Research Square

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Differential expression of circRNA in amyotrophic lateral sclerosis and validation of identified circRNA biomarker

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Research Article

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Abstract

Amyotrophic lateral sclerosis (ALS) is a rapidly progressive adult-onset neurodegenerative disease that is often diagnosed with a delay due to initial non-specific symptoms. Therefore, reliable and easy-to-obtain biomarkers are in desperate need for earlier and more accurate diagnostics. Circular RNAs (circRNAs) have been already proposed as potential biomarkers for several neurodegenerative diseases. In this study, we further investigated the usefulness of circRNAs as potential biomarkers for ALS. We first performed a microarray analysis of circRNAs on peripheral blood mononuclear cells of a subset of ALS patients and controls. Among the differently expressed circRNA by microarray analysis, we selected only the ones with a host gene that harbors the highest level of conservation and genetic constraints. This selection was based under the hypothesis that genes under selective pressure and genetic constraints could have a major role in determining a trait or disease. Then we performed a linear regression between ALS cases and controls using each circRNA as a predictor variable. With an FDR threshold of 0.1, only six circRNAs passed the filtering and merely one of them remained statistically significant after Bonferroni correction: hsa_circ_0060762 and its host gene CSE1L. Finally, we observed a significant difference in expression levels between larger sets of patients and healthy controls for both hsa_circ_0060762 and CSE1L . In addition, receiver operating characteristics curve analysis showed diagnostic potential for CSE1L and hsa_circ_0060762 . Hsa_circ_0060762 thus represent a novel potential peripheral blood circRNA biomarker for ALS.

Introduction

Amyotrophic lateral sclerosis (ALS) is a rapidly progressive adult-onset neurodegenerative disorder where both upper and lower motor neurons are affected [1]. Majority of the patients die within 3 years of first symptoms [2]. Current diagnosis is based on clinical examination (El-Escorial criteria) [3] and neurophysiological examination (Awaji criteria) [4], while recently the ALS diagnostic index was also described [5]. Despite all this, establishing the correct diagnosis can still take one year or more [6]. Therefore, reliable and easy-to-obtain biomarkers are in desperate need for earlier and more accurate diagnoses. Our research group previously described the potential use of circular RNA (circRNA) expression levels in ALS patients as biomarkers [7]. CircRNAs represent a class of non-coding RNAs that are formed during precursor mRNA processing via back-splicing events [8]. They have several biologically diverse functions such as miRNA sponges, protein-coding RNAs, and transcriptional regulators [9]. They have been already associated with numerous diseases of the nervous system such are Parkinson's disease [10,11], Alzheimer's disease [12,13], glioblastoma [14,15], multiple sclerosis [16], and epilepsy [17].

A previous work [18] demonstrated the importance of highlighting genes and critical genomic regions subjected of strong purifying selection, showing that such regions are enriched for disease-causing variants and thus should be prioritized in a genetic study that aims to find the causal actors for a disease. The gene prioritization approaches will help identify the parts of the human genome increasingly likely to harbor mutations that influence the risk of disease [19].

Here, we wanted to use an approach based on metrics of constraints for the selection of potential circRNAs biomarkers. We focused on circRNA host genes that are under selective pressure and have strong genetic constraints as these genes could have a predominant role in determining the disease.

Selection of circRNAs for the analysis was based on p-value, fold change, and function of the host gene. A statistically significant difference (after Bonferroni correction) in expression between patients and controls was observed for *hsa_circRNA_060762* and its host gene *CSE1L*. This approach showed great potential for use as blood-based biomarkers for ALS.

Materials And Methods

Samples

Patients were diagnosed with ALS at the Institute of Clinical Neurophysiology, University Medical Centre Ljubljana, Slovenia. Sixty patients (30 females and 30 males) and 25 age- and sex-matched healthy controls were included in the study. Detailed clinical characteristics are shown Table 1.

Table 1. Detailed clinical characteristics of included patients and healthy controls.

	Samples	
Characteristics	ALS (n=60)	Healthy controls (n=25)
Sex (M/F)	30/30	15/10
Age (years)[a]	67 (35-92)	56 (47-73)
Age at onset (years)	65 (35-92)	/
ALS onset (spinal/bulbar/mixed)	45/13/2	/
Disease duration (years)[b]	1.5 (0.0-5.5)	/
Survival time (years)[c]	2.0 (0.5-5.0) n=27	/
Level of functional impairment[d]	34 (20-48)	/
Rate of progression[e]	-1.11 (-0.03 - -4.19)	/

[a] Age at the time of blood collection.

[b] Time from symptom onset to blood collection.

[c] Time from symptom onset to death.

[d] ALS-FRS-R (ALS functional rating scale revised) points at the time of blood collection.

[e] Slope of the linear regression line for ALS-FRS-R points.

RNA extraction

Peripheral blood mononuclear cells (PBMCs) were isolated from fresh blood. Ficoll density centrifugation (GE Healthcare, Sweden) was used to collect the cells that were afterwards stored at − 80 °C in Qiazol reagent (Qiagen, Germany). Total RNA was extracted from stored cells using miRNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. The concentration and purity of total RNA were measured with NanoDrop ND-1000 (ThermoFisher, USA).

Microarray analysis

Microarray analysis of circRNA expression was performed on a subset of 20 samples — 12 patients (6 females, 6 males) and 8 age- and sex-matched controls. Samples were prepared and processed as previously described [7].

qPCR

Total RNA was reverse transcribed to cDNA using SuperScript VILO Master Mix (ThermoFisher, USA). Expression levels circRNAs were measured by real-time quantitative PCR (qPCR) using Sybr Select Master Mix (ThermoFisher, USA) on the Rotor Gene Q 5plex HRM platform (Qiagen, Germany) in duplicate for each sample. Used primers are shown in Table 2. Primers were synthesized by IDT (USA) or Qiagen (Germany) (QuantiTect primers). *RPS17* and *RPL13A* were used as reference genes and the data were analyzed using the comparative cycle threshold method ($2^{\Delta\Delta Ct}$).

Table 2. List of qPCR primers.

Target RNA	Primer sequence (5' – 3')
hsa_circ_0060762	F: CCAAAATTCACCTGGCACAG
	R: GGTGGTCATTTGCTTTGG
CSE1L	QuantiTect: Hs_CSE1L_1_SG (Cat. No. QT00015498)
RPS17	F: CCATTATCCCCAGCAAAAAG
	R: GAGACCTCAGGAACATAATTG
RPL13A	QuantiTect: Hs_RPL13A_1_SG (Cat. No. QT00089915)

Statistical analysis

Among the differently expressed circRNA by microarray analysis, we collected only the ones with a host gene that harbors any evidence of genetic constraints. This selection was based under the hypothesis that genes under selective pressure and genetic constraints could have a major role in determining a trait or disease. In particular, we collected the loss of function intolerance score (pLI) from gnomAD [20], and the DSC and SSC score for Europeans [21]. Then we created a "conserved" set of genes that follow these criteria: pLI=1 (highest probability of loss of function intolerance), DSC score < -2 and SSC score < -2 [21]. These stringent criteria aim to selected the genes with the highest level of genetic constrains and evidence of ongoing purifying selection. Linear regression analyses were performed using the expression level as a predictor and the status (cases and controls) as the response. For regression analyses, we used only the set of genes labelled as "conserved set". False discovery rate and Bonferroni correction on regression p-values were calculated using R [22].

All experimental data were analyzed using SPSS software 24.0 (SPSS, USA). Differences in expression levels between patients and healthy controls were assessed using the Mann-Whitney U test. Spearman's rank correlation was used to determine the correlations between circRNA/gene expression levels and clinical data. Diagnostic potential of circRNA and host gene was assessed with ROC curve analysis. A p-value < 0.05 and AUC metric > 0.5 was considered to be statistically significant.

Results

genes. Then we performed the regression analyses using the circRNA mapped on these genes (total of 95 from an initial set of 10161). Then, after Bonferroni correction, the only circRNA that was significantly associated (p-value≤0.05/95) with ALS status was *hsa_circRNA_060762* that is encoded in *CSE1L* gene (see Fig. 1). Afterwards, we also estimated the false discovery rate (FDR) for each circRNA analyzed among the “conserved set” of genes, using, in this case, a less stringent cut-off of false discovery rate of 0.1. We found that the following circRNA and genes are associated with ALS status: *UPF2*, *XPOI*, *KPNB1* and *MED13*, in addition to *CSE1L* (Table 3). Despite not being statistically significant (after Bonferroni correction), we can consider these genes as possible candidates for future analyses of gene-gene interaction.

Table 3. Constraints metrics and regression statistics of genes with circRNA showing a FDR<0.1).

GENE_ID	GENE_NAME	DSC_score	SSC_score	pLI	R2	p-value	FDR	p-value (Bonferroni)	circRNA
ENSG00000108510	*MED13*	-2.36	-2.51	1	0.35	0.005521	0.087	0.524	*hsa_circRNA_405614*
ENSG00000108424	*KPNB1*	-2.50	-2.61	1	0.35	0.005477	0.087	0.52	*hsa_circRNA_102111*
ENSG00000151461	*UPF2*	-2.20	-2.71	1	0.39	0.003135	0.074	0.297	*hsa_circRNA_007448*
ENSG00000082898	*XPO1*	-2.30	-2.84	1	0.40	0.002702	0.074	0.256	*hsa_circRNA_102732*
ENSG00000151461	*UPF2*	-2.20	-2.71	1	0.48	0.000621	0.029	0.059	*hsa_circRNA_100548*
ENSG00000124207	*CSE1L*	-2.42	-2.98	1	0.62	0.000034	0.003	0.003	*hsa_circRNA_060762*

Expression of circRNA and host gene

The only circRNA that remained significant after Bonferroni correction was *hsa_circRNA_060762*. We determined the expression levels of *hsa_circ_0060762* and its host gene *CSE1L* (Fig. 2). Both circRNA and its host gene were significantly downregulated in sixty ALS patients compared to healthy controls, expression of *hsa_circ_0060762* by 2.5-fold and expression of *CSE1L* by 1.8-fold.

Associations between clinical variables and circRNA expression

Using the Spearman rank correlation test, we observed no statistically significant association between circRNA expression and clinical parameters (Table 4). There is a slight positive correlation between the expression of circRNA and its host gene.

Table 4. Correlations between circRNA and gene expression levels in ALS patients and association with clinical variables.

	Sex	ALS onset	Age at the time of blood collection	Age at onset	Disease duration
Sex	-	0.289*	0.176	0.146	-0.166
ALS onset		-	0.187	0.202	-0.294*
Age at the time of blood collection			-	0.991**	-0.037
Age at onset				-	-0.113
Disease duration					-
Survival time					
Level of functional impairment					
Rate of progression					
CSE1L					
Has_circ_0060762					

	Survival time	Level of functional impairment	Rate of progression	*CSE1L*	*Has_circ_0060762*
Sex	0.073	0.077	-0.165	-0.133	-0.051
ALS onset	-0.211	0.288*	-0.225	-0.098	0.194
Age at the time of blood collection	0.160	-0.177	-0.241	0.056	-0.032
Age at onset	0.107	-0.148	-0.307	0.072	-0.033
Disease duration	0.817**	-0.418**	0.593**	0.043	0.053
Survival time	-	-0.451*	0.652**	-0.173	0.162
Level of functional impairment		-	-0.177	0.116	0.147
Rate of progression			-	-0.108	0.026
CSE1L				-	0.289*
Has_circ_0060762					-

Diagnostic potential

We performed receiver operating characteristics (ROC) curve analysis to evaluate the diagnostic potential of *hsa_circ_0060762* and *CSE1L*. The curves for circRNA and its host gene are similar, resulting in an area under the curve (AUC metric) of approximately 0.75, together with 82.5% sensitivity and 62.5% specificity for the optimal cut-off point (Fig. 3)

Discussion

ALS is a rapidly progressing neurodegenerative disease that is often diagnosed with a delay due to initial non-specific symptoms. Several types of biomarkers were already proposed (miRNAs, mRNAs, proteins, various metabolites) in cerebrospinal fluid, leukocytes, serum, and plasma [23]. None of them is routinely used in the diagnostic at the moment, although some show great potential for further validation. CircRNAs have been already proposed as potential biomarkers for several diseases, including Alzheimer's disease [24], multiple sclerosis [16], major depressive disorder [25], numerous cancers [26], and ALS itself [7].

Here, we further investigated the usefulness of circRNAs as potential biomarkers for ALS. Our framework was based on a gene prioritization approach in order to select the circRNAs with host genes that showed the highest level of conservation and genetic constraints. Because, according to our hypothesis, these

conserved genes should be the ones in which any kind of variation should have a more substantial effect on the disease compared to the non-conserved type of genes. Then we performed a linear regression between ALS cases and controls using each circRNA as a predictor variable. With an FDR threshold of 0.1, only six circRNAs passed the filtering and merely one of them remained statistically significant after Bonferroni correction: *hsa_circ_0060762* and its host gene *CSE1L.* Finally, we observed the significant difference in expression levels between patients and healthy controls for both *hsa_circ_0060762* and *CSE1L*. *Hsa_circ_0060762* is encoded in *CSE1L* gene, which is found in the "conserved set" of ALS genes with the highest level of genetic constraints. *CSE1L* in humans encodes an exporting factor for importin-α. In the spinal cord of mice model of ALS, an altered localization of two proteins of nucleocytoplasmic transport system, importin-α and importin-β, was detected using immunohistochemistry [27]. An abnormal transporter protein distribution was also detected in spinal cords of patients with a sporadic and familial form of ALS [28]. Furthermore, reduced levels of CSE1L were reported in the brains of patients with frontotemporal lobar degeneration (FTLD) [29], the disease which shares many clinical, pathological and genetic characteristics with ALS, including nuclear trafficking impairment [30,31].

In this study, we report for the first time the reduced expression of *has_circ_0060762* and it's host gene *CSE1L* in peripheral blood mononuclear cells of patients with ALS. In addition, receiver operating characteristics curve analysis showed some diagnostic potential for *CSE1L* and *hsa_circ_0060762*. *Hsa_circ_0060762* thus represent a novel potential circRNA biomarker for ALS, together with three other circRNAs that we have previously reported in connection with ALS [7]. Nevertheless, all described potential circRNA biomarkers for ALS need validation and comparison with other neurodegenerative diseases before any conclusion is made on their usefulness as ALS biomarkers. Also, some limitations in this study like samples size and reduced power to detect circRNA with smaller effect have to be considered.

In conclusion, we showed that circRNAs have the potential to be effective blood-based circulating ALS disease biomarkers. However, an extensive validation based on diverse sets of healthy and diseased cases, preferably with a larger number of samples in each group, has to be performed, before we can classify them as useful biomarkers for ALS.

Declarations

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Conflict of interests

The authors declare no conflict of interest.

Availability of data and materials

The datasets during and/or analysed during the current study available from the corresponding author on reasonable request.

Code Availability Not applicable

Authors' Contributions

Conceptualization, D.G.; formal analysis and methodology, M.M.; investigation and writing—original draft preparation, A.D.; resources, B.K.; writing - review & editing and supervision, M.R-G. All authors have read and agreed to the published version of the manuscript.

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Ethics approval

The study was approved by the National Medical Ethics Committee of Republic of Slovenia. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Consent to participate

Informed consent was obtained from all individual participants included in the study.

Consent for publication

Informed consent was obtained from all individual participants included in the study.

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Figures

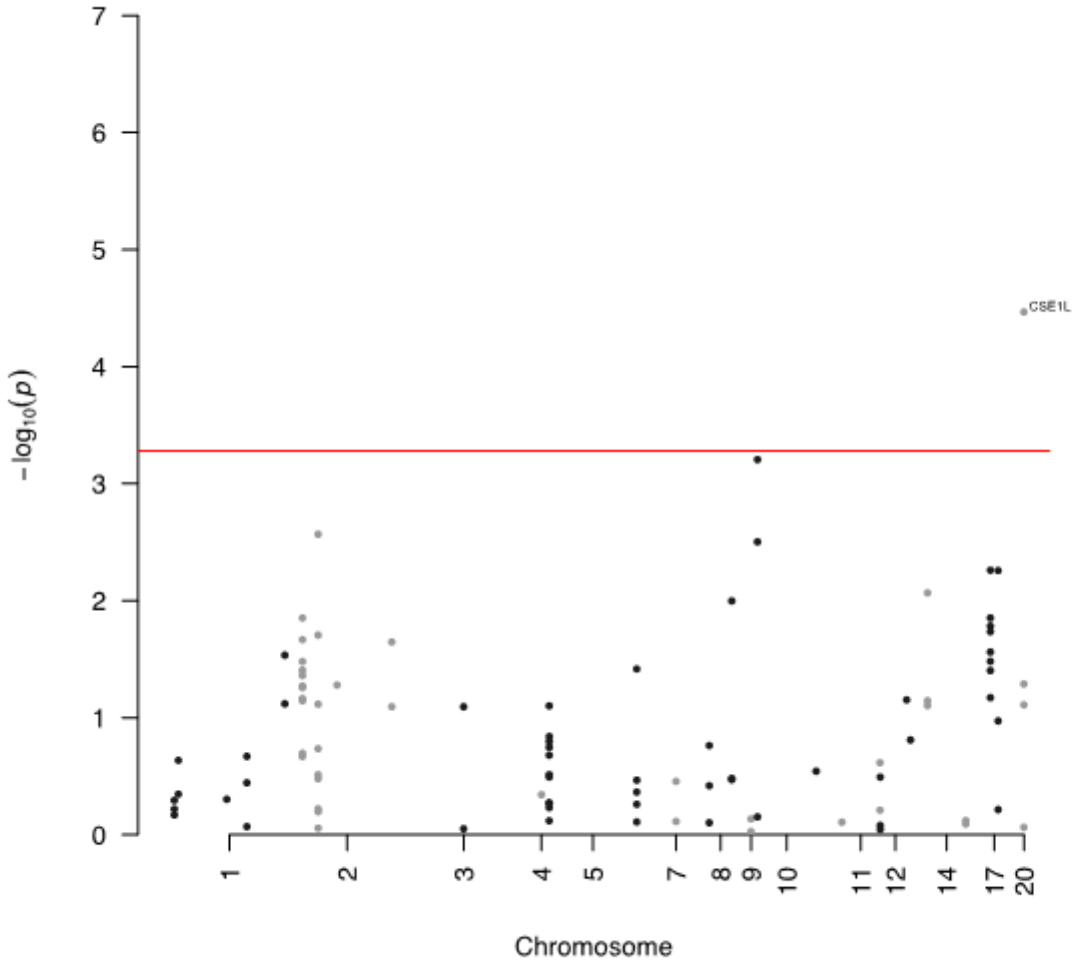


Figure 1

Manhattan plot of circRNA inside the “conserved set” of genes, the red line represents the significance threshold after Bonferroni correction

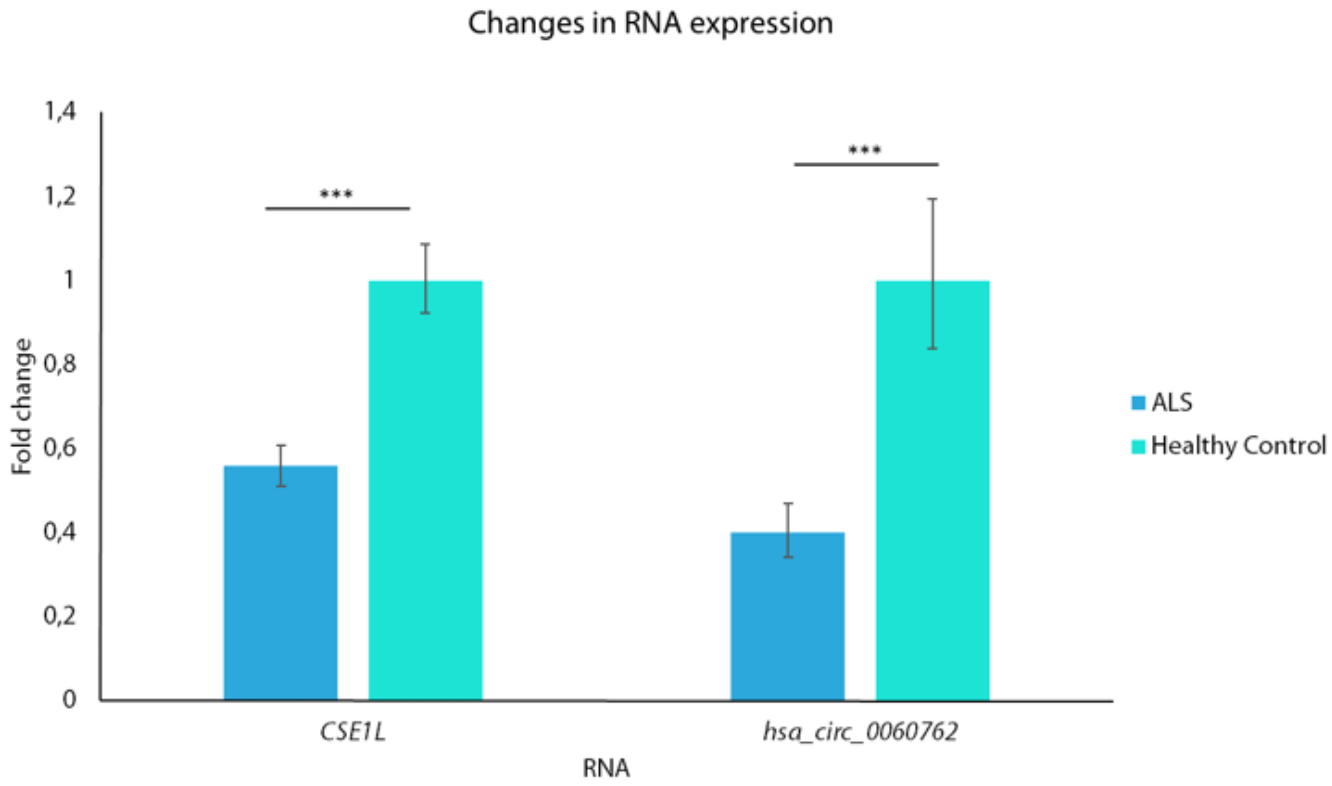
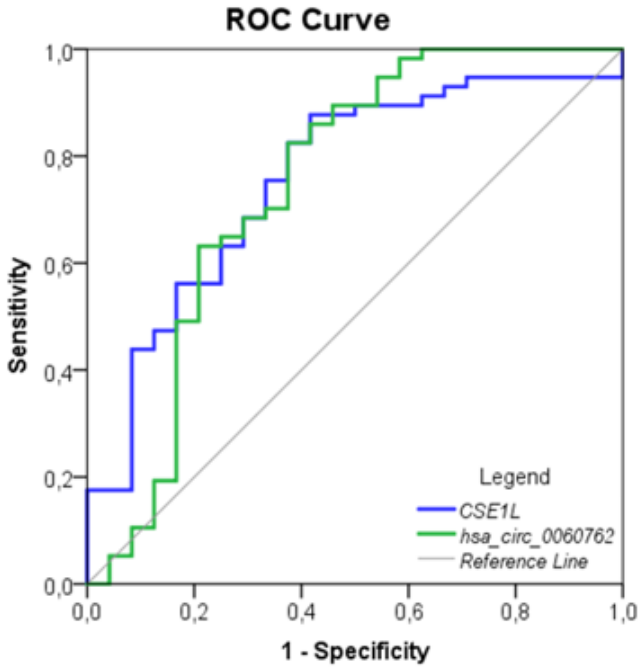


Figure 2

Expression levels of hsa_circ_0060762 and CSE1L

A



B

circRNA	AUC	SE	p-value	95 % confidence interval AUC	Sensitivity[1]	Specificity[1]	d^2
CSE1L	0.758	0.059	< 0,001	0.642 - 0.874	0.825	0.625	0.414
hsa_circ_0060762	0.746	0.070	< 0,001	0.609 - 0.884	0.825	0.625	0.414

[1]Sensitivity and specificity in the optimal cut-off point.

[2]Distance between ideal point (0, 1) and optimal cut-off point.

Figure 3

Diagnostic potential of hsa_circ_0060762 and CSE1L. (A) ROC curves. (B) Detailed information of shown ROC curves