Supplementary information

Levels of circulating miR-133a are elevated in sepsis and predict mortality in critically ill patients

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Inventory of Supporting Information

Supplementary Data set

Supplementary Materials and Methods

Supplementary References

Supporting Materials and Methods

Mouse model of polymicrobial sepsis

Male C57Bl/6 mice (6–8 wk of age) were purchased from The Jackson Laboratory (Bar Harbor, ME). The mice (n=14) were subjected to cecal pole ligation and puncture (CLP) surgery, as described previously (1). Blood was taken before and 24 h after surgery for analysis of miR-133a serum concentrations. Only sex-matched animals were compared. Animals received human care according to European, national and institutional regulations. The specific experiments described within this manuscript were approved by the "*Landesamt für Natur, Umwelt und Verbraucherschutz NRW, Germany* (Aktenzeichen 10429G1 (09.152)).

Characteristics of sepsis and non-sepsis patients

For the categorization as sepsis or non-sepsis patients, the criteria proposed by the American College of Chest Physicians and the Society of Critical Care Medicine Consensus Conference Committee for severe sepsis and septic shock were used (2,3). 138 of the 223 patients included in this study conformed to the criteria of bacterial sepsis (Table 1). Pneumonia represented the most prominent origin of infection (Table 2). In non-sepsis patients cardiopulmonary diseases (myocardial infarction, pulmonary embolism, and cardiac pulmonary edema), decompensated liver cirrhosis or other critical conditions represented the predominant etiologies. As expected, sepsis patients displayed significantly higher levels of routinely used biomarkers of inflammation (that is, C-reactive protein, procalcitonin, white blood cell count; Table 1 and data not shown). Furthermore, sepsis patients were more often in need of mechanical ventilation as compared to the non-sepsis patients (Table 1). Of note, both patient cohorts did not differ in Acute Physiology and Chronic Health

Evaluation II score (APACHE II), Sequential Organ Failure Assessment (SOFA) and Simplified Acute Physiology Score 2 score (SAPS 2), vasopressor demand, or laboratory parameters indicating liver or renal dysfunction (data not shown).

miRNA isolation from serum

400 µl serum (humans) / 70 µl serum (mice) was spiked with miScript miRNA mimic SV40 (2 µM, 1 µl/100 µl serum, Qiagen, Hilden, Germany) for sample normalization. 800 µl phenol (Qiazol, Qiagen, Hilden, Germany) and 200 µl chloroform were added to the sample and mixed vigorously for 15 sec followed by an incubation at room temperature for 5 min. Samples were centrifuged for 15 min at 12,000 g at 4°C until complete phase separation. The aqueous phase, containing total RNA, was precipitated with 500 µl 100% isopropanol and 2 µl glycogen (Fermentas, St. Leonroth, Germany) overnight at -20°C. After centrifugation at 4 °C for 30 min (12,000 g) the pellets were washed once with 70 % ethanol. Precipitated RNA was resuspended in 30 µl RNase free water (Ambion, Austin, TX). To asses the quality of RNA, the samples were measured with a NanoDrop spectrophotometer (NanoDrop, Thermo Fisher Scientific, Waltham, MA), and a smallRNA assay for Agilent´s Bioanalyzer was performed (Agilent Technologies, Böblingen, Germany) (4,5).

miRNA-Isolation from tissue

Total RNA was purified from liver tissue using Trizol reagent (Invitrogen) and miRNeasy Mini kit (Qiagen) according to the manufacturer's protocol, and was resuspended in suitable amounts of H_2O.

Cell Culture and Transfection

The monocyte cell line U937 cells (5) were cultured in RPMI medium with 10% fetal bovine serum, 300 mg/L L-glutamine and penicillin/streptomycin. For differentiation, cells were seeded on 6-well-plates and stimulated with 20 nmol/ml PMA for 24 hours. For transfection the Hiperfect transfection reagent (Qiagen) with miRNA specific plasmids (Qiagen) was used according to the manufacturer's instructions. Seventy-two hours after transfection cells were harvested for RNA isolation.

I*n silico*-analysis of potential miRNA target genes

The microRNA databases and target prediction tool TargetScan (http://www.targetscan.org/index.html) was used to identify potential microRNA targets.

Quantitative real-time PCR

Quantitative real-time Polymerase chain reaction (PCR) was performed as recently described (5). In detail, 5 µl of extracted total RNA was used to synthesize complementary Deoxyribonucleic acid (cDNA) utilizing miScript Reverse Transcriptase Kit (Qiagen) according to the manufacturer's protocol and was diluted in suitable amounts of H_2O. The rest of the protocol was conducted via the miScript Reverse Transcription Kit according to manufacturer's protocol (Qiagen). cDNA samples (2 µl) were used for quantitative real-time PCR in a total volume of 25 µl using the miScript SYBR Green PCR Kit (Qiagen) and miRNA specific primers (Qiagen) on a qPCR machine (Applied Biosystems 7300 Sequence Detection System, Applied Biosystems, Foster City, CA). All real-time PCR reactions were

performed in duplicates. Data were generated and analyzed using the SDS 2.3 and RQ manager 1.2 software packages (Applied Biosystems, Foster City, CA).

Statistical analysis

Statistical analyses were performed as described previously (4, 5). In brief, data are displayed as median and range considering the skewed distribution of most parameters. Differences between two groups were assessed by Mann-Whitney- U-test and multiple comparisons between more than two groups have been conducted by Kruskal-Wallis- ANOVA and Mann-Whitney-U-test for post hoc analysis. Box plot graphics illustrate comparisons between subgroups and display a statistical summary of the median, quartiles, range and extreme values. The whiskers extend from the minimum to the maximum value excluding outside and far out values which are displayed as separate points. An outside value (indicated by an open circle) was defined as a value that is smaller than the lower quartile minus 1.5-times the interquartile range, or larger than the upper quartile plus 1.5-times the interquartile range. A far out value was defined as a value that is smaller than the lower quartile minus three times the interquartile range, or larger than the upper quartile plus three times the interquartile range. All values, including “outliers”, have been included for statistical analyses. Correlations between variables have been analysed using the Spearman correlation test, and values of $P < 0.05$ were considered statistically significant. Kaplan Meier curves were plotted to display the impact on survival. Receiver operating characteristic (ROC) curve analysis and the derived area under the curve (AUC) statistic provide a global and standardized appreciation of the accuracy of a marker for predicting an event (presence of sepsis, mortality). ROC curves were generated by plotting sensitivity against 1-specificity. The prognostic value of the variables was tested in the Cox regression model as recently described

(3). All statistical analyses on human samples were performed with SPSS version 12.0 (SPSS, Chicago, IL).

Differences in mice between before and after surgery were assessed by Wilcoxon matched pairs test after the Shapiro-Wilk-test did not show a Gaussian distribution ($p < 0.01$). Graphical presentations in mice were performed with GraphPad Prism 5 (Graph-Pad, San Diego, CA)

Supplementary Table 1. Correlations of miR-133a serum concentrations at admission day with other laboratory markers

	ICU patients	
Parameter	**r**	**p**
Markers of liver function		
gGT	0.314	<0.001
AP	0.252	<0.001
Bilirubin total	0.164	0.016
Bilirubin direct	0.330	<0.001
AST	0.389	<0.001
ALT	0.265	<0.001
INR	0.163	0.017
Markers of inflammation		
CRP	0.237	<0.001
Procalcitonin	0.248	0.002
IL-6		
IL-10	0.268	0.003
TNF	0.439	0.001
ATIII	-0.254	0.035
D-Dimers	0.243	0.037
Markers of renal function		
Cystatin C GFR	-0.217	0.014
Others variables		
LDH	0.447	<0.001
suPAR	0.448	<0.001
Ghrelin	-0.339	0.006
Resistin	0.318	0.008
APRIL	0.355	<0.001
Duration of Ventilation	0.143	0.051
p(max)	0.285	0.014
Days on ICU	0.137	0.043
Clinical scoring		
APACHE-II	0.149	0.041
SOFA	0.253	0.004

r, correlation coefficient; p, p-value; Spearman rank correlation; GGT, Gamma glutamyl transpeptidase; AP, Alkaline phosphatase; AST, Aspartate transaminase; ALT, Alanine transaminase; INR, International Normalized Ratio; CRP, C-reactive protein; IL, Interleukin; TNF, Tumour necrosis factor; AT III, Antithrombin III; GFR, Glomerular filtration rate; LDH, Lactate Dehydrogenase, suPAR, soluble urokinase-type plasminogen activator receptor; APRIL, A Proliferation Inducing Ligand; ICU, Intensive care unit; p(max), maximum pressure; APACHE-II, Acute Physiology and Chronic Health Evaluation II; SOFA, Sequential Organ Failure Assessment

Supplementary Table 2. miR-133a target genes

Target gene	Gene name	inflammation	sepsis	TNF	immune cells
EGFR	epidermal growth factor receptor	543	32	269	134
BCL2L1	BCL2-like 1	173	23	415	121
SP1	Sp1 transcription factor	193	11	172	209
TRAF3	TNF receptor-associated factor 3	29	0	335	15
CTGF	connective tissue growth factor	215	1	81	69
TGFB2	transforming growth factor, beta 2	152	1	119	57
SIRT1	sirtuin 1	185	8	41	33
GDNF	glial cell derived neurotrophic factor	132	1	66	16
PML	promyelocytic leukemia	65	20	45	97
IGF1R	insulin-like growth factor 1 receptor	62	6	45	22
CHP	calcium binding protein P22	65	17	22	10
FURIN	furin (paired basic amino acid cleaving enzyme)	43	2	52	14
CYLD	cylindromatosis (turban tumor syndrome)	42	2	51	5
CD47	CD47 molecule	62	2	29	61
TPM3	tropomyosin 3	69	5	12	16
PPP2CA	protein phosphatase 2, catalytic subunit, alpha isozyme	45	6	33	23
PPP2CB	protein phosphatase 2, catalytic subunit, beta isozyme	45	6	33	23
CNP	2',3'-cyclic nucleotide 3' phosphodiesterase	57	11	16	13
ADCYAP1	adenylate cyclase activating polypeptide 1 (pituitary)	53	10	17	6
FGF1	fibroblast growth factor 1 (acidic)	54	0	25	22
COL1A1	collagen, type I, alpha 1	45	1	14	15
FLT1	fms-related tyrosine kinase 1	41	3	14	28
TNFRSF10D	TNF receptor superfamily, member 10d	5	0	46	2
SP3	Sp3 transcription factor	25	0	25	35
ELAVL1	ELAV (embryonic lethal, abnormal vision, Drosophila)-like 1	25	1	24	9
HLF	hepatic leukemia factor	28	3	16	10
MMP14	matrix metallopeptidase 14 (membrane-inserted)	30	0	15	7
TFG	TRK-fused gene	13	3	25	6
AQP1	aquaporin 1 (Colton blood group)	27	2	10	2
DAPK2	death-associated protein kinase 2	17	0	20	9
ADAMTS5	ADAM metallopeptidase with thrombospondin type 1 motif, 5	19	1	15	2
FGFR1	fibroblast growth factor receptor 1	18	0	12	14
GCLC	glutamate-cysteine ligase, catalytic subunit	17	3	10	3
POU2F1	POU class 2 homeobox 1	12	1	13	14
AGRP	agouti related protein homolog (mouse)	18	1	7	0
PKHD1	polycystic kidney and hepatic disease 1 (autosomal recessive)	15	9	2	1
LCORL	ligand dependent nuclear receptor corepressor-like	12	6	7	2
CRK	v-crk sarcoma virus CT10 oncogene homolog (avian)	16	1	5	4
SGK1	serum/glucocorticoid regulated kinase 1	21	0	1	4
PAX7	paired box 7	14	0	7	1
MAP3K3	mitogen-activated protein kinase kinase kinase 3	7	0	14	1
UNK	unkempt homolog (Drosophila)	10	1	10	6
NFAT5	nuclear factor of activated T-cells 5, tonicity-responsive	8	2	10	5
SIGLEC1	sialic acid binding Ig-like lectin 1, sialoadhesin	17	0	3	23
CYCD1	CyclinD1	10	0	12	2
PHIP	pleckstrin homology domain interacting protein	16	0	1	2
INSR	insulin receptor	10	0	6	4
MCL1	myeloid cell leukemia sequence 1 (BCL2-related)	4	0	12	8
PDE7A	phosphodiesterase 7A	11	0	5	2
ELF3	E74-like factor 3	14	0	2	5
GLS	glutaminase	6	1	8	3

(numbers indicate PubMed hits)

Supporting References

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