-reduction process, canonical glycolysis, gluconeogenesis, complement activation and alternative pathway, carbohydrate metabolic process, tumor necrosis factor-mediated signaling pathway, ATP metabolic process, glutathione biosynthetic process, glycolytic process, complement activation, acute-phase response, Wnt signaling pathway, planar cell polarity pathway, cellular amino acid metabolic process, regulation of immune response, negative regulation of cell adhesion, NIK/NF-kappaB signaling, glucose metabolic process, complement activation and classical pathway
T3 (2019.05.10)	44.3	Yes	Partial response	2019.04.19	Pemetrexed:881 mg (first day)	T3: cell-cell adhesion, oxidation-reduction process, cellular amino acid metabolic process, platelet degranulation, carbohydrate metabolic process, proteolysis, NIK/NF-kappaB signaling, Wnt signaling pathway and planar cell polarity pathway, canonical glycolysis, tumor necrosis factor-mediated signaling pathway, gluconeogenesis, negative regulation of endopeptidase activity, Fc-epsilon receptor signaling pathway, glucose metabolic process, proteolysis involved in cellular protein catabolic process, glycolytic process, T cell receptor signaling pathway, metabolic process, blood coagulation, cell adhesion, Fc-gamma receptor signaling pathway involved in phagocytosis, innate immune response, regulation of complement activation, complement activation and alternative pathway

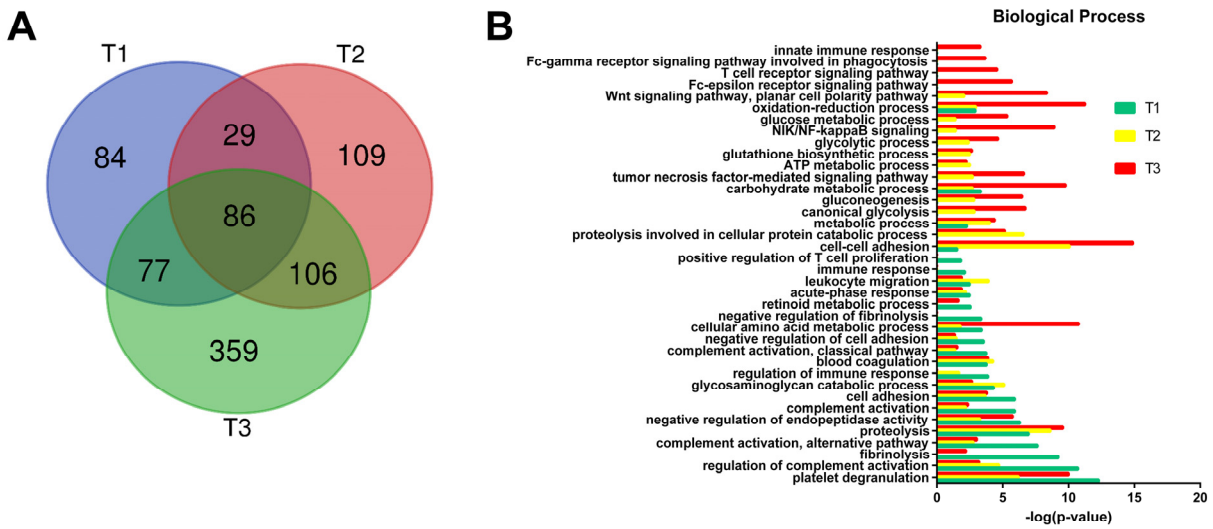


Fig. 6 Proteomic analysis of urine samples in patient P6. **A**, overlap evaluation of the differential proteins identified at three time points, T1, T2, and T3. **B**, biological processes of differential proteins at three time points T1, T2 and T3.

The changed biological processes at the three time points show that during the overall treatment process, immune-related biological processes changed significantly. The changes reflected by urinary protein are consistent with a partial response. This indicates that the combination of these drugs (pemetrexed and cisplatin) had a good therapeutic effect in patient P6.

Changes in the urine proteome and functional analysis in patient P7. The urine samples of patient P7 at two time points before and after drug treatment were collected and analyzed. More details about the urine samples and the clinical symptoms of the patient are shown in Table 7. Compared to those seen before drug treatment, a total of 1,215 urinary proteins were identified. After the screening, 212 differential urinary proteins were identified (Table 1).

The differential proteins were analyzed by the DAVID database and classified based on biological processes. Changes in biological processes are shown in Table 8 and Fig. 7. Unlike

other patients, patient P7 developed symptoms of severe fever and pneumonia after the initial treatment and died a few days later. The patient's death may have been caused by adverse events following treatment with sintilimab. Sintilimab is a human immunoglobulin G4 monoclonal antibody that specifically binds PD-1 molecules on the surface of T cells, thereby activating lymphocytes to treat tumors. However, a variety of immune-related adverse events (irAEs) may also occur during treatment with similar drugs, such as immune-related pneumonia, myocarditis, nephritis, renal insufficiency, and hepatitis [42]. There have been reports of three cases of fatal pneumonia with such drugs in clinical trials [43]. Of all the biological process affected, as reflected by changed urinary proteins, platelet degranulation, gluconeogenesis, leukocyte migration, retinoid metabolic processes, cell adhesion, movement of cell or subcellular component, negative regulation of endopeptidase activity, extracellular matrix organization, cellular response to tumor necrosis factor, and glycosaminoglycan catabolic process changed most significantly.

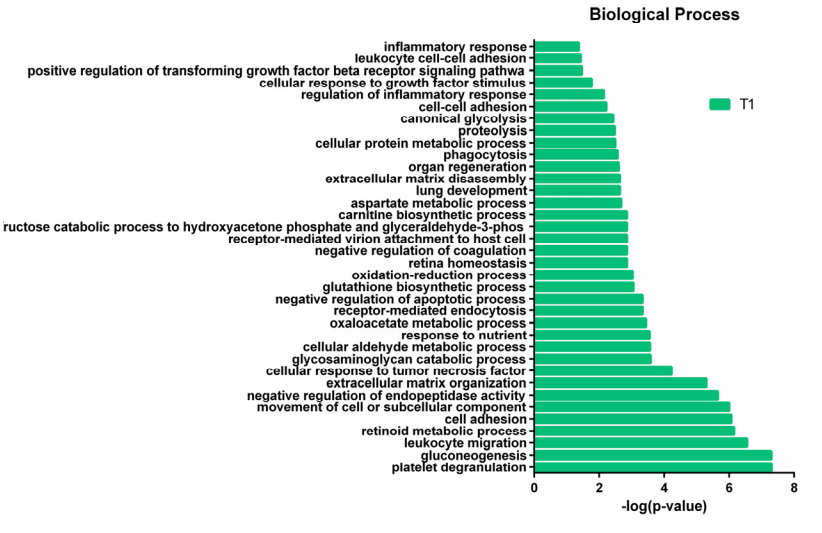


Fig. 7 Biological processes of differential proteins in patient P7.

366 Table 8. The clinical information of patient P7 and the changed biological processes.

Urine collection time	Target focus length (mm)	Non-target focus	Clinical manifestations	Treatment duration	Medication use	Biological processes change significantly (in part)
Control (2018.11.20)	15.2	Yes	Baseline	None	None	None
T1 (2018.12.11)	-	-	Death	2018.11.20	Pemetrexed: 866mg, carboplatin: 629mg, sintilimab: 200mg	T1: platelet degranulation, gluconeogenesis, leukocyte migration, retinoid metabolic process, cell adhesion, movement of cell or subcellular component, negative regulation of endopeptidase activity, extracellular matrix organization, cellular response to tumor necrosis factor, glycosaminoglycan catabolic process, cellular aldehyde metabolic process, response to nutrient, oxaloacetate metabolic process, receptor-mediated endocytosis, negative, regulation of apoptotic process, glutathione biosynthetic process, oxidation-reduction process, retina homeostasis, negative regulation of coagulation, receptor-mediated virion attachment to host cell, fructose catabolic process to hydroxyacetone phosphate and glyceraldehyde-3-phosphate, carnitine biosynthetic process, aspartate metabolic process, lung development, extracellular matrix disassembly, organ regeneration, phagocytosis, cellular protein metabolic process, proteolysis, canonical glycolysis

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The patient experienced severe adverse reactions after a period of treatment with sintilimab, pemetrexed, and carboplatin, but significant changes in a large number of immune and cytokine-related biological processes were not observed in the urine samples collected after symptoms appeared. It is speculated that the reason may be that the occurrence of irAEs was very sudden and rapid. Prior to this, the biological processes in the body were still in a relatively normal state, and the urine proteome in patient P7 was not affected.

Changes in the urine proteome and functional analysis in patient P8. The urine samples of patient P8 at three time points before and after drug treatment were collected and analyzed. More details about the urine samples and the clinical symptoms of the patient are shown in Table 9. Compared to those seen before drug treatment, a total of 1,248 urinary proteins were identified. After the screening, 408 differential urinary proteins were identified, of which 194 and 329 were identified at the time points T1 and T2, respectively (Table 2). A Venn diagram showed overlapping differential urinary proteins at two time points (Fig. 8A). One hundred and fifteen proteins changed at both time points, and 79 and 214 unique differential urinary proteins changed at the T1 and T2 time points, respectively.

The differential proteins identified at the T1 and T2 time points were analyzed by the DAVID database and classified based on biological processes. Changes in biological processes are shown in Table 9 and Fig. 8B. Some common biological processes were significantly affected, such as negative regulation of endopeptidase activity, platelet degranulation, glycosaminoglycan catabolism, cellular protein metabolic process, fibrinolysis, carbohydrate metabolism process, positive regulation of nitric oxide biosynthesis process, gluconeogenesis, canonical glycolysis, glycolysis process, proteolysis, retinoid metabolism process, cell-cell

adhesion, glycosaminoglycan metabolism process, glutathione metabolism process, acute phase response, ATP biosynthesis process, complement activation and classical pathway, complement activation and alternative pathway, complement activation, leukocyte cell-cell adhesion, and regulation of complement activation. These biological processes included immune responses and reactions related to tumor growth. At the T1 time point, some metabolic-related biological processes were significantly affected, and other biological processes related to the immune response were also affected. At that time, the patient was evaluated as having stable disease. At the T2 time point, the degree of change in biological processes related to tumor growth was significant and was higher than the degree of changes in biological processes related to immunology. Meanwhile, the number of changes immunologically related biological processes was large. The patient was evaluated as having stable disease.

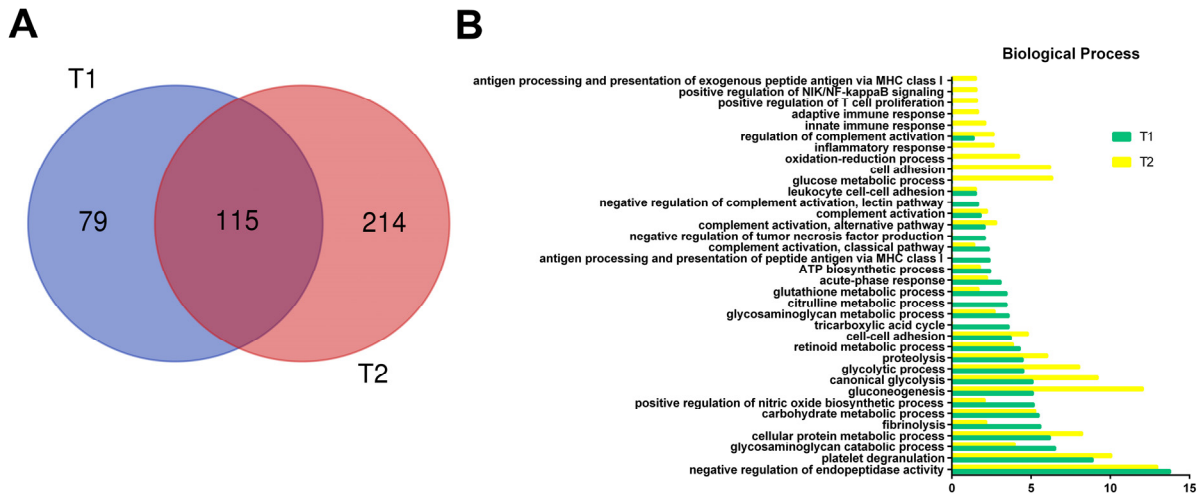

Fig. 8 Proteomic analysis of urine samples in patient P8. **A**, overlap evaluation of the differential proteins identified at two time points, T1 and T2. **B**, biological processes of differential proteins at two time points T1 and T2.

408 Table 9. The clinical information of patient P8 and the changed biological processes at different time points.

Urine collection time	Target focus length (mm)	Non-target focus	Clinical manifestations	Treatment duration	Medication use	Biological processes change significantly (in part)
Control (2019.01.25)	110.4	Yes	Baseline	None	None	None
T1 (2019.03.09)	106.9	Yes	Stable disease	2019.02.14	Pemetrexed and: 906mg (first day); cisplatin: 136mg (first day)	T1: negative regulation of endopeptidase activity, platelet degranulation, glycosaminoglycan catabolic process, cellular protein metabolic process, fibrinolysis, carbohydrate metabolic process, positive regulation of nitric oxide biosynthetic process, gluconeogenesis, canonical glycolysis, glycolytic process, proteolysis, retinoid metabolic process, cell-cell adhesion, tricarboxylic acid cycle, glycosaminoglycan metabolic process, citrulline metabolic process, glutathione metabolic process, acute-phase response, ATP biosynthetic process, antigen processing and presentation of peptide antigen via MHC class I, complement activation, classical pathway, negative regulation of tumor necrosis factor production, complement activation and alternative pathway, complement activation, negative regulation of complement activation, lectin pathway, leukocyte cell-cell adhesion
T2 (2019.04.09)	106.7	Yes	Stable disease	2019.03.29	Pemetrexed and: 680mg (first day); cisplatin: 136mg (first day)	T2:negative regulation of endopeptidase activity, gluconeogenesis, platelet degranulation, canonical glycolysis, cellular protein metabolic process, glycolytic process, glucose metabolic process, cell adhesion, proteolysis, carbohydrate metabolic process, cell-cell adhesion, oxidation-reduction process, glycosaminoglycan catabolic process, retinoid metabolic process, complement activation, alternative pathway, glycosaminoglycan metabolic process, regulation of complement activation, inflammatory response, acute-phase response, complement activation, fibrinolysis, innate immune response, positive regulation of nitric oxide biosynthetic process, ATP biosynthetic process, glutathione metabolic process, adaptive immune response, positive regulation of T cell proliferation, positive regulation of NIK/NF-kappaB signaling, leukocyte cell-cell adhesion, antigen processing and presentation of exogenous peptide antigen via MHC class II

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The significantly affected biological process at two time points show that during the treatment process, the changes in the metabolic biological processes of tumors were more significant than the changes in other processes; this is consistent with the clinical assessment of the patient, which indicated that combination treatment with pemetrexed and cisplatin had an effect in patient P8. This is consistent with the evaluation of stable disease, and the efficacy of the drug may require follow-up observation.

Methods

Ethics statement. This study was approved by the Ethics Committee of Peking Union Medical College Hospital. All methods used in this protocol were carried out in accordance with relevant guidelines and regulations. All participants signed informed consent forms.

Patient samples. A total of 23 urine samples were collected from 8 patients with advanced lung cancer (patients with unresectable stage IIIB-IV lung cancer) from Peking Union Medical College Hospital. The clinical data of all patients are summarized in Table 10. Lung cancer staging was based on the eighth edition of the International Lung Cancer TNM Staging Standard[37]. RECIST 1.1 were used to evaluate responses[38].

Table 10 Clinical profiles of patients with advanced lung cancer.

Patient ID	Age, years	Sex	Type of lung cancer	Clinical stage
P1	53	Male	Lung adenocarcinoma	IVA
P2	62	Male	Infiltrating adenocarcinoma of the upper left lung	IVA
P3	83	Male	Differentiated squamous cell carcinoma in the right lung	IIIB
P4	53	Male	Differentiated squamous cell carcinoma of the left lung	IIIB
P5	64	Male	Large cell neuroendocrine carcinoma	IIIB
P6	59	Female	Adenocarcinoma of the right upper lung	IVA

P7	70	Female	Lung poorly differentiated adenocarcinoma	IVA
P8	64	Male	Lung adenocarcinoma	IVA

Sample preparation. Urine samples were collected at different time points after different drug treatments from all patients. In addition, the control samples were collected from patients at the time they were diagnosed with advanced lung cancer and had not received treatment. All samples were morning and midstream urine samples. After collection, urine samples were centrifuged to remove cell debris and impurities and temporarily stored in a -80 °C refrigerator for later use. LC-MS/MS analysis was conducted. Briefly, 20 ml urine was thawed and centrifuged at 12,000 g for 30 minutes, and then the supernatants were transferred and mixed with three volumes of precooled ethanol in a refrigerator at -20 °C for 12 hours. The supernatant was discarded, the precipitates were dried, and lysis buffer (8 mol/L urea, 2 mol/L thiourea, 50 mmol/L Tris, and 25 mmol/L dithiothreitol) was added until the precipitates were dissolved. After centrifugation, the supernatant was retained and measured by Bradford assay. The FASP method was used to digest all the protein samples[39]. One hundred micrograms of each sample was added to a 10 kDa filter (Pall, Port Washington, NY, USA), and then the urea buffer solution (UA, 8 mol/L urea and 0.1 mol/L Tris-HCl, pH 8.5) and the 25 mmol/L NH4HCO3 solution were added sequentially to wash the urinary protein several times. Then, 100 μl NH4HCO3 solution and dithiothreitol (final concentration was 20 mmol/L) were added and incubated at 37 °C for 1 hour. Combined with the 100 μl NH4HCO3 solution, iodoacetamide (final concentration was 50 mmol/L) was added, and the mixture was placed in the dark for 30 minutes. After centrifugation at 14,000 g for 40 minutes, the UA solution and NH4HCO3 solution were again used to wash the protein several times. Then, trypsin was added to the protein (the ratio of enzyme to protein was 1:50) and mixed, and the protein was digested at 37 °C overnight. After

centrifugation at 14,000 g for 40 minutes, the filtrate was the peptide mixture. The peptide mixture was desalted with Oasis HLB cartridges (Waters, Milford, MA), evaporated to dryness in vacuo and diluted to 0.5 μg/μL with 0.1% formic acid (FA). The concentrations were determined by a BCA assay.

LC-MS/MS analysis. Two microliters of the peptide from each sample was loaded into a trap column at a flow rate of 0.3 ml/min and then separated with a reverse-phase C18 analytical column. Peptides were eluted with a gradient extending from 4% to 28% buffer B (0.1% formic acid in 80% acetonitrile) for 90 min and then analyzed with an Orbitrap Fusion Lumos Tribrid Mass Spectrometer (Thermo Fisher Scientific, USA). The MS data were acquired using the following parameters: spray voltage was 2.4 kV; ion transfer tube temperature was 320 °C; first-level full scan was set to 350-1,550 m/z, resolution was 60,000; secondary scan was set to 200-2,000 m/z, resolution was 30,000; cycle time was 3 s; and HCD collision energy was 30%. Each peptide sample was analyzed three times.

The raw data collected by the mass spectrometer were analyzed by Proteome Discoverer software (version 2.1, Thermo Fisher) to convert the format. All data files were searched using Mascot software (version 2. 4.1; Matrix Science, UK) against the SwissProt database (taxonomy: Homo; containing 20,346 sequences). The search criteria included trypsin digestion; the parent ion tolerance was 10 ppm; the fragment ion mass tolerance was 0.02 Da; two missed trypsin cleavage sites were allowed; the carbamidomethylation of cysteine was set as a fixed modification; and the oxidation of methionine was considered a variable modification. After the retrieval was completed, all the .dat files were exported and processed using Scaffold software (version 4.7.5, Proteome Software Inc., Portland, OR). The parameters were set as follows: both

protein and peptide identifications were accepted at a false discovery rate (FDR) of less than 1%; each protein contained at least 2 unique peptides; different samples were performed after normalization of total spectra; and spectral counting was used to compare protein abundance at different time points according to previously described procedures[40,41].

Statistical analysis. For each sample, three technical replicates were performed for statistical analysis. Urine samples from before and after treatment were compared for each patient. The selected criteria of differential urinary proteins were set as follows: fold change ≥2 or ≤0.5; and P-value <0.05. Comparisons between two time points were conducted using a two-sided paired t-test. The statistical analysis was performed with GraphPad Prism version 7.0 (GraphPad, San Diego, CA) software. The differential proteins were analyzed by DAVID 6.8 (https://david.ncifcrf.gov/). The proteins were described according to biological processes.

Conclusion

In this study, patients with advanced lung cancer were treated with different drugs and their urine proteomes were compared before and after treatment. It was found that pathophysiological changes in patients with advanced lung cancer could be reflected by changes in urinary protein after different drug treatments. In addition, the changes in urinary protein could reflect different biological processes in patients after the same drug treatment, and the clinical condition of patients was consistent with these changes. These findings may provide additional information for clinical treatment.

Data availability

All the data supporting the findings of this study are available within the article and its supplementary information files and from the corresponding authors upon reasonable request.

References

1 Bray, F. *et al.* Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 68, 394-424, doi:10.3322/caac.21492 (2018).

2 Torre, L. A., Siegel, R. L., Ward, E. M. & Jemal, A. Global Cancer Incidence and Mortality Rates and Trends--An Update. *Cancer Epidemiol Biomarkers Prev* 25, 16-27, doi:10.1158/1055-9965.EPI-15-0578 (2016).

3 Torre, L. A., Siegel, R. L. & Jemal, A. Lung Cancer Statistics. *Adv Exp Med Biol* 893, 1-19, doi:10.1007/978-3-319-24223-1_1 (2016).

4 Miller, K. D. *et al.* Cancer treatment and survivorship statistics, 2016. *CA Cancer J Clin* 66, 271-289, doi:10.3322/caac.21349 (2016).

5 Lemjabbar-Alaoui, H., Hassan, O. U., Yang, Y. W. & Buchanan, P. Lung cancer: Biology and treatment options. *Biochim Biophys Acta* 1856, 189-210, doi:10.1016/j.bbcan.2015.08.002 (2015).

6 Vasconcellos, V. F. *et al.* Cisplatin versus carboplatin in combination with third-generation drugs for advanced non-small cell lung cancer. *Cochrane Database Syst Rev* 1, CD009256, doi:10.1002/14651858.CD009256.pub3 (2020).

7 Seto, T. *et al.* Erlotinib alone or with bevacizumab as first-line therapy in patients with advanced non-squamous non-small-cell lung cancer harbouring EGFR mutations (JO25567): an open-label, randomised, multicentre, phase 2 study. *Lancet Oncol* 15, 1236-1244, doi:10.1016/S1470-2045(14)70381-X (2014).

8 Laskin, J. *et al.* Safety and efficacy of first-line bevacizumab plus chemotherapy in elderly patients with advanced or recurrent nonsquamous non-small cell lung cancer: safety of avastin in lung trial (MO19390). *J Thorac Oncol* 7, 203-211, doi:10.1097/JTO.0b013e3182370e02 (2012).

9 Tassinari, D. *et al.* Bevacizumab in the treatment of advanced, non-squamous non-small cell lung cancer: an evidence-based approach. *Oncology* 80, 350-358, doi:10.1159/000328781 (2011).

10 La-Beck, N. M., Nguyen, D. T., Le, A. D., Alzghari, S. K. & Trinh, S. T. Optimizing Patient Outcomes with PD-1/PD-L1 Immune Checkpoint Inhibitors for the First-Line Treatment of Advanced Non-Small Cell Lung Cancer. *Pharmacotherapy*, doi:10.1002/phar.2364 (2020).

11 Herbst, R. S. *et al.* Pembrolizumab versus docetaxel for previously treated, PD-L1-positive, advanced non-small-cell lung cancer (KEYNOTE-010): a randomised controlled trial. *Lancet* 387, 1540-1550, doi:10.1016/S0140-6736(15)01281-7 (2016).

12 YouHe, G. Urine ―an untapped goldmine for biomarker discovery? *Science China (Life Sciences)*, 10 (2013).

13 Zhao, M., Wu, J., Li, X. & Gao, Y. Urinary candidate biomarkers in an experimental autoimmune myocarditis rat model. *J Proteomics* 179, 71-79, doi:10.1016/j.jprot.2018.02.032 (2018).

14 Zhang, F., Wei, J., Li, X., Ma, C. & Gao, Y. Early Candidate Urine Biomarkers for Detecting Alzheimer's Disease Before Amyloid-beta Plaque Deposition in an APP (swe)/PSEN1dE9 Transgenic Mouse Model. *J Alzheimers Dis* 66, 613-637, doi:10.3233/JAD-180412 (2018).

15 Zhang, F., Ni, Y., Yuan, Y., Yin, W. & Gao, Y. Early urinary candidate biomarker

discovery in a rat thioacetamide-induced liver fibrosis model. *Science China Life Sciences* 61, 1369-1381 (2018).
16 Ni, Y., Zhang, F., An, M., Yin, W. & Gao, Y. Early candidate biomarkers found from urine of glioblastoma multiforme rat before changes in MRI. *Sci China Life Sci* 61, 982-987, doi:10.1007/s11427-017-9201-0 (2018).
17 Wu, J., Li, X. & Gao, Y. Early detection in urinary proteome for the effective early treatment of bleomycin-induced pulmonary fibrosis in a rat model. *Proteomics Clin Appl* 11, 11-12 (2017).
18 Zhang, L., Li, Y. & Gao, Y. Early changes in the urine proteome in a diethyldithiocarbamate-induced chronic pancreatitis rat model. *Journal of proteomics* 186, 8-14 (2018).
19 Yameng, Z., Yufei, G. & Youhe, G. Early changes in the urine proteome in a rat liver tumor model. *bioRxiv*, 568246 (2019).
20 Zhang, L., Li, Y., Meng, W., Ni, Y. & Gao, Y. Dynamic urinary proteomic analysis in a Walker 256 intracerebral tumor model. *Cancer Med* 8, 3553-3565, doi:10.1002/cam4.2240 (2019).
21 Gao, Y. *Urine: Promising Biomarker Source for Early Disease Detection*. (Springer, 2019).
22 Grasmann, G., Smolle, E., Olschewski, H. & Leithner, K. Gluconeogenesis in cancer cells - Repurposing of a starvation-induced metabolic pathway? *Biochim Biophys Acta Rev Cancer* 1872, 24-36, doi:10.1016/j.bbcan.2019.05.006 (2019).
23 Kubes, P. The enigmatic neutrophil: what we do not know. *Cell Tissue Res* 371, 399-406, doi:10.1007/s00441-018-2790-5 (2018).
24 Chaturvedi, S., Hoffman, R. M. & Bertino, J. R. Exploiting methionine restriction for cancer treatment. *Biochem Pharmacol* 154, 170-173, doi:10.1016/j.bcp.2018.05.003 (2018).
25 Chen, K. *et al.* Genetic variants in RUNX3, AMD1 and MSRA in the methionine metabolic pathway and survival in nonsmall cell lung cancer patients. *Int J Cancer* 145, 621-631, doi:10.1002/ijc.32128 (2019).
26 Yang, M. & Vousden, K. H. Serine and one-carbon metabolism in cancer. *Nat Rev Cancer* 16, 650-662, doi:10.1038/nrc.2016.81 (2016).
27 Cavallaro, U. & Christofori, G. Cell adhesion in tumor invasion and metastasis: loss of the glue is not enough. *Biochim Biophys Acta* 1552, 39-45, doi:10.1016/s0304-419x(01)00038-5 (2001).
28 Lawrence, T., Gilroy, D. W., Colville-Nash, P. R. & Willoughby, D. A. Possible new role for NF-kappaB in the resolution of inflammation. *Nat Med* 7, 1291-1297, doi:10.1038/nm1201-1291 (2001).
29 Duchartre, Y., Kim, Y. M. & Kahn, M. The Wnt signaling pathway in cancer. *Crit Rev Oncol Hematol* 99, 141-149, doi:10.1016/j.critrevonc.2015.12.005 (2016).
30 Yang, Y., Sun, M., Wang, L. & Jiao, B. HIFs, angiogenesis, and cancer. *J Cell Biochem* 114, 967-974, doi:10.1002/jcb.24438 (2013).
31 Perrotta, C. *et al.* Nitric Oxide Generated by Tumor-Associated Macrophages Is Responsible for Cancer Resistance to Cisplatin and Correlated With Syntaxin 4 and Acid Sphingomyelinase Inhibition. *Front Immunol* 9, 1186, doi:10.3389/fimmu.2018.01186 (2018).
32 Dobrotkova, V., Chlapek, P., Mazanek, P., Sterba, J. & Veselska, R. Traffic lights for

retinoids in oncology: molecular markers of retinoid resistance and sensitivity and their use in the management of cancer differentiation therapy. *BMC Cancer* 18, 1059, doi:10.1186/s12885-018-4966-5 (2018).
33 Kwaan, H. C. & Lindholm, P. F. Fibrin and Fibrinolysis in Cancer. *Semin Thromb Hemost* 45, 413-422, doi:10.1055/s-0039-1688495 (2019).
34 DeBerardinis, R. J. & Chandel, N. S. Fundamentals of cancer metabolism. *Sci Adv* 2, e1600200, doi:10.1126/sciadv.1600200 (2016).
35 Hanahan, D. & Weinberg, R. A. Hallmarks of cancer: the next generation. *Cell* 144, 646-674, doi:10.1016/j.cell.2011.02.013 (2011).
36 Pavlova, N. N. & Thompson, C. B. The Emerging Hallmarks of Cancer Metabolism. *Cell Metab* 23, 27-47, doi:10.1016/j.cmet.2015.12.006 (2016).
37 Amin M, E. S. G. F. S. R. G. W. M. e. a. AJCC cancer staging manual. 8th edition *New York, NY: Springer* (2017).
38 Eisenhauer, E. A. *et al.* New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). *Eur J Cancer* 45, 228-247, doi:10.1016/j.ejca.2008.10.026 (2009).
39 Wisniewski, J. R., Zougman, A., Nagaraj, N. & Mann, M. Universal sample preparation method for proteome analysis. *Nat Methods* 6, 359-362, doi:10.1038/nmeth.1322 (2009).
40 Old, W. M. *et al.* Comparison of label-free methods for quantifying human proteins by shotgun proteomics. *Mol Cell Proteomics* 4, 1487-1502, doi:10.1074/mcp.M500084-MCP200 (2005).
41 Schmidt, C. *et al.* Mass spectrometry-based relative quantification of proteins in precatalytic and catalytically active spliceosomes by metabolic labeling (SILAC), chemical labeling (iTRAQ), and label-free spectral count. *RNA* 20, 406-420, doi:10.1261/rna.041244.113 (2014).

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Author contributions

Z.Y.M., W.Y.Y and G.Y.H. conceived and designed the experiments; Z.Y.M and H.Z.Q performed the experiments; Z.Y.M., H.Z.Q and W.J. analyzed the data, Z.Y.M. wrote the paper.

Competing interests

All the authors declare no competing interests.

Figure legends

Fig. 1 Proteomic analysis of urine samples in patient P1. A, overlap evaluation of the differential proteins identified at three time points, T1, T2, and T3. B, biological processes of differential proteins at three time points, T1, T2 and T3.

Fig. 2 Proteomic analysis of urine samples in patient P2. A, overlap evaluation of the differential proteins identified at three time points, T1, T2, and T3. B, biological processes of differential proteins at three time points T1, T2 and T3.

Fig. 3 Biological processes of differential proteins in patient P3.

Fig. 4 Biological processes of differential proteins in patient P4.

Fig. 5 Biological processes of differential proteins in patient P5.

Fig. 6 Proteomic analysis of urine samples in patient P6. A, overlap evaluation of the differential proteins identified at three time points, T1, T2, and T3. B, biological processes of differential proteins at three time points T1, T2 and T3.

Fig. 7 Biological processes of differential proteins in patient P7.

Fig. 8 Proteomic analysis of urine samples in patient P8. A, overlap evaluation of the differential proteins identified at two time points, T1 and T2. B, biological processes of differential proteins at two time points T1 and T2.

Tables

Figures

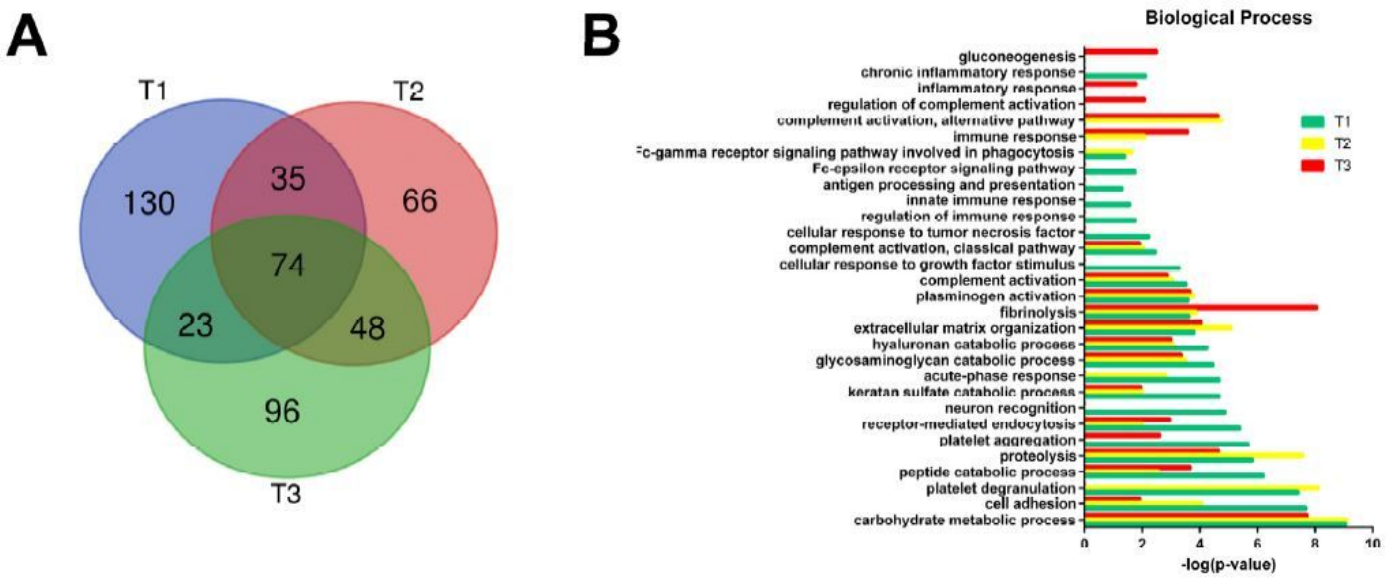

Figure 1

Proteomic analysis of urine samples in patient P1. A, overlap evaluation of the differential proteins identified at three time points, T1, T2, and T3. B, biological processes of differential proteins at three time points, T1, T2 and T3.

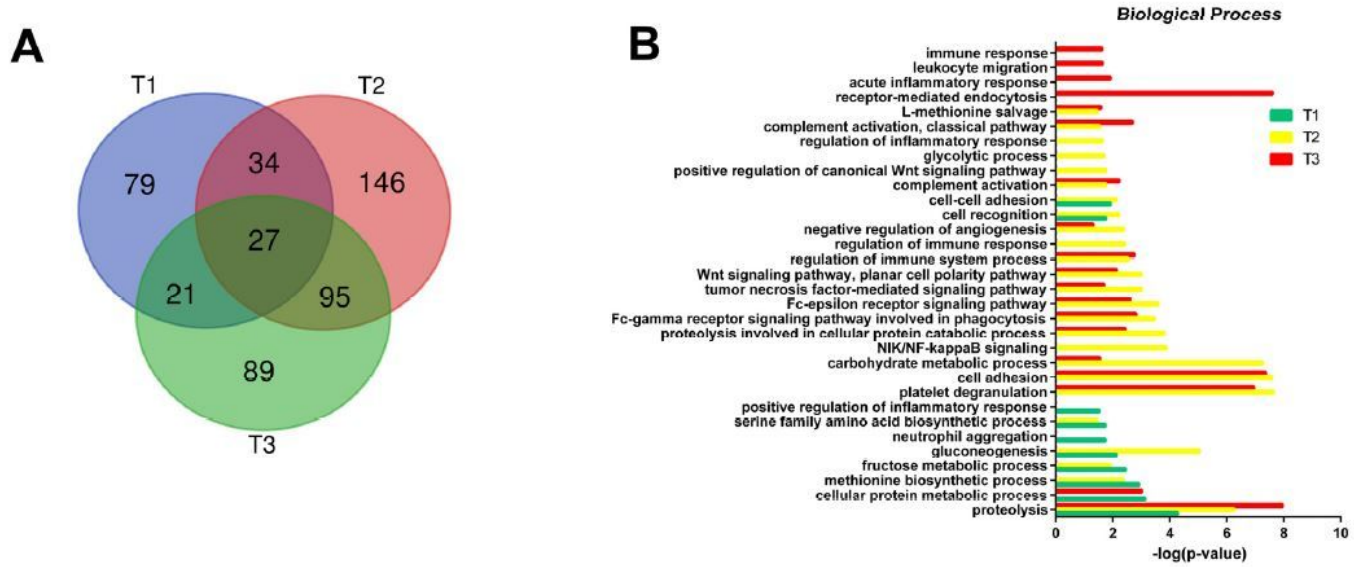

Figure 2

Proteomic analysis of urine samples in patient P2. A, overlap evaluation of the differential proteins identified at three time points, T1, T2, and T3. B, biological processes of differential proteins at three time points T1, T2 and T3.

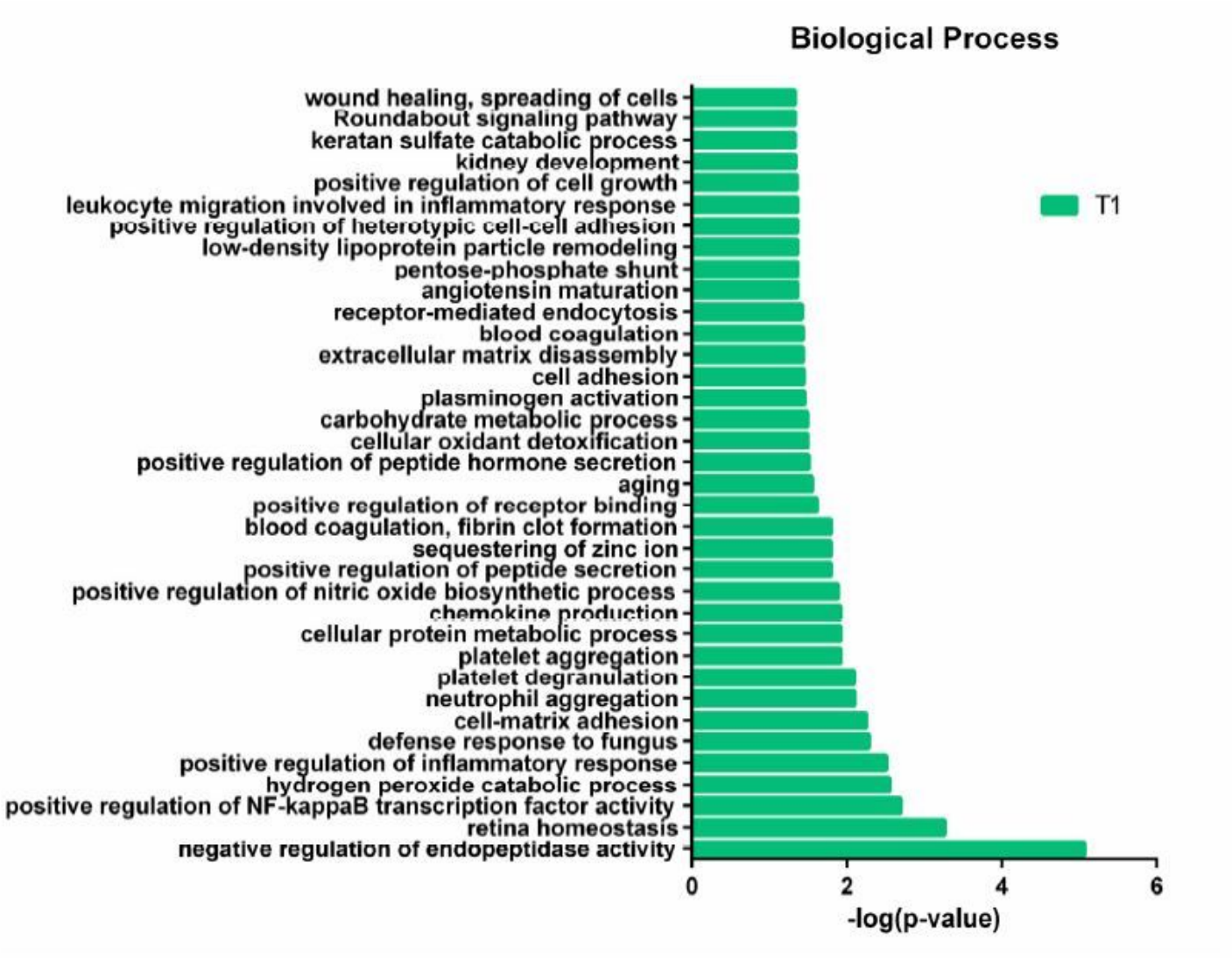

Figure 3

Biological processes of differential proteins in patient P3.

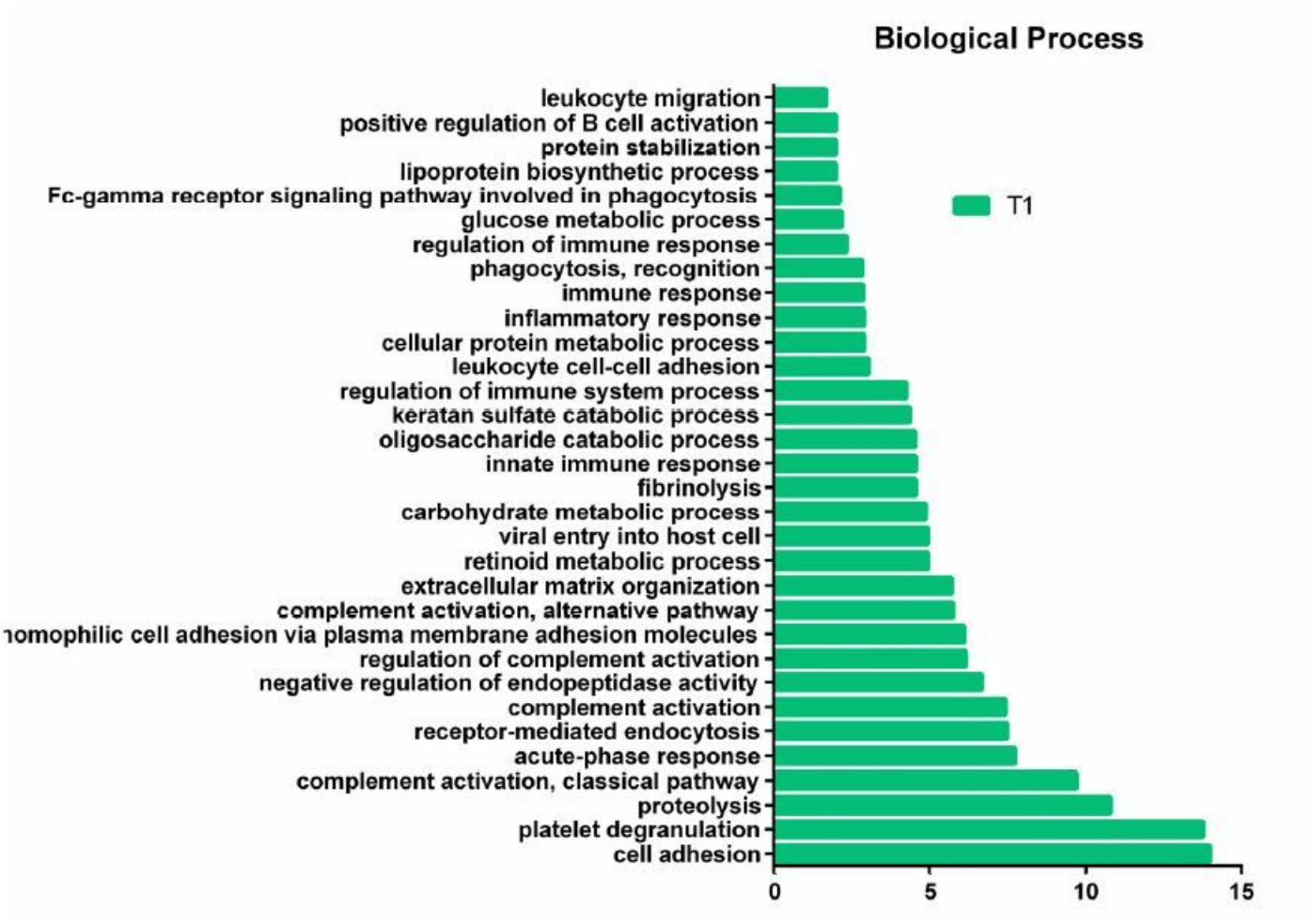

Figure 4

Biological processes of differential proteins in patient P4.

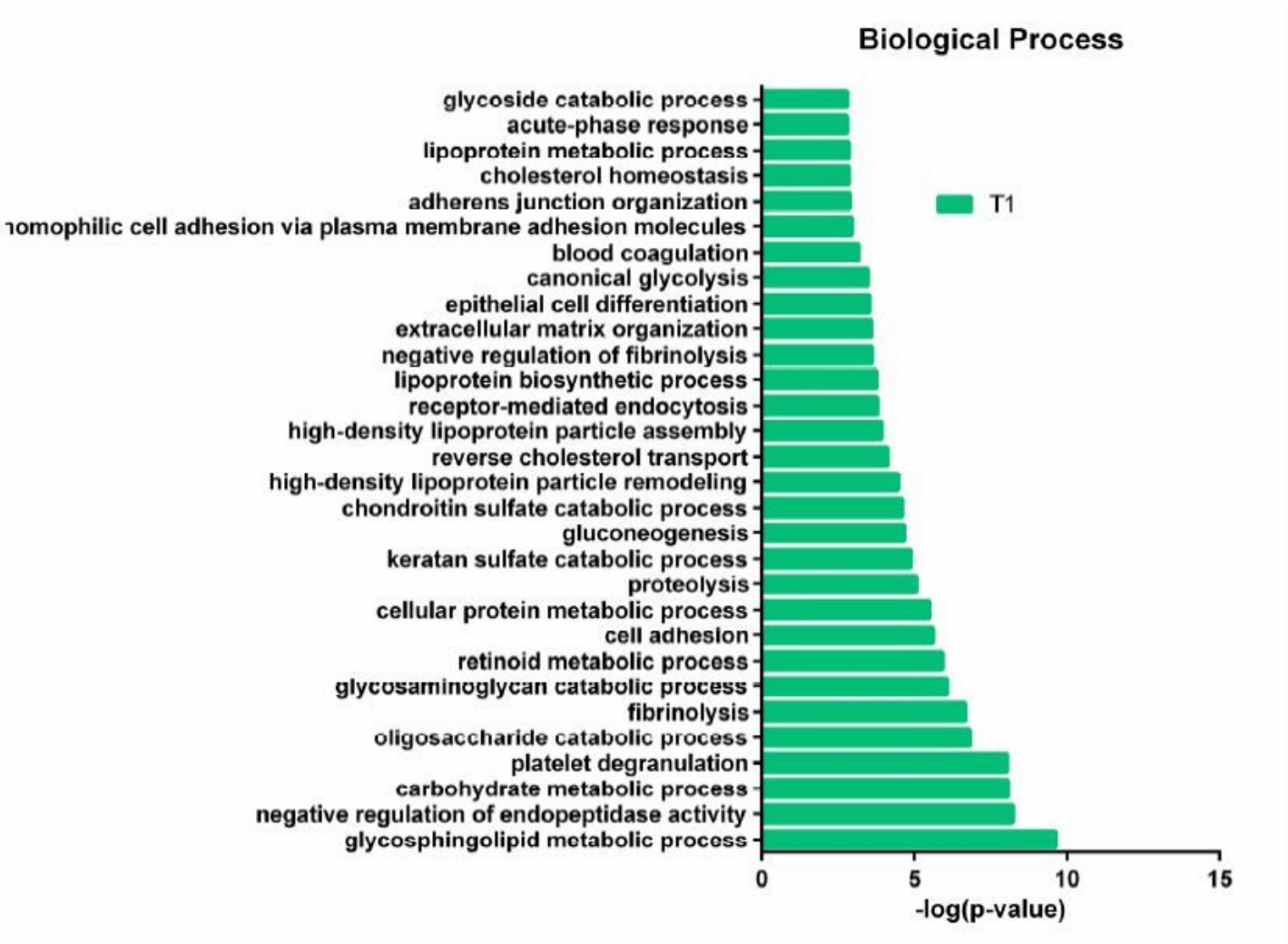

Figure 5

Biological processes of differential proteins in patient P5.

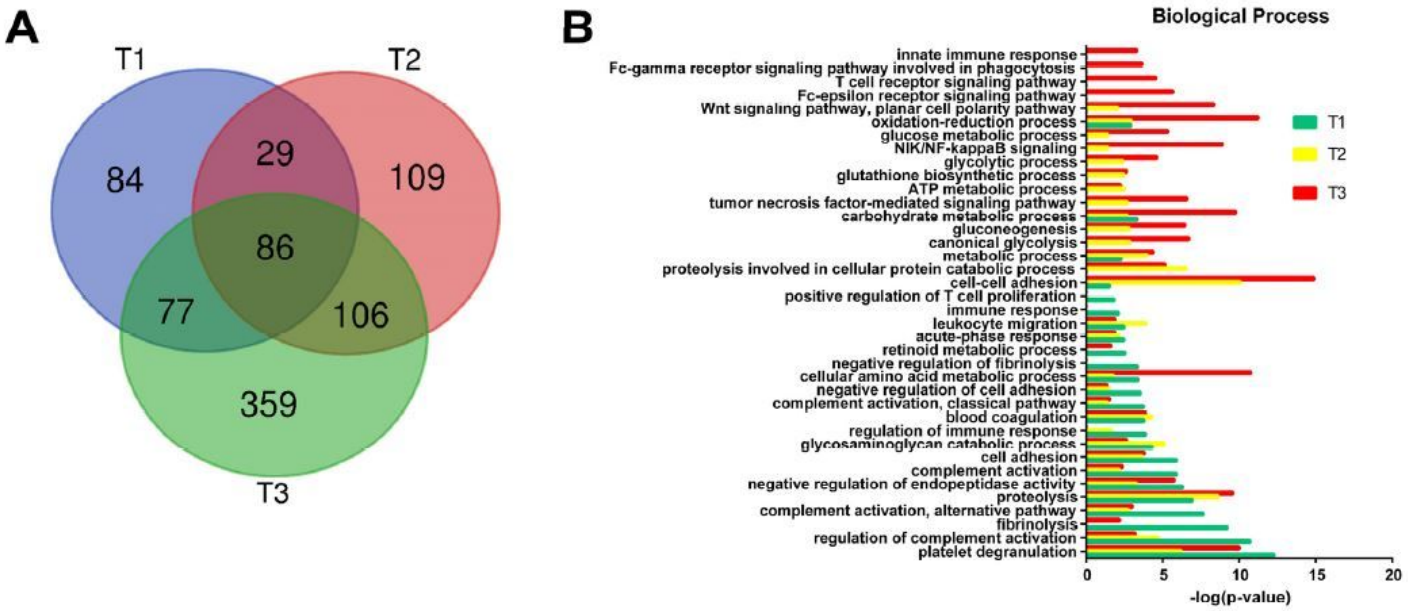

Figure 6

Proteomic analysis of urine samples in patient P6. A, overlap evaluation of the differential proteins identified at three time points, T1, T2, and T3. B, biological processes of differential proteins at three time points T1, T2 and T3.

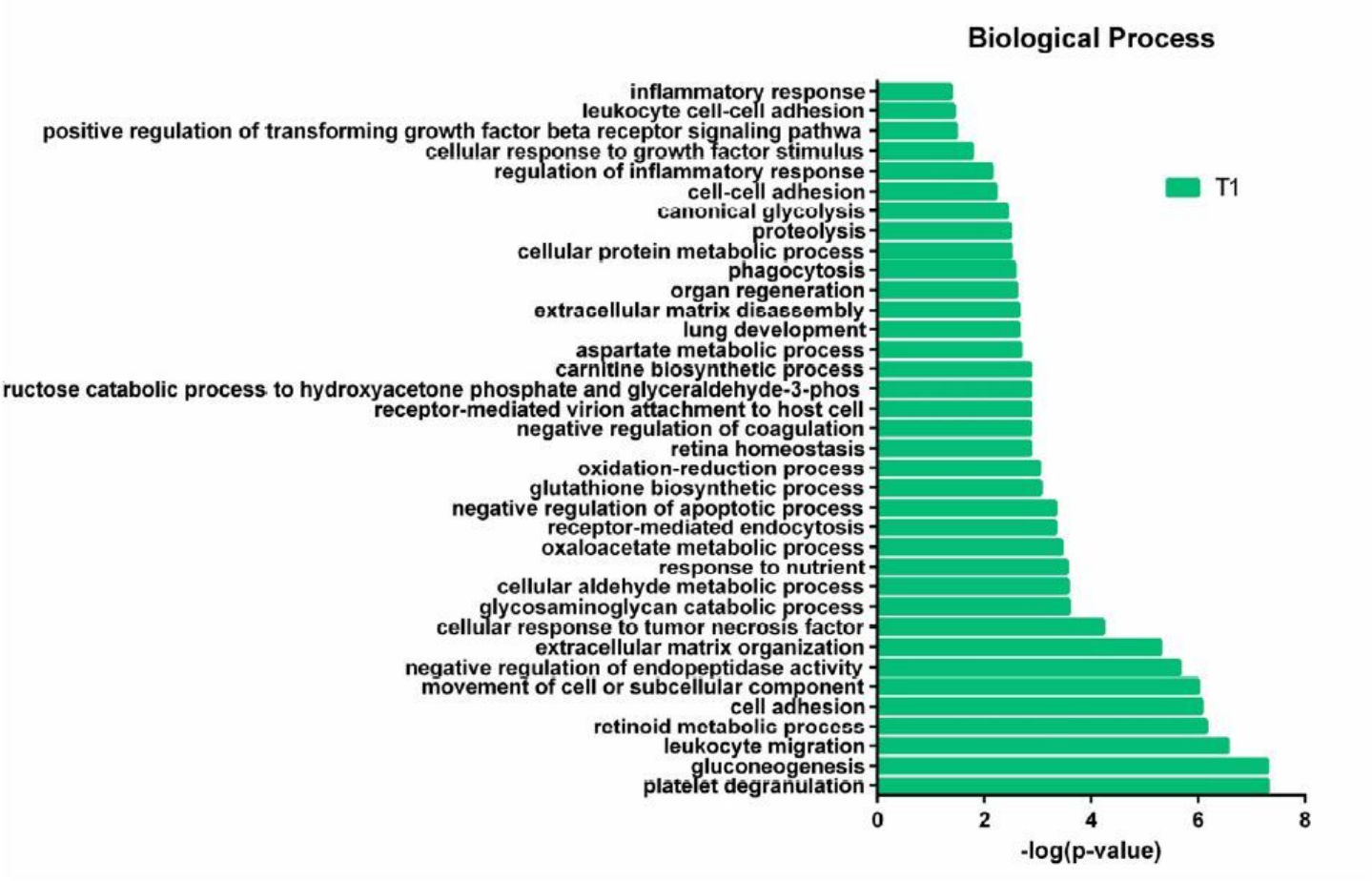

Figure 7

Biological processes of differential proteins in patient P7.

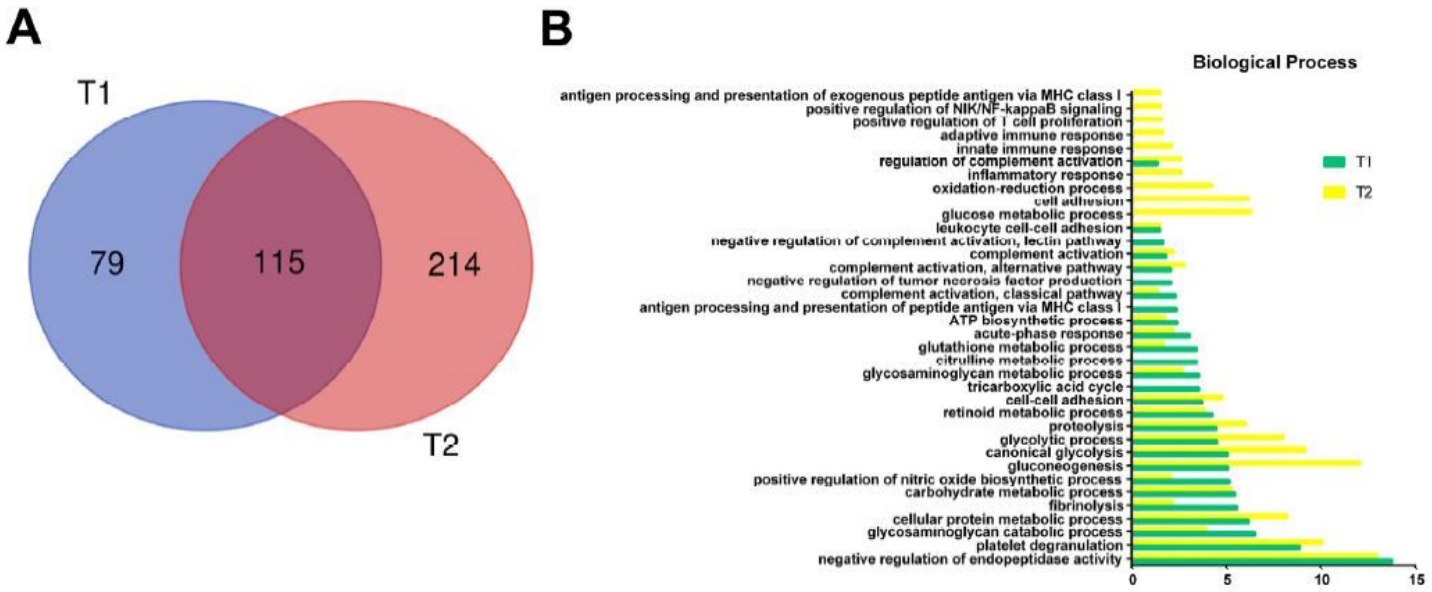

Figure 8

Proteomic analysis of urine samples in patient P8. A, overlap evaluation of the differential proteins identified at two time points, T1 and T2. B, biological processes of differential proteins at two time points T1 and T2.