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Thiol-based Drugs Effectively Reduce the Circulating Poly-IgA Immune Complex but Reduce the Soluble CD89-IgA Complex with Different Rates in the Peripheral Blood of IgA Nephropathy

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Abstract

Background and objectives: Immunoglobulin A nephropathy (IgAN) is the most common type of glomerulonephritis and is due to the deposit of circulating IgA immune complex in kidney. There is evidence showing that reduction of disulfides may be able to break down IgA immune complexes. The goal of this research is to measure and compare the reduction rates of a representative thiol-based reducer on two types of IgA immune complex in-vitro by using plasma or serum samples from IgAN patients, other kidney diseases and healthy controls.

Methods: The thiol-based drug, n-acetylcysteine (NAC), was used as an representative after being compared with cysteine (CYS), and cysteamine (CA) for reaction conditions. Using plasma or serum samples from different patient groups, two types of IgA immune complex were quantified with house-developed ELISA kits prior and post NAC reduction. One is the poly-IgA immune complex which can be captured by CD89, the IgA Fc receptor, hence it is not combined with CD89 in the circulation, denoted as Poly-IgA-IC; the other is the IgA immune complex which has already been combined with CD89 in the circulation, called soluble CD89-IgA immune complex, denoted as CD89-IgA-IC. Both types were quantified with ELISA kits in parallel. The reduction rates were summarized and compared within each sample group. At last ROC analysis were performed to assess the clinical utility as a novel biomarker for IgAN using the CD89-IgA-IC residuals after reduction.

Results: N-acetylcysteine and cysteamine achieved reduction saturation of Poly-IgA-IC using 5 mM under room temperature or 37◦C for one hour, while Cysteine didn't achieve saturation even at 10 mM with a relatively smaller reduction rate. Using 10 mM NAC as a reducer, plasma or serum samples of three patient groups, IgA nephropathy (IgAN), presumably none-IgAN kidney disease (KD) and healthy control group (HC) were tested for Poly-IgA-IC and CD89- IgA-IC reduction in parallel. For Poly-IgA-IC, the reduction rates showed no difference among the groups, with an average of 82% for IgAN and KD and 78% for HC. On the other hand, for CD89- IgA-IC, a large number of IgAN and some KD samples had greater portions of none-reduced residuals and had much smaller reduction rates than HC, suggesting that CD89-IgA-IC might consist of the reducible and the irreducible subtypes. The reducible type is related to the normal immunity and the irreducible type might be clinically related to IgAN. This was indicated by the ROC analysis using the irreducible CD89-IgA-IC residues to predict the biopsy results, whether IgAN was diagnosed, with an AUC of 0.69, an specificity of 66%, and an sensitivity of 73%.

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Conclusions: IgA immune complex consists of two types, Poly-IgA-IC and CD89-IgA-IC. This in-vitro study showed that in the plasma or sera, thiol-based compounds can effectively reduce the Poly-IgA-IC for all tested samples but can only effectively reduce CD89-IgA-IC in less than half of IgAN samples. Re-purposing of these drugs such as NAC may be a novel approach to IgA nephropathy treatment and management. For CD89-IgA-IC type, the irreducible residuals in a large number of IgAN samples and in some presumably none-IgAN KD samples might be clinically related to IgAN and need for further investigation.

Keywords: IgA nephropathy; Thiol-based Drug; N-acetylcysteine; CD89; IgA Immune Complex

Abbreviations: 4PL: Four Parameter Logistic Model; AUC: Area Under the Curve; BSA: Bovine Serum Albumin; CA: Cysteamine; CD89-IgA-IC: Soluble CD89-IgA Immune Complex; CYS: Cysteine; IgAN: IgA Nephropathy; CV: Coefficient of Variation; mIgA: monomeric IgA; NAC: N-acetylcysteine; PBS: Phosphate-Buffered Saline; pIgA: Polymeric IgA; Poly-IgA-IC: Polymeric IgA Immune Complex (which is not combined with CD89 in the circulation); ROC: Receiver Operating Characteristic Curve.

Introduction

Immunoglobulin A nephropathy (IgAN), an autoimmune disease, is the most common type of glomerulonephritis and is due to the deposition of the circulating IgA immune complex in kidney [1]. The mainstream understanding of IgA pathogenesis is the multi-step hit hypothesis and the immune complex formation is due to the circulating galactose deficit IgA1 (Gd-IgA1) targeted by the specific autoantibodies IgG [2, 3]. Along this line, conventional IgAN treatments include anti-hypertensive supportive therapy and immunosuppression, and the promising future alternative treatments include inhibition of BAFF/APRIL signaling, depletion of plasma cells producing Gd-IgA1, IgA deposit clearance, modulation of mucosal immunity, complement pathway blockade and so on [1]. On the other hand, there was strong evidence that IgA immune complex could also form through the intermolecular disulfides and IgA deposit was shown in a mouse model, suggesting that the disulfide reducing compounds may be able to breakdown IgA immune complex in the circulation and in the deposit, and the compounds with the thiol (-SH) functional group are typical disulfide reducers and were proposed as a novel approach to IgAN treatment[4]. Since the thiol-based drugs have been in use for decades and they provide insights into the development of new treatments[5], re-purposing these drugs for IgAN treatment might be a promising alternative. In this in-vitro study, we tested and compared the efficiencies or the reduction rates of the representative thiol-based drugs in breaking down two types of circulating IgA immune complexes in plasma or serum among IgAN patients, other kidney diseases, and health controls.

Materials and Methods

Subjects

Study subjects came from three sources. One consisted of the plasma of 54 kidney disease patients collected in First People's Hospital of Chengdu. The other consisted of the sera of 120 kidney disease patients collected in The Third Affiliated Hospital of Southern Medical University. The plasmas of 16 healthy controls (HC) were also included. Based on the biopsy results, the study set consisted of 16 HC, 68 IgA nephropathy samples (IgAN), and 125 presumably (some with biopsy results unavailable) none-IgAN kidney diseases (KD). Some patients might have multiple blood draws at different time points. Plasma or sera were extracted to aliquots following standard clinical lab procedure. This study was approved by the local ethical committees, and informed consent forms were signed.

Thiol-Containing Reducers

NAC (N-Acetyl-L-cysteine, Sigma, Cat #: A7250-5G, 616-91-1), CYS (L-Cysteine, Sigma, Cat #: 168149-2.5G, 52-90-4), and CA (Cysteamine, Aladdin, Cat #: C106461-1G, 60-23-1) were dissolved in Tris Buffered Saline (TBS) to concentrations 5 mM and 10 MM respectively, and were to be used for CD89-IgA-IC tests. In addition, NAC was also dissolved in Phosphate Buffered Saline (PBS) to 10 mM and was to be used for Poly-IgA-IC tests.

Measuring Poly-IgA-IC

Measuring Poly-IgA-IC was based on the protocol described in Zhang X, et. al. [8]. Ninety-six- well microtiter plates (Thermo, Nunc MaxiSorp, Cat #: 446639) were coated for three hours at room temperature with 100ul (5ug/mL) of the recombinant CD89 developed in-house in PBS buffer, pH7.4. The plates were washed with PBS containing 0.05% Tween 20 (PBST) three times and blocked with 200 ul/well of 2% BSA for 2h at room temperature. The plasma aliquots were treated with a ratio of 1:200 using the 10 mM NAC, which was prepared using PBS as in the above, at 37°C. Then a standard curve dilution series, the untreated plasma samples diluted to 1:200 in PBS with 0.5% BSA, and the treatment samples were added and incubated for 2h at room temperature. After washing, the HRP-conjugated goat clonal anti-human IgA antibody diluted in TBST containing 0.5% BSA was added and incubated for 1 hour at room temperature. The color was developed using Peroxide solution A and TMB solution B (Sino Biological) and the absorbance was measured at 405 nm with a micro-plate reader (Tecan, INFINITE F50). The

standard curve was replaced by the recombinant Poly-IgA developed in-house as described in Xie X, et. al. [2], starting at 500 ng/mL with 2X dilutions. The unit of the measured result is denoted as U/mL, corresponding to the concentration of the recombinant Poly-IgA contained in one unit of the standard (1 ug/mL).

Measuring CD89-IgA-IC

Ninety-six-well microtiter plates (Beaver, Cat #: 40304) were coated overnight at 4°C with 100ul (2ug/mL) of rabbit clonal anti-human CD89 (Sino Biological, 10414-R002) in Citrate Buffered Saline (CBS), pH9.6. The plates were washed with TBS containing 0.05% Tween 20 (TBST) three times and blocked with 200 ul/well of 2% BSA for 2h at room temperature. The plasma aliquots were treated with a ratio of 1:50 using the 5 mM or the 10 mM NAC, CYS, or CA, which were prepared using TBST containing 0.1% BSA, at room temperature or 37°C respectively. Then a standard curve dilution series, the untreated plasma samples diluted to 1:50 in TBST containing 0.1% BSA, and the treated samples were added and incubated for 2h at room temperature. From here on, the steps were the same as in the previous section. The standard curve was the commercial human IgA serum (Yuduobio, Cat #: LY210) starting at 16 ug/mL with 2X dilutions. The unit of the measured result is denoted as U/mL, corresponding to the concentration of CD89-IgA-IC contained in one unit of the standard (1 ug/mL).

Data Analysis

Data analysis and plots were mostly implemented by R scripts developed in-house using RStudio 2022.07.1 with R version 4.0.5 on the Mac platform with OS version darwin17.0. ROC was based on *prediction and performance* in R package *ROCR*. Standard curves were the 4PL (4-parameter logistic) regression models by calling *drm* with modeling function LL.4() in the R package *drc*.

Results

Reducing Reaction Conditions and Selection of NAC as the Representative

Two representative samples were used for the purpose of determining the reaction concentration and the temperature: A1H19P0076 is an IgAN sample with high Poly-IgA-IC concentration before the reduction and HC0021 is a healthy control. Three reducers were applied at 5 mM and 10 mM under room temperature and 37◦C respectively. The samples were paired using prior- and post-reduction aliquots, which were then tested on the same ELISA plate. The reduction rate (percentage) was calculated by (Cprior-Cpost)/Cprior x 100%, where Cprior, Cpost are the measured concentrations of prior- and post-reduction by the ELISA method. The results are shown in Table 1. It shows that both NAC and CA were saturated at 5 mM because 10 mM didn't increase the reduction rates. However, CYS was not saturated because 10 mM gave much higher rates than 5 mM. On the other hand, both concentrations of the same compound gave comparable rates between two temperatures except the unsaturated CYS 5 mM. In conclusion, NAC reduced about 80% Poly-IgA-IC for the IgAN sample across all conditions, but CA reduced about 60%. As for the healthy object HC0021, only 10 mM in room temperature was tested and both NAC and CA reduced about 50%. CYS was not saturated and higher concentrations needed to be tested. Hence NAC 10 mM was used for subsequent tests.

Table 1: Percent of Poly-IgA-IC reduction using 3 thiol-based compounds NAC, CA, CYS. A1H19P0076 is a pre-selected KD sample with high Poly-IgA-IC, HC0021 is an HC sample. Reduction reactions were for concentrations 5 mM and 10 mM in room temperature and 37 °C respectively.

SampleID	Reducer	Conc.(mM)	% Reduced (25°C)	% Reduced (37°C)
A1H19P0076	CA	5	64	62
A1H19P0076	CA	10	64	57
A1H19P0076	CYS	5	4	20
A1H19P0076	CYS	10	34	38
A1H19P0076	NAC	5	79	78
A1H19P0076	NAC	10	83	81
HC0021	CA	10	47	NA
HC0021	CYS	10	33	NA
HC0021	NAC	10	56	NA

Table 2: The statistics of the Poly-IgA-IC reduction rates (%) with NAC for plasma and serum. The average reductions via NAC for plasma of KD and HC are around 80% while for sera it is around 90%. The narrow ranges from Min to Max and the small C.V. around 5% within each group indicate that all samples were reduced by almost equal rates.

Type	Group	n	Min	Max	Average	STD	C.V.
Plasma	HC	15	71.42	84.21	78.3	3.89	4.97
Plasma	IgAN or KD	31	72.82	89.99	81.76	4.12	5.04
Serum	IgAN or KD	114	76.57	97.58	89.99	4.42	4.91

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NAC Effectively Reduced Poly-IgA-IC with Comparable Rates among IgAN, KD and HC Groups

For Poly-IgA-IC, 160 plasmas or sera were tested prior and post NAC reduction (10 mM, 37°C). The summary statistics with respect to sample types are presented in Table 2. It showed that the Poly-IgA-IC of all plasma samples regardless of HC and the disease types was reduced significantly by 80%, comparably, and that of sera was reduced by 90%. For each type, the narrow ranges from the minimum to the maximum is small and and the C.V. is around 5%, indicating that the Poly-IgA-IC of all plasma and sera samples was almost completely reduced by 80 − 90%.

NAC Did Not completely Reduce CD89-IgA-IC in a majority of IgAN Samples and in some Presumably none-IgAN KD Samples

For CD89-IgA-IC, 209 plasma or sera were tested prior and post NAC reduction (10 mM, 37°C). Since the reduction rates showed very diverse ranges, summary statistics was applied with respect to three sample groups HC, IgAN, and KD (none-IgAN kidney diseases) and is presented in Table 3. For HC (n=16) , CD89-IgA-IC was reduced significantly by an average of 76.75%, ranging from 63% to 98% with CV 13.49%, hence all HC samples were reduced uniformly at rates comparable to the previous type Poly-IgA-IC. However, the reduction rates for the kidney disease samples decreased significantly and were even more so for IgAN samples, suggesting CD89-IgA-IC in disease samples is much harder to be reduced by NAC, and a majority of IgAN samples were even harder. In Table 4, for IgAN samples, the average reduction rate (n=97) is 24.34%, the range goes from 0% (negative percentages were truncated to 0 for more meaningful summary) to 97% with the largest CV (134.10%) among the three groups. For KD (presumably none-IgAN kidney disease) samples, the average reduction rate (n=96) is 47.23%, the range goes from 0% (also truncated the negative values to 0) to 99% with a larger CV (75.94%) than HC. Figure 1 shows the histogram of the reduction rates for the HC, IgAN and KD groups, it shows that 73 (75%) samples in the IgAN group have reduction rates less than 50%, while it is 48 (50%) samples in the KD group and all HC samples have reduction rates greater than 60%. Moreover, 51% IgAN and 26% KD samples were rarely reduced by NAC with rates < 10%. From another point of view, Table 4 lists the summary statistics of CD89-IgA-IC for the three groups, prior and post NAC reduction. Prior to NAC reduction, the average CD89-IgA-IC concentrations (unit: U/mL) are: 145.24 (HC), 87.31 (IgAN) and 119.26 (KD) , while after NAC reduction, the averages are: 18.69 (HC), 44.76 (IgAN) and 33.75 (KD). Although the IgAN group has the least total CD89-IgA-IC while the HC group has the highest prior to the NAC reduction, the IgAN group has the highest irreducible residuals while the HC group has the least after the reduction. This is due to the different reduction efficiencies among them, and on average, the unreduced residual accounts for 51.27% of the total CD89-IgA-IC for IgAN, 28.30% for KD, and 12.87% for HC. Therefore, NAC reduction is the least efficient for the IgAN

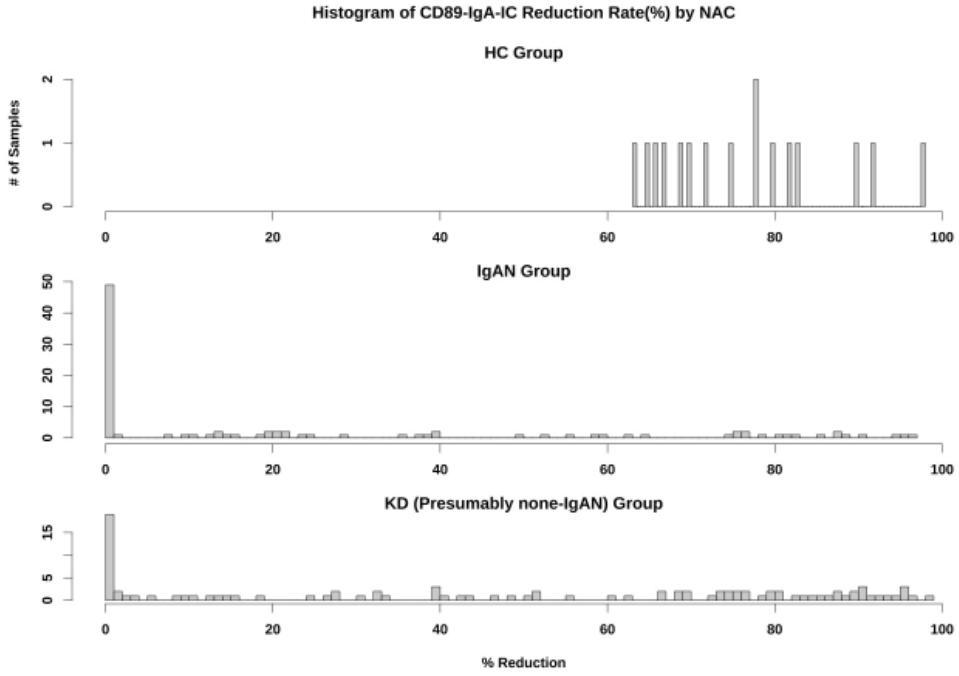

Figure 1: Histograms of the CD89-IgA-IC reduction rates with NAC in the HC, IgAN and KD groups. For IgAN, 73 (75%) samples in the IgAN group have reduction rates less than 50%, while it is 48 (50%) samples in the KD group, and all HC samples have reduction rates greater than 60%. The immune complexes of type CD89-IgA-IC in 50% IgAN and 26% KD samples were barely reduced by NAC (rates < 10%).

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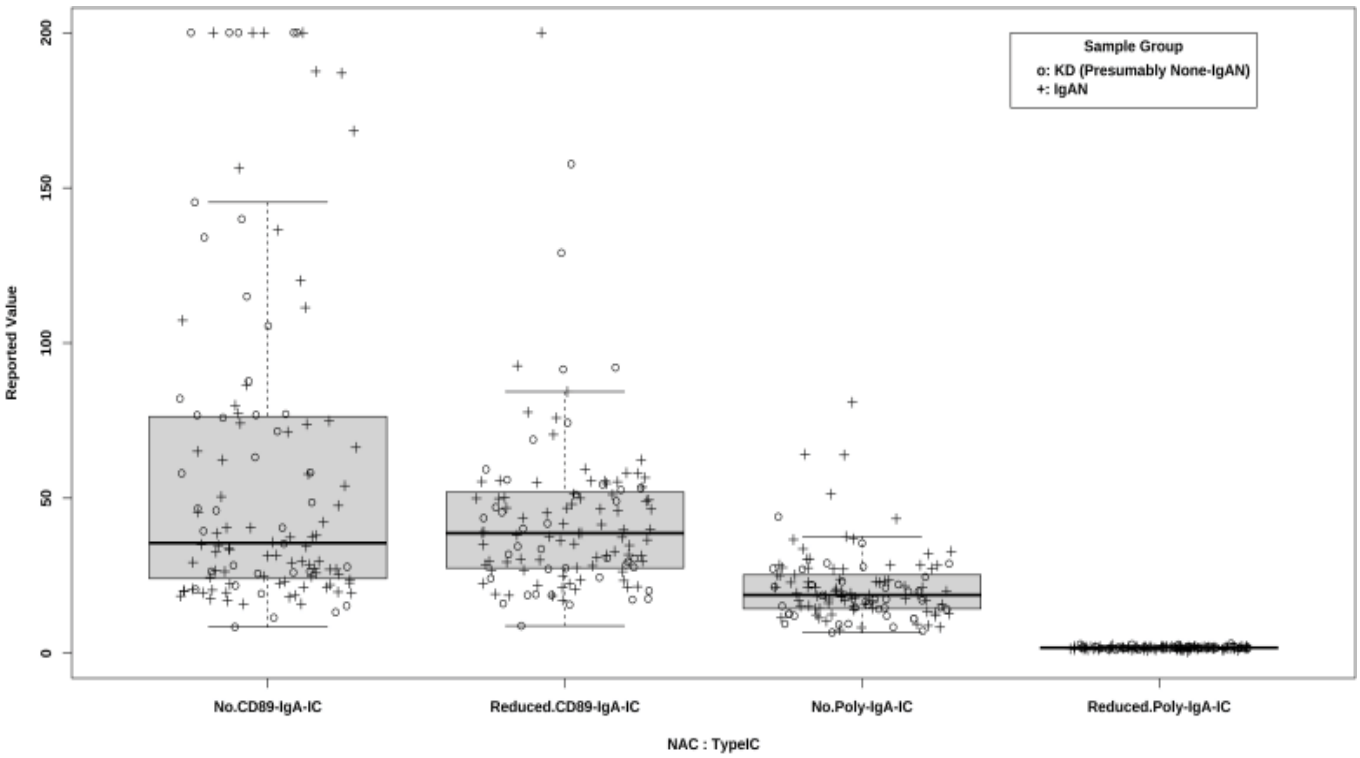

Figure 2: Box plots of two types of IgA immune complex prior (annotated as No in the axis label) and post reduction (annotated as Reduced in the axis label) by NAC. The raw data points annotated by the sample groups are superimposed, truncated above at 200 for displaying purpose. It shows that the Poly-IgA-IC type was completely reduced, while the CD89-IgA-IC type largely remained intact after the reduction. CD89-IgA-IC post reduction has a slightly larger median value than prior reduction and most IgAN samples and some KD ones have large irreducible residues.

Table 3: The summary of the CD89-IgA-IC reduction rates (%) by NAC. The average reduction rate for the IgAN group is 24.34%, which is the least among the three groups. The KD (presum- ably none-IgAN) group is 47.23% while the HC group is 76.75%.

Group	n	Min	Max	Average	Std	CV
HC	16	63	98	76.75	10.36	13.49
IgAN	97	0	97	24.34	32.64	134.1
KD	96	0	99	47.23	35.86	75.94

Table 4: The summary of the CD89-IgA-IC test results prior (Reduced=No) and post (Reduced=Yes) NAC reduction within group HC, IgAN and KD (presumably none-IgAN).

Group	Reduced	Min	Max	Average	Std	CV
HC	No	10.49	1427.77	145.24	314.52	216.55
HC	Yes	8.46	49.24	18.69	12	64.19
IgAN	No	15.91	1145.37	87.31	161.31	184.75
IgAN	Yes	12.05	489.11	44.76	52.54	117.39
KD	No	8.49	1471.31	119.26	196.67	164.92
KD	Yes	8.85	157.68	33.75	27.8	82.35

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group, the most efficient for the HC group, while the KD group is in between. The reduction efficiency is dramatically different among the groups. Figure 2 shows the box plots of two types of IgA immune complexes prior and post reduction by NAC. It shows that the Poly-IgA-IC type was completely reduced, while the CD89-IgA-IC type largely remained intact after the reduction. CD89-IgA-IC post reduction has a slightly larger median than prior reduction and most IgAN samples and some KD ones have large irreducible residues. At last, two cases with the highest CD89-IgA-IC residuals post NAC reduction are reviewed. One case has prior and post reduction residuals 811.47 and 489.11 respectively, with reduction rate 40%, and is a 40-year-old male with fever, renal dysfunction, and proteinuria for 3 months, and was diagnosed as IgAN by kidney biopsy. The pathological analysis reported IgA++, C3++, C1q-, λ++, diffuse mesangial deposition (block-like), without classification (fewer glomeruli). The patient has a medical history of 30 years of hepatitis B and has been treated with entecavir for 2 years. The other case has 175.93 (prior), 131.33 (post) with a 25% reduction and is a 35-year-old female, with proteinuria for 7 months but with no further biopsy result.

In summary, there were much higher CD89-IgA-IC residuals after NAC reduction in a large population of IgAN samples and in some presumably none-IgAN KD samples while it was neglectful for all HC subjects. Case studies showed that there were very high CD89-IgA-IC residuals even though the reduction rates were not neglectful in some IgAN patients or kidney diseases. We propose that the circulating CD89-IgA-IC may have different types, one type is for normal immunity, abundant in HC, and can be effectively reduced by NAC; the other type is the residuals after NAC reduction, which could not be dissolved and might have clinical implications for IgAN. It will be intriguing to investigate it in the future.

The NAC-irreducible CD89-IgA-IC Residual is a Novel Biomarker for IgAN Diagnosis

Soluble CD89 mainly consisting of CD89-IgA-IC is not specific for IgAN diagnosis from the literature. However, the NAC-irreducible residuals of soluble CD89-IgA-IC have strong prediction power for IgAN diagnosis and hence might be clinically related to IgAN. ROC was applied to the prior and post reduction test results respectively. Here the IgAN group was regarded as the positive group and the other two groups are negative. The total CD89-IgA-IC without reduction gave an AUC of 0.51 and therefore it is close to random and is not specific for IgAN. On the contrary, the irreducible CD89-IgA-IC after NAC reduction gave an AUC of 0.69, a specificity of 66%, and a sensitivity of 73%. Therefore, the irreducible CD89-IgA-IC residual can be used as a novel biomarker for IgAN diagnosis. In parallel, ROC analysis was also applied to Poly-IgA-IC (no reduction), the AUC is also 0.69, the specificity is 67%, and the sensitivity is 63%. Hence as a biomarker for IgAN, the irreducible CD89-IgA-IC is comparable to Poly-IgA-IC. Note that almost all Poly-IgA-IC was reduced by NAC for all sample groups and hence ROC was not applied.

Discussion

We have investigated NAC reduction rates of two types of IgA immune complex in plasma or sera, one is Poly-IgA-IC which is not combined with CD89 in the circulation and so it can be enriched by the recombinant CD89 with the house-developed ELISA kit; the other is CD89- IgA-IC which has been combined with CD89 in the circulation and so it can only be enriched by anti-CD89 antibody with another house-developed ELISA kit. The reduction rates showed very different profiles between them. For Poly-IgA-IC, NAC reduced this type of complexes with very high efficiencies regardless sample groups so that the remained residuals were neglectful for HC, KD and IgAN samples. This indicates that the NAC and other thiol-based drugs might be administrated to IgAN patients to dissolve the circulating and the deposited Poly-IgA-IC. On the other hand, for CD89-IgA-IC, a large population of IgAN and some presumably none-IgAN KD samples showed significant amount of irreducible residuals, which were demonstrated to be clinically related to the biopsy diagnosed IgAN with an AUC of 0.69. Hence we proposed that CD89-IgA-IC might have two types, NAC-reducible and NAC-irreducible. The reducible type is related to the normal immunity because CD89-IgA-IC in all HC samples was abundant and largely reducible, while the irreducible type is clinically related to IgAN. Soluble CD89, mainly appearing as CD89-IgA-IC in the circulation, has long been investigated as a biomarker for IgAN[9]. The results have been controversial. CD89 is human myeloid IgA Fc receptor expressed on the membranes of monocyte immune cells such as neutrophils, eosinophils and monocytes/macrophages, and cross-linking of CD89 on these cells by Poly-IgA-FC or anti-CD89 monoclonal antibodies can trigger various immunological effector functions [10]. CD89 plays double roles in mediating and promoting immunity. It was shown that monomeric (mIgA) and polymeric IgA (pIgA) had a similar association with CD89 but mIgA dissociated more rapidly from CD89 than pIgA, and the anti-inflammatory role of IgA is through the competition of mIgA for CD89 with pIgA, while the linkage of CD89 with pIgA triggers immune cell activation [11, 12]. In IgAN, the circulating CD89-IgA-IC is elevated [13], and it is also associated with histologic inflammation in children IgAN[16]. However, our data in this research showed that the average total CD89-IgA-IC in the IgAN group is even smaller than HC and KD and hence it has no association with IgAN. Nevertheless, the irreducible CD89-IgA-IC portion after

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NAC reduction was shown to be clinically association with IgAN. Soluble CD89-IgA-IC was also shown to be without susceptibility in IgAN but was associated with disease progression [14]. Another research in a Korean population showed that the circulating CD89-IgA-IC does not predict deterioration of kidney function in IgAN[15]. These seemly conflicting results suggest that CD89-IgA-IC might have different subtypes and it requires for further investigation. At last, in the clinical trials of thiol-based drugs re-purposed to IgAN treatment, quantifying the irreducible CD89-IgA-IC may provide a companion test for patient enrollment because a large irreducible CD89-IgA-IC residual may indicate an improper administration of the reducers.

Declaration

Conflict of Interest

Tang Y, Chen M, Xie X, Lv JC, and Zhang H claim no conflict of interest. Jin J is a scientific advisor, Rao A is a co-founder, and all others are employees, of Shenzhen Luwei (Biomanifold) Biotechnology Limited, Shenzhen, China. A patent application is pending in China.

Ethics Approval

This study was approved by the local Ethical Committees.

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