Supplemental materials

The Role of TNF Superfamily Member 13 in the Progression of IgA Nephropathy

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Supplemental tables

Supplemental Table 1. Baseline characteristics of the control groups

	Controls for SNP (n=1068)	Controls for plasma (n=163)
Age (years)	61.1 ± 9.3	30.1 ± 6.4
Male sex (%)	35.6	79.8
Current smoker (%)	16.1	–*
Systolic blood pressure (mmHg)	114 ± 11.2	–*
Diastolic blood pressure (mmHg)	74 ± 7.0	–*
Hypertension (%)	0	0
Diabetes mellitus (%)	0	0
Dyslipidemia (%)	0	0
Cardiovascular disease (%)	0	0
Malignancy (%)	0	0
Serum creatinine (mg/dL)	0.9 ± 0.12	0.9 ± 0.12
Estimated GFR (mL/min/1.73 m^2)	76.5 ± 1.02	111.1 ± 1.24
Abnormality in dipstick test (%)	0	0

*Data on smoking and blood pressure were not available.

Supplemental Table 2. SNP frequencies in the *TNFSF13* gene in all subjects, including both IgAN patients and healthy individuals

	Allele frequency		Genotype frequency		Hardy-Weinberg equilibrium		
SNP position	Allele	N (%)	Genotype	N (%)	Observed	Expected	*P*
rs11552708	A	1190 (37.6)	A/A	224 (14.2)	0.469	0.469	1.00
	G	1976 (62.4)	A/G	742 (46.9)			
			G/G	617 (39.0)			
rs3803800	A	1072 (33.9)	A/A	183 (11.6)	0.446	0.448	0.90
	G	2094 (66.1)	A/G	706 (44.6)			
			G/G	694 (43.8)			

Supplemental Table 3. Risk of IgA nephropathy according to *TNFSF13* genotype

SNP position	Comparison	OR (95% CI)	*P*
rs11552708	GG vs. AG and AA	1.19 (0.958–1.471)	0.12
	AA vs. GG and AG	0.80 (0.597–1.083)	0.15
rs3803800	GG vs. AG and AA	0.65 (0.534–0.801)	<0.001
	AA vs. GG and AG	1.40 (1.020–1.924)	0.04

OR, odds ratio; CI, confidence interval.

Supplemental Table 4. Serum IgA levels of IgAN patients according to the genotypes of TNFSF13 or plasma TNFSF13 groups

Grouping criteria	Group (n)*	Mean IgA (mg/dL)	*P*
rs11552708	AA (27)	325.4 ± 96.0	1 (Reference)
	AG (99)	302.9 ± 106.3	0.19
	GG (91)	333.3 ± 111.9	0.66
rs3803800	AA (24)	365.4 ± 139.0	1 (Reference)
	AG (97)	317.2 ± 114.5	0.01
	GG (96)	326.5 ± 111.2	0.03
Plasma TNFSF13	Undetectable (100)	298.0 ± 79.6	1 (Reference)
	1^{st} tertile (65)	288.9 ± 98.9	0.56
	2^{nd} tertile (64)	311.0 ± 95.4	0.40
	3^{rd} tertile (79)	327.0 ± 112.6	0.05

*Among the SNP and plasma TNFSF13 cohorts, the total numbers of patients having serum IgA levels were 217 and 308, respectively.

Supplemental Table 5. Estimated GFR levels according to the TNFSF13 groups

Disease	TNFSF13 group	eGFR (mL/min/1.73 m^2)	*P*
IgA nephropathy	Undetectable (n=128)	94.3 ± 24.1	–
	1st tertile (n=94)	92.4 ± 24.8	0.59
	2nd tertile (n=94)	79.9 ± 26.3	<0.001
	3rd tertile (n=94)	65.1 ± 32.2	<0.001
Membranous nephropathy	Undetectable (n=16)	91.2 ± 17.36	–
	Low (n=6)	66.2 ± 35.50	0.15
	High (n=6)	81.4 ± 41.21	0.86
Lupus nephritis	Undetectable (n=18)	108.1 ± 24.93	–
	Low (n=10)	66.5 ± 23.02	<0.001
	High (n=11)	68.4 ± 50.75	0.03
Diabetic nephropathy	Undetectable (n=9)	70.6 ± 33.45	–
	Low (n=15)	52.4 ± 26.86	0.12
	High (n=15)	31.6 ± 23.34	0.002

Statistical difference was calculated when the undetectable group was assigned as a reference.

eGFR, estimated glomerular filtration rate.

Supplemental Table 6. Proportions of each pathologic score in biopsied kidney tissue according to plasma TNFSF13 levels

	M score		E score		S score		T score	
TNFSF13 group	0 (%)	1 (%)	0 (%)	1 (%)	0 (%)	1 (%)	0 (%)	1&2 (%)
Undetectable	50.0	50.0	92.9	7.1	44.6	55.4	89.3	10.7
1st tertile	55.4	44.6	90.8	9.2	58.5	41.5	90.8	9.2
2nd tertile	47.8	52.2	81.2	18.8	49.3	50.7	84.1	15.9
3rd tertile	50.0	50.0	72.6	27.4	45.2	54.8	64.5	35.5

M, mesangial hypercellularity; E, endocapillary hypercellularity; S, segmental glomerulosclerosis; and T, interstitial fibrosis/tubular atrophy.

Supplemental Table 7. List of genes with a significant fold change between rhTNFSF13-treated and non-treated groups

Group	Gene	Signal in non-treated group	Signal in treated group	Fold change (log_2)	p value	q value
IgAN	HPX	9.09646	0.40384	-4.49345	0.00005	0.002734
	RPS17L	0.67065	4.43142	2.72414	0.00005	0.002734
	PTGDS	2.30218	0.37542	-2.61643	0.00050	0.016872
	ACE	0.23248	0.74707	1.68417	0.00065	0.020223
	SFTPB	0.08134	1.16815	3.84414	0.00330	0.061662
	PTGES	0.56062	2.88451	2.36324	0.00395	0.069242
	SLC39A5	0.45190	0.05574	-3.01916	0.00520	0.082416
	COL1A1	0.57193	0.19735	-1.53505	0.00925	0.118341
	PGC	0.64948	0.08720	-2.89686	0.01020	0.125674
	MMP9	76.8909	170.048	1.14505	0.01180	0.136376
	LINC00504	9.08985	2.94110	-1.62790	0.01445	0.152327
	MYEOV	0.26807	0.81437	1.60306	0.01625	0.162997
	MARCO	2.48468	9.91467	1.99651	0.01815	0.173358
	MT2A	56.9543	128.268	1.17128	0.02040	0.185724
	RAI14	0.68641	1.39117	1.01916	0.02060	0.186885
	GUSBP2	7.22469	2.24432	-1.68666	0.02305	0.199320
	MT1G	18.7250	51.8838	1.47032	0.02615	0.214432
	MT1F	27.1045	57.7494	1.09127	0.03025	0.232944
	WDR78	0.68383	0.23719	-1.52758	0.03030	0.233144
	ZP3	3.79852	13.8344	1.86475	0.03125	0.237222
	CSF3	0.47558	1.27211	1.41945	0.03695	0.260909
	CLEC5A	27.8384	49.2863	0.82411	0.03765	0.263686
	MEGF11	0.48358	0.14984	-1.69031	0.03935	0.270236
	SPP1	15.4633	34.1342	1.14237	0.04085	0.275909
	IGHV4-59	124.535	71.2739	-0.80511	0.04340	0.285336
	PCSK4	0.54217	0.23325	-1.21684	0.04690	0.298432
	MT1H	13.8138	40.8222	1.56324	0.04695	0.298603
	GOLGA8N	0.20717	0.70012	1.75681	0.04825	0.303281

	STAC	0.24573	0.52295	1.08963	0.04900	0.306081
Healthy	SEPT4	0.48044	0.15133	-1.66664	0.00020	0.008447
	FAM153B	0.12302	0.54051	2.13545	0.00025	0.009992
	KRT5	0.24389	1.40768	2.52903	0.00090	0.025593
	LRRC49	0.27181	0.77218	1.50633	0.00090	0.025593
	COL1A1	0.79667	2.96232	1.89468	0.00100	0.027615
	COL3A1	0.56214	1.89629	1.75418	0.00185	0.042263
	COL1A2	0.69296	1.79058	1.36959	0.00245	0.051277
	RNASE1	0.68189	0.12651	-2.43030	0.00310	0.059343
	FXYD3	0.18091	0.68417	1.91909	0.00315	0.059915
	GLYATL1	0.83781	3.47243	2.05126	0.00490	0.079535
	COPZ2	0.94446	3.14116	1.73374	0.00525	0.082903
	AGR2	0.25103	1.36467	2.44263	0.00610	0.091365
	IFI27	0.20911	0.58851	1.49278	0.00800	0.108218
	KRT8	0.73335	2.31664	1.65947	0.01245	0.140530
	KANK2	0.59117	0.21878	-1.43409	0.01410	0.150317
	RP11-809C9.2	0.46133	0.07401	-2.63993	0.01540	0.158162
	FBXL16	1.78660	0.52204	-1.77499	0.01545	0.158528
	KRT19	0.21865	0.67681	1.63015	0.01725	0.168463
	AL590560.1	0.54561	0	–	0.01745	0.169539
	CPE	0.08193	0.41190	2.32979	0.01930	0.179879
	RPS17L	0.46042	1.06056	1.20381	0.01945	0.180516
	TTC23L	0.12744	0.42628	1.74197	0.02005	0.183792
	FAM174B	0.48397	1.32985	1.45827	0.02435	0.205711
	DMKN	0.93046	1.39351	0.58271	0.02735	0.220166
	WDR78	0.75787	0.27154	-1.48078	0.02965	0.230087
	SPINK1	0.13585	0.75920	2.48244	0.03115	0.236764
	AL355490.1	0	0.97343	–	0.03285	0.244310
	TAC4	0.51114	0.18438	-1.47106	0.03360	0.247650
	PLG	0.19967	0.54566	1.45041	0.03710	0.261443
	SAMD12	0.82994	2.08533	1.32919	0.03730	0.262351

SFTPB	3.25750	0.07099	-5.52005	0.03745	0.262809
PLS1	0.30807	0.65260	1.08294	0.03820	0.265973
SCIN	0.23515	0.52309	1.15350	0.03925	0.26974
CLIP3	0.41376	0.15797	-1.38914	0.04150	0.278645
PTK7	0.24779	0.51912	1.06694	0.04165	0.279095
GUSBP2	2.52779	28.6756	3.50388	0.04385	0.286784
MTUS1	0.31675	0.57708	0.86540	0.04390	0.286844
MGST1	1.22074	2.51367	1.04203	0.04885	0.305613

IgAN, IgA nephropathy.

Supplemental Table 8. Shared gene sets retrieved from healthy individuals and IgAN patients

Gene set category	Name	Size	Enrichment score	FDR	Remarks
3	V$SEF1_C	5	1.254	0.0055	Genes with promoter regions [-2kb, 2kb] around transcription start site containing motif AACACGGATATCTGTGGTY
3	GGATCCG,MIR-127	11	1.238	0.0050	Targets of MIR-127
6	RAPA_EARLY_UP.V1_UP	149	1.306	0.0000	Genes up-regulated in BJAB cells by everolimus
6	ESC_J1_UP_LATE.V1_DN	162	1.154	0.0118	Genes down-regulated during late stages of differentiation of embryoid bodies from J1 embryonic stem cells
6	PTEN_DN.V1_UP	142	1.329	0.0000	Genes up-regulated upon knockdown of PTEN by RNAi

FDR, false discovery rate.

Supplemental Table 9. List of glycosyltransferase genes used in the Gene set enrichment analysis (GSEA)

Order	Gene name	Corresponding protein name
1	GALNT2	Polypeptide N-acetylgalactosaminyltransferase 2
2	C1GALT1	Glycoprotein-N-acetylgalactosamine 3-beta-galactosyltransferase 1
3	C1GALT1C1	C1GALT1-specific chaperone 1
4	ST6GALNAC1	Alpha-N-acetylgalactosaminide alpha-2,6-sialyltransferase 1
5	ST6GALNAC2	Alpha-N-acetylgalactosaminide alpha-2,6-sialyltransferase 2
6	ST6GALNAC4	Alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3-N-acetyl-galactosaminide alpha-2,6-sialyltransferase
7	ST6GALNAC6	Alpha-N-acetylgalactosaminide alpha-2,6-sialyltransferase 6
8	ST3GAL1	CMP-N-acetylneuraminate-beta-galactosamide-alpha-2,3-sialyltransferase 1

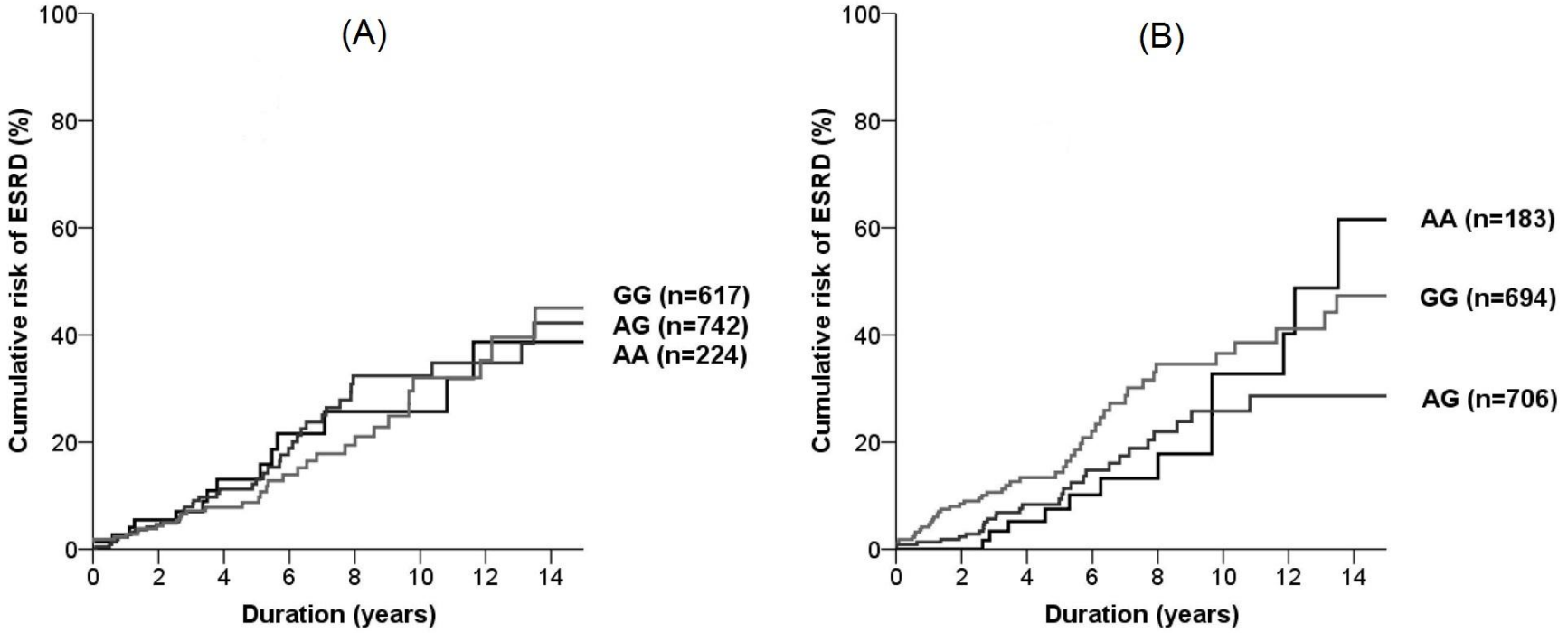
Supplemental Table 10. Run summary for RNA sequencing

	Patients with IgA nephropathy						Healthy Individuals					
Information	A (-) *	A (+)	B (-)	B (+)	C (-)	C (+)	D (-)	D (+)	E (-)	E (+)	F (-)	F (+)
Read length (bp)	2 × 100	2 × 100	2 × 100	2 × 100	2 × 100	2 × 100	2 × 100	2 × 100	2 × 100	2 × 100	2 × 100	2 × 100
No. of reads	72,188,382	79,297,706	66,200,334	70,079,474	59,658,398	68,122,150	72,370,924	60,267,206	69,431,104	72,931,698	68,365,322	63,590,854
% of reads on genome	87.9	90.1	87.5	87.9	89.1	88.6	88.9	89.1	89.2	88.4	87.5	87.5
% of reads on transcriptome	81.8	87.8	88.8	92.0	90.0	90.5	93.6	94.2	94.9	94.9	89.4	87.1

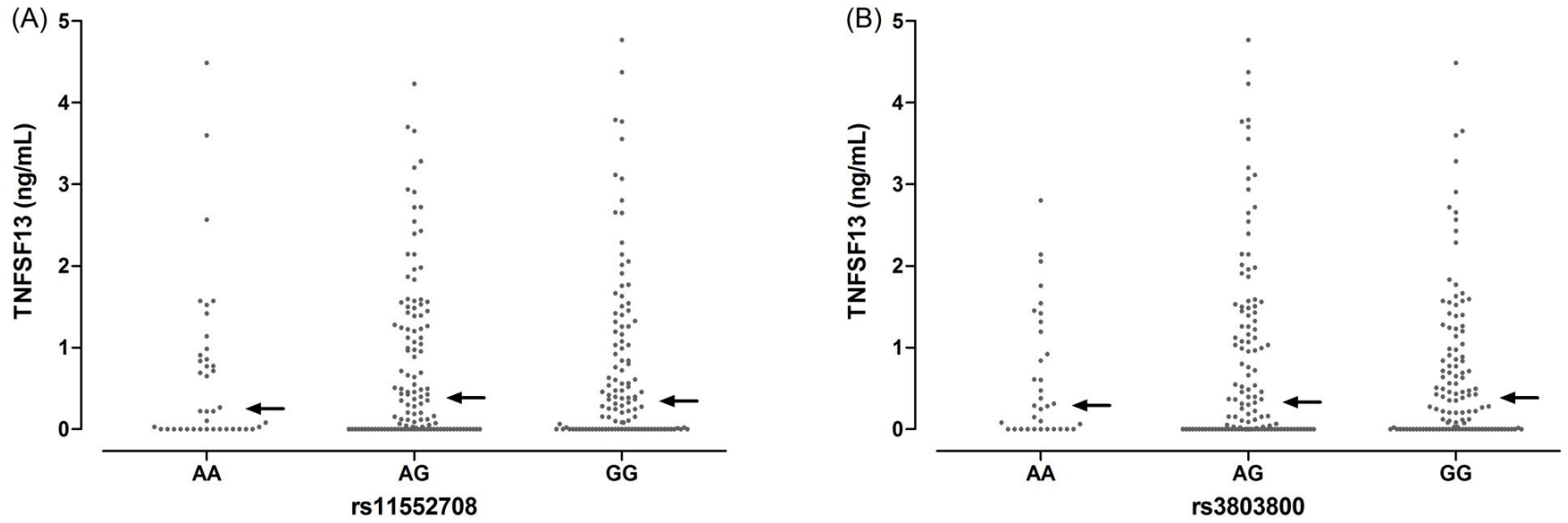
* Each subject is assigned a letter code. “(-)” and “(+)” denote the non-treated group and rhTNFSF13-treated group, respectively.

Supplemental figures

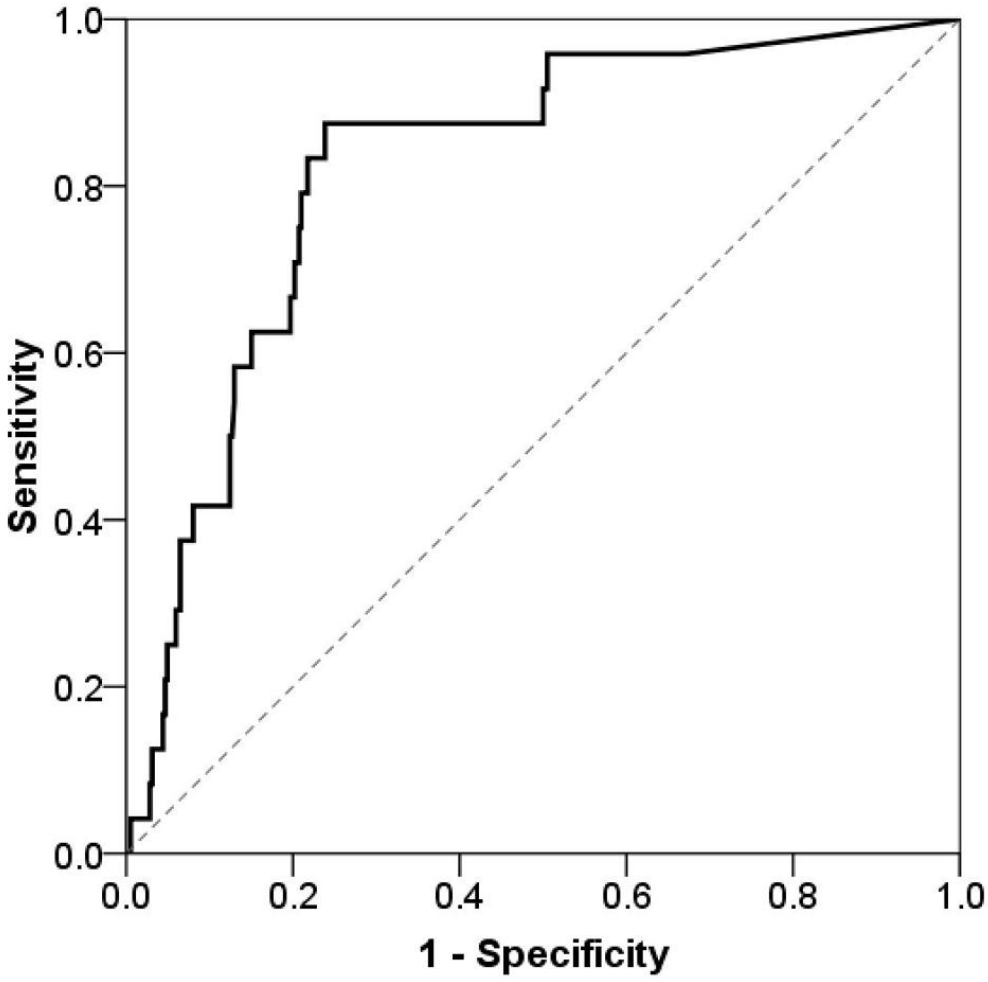
Supplemental Figure 1. Cumulative risk curves for ESRD according to the genotypes in rs11552708 (A) and rs3803800 (B).



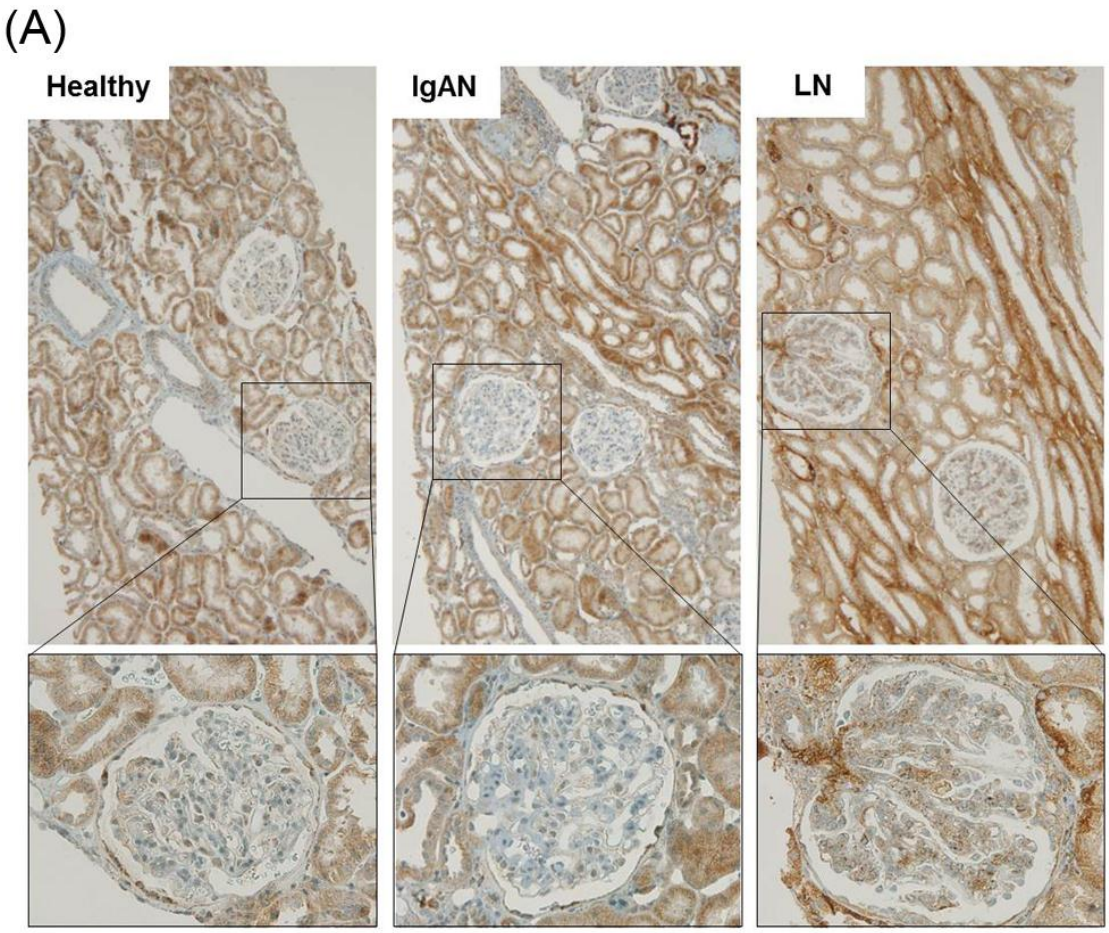
Supplemental Figure 2. Plasma TNFSF13 levels according to the genotypes in rs11552708 (A) and rs3803800 (B). Arrows indicate median values.

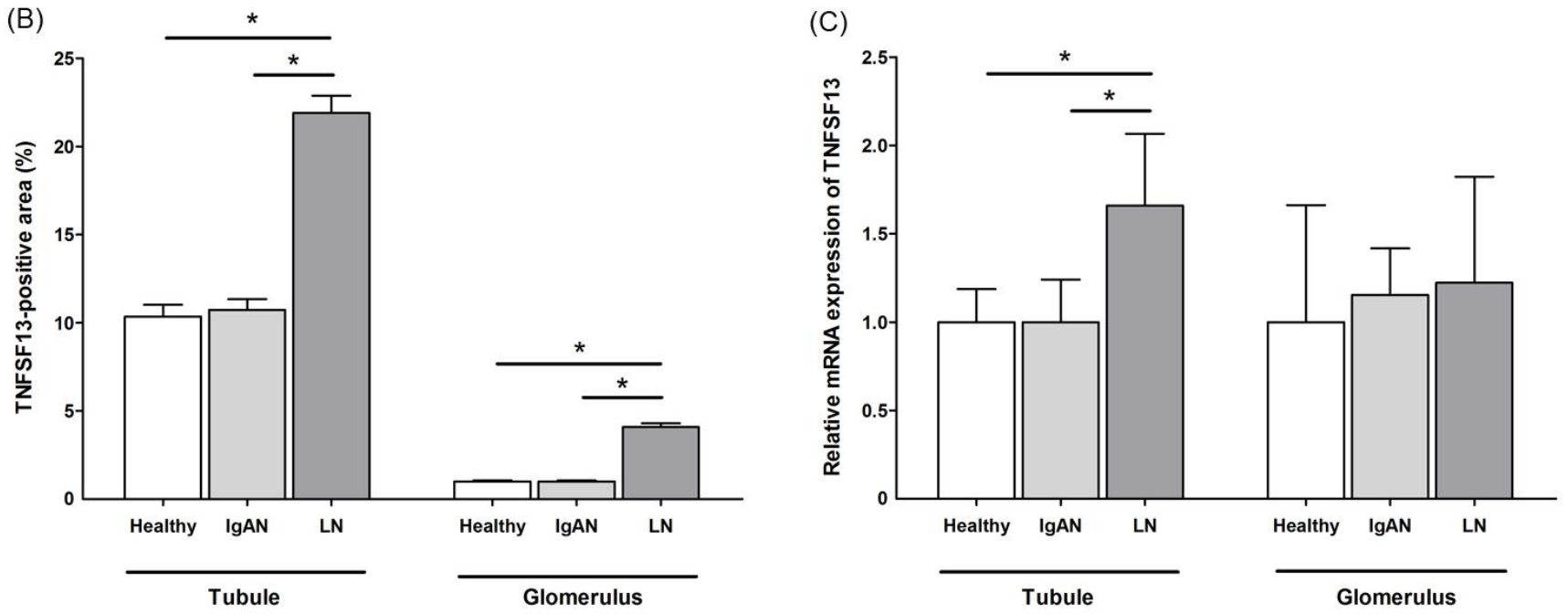


Supplemental Figure 3. ROC curve of plasma TNFSF13 predicting 5-year ESRD risk.

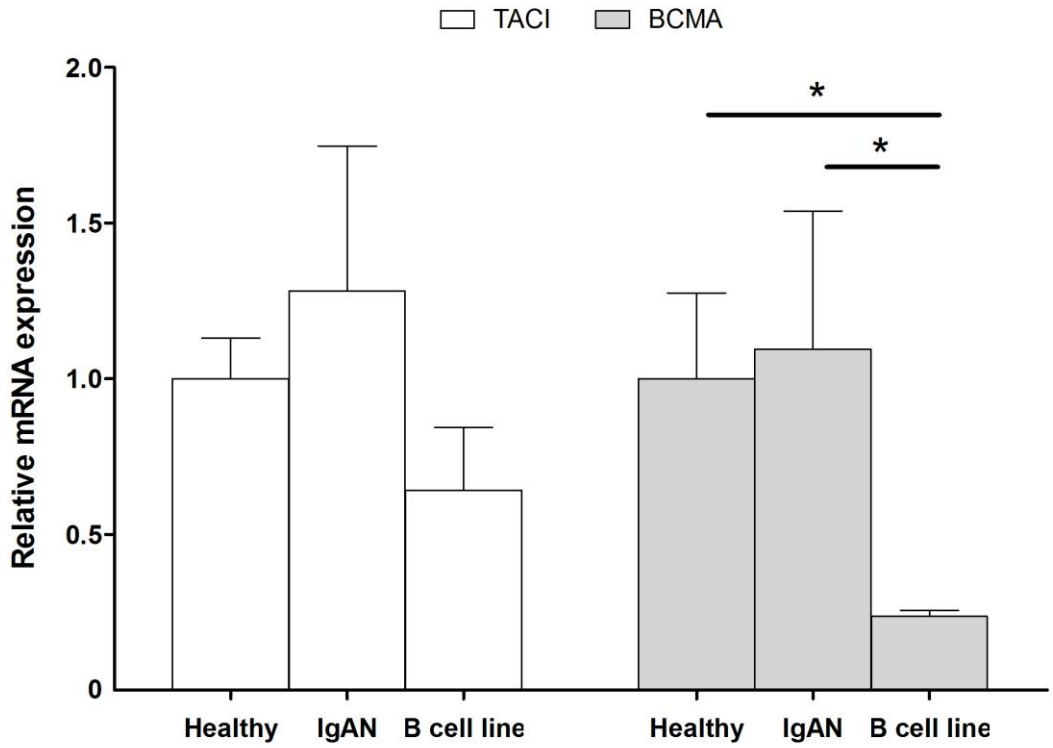


Supplemental Figure 4. Immunohistochemical staining for TNFSF13 in kidney tissues. (A) Representative slides from healthy individuals and patients with IgA nephropathy and lupus nephritis. (B) Semiquantitative immunostaining scores for each group. (C) Comparison in the expression of *TNFSF13* between kidney tissues of healthy individuals, IgA nephropathy patients, and lupus nephritis patients. **P*<0.05. IgAN, IgA nephropathy; LN, lupus nephritis.

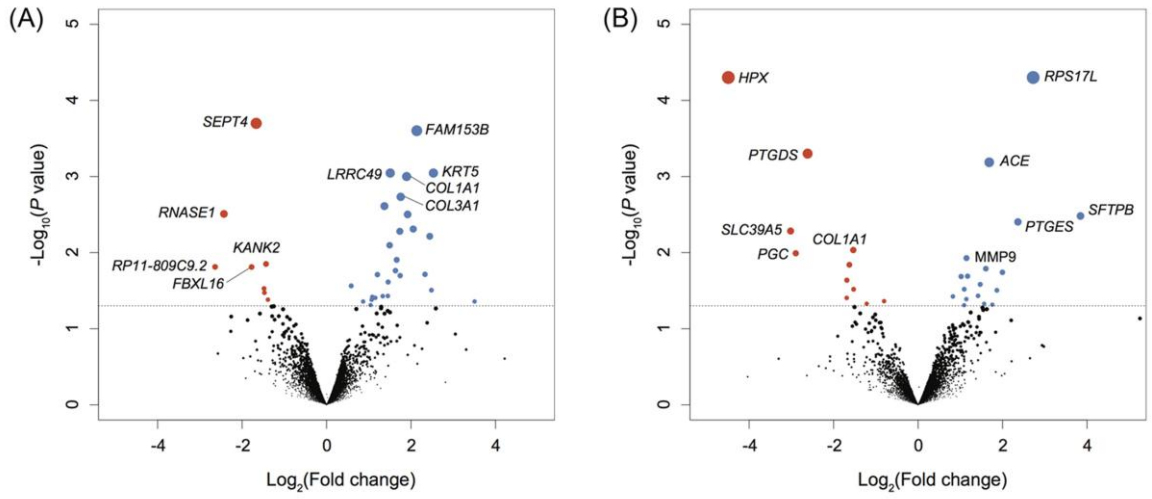



(B)
TNFSF13-positive area (%)
25
20
15
10
5
0
*
*
*
*
Healthy
IgAN
LN
Healthy
IgAN
LN
Tubule
Glomerulus
(C)
Relative mRNA expression of TNFSF13
2.5
2.0
1.5
1.0
0.5
0
*
*
Healthy
IgAN
LN
Healthy
IgAN
LN
Tubule
Glomerulus

Supplemental Figure 5. Expression of TNFSF13 receptors in healthy individuals, patients with IgA nephropathy, and B cell lines.



Supplemental Figure 6. Volcano plots showing the fold change of gene expression between the TNFSF13-treated group and non-treated group. (A) Healthy individuals. (B) Patients with IgA nephropathy. Dot size is proportional to -Log_{10} *P*. Dashed line indicates the point where the *P* value is 0.05. Figure shows the most significant 5 genes with increased or decreased expression (blue point, increase in expression; red point, decrease in expression); additional data can be found in Supplemental Table 7.



Detailed methods

Patients and data collection

The study protocol complies with the Declaration of Helsinki and received full approval from the institutional review board at the Seoul National University Hospital (no. H-1306-108-500). Data on 637 patients with primary IgAN were retrospectively recruited from 4 tertiary referral hospitals (Seoul National University Hospital; Yonsei University Medical Center; Seoul National University Boramae Medical Center; and Gachon University Gil Hospital) between July 1983 and May 2013. All patients were diagnosed by kidney biopsy and provided written informed consent for the donation and use of their specimens (e.g., genomic DNA, blood, and kidney tissues) in the current study. All the samples were immediately recruited, stored, and monitored by the Seoul National University Hospital Human Biobank.

The clinical parameters recorded included the following: age, sex, smoking (current vs. none), diabetes, hypertension, and autoimmune disease. Blood parameters recorded were creatinine, IgA, hepatitis B antigen, and anti-hepatitis C antibody. Glomerular filtration rates were calculated using the CKD-EPI creatinine equation.[1] Hematuria and proteinuria were semi-quantitatively scored from negative to ≥+3 with a dipstick test. All of these baseline parameters were assessed when the kidney biopsy was performed. Based on the Oxford classification,[2] biopsied slides were reviewed (n=253) and scored using following criteria: mesangial hypercellularity (M0 and M1), endocapillary hypercellularity (E0 and E1), segmental glomerulosclerosis (S0 and S1), and interstitial fibrosis/tubular atrophy (T0, T1, and T2). The primary outcome was the development of ESRD. For additional outcomes, doubling of serum creatinine was also examined.

We established control groups to compare genetic polymorphisms and plasma levels of TNFSF13 with the patient group. With regard to genetic polymorphisms, a total of 1,068 healthy individuals were selected for the control group from the Illumina cohort. For the healthy control group for the plasma TNFSF13 levels, a total of 163 healthy individuals were recruited at the time of health checkup. All of these individuals had a serum creatinine less than 1.3 mg/dL and did not have proteinuria, hematuria, or a history of hypertension, diabetes, dyslipidemia, cardiovascular disease, or cancer. Additionally, we set up disease controls and retrieved plasma samples of patients with other kidney diseases, such as membranous nephropathy (n=28), lupus nephritis (n=39), and diabetic nephropathy (n=39). All of them were diagnosed at the time of kidney biopsy.

Plasma was acquired from whole blood samples after centrifugation at 3,000 rpm for 10 minutes at 4°C. The TNFSF13 level was then measured using an ELISA kit (eBioscience, San Diego, CA, USA).

Genotyping analysis

Genomic DNA was extracted from whole blood samples using a QIAamp DNA blood kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The study subjects (515 IgAN patients) were genotyped using the TaqMan fluorogenic 5' nuclease assay (Applied Biosystems, Foster City, CA, USA) for two single-nucleotide polymorphisms (SNPs): rs11552708 and rs3803800. All reactions were performed using a Dual 384-Well GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA) and the endpoint fluorescent readings were performed on an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Duplicate samples and negative controls were included to ensure the accuracy of genotyping.

Immunohistochemistry of kidney tissues

Biopsied kidney tissues (4 μm-thick section) from IgAN patients, lupus nephritis patients, and healthy individuals were stained with anti-TNFSF13 antibody. Tonsilar tissues were used as a positive control. Immunohistochemistry was conducted using a Ventana Benchmark XT automated staining system (Ventana Medical Systems, Tucson, AZ, USA). After heat-induced epitope retrieval, tissues were incubated with mouse anti-human TNFSF13 monoclonal antibody (1:100, Novus Biologicals, Littleton, CO, USA) for 1 hour at room temperature. Then, a secondary antibody in the OptiView DAB IHC Detection kit (Ventana Medical Systems, Tucson, AZ, USA) was applied for 16 minutes at room temperature. Slides were counterstained with hematoxylin II for 4 minutes, followed by the addition of Bluing Reagent (Ventana Medical Systems, Tucson, AZ, USA) for 4 minutes at 37.1°C. All morphometric parameters were determined using a microscope coupled to a computerized morphometry system (Qwin3, Leica, Rijswijk, Netherlands).

Quantitative PCR of *TNFSF13* in kidney tissues

Unfixed biopsy cores were transferred to RNase inhibitor and microdissected into glomerular and tubular specimens. RNA was isolated from each kidney tissue compartment with an RNeasy Micro Kit (Qiagen, Hilden, Germany). Subsequently, RNA was converted to cDNA with a Promega A3500 kit (Promega Biotech, Madison, WI, USA). Relative RNA expression was analyzed using the $2^{-\Delta\Delta CT}$ method on a 7500 Real Time PCR System (Applied Biosystems, Foster City, CA, USA). Primers used for amplification of the *TNFSF13* gene were as follows: sense, 5'-CCTTGCTACCCCACTCTTG-3'; and antisense, 5'-ACACTCAGAATATCCCCTTGG-3'. For a reference gene, the pre-developed TaqMan reagent *GAPDH* gene was used (Applied Biosystems, Foster City, CA, USA).

B cell isolation and TNFSF13 treatment

Heparinized whole blood was pretreated with the RosetteSep® (StemCell Technologies, Vancouver, Canada) and isolated using a Ficoll-density gradient. Subsequently, B cells were isolated with a FACS sorter (FACS Aria III, BD Biosciences, San Jose, CA, USA) by using CD19 staining or positive/negative magnetic bead sorting methods (Miltenyi Biotec Inc., Auburn, CA, USA). The cell purity following the isolation of B cells was identified as greater than 96%.

Isolated B cells were cultured at a density of 3×10^5 cells per 200-μL well in 96-well round-bottom plates. The complete medium used was RPMI 1640 that was supplemented with 10% heat-inactivated fetal bovine serum and penicillin-streptomycin. As a stimulant, we used 1 μg/mL CD40L (R&D Systems, Minneapolis, MN, USA) and 1 μg/mL LPS (Sigma-Aldrich, Saint Louis, MO, USA) for all groups. We used 0.2 or 1.0 μg/mL recombinant human TNFSF13 (rhTNFSF13), receptor blockers, such as recombinant human Fc chimera for transmembrane activator and CAML interactor (TACI) and B-cell maturation antigen (BCMA), or isotype control (i.e., recombinant human immunoglobulin G_1 Fc) (all products were purchased from R&D Systems) to determine the effect of TNFSF13. After 4 days, cells were harvested for subsequent experiments, including flow cytometry and ELISA. In certain experiments (i.e., to conduct real-time quantitative PCR for identifying glycosyltransferase gene changes), a human B lymphoma cell line that produces IgA1 (DAKIKI; ATCC, Manassas, VA, USA) was also used. The protocol for cell culture was the same as stated above.

Flow cytometry of B cells

Cultured cells were harvested and washed with 0.5% BSA and 0.05% sodium azide in PBS. Initially, we used the Fc receptor blocking reagent (Miltenyi Biotec) in all the tubes.

Subsequently, surface staining of APC-CD19, FITC-IgA (Miltenyi Biotec) and TRITC-lectin was performed. To specify binding to GalNAc, the lectin derived from *Helix aspersa* (HAA) was used. Isotype control and fluorescence minus one control tubes were included. To measure the viable proportion of B cells, cells were harvested and stained with antibodies against IgA and lectin, annexin V, and 7-aminoactinomycin (7-AAD) according to the manufacturer's protocol (BD Biosciences). Cells were analyzed with the FlowJo software (version 10.0.7; FlowJo LLC, Ashland, OR, USA).

Measurement of IgA and GdIgA levels

Ninety-six well plates were coated overnight with 3 μg/mL $F(ab')_2$ fragments of goat anti-human IgA antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) in carbonate buffer at 4°C. After washing, plates were blocked for 3 h at 37°C with 2% bovine serum albumin in PBS containing 0.05% Tween-20 (v/v). After washing, samples diluted in PBS were added to each well and incubated for 2 h at room temperature. After washing, the captured IgA was then detected with biotin-labeled $F(ab')_2$ fragments of goat IgG anti-human IgA antibody (InvivoGen, San Diego, CA, USA) for 2 h at room temperature. After washing, biotin binding was detected with streptavidin-horseradish peroxidase conjugate (Sigma-Aldrich, Saint Louis, MO, USA), which was diluted to 1:500 in blocking buffer for 1 h at room temperature. The reaction was developed with the peroxidase chromogenic substrate o-phenylenediamine-H_2O_2 (Sigma-Aldrich, Saint Louis, MO, USA) at room temperature. The color reaction was stopped with 2N H_2SO_4, and the OD of each sample was determined at 490 nm. For GdIgA measurement, we purified GdIgA from an IgAN patient's plasma using the immobilized Jacalin (Thermo Scientific, Rockford, IL, USA). The steps before sample loading were identical to the above protocol. Then, the captured IgA plates were desialylated with 10 mU/mL neuraminidase (Roche, Mannheim, Germany) in 10 mM sodium acetate

buffer (pH 5.2) for 3 h at 37°C. After washing, plates were incubated with biotin-labeled HAA lectin (EY Laboratories, Mateo, CA, USA) for 3 h at 37°C, which was diluted to 1:500 in blocking buffer. The steps for developing and stopping the reaction were the same as used in the IgA ELISA. We expressed the results in U/mL (1 U of GdIgA was defined as 3.7 μg of standard). Then, this value was expressed as U/mg IgA after normalization by total IgA amounts.

Quantitative PCR in B cell experiment

The isolation of RNA and its conversion into cDNA were performed using primary B cells and a B lymphoma cell line in accordance with the methods stated in the above kidney tissue section. Relative RNA expression in the reference, *GAPDH*, was analyzed using the $2^{-\Delta\Delta CT}$ method. Primers used for amplification were as follows: *TNFRSF13B* gene for TACI (sense, 5'-CACCCTAAGCAATGTGC-3'; antisense, 5'-TGGGACTCAGAGTGCC-3'); *TNFRSF17* gene for BCMA (sense, 5'-TTACTTGTCCTTCCAGGCTGTTCT-3'; antisense, 5'-CATAGAAACCAAGGAAGTTTCTACC-3'); *C1GalT1* gene (sense, 5'-TCATGCAAGGCATTCAGATG-3'; antisense, 5'-ATGGGTTCTTCAGGGTCGTA-3'); and *ST6GalNAcII* gene (sense, 5'-AAGCTGCTACATCCGGACTTCA-3'; antisense, 5'-GGGACAGATCGTGGTTTGCATA-3').

RNA sequencing and its analysis

For RNA sequencing, we enrolled 6 subjects (3 IgAN patients and 3 healthy individuals) and cultured their B cells in RPMI 1640 complete medium in the presence or absence of 0.2 μg/mL of rhTNFSF13 for 24 h. Transcriptome libraries were prepared according to the protocol for Illumina's TruSeq Stranded mRNA Sample Prep kit (Illumina, San Diego, CA, USA) using 100-500 ng of total RNA from cultured B cells. Poly(A) RNA was isolated using

AMPure XP beads (Beckman Coulter, Brea, CA, USA) and fragmented with the Ambion Fragmentation Reagents kit (Life Technologies, Carlsbad, CA, USA). cDNA synthesis, end-repair, A-base addition, and ligation of the Illumina indexed adapters were performed according to the manufacturer's protocol. Libraries were size-selected for 250–300 bp cDNA fragments on a 3% 3:1 agarose gel. Bands were recovered using QIAEX II gel extraction reagents (Qiagen, Hilden, Germany) and amplified using Phusion DNA polymerase (New England Biolabs, Ipswich, MA, USA) for 14 cycles. Library qualities for product size and concentration were measured on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Paired-end libraries were sequenced with an Illumina HiSeq 2000 with a read length of 100 bp. Sequenced reads were mapped against the human reference genome (Ensemble GRCh37 Release 72) using TopHat2 version 2.0.11 (http://tophat.cbcb.umd.edu/) with the option -r 169, --mate-std-dev 47, and --library-type fr-firststrand.[3] Transcriptomes from each sample were assembled by Cufflinks version 2.2.1, using default settings and merged by Cuffmerge.[4] Differential gene expression analysis was performed using Cuffdiff. Differentially expressed genes were evaluated according to the false discovery rate (Benjamini and Hochberg approach). Additional information on the reads is available in Supplemental Table 10. Subsequently, we performed the gene set enrichment analysis using the same RNA sequencing dataset by the SeqGSEA R package (version 3.1) and MSigDB gene sets.[5]

Statistical analysis

All analyses and calculations were performed using SPSS (version 21.0; IBM, Armonk, NY, USA) and STATA (version 12.0; StataCorp LP, College Station, TX, USA). Data are presented as the mean ± standard deviation for continuous variables and as proportions for categorical variables. Based on variable distributions determined using histograms, the

variables with non-normal distributions are expressed as medians (interquartile ranges). The comparisons were evaluated using the chi-square test for categorical variables, ANOVA for normally distributed continuous variables (LSD post hoc analysis between two groups), and the Kruskal-Wallis test for non-normally distributed continuous variables (Mann-Whitney *U* test between two groups). Odds ratios were calculated using logistic regression analysis. A log-rank test was initially applied to compare the outcomes between groups. The Cox proportional hazard model, both with and without the adjustment of covariates, was used to calculate the hazard ratios for the outcomes. The fractional polynomials method was applied to account for a possible nonlinear relationship between TNFSF13 and outcomes. With this non-linear relationship in mind, we determined a threshold value of TNFSF13 that predicted outcomes by calculating the Youden index of the receiver operating characteristic (ROC) curve,[6] after adjustment of age, sex, creatinine, and proteinuria. A *P* value of less than 0.05 was considered significant.

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