A pilot assessment of xanthine oxidase activity in plasma from patients with hematological malignancies using a highly sensitive assay

Title:

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Short Running Title:

Plasma XO activity in hematological malignancies

Keywords

xanthine oxidase (XO), graft-versus-host disease (GVHD), allogeneic hematopoietic stem cell transplantation (allo-HSCT), hematological malignancy, uric acid

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Abstract

Xanthine oxidase (XO) is a rate limiting enzyme for production of uric acid. The activity of XO in plasma is reported to elevate in patients with familial hypercholesterolaemia and cardiovascular diseases. Such an increase in plasma XO activity was also demonstrated in patients with a variety of cancers. However, there have been few reports evaluating the plasma XO activity in patients with hematological malignancies during course of allogeneic hematopoietic stem cell transplantation (allo-HSCT) and chemotherapies. We explored the relationship between plasma XO activity and clinical symptoms/biochemical parameters in 10 patients with hematological malignancies. The XO activity was measured by a high-sensitivity fluorescence technique. Plasma XO activity in hematological malignancies before chemotherapy was not elevated compared to healthy subjects. In patients receiving allo-HSCT, no apparent increase in plasma XO activity was observed at the appearance of graft-versus-host disease (GVHD). Noticeably, however, the value of plasma XO activity was closely associated with that of serum level of liver transaminases in the course of allo-HSCT and chemotherapies. To our knowledge, the present study is the first to investigate consecutively the value in plasma XO activity in patients with hematological malignancies. Despite small numbers analyzed, value of plasma XO activity was tightly associated with that of serum level of liver transaminases in the course of allo-HSCT and chemotherapies, suggesting that the value may reflect liver damage related with a variety of therapeutic interventions.

1. Introduction

Xanthine oxidoreductase (XOR) is the key enzyme in the production of uric acid. XOR is abundantly expressed as xanthine dehydrogenase (XDH) mainly in liver and intestine. XDH is physiologically converted to xanthine oxidase (XO) by proteases on the vasculature in the blood. Noticeably, when liver is suffered from hypoxia/inflammation, XDH is released into circulation and is subsequently converted into XO.[1-2] Elevated plasma XO level has been reported in familial hypercholesterolaemia and cardiovascular diseases.[3-4] Elevated plasma XO level has also been reported in patients with a variety of malignancies including breast cancer, lung cancer and non-Hodgkin's lymphoma.[5] Of note, it has been shown that local XO activities were elevated by hypoxia, inflammatory cytokines and glucocorticoids.[6-7] Very recently, we reported that the value of plasma XO activity was significantly correlated with indices of insulin resistance in patients with type 2 diabetes mellitus (T2DM) and metabolic syndrome (MetS), suggesting that the value of plasma XO activity possibly providing a clue to uncover hidden risks for metabolic derangement in clinics.[8]

Regarding hematological malignancies, high value of XO activity in plasma has been reported in patients with non-Hodgkin's lymphoma and acute lymphoblastic lymphoma, tempting to speculate that plasma XO activity would reflect disease activity or therapeutic efficacy also in hematological malignancies.[5] Allogeneic hematopoietic stem cell transplantation (allo-HSCT) effectively eradicates a line of hematological malignancies, but graft-versus-host disease (GVHD) remains lethal in some cases. The clinical manifestations of acute GVHD mainly occur in the skin, gastrointestinal tract, lung and liver.[9] However, there seems no validated predictive biomarker for acute GVHD.[10] Of note, susceptible organs of GVHD such as liver and

intestine are the major production sites of XDH, a biological precursor of XO. In this context, to explore the clinical implication of plasma XO activity in patients with hematological malignancies, we performed an in-depth pilot assessment of plasma XO activity in the clinical course of 10 patients with hematological malignancies.

2. Materials and Methods

Participants

The present study was approved by the institutional ethical committee (approved number: 992, institutional ethical committee of University of the Ryukyus on September 2nd, 2016). Written informed consent was obtained from all participants. A line of studies was conducted in accordance with the Helsinki Declaration. Thirty-five patients with hematological malignancies who received treatment at Ryukyu University Hospital during the period from October 2016 to August 2017 were enrolled in this study. Among the patients studied, in-depth assessments were performed for 10 patients whose plasma XO activity and clinical parameters were measured consecutively at least 5 times during the study period. Among these 10 patients, 4 patients underwent allo-HSCT and 6 patients received chemotherapy during the study period. To examine the possible difference in the value of plasma XO activity between patients with hematological malignancies and healthy subjects, we enrolled 5 healthy volunteers. Plasma XO activities were compared to those of 5 patients at the pre-treatment status.

Sample preparation for assessing XO activity in plasma

Blood was collected into 5-mL tubes containing heparin from participants regardless of before and after a meal time. Blood collection tubes were purchased from TERUMO Corporation (Venoject II vacuum blood collection tube, Japan).

Samples were centrifuged at 1500 × g for 15 min at 4°C for isolation of plasma. Centrifuge was performed within 1 hour after blood collection to avoid the leak of hypoxanthine from erythrocytes into plasma. Supernatants were subsequently transferred to new tubes and were pooled at -80°C as plasma samples.

Measurement of XO activity in plasma

Plasma XO activity was determined using a fluorometric assay measuring the conversion of pterin to isoxanthopterin with a considerably high sensitivity [11-12]. Briefly, the enzyme reaction was initiated by mixing 50 µl of plasma with 50 µl of 0.05 mol/L Tris-HCl buffer (pH 9.0) containing 100 µmol/L pterin and 1% DMSO. For blank sample, 0.05 mol/L Tris-HCl buffer (pH 9.0) was used instead of plasma. After incubation for 3 hours at 37°C, 100 µl of 4% $HClO_4$ was added to stop reaction. The resulting mixture was vigorously shaken and centrifuged at 15000 × g for 10 min at room temperature. 150 µl of the supernatant was neutralized with 6 µl of 5 mol/L K_2CO_3 and centrifuged at 15000 × g for 10 min at room temperature. 10 µl of supernatant was subjected fluorometric analysis.

Isoxanthopterin concentration in each sample was measured by high performance liquid chromatography (HPLC) with a fluorescence detector. The HPLC system were Nexera X2 (Shimazu, Kyoto, Japan) and fluorescence detector RF-20Axs (Shimazu, Kyoto, Japan) using an YMC-Triart C18 3-µm column (YMC, Kyoto, Japan). The mobile phase was 20 mmol/L potassium phosphate buffer, the flow-rate was 1.0 mL/min and the excitation and emission wavelengths were 345 and 410 nm, respectively. For standard solution, 10 - 2000 nmol/L of isoxanthopterin solution diluted with 0.1 N NaOH or 0.05 mol/L Tris-HCl buffer (pH 9.0) was used. Area under the curve in plasma sample and blank sample was calculated, and the concentration was calibrated by standard linear regression. Isoxanthopterin concentration

produced by plasma was given by subtracting concentration of sample from that of blank. Plasma XO activity was expressed as pmol isoxanthopterin/minute/mL (pmol IXP/min/mL). Patients who routinely use XO inhibitors were carefully excluded. In case patients had taken febuxostat, a representative XO inhibitor, to prevent tumor lysis syndrome, plasma XO activity was measured after the discontinuation of febuxostat.

Metabolic parameters

Anthropometric variables were measured in the standing position. Body mass index (BMI) was calculated as weight (in kilograms) divided by the square of height in meters. Serum biochemical parameters including aspartate aminotransferase [AST], alanine aminotransferase [ALT], creatinine [CRE], uric acid [UA], total bilirubin [T-BIL], lactate dehydrogenase [LDH], soluble interleukin-2 receptor [sIL-2R], C-reactive protein [CRP] were measured by conventional automated analyzers. The estimated glomerular filtration rate (eGFR) was calculated according to the estimation formula.

Statistical Analysis

Statistical analyses were performed using a standard software package (JMP version 14; SAS Institute Inc. Cary. NC). All data were examined for normality by the Shapiro-Wilk test. Logarithmic transformation of variables was performed, where applicable. Continuous variables were compared with the Wilcoxon rank sum test. The correlation between variables was calculated using Pearson's correlation coefficient for data showing normal distribution or log-normal distribution. Spearman's rank correlation coefficient was used for data which did not show either normal distribution or log-normal distribution. To further analyze the correlation among plasma XO activity and a series of clinical parameters, interquartile range

(IQR) analysis was performed. *P* values of less than 0.05 were considered as statistically significant.

3. Results

Profile of patients

Profile of patients is summarized in Table 1. Among patients undergoing allo-HSCT, 2 patients had adult T-cell leukemia lymphoma (ATL), one patient had myelodysplastic syndrome (MDS), and one patient had acute lymphoblastic leukemia (ALL). Among patients treated with chemotherapies, 3 patients had diffuse large B-cell lymphoma (DLBCL), one patient had anaplastic large cell lymphoma (ALCL), one patient had ATL, and one patient had primary mediastinal lymphoma (PMBL).

Plasma XO activity in patients who underwent allo-HSCT patients

Figure 1 (A) summarizes the value of plasma XO activity and clinical manifestations in 4 patients who underwent allo-HSCT (case1-4), showing the results of serial measurement of clinical parameters up to 16 weeks after allo-HSCT, just because most of acute GVHD develops during the first 100 days after the allo-HSCT.[9] All 4 cases developed grade 1 or 2 acute GVHD with skin symptoms such as erythema and diarrhea. In cases 1 and 2, there was no increase in plasma XO activity during the clinical course. Notably, in cases 1 and 2, no apparent changes were observed in liver transaminases (AST and ALT). On the other hand, in cases 3 and 4, the value of both plasma XO activity and liver transaminase (AST and ALT) were tightly associated and concomitantly elevated.

Plasma XO activity in the course of chemotherapies

Figure 1 (B) summarizes plasma XO activity in 6 patients who received chemotherapy (case 5-10). Observation period for the plasma XO activity is 32 weeks, covering post-chemotherapy follow-up periods. No appreciable elevations in plasma XO activity, AST and ALT were observed in cases 5, 6 and 9. On the other hand, in case 7, 8 and 10, the value of both plasma XO activity and liver transaminase (AST and ALT) were tightly associated and concomitantly elevated.

Plasma XO activity and levels of serum sIL-2R

In cases 9 and 10, plasma XO activities were measured prior to chemotherapy and in the subsequent clinical course. In both cases, levels of sIL-2R, a representative tumor marker for hematological malignancies, was declined rapidly after the chemotherapies. Of note, value of plasma XO activity was stable in case 9. On the other hand, in case 10, the value of plasma XO activity was concomitantly elevated with those of liver transaminases (AST and ALT).

Comparison of XO activities in plasma between patients with hematological malignancies and healthy controls

Among enrolled subjects, XO activities in plasma were assessed in 5 patients with hematological malignancies before receiving any chemotherapies. There were no apparent differences in plasma XO activities between 5 patients with hematological malignancies and 5 healthy volunteers as described in Materials and Methods (Table 2).

Correlation between plasma XO activity and a variety of clinical parameters

Figure 2 shows correlations between plasma XO activity and a line of clinical parameters in all of blood samples (n=83) from 10 patients analyzed in the present study. Significant correlations were observed between the value of plasma XO activity and that of liver transaminases (AST; $r=0.418$, $p<0.001$, ALT; $r=0.676$,

p<0.001, respectively). In addition, the value of plasma XO activity was weakly but significantly correlated with that of LDH (r=0.266, p=0.015), UA (r=0.323, p=0.003) and CRP (r=0.318, p=0.003).

Correlation between the value of plasma XO activity and that of a line of clinical parameters via interquartile range (IQR) analyses

For a series of clinical parameters correlated with the value of plasma XO activity shown in Figure 2, analysis using interquartile range (IQR) was also carried out to further confirm whether such a correlation was similarly observed within intra-individual variations. IQR reflects physiological stability as well as pathological fluctuation in each case. Figure 3 clearly shows a significantly positive correlation between plasma XO activity and liver transaminase even in the IQR analyses (AST; r = 0.718, p = 0.019, ALT; r = 0.876, p = 0.001).

4. Discussion

The major findings in the present study are as follows. In patients with hematological malignancies, the value of plasma XO activity was tightly associated with that of serum level of liver transaminases (AST and ALT) in the course of allo-HSCT and chemotherapies, suggesting that the value of plasma XO activity may reflect liver damage due to chemotherapies and related clinical interventions. It is well known that serum value of AST and ALT are elevated by a considerable release from liver on tissue damage or inflammation. During such situations, XDH, predominantly contained in the liver, is also released into the circulation, and the value of plasma XO activity concomitantly increases through protease-mediated transformation into XO.[1-2] Since the present study included a small size of patients, further studies in a large number are warranted to fully understand the clinical

implication of plasma XO activity in patients with hematological malignancies. However, to our knowledge, the present study is the first to assess the value in plasma XO activity consecutively in patients with hematological malignancies. Importantly, the present study demonstrated that the value of plasma XO activity in pre-treated patients with hematological malignancies was not elevated as compared to that of healthy volunteers. In a limited number of previous reports, plasma XO activities were reported to elevate in patients with non-Hodgkin's lymphoma and acute lymphocytic leukemia.[5] However, these reports payed no attention to the possible relationship between plasma XO activity and liver transaminases. It is therefore likely that the value of plasma XO activity in such patients was evaluated during the course of chemotherapies, thereby possibly reflecting liver damage-induced elevation of plasma XO activity.

XO is a major ROS-generating enzyme, being involved in a wide variety of pathophysiological conditions. Notably, in a mice model of influenza virus-driven pneumonia, suppression of infection-induced exaggerated ROS in lung by XO inhibitors markedly reduced the death rate in mice.[13] Although role of XO in health and disease is attracting growing attention, plasma XO activities in humans is considerably low as compared to those in rodents.[14-15] Therefore, the sensitivity of the measurement is critical to precisely evaluate the value of XO activity.[14-16] In most of previous reports, however, methods for assessing plasma XO activity showed difficulties in detectable ranges.[17-19]

To overcome this issue, in the present study, we measured XO activities using considerably-high sensitive fluorometric assay, thereby precisely estimating the plasma XO activities in all samples studied.

Our recent study demonstrated that the value of plasma XO activity is not affected by the time point, diet or exercise.[8] The IQR analyses in the present study further confirmed that plasma XO activity was stable in each individual as far as levels of liver transaminases were constant. It is therefore reasonable to speculate that the value of plasma XO activity would be unique in each individual, potentially providing a novel avenue to estimate the responsiveness to therapies, conditions of diseases and pathophysiology in patients with hematological malignancies.

The present study has some limitations. Because the number of patients studied was limited, further extensive studies are required to strengthen our findings. Studies including a wide variety of hematological malignancies are also warranted to see whether our findings are universal phenomena. However, to the best of our knowledge, the present study is the first demonstration of consecutive analyses for plasma XO activities in hematological malignancies in humans. Despite small numbers analyzed, the value was not elevated as compared to healthy controls, but was tightly associated with the serum level of liver transaminase during the course of allo-HSCT or a series of chemotherapies, thereby providing a potential usefulness of plasma XO activities for assessing liver damages in a variety of therapies against hematological malignancies.

5. Conclusion

Plasma XO activity was tightly associated with that of serum level of liver transaminases in the course of allo-HSCT and chemotherapies, suggesting that the value may directly reflect liver damage related with a variety of therapeutic interventions in the patients with hematological malignancies.

Contributions

N. Hokama and S. Sunagawa designed the present study, collected the data and performed statistical analysis. T. Shirakura designed the study and reviewed the manuscript. S. Morishima, S. Nakachi, Y. Nishi recruited patients, conducted laboratory tests and reviewed the manuscript. T. Shirakura, C. Matsui, N. Hase, Y. Murayama and M. Tamura performed assays for xanthine oxidase activity. S. Okamoto, M. Shimabukuro and K. Nakamura reviewed the manuscript and provided a series of invaluable advices. H. Masuzaki designed the present study and wrote the manuscript. All authors contributed to the entire process of manuscript preparation and approved the final version. We thank I. Nomura, C.Horiguchi and T. Ikematsu for technical assistance. We are also grateful to M. Hirata, I. Asato, H. Kaneshiro, T. Uema and C. Noguchi for secretarial assistance.

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Figure legends

Figure 1 (A) Serial measurements of clinical parameters in 4 cases with allogeneic hematopoietic stem cell transplantation.

After the engraftment, all patients showed skin symptoms and intestinal symptoms as graft-versus-host diseases (GVHD). In patients without apparent changes in serum levels of liver transaminase (AST and ALT), there was no significant elevation in plasma XO activity (case 1 and case 2). On the other hand, in case 3 and case 4, plasma XO activity and serum levels of liver transaminase (AST and ALT) were concomitantly elevated. Black circles, diamonds, triangles, and crosses in the figure indicate xanthine oxidase activity (XOA), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and soluble form of interleukin-2 receptor (sIL-2R), respectively. The arrow line indicates the period of symptoms. The broken line indicates skin symptoms and the solid line indicates diarrhea.

Figure 1(B) Serial measurements of clinical parameters in 6 cases treated with chemotherapy.

In patients without apparent elevation in serum levels of liver transaminase (AST and ALT) (case 5, case 6, and case 9), there was no significant elevation in plasma XO activity. On the other hand, plasma XO activity value and serum levels of liver transaminase (AST and ALT) were concomitantly elevated in case 7, case 8, and case 10. Black circles, diamonds, triangles, and crosses in the figure indicate xanthine oxidase activity (XOA), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and soluble form of interleukin-2 receptor (sIL-2R), respectively. Arrows indicate chemotherapies.

Figure 2 Correlation between the level of plasma XO activity and a line of clinical parameters

Data were analyzed by Spearman's rank correlation coefficient. *P* values of less than 0.05 were considered as statistically significant. XOA: xanthine oxidase activity, AST: aspartate aminotransferase, ALT: alanine aminotransferase, T-BIL: total bilirubin, CRE: creatinine, eGFR: estimated glomerular filtration rate, LDH: lactate dehydrogenase, sIL-2R: soluble interleukin-2 receptor. UA: uric acid, CRP: C-reactive protein.

Figure 3 Correlation between the level of plasma XO activity and a line of clinical parameters via the interquartile range (IQR) analyses

The correlation between variables was calculated using Pearson's correlation coefficient for data showing normal distribution or log-normal distribution. Spearman's rank correlation coefficient was employed for data which did not show either normal distribution or log-normal distribution. *P* values of less than 0.05 were considered as statistically significant. IQR: interquartile range, XOA: xanthine oxidase activity, AST: aspartate aminotransferase, ALT: alanine aminotransferase, LDH: lactate dehydrogenase, UA: uric acid, CRP: C-reactive protein.

Table 1. Characteristics of patients studied

Profiles of patients undergoing allogeneic hematopoietic stem cell transplant

Case	Sex	Age	Diagnosis	Conditioning regimen	GVHD prophylaxis
1	Female	48	ATL	BU-CY	CYA-sMTX
2	Female	61	ATL	Flu-BU-TBI	FK-sMTX
3	Female	47	MDS	Flu-BU-TBI	CYA-sMTX
4	Male	27	ALL	CY-TBI	CYA-sMTX

Profiles of patients treated with chemotherapies

Case	Sex	Age	Diagnosis	Chemotherapy during the study period
5	Female	57	DLBCL	R-CHOP 3 course
6	Female	78	DLBCL	R-THP-COP 2 course
7	Female	79	DLBCL	R-THP-COP 1 course
8	Male	47	ALCL	THP-COP 3 course + BV 3 course + DeVIC 3 course
9	Male	69	ATL	CHOP 6 course
10	Female	30	PMBL	DA-EPOCH 6 course

GVHD: graft-versus-host disease, ATL: adult T cell leukemia, MDS: myelodysplastic syndrome, ALL: acute lymphoblastic leukemia, DLBCL: diffuse large B cell lymphoma, ALCL: anaplastic large cell lymphoma, PMBL: primary mediastinal B-cell lymphoma, BU-CY: busulfan and cyclophosphamide, Flu-BU-TBI: fludarabine, busulfan and total body irradiation, CY-TBI: cyclophosphamide and total body irradiation, CYA: cyclosporine A, FK: tacrolimus, sMTX: short term methotrexate, R-CHOP: rituximab, cyclophosphamide, doxorubicin, vincristine, prednisolone, R-THP-COP: rituximab, pirarubicin, cyclophosphamide, vincristine, prednisolone, THP-COP: pirarubicin, cyclophosphamide, vincristine, prednisolone, BV: brentuximab vedotin, DeVIC: dexamethasone, etoposide, Ifosfamide, Carboplatin, CHOP: cyclophosphamide, doxorubicin, vincristine, prednisolone, DA-EPOCH: dose-adjusted etoposide, doxorubicin, cyclophosphamide, vincristine, prednisolone.

Table 2. Comparison of plasma XOA between patient with hematological malignancies and healthy controls

	Healthy controls (n=5)			Hematological malignancies (n=5)			*P* value
XOA (pmol IXP/min/mL)	0.13	±	0.07	0.12	±	0.12	0.402
Age (years)	54.4	±	12.78	58.2	±	19.56	0.676
BMI (kg/m^2)	23.6	±	1.45	20.2	±	2.14	0.036
AST (IU/l)	19.2	±	7.23	17	±	2.24	0.834
ALT (IU/l)	19.2	±	10.62	10	±	3.67	0.091
CRE (mg/dl)	0.61	±	0.18	0.72	±	0.17	0.295
UA (mg/dl)	4.9	±	1.11	5.8	±	1.11	0.295

Data are expressed as mean ± standard deviation. Data were analyzed by Wilcoxon rank sum test.

XOA: xanthine oxidase activity, BMI: Body Mass Index, AST: aspartate aminotransferase, ALT: alanine aminotransferase, CRE: creatinine, UA: uric acid.

Figure 1

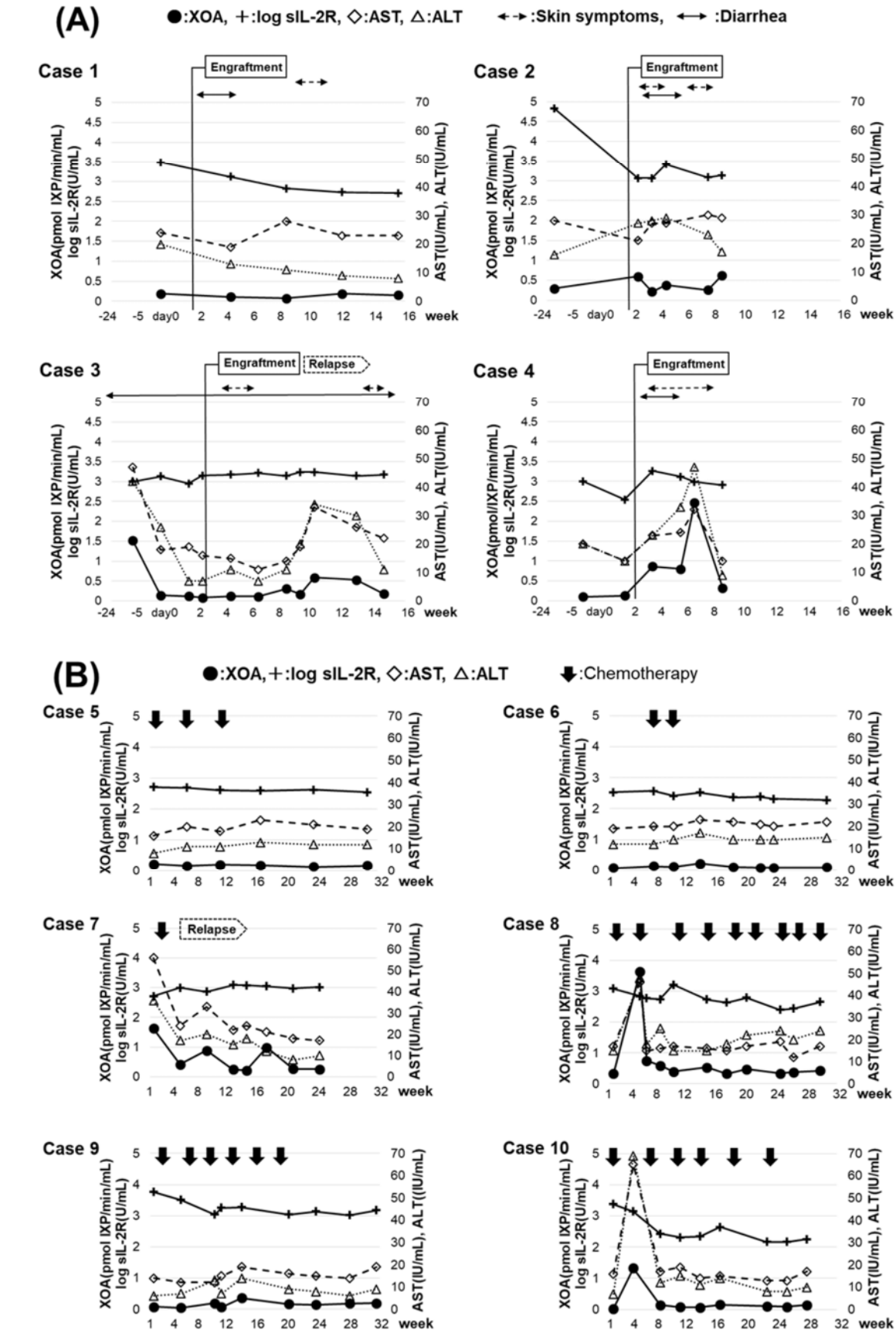

(A)
●:XOA, ＋:log sIL-2R, ◇:AST, △:ALT
:Skin symptoms,
:Diarrhea
Case 1
Engraftment
Case 2
Engraftment
Case 3
Engraftment
Relapse
Case 4
Engraftment
XOA(pmol IXP/min/mL)
log sIL-2R(U/mL)
AST(IU/mL), ALT(IU/mL)
-24 -5 day0 2 4 6 8 10 12 14 16 week
(B)
●:XOA,＋:log sIL-2R, ◇:AST, △:ALT
:Chemotherapy
Case 5
Case 6
Case 7
Relapse
Case 8
Case 9
Case 10
XOA(pmol IXP/min/mL)
log sIL-2R(U/mL)
AST(IU/mL), ALT(IU/mL)
1 4 8 12 16 20 24 28 32 week

Figure 2

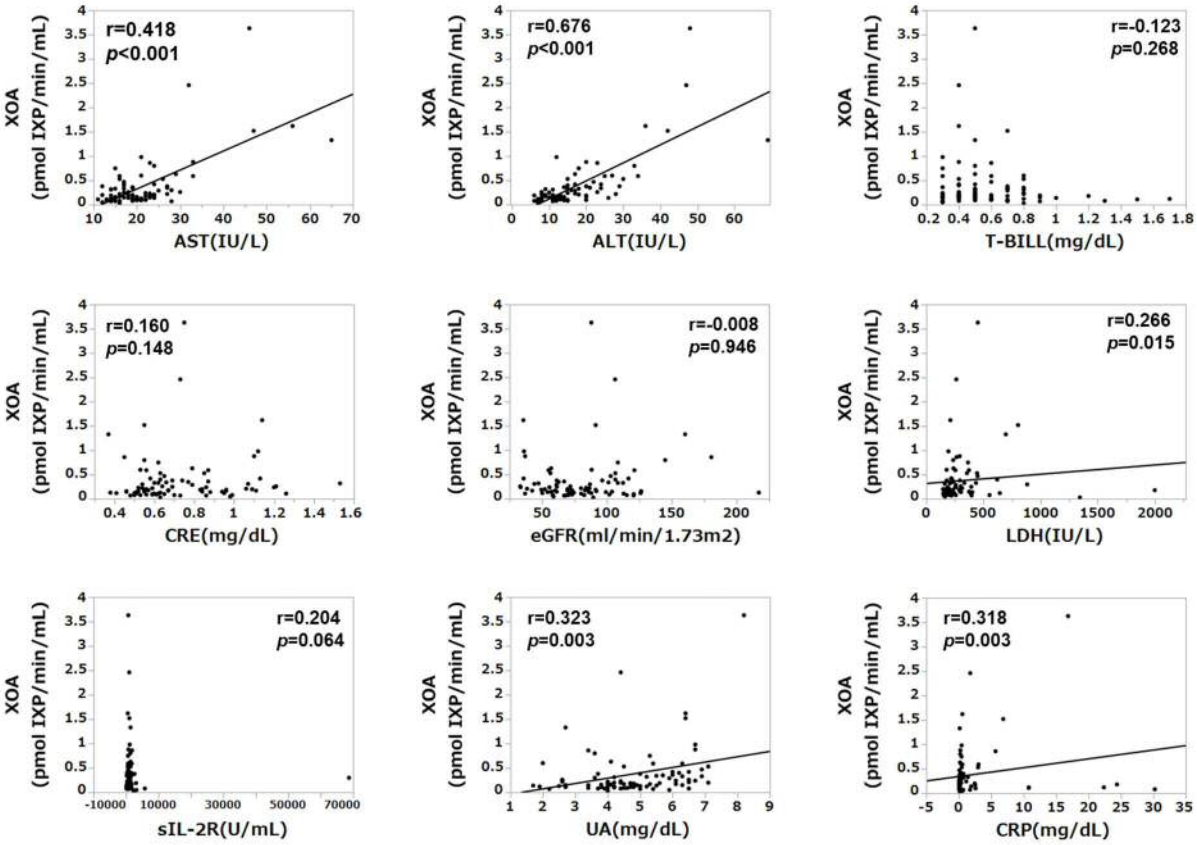
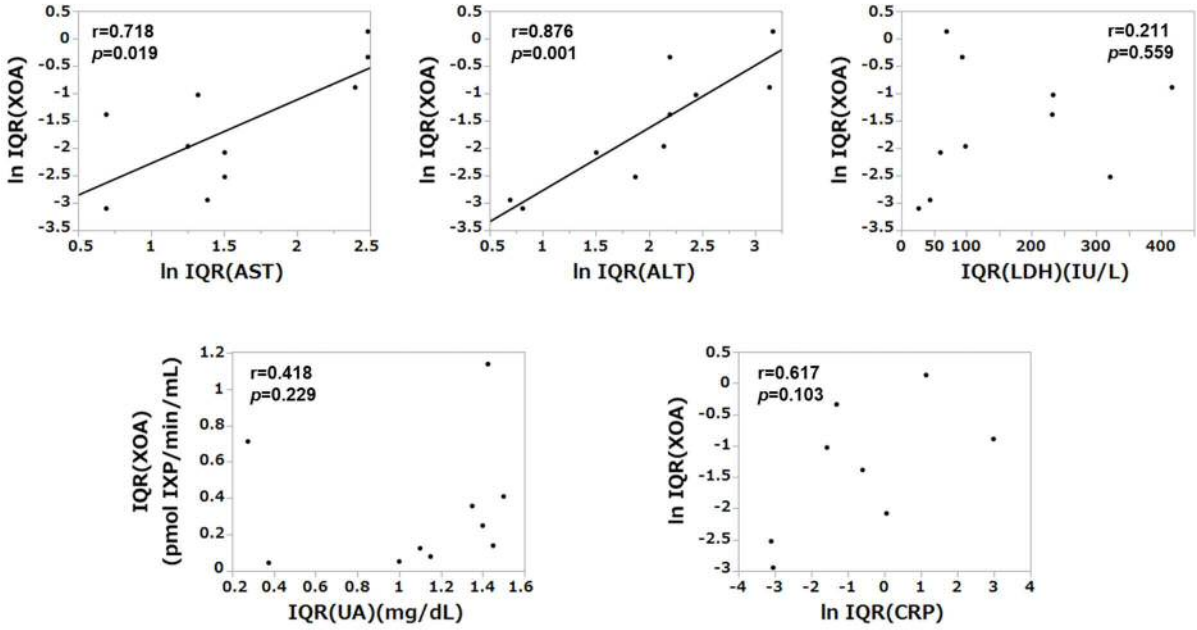

XOA (pmol IXP/min/mL)
r=0.418
p<0.001
AST(IU/L)
r=0.676
p<0.001
ALT(IU/L)
r=-0.123
p=0.268
T-BILL(mg/dL)
r=0.160
p=0.148
CRE(mg/dL)
r=-0.008
p=0.946
eGFR(ml/min/1.73m2)
r=0.266
p=0.015
LDH(IU/L)
r=0.204
p=0.064
sIL-2R(U/mL)
r=0.323
p=0.003
UA(mg/dL)
r=0.318
p=0.003
CRP(mg/dL)

Figure 3


r=0.718
p=0.019
ln IQR(XOA)
ln IQR(AST)
r=0.876
p=0.001
ln IQR(XOA)
ln IQR(ALT)
r=0.211
p=0.559
ln IQR(XOA)
IQR(LDH)(IU/L)
r=0.418
p=0.229
IQR(XOA)
(pmol IXP/min/mL)
IQR(UA)(mg/dL)
r=0.617
p=0.103
ln IQR(XOA)
ln IQR(CRP)