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Original Article

The Role and Underlying Mechanism of Exosomal CA1 in Chemotherapy Resistance in Diffuse Large B Cell Lymphoma

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Chemotherapy resistance plays a major role in treatment failure of diffuse large B cell lymphoma (DLBCL). Exosomes are closely related to tumor drug resistance. Herein, the expression of exosomal proteins in DLBCL and their roles in chemotherapy resistance of DLBCL are explored. Tandem mass tag labeling proteomics was used to perform proteomic profiling in exosomes from DLBCL patients' serum. The expression of carbonic anhydrase 1 (CA1) in parental, chemo-resistant DLBCL cells and DLBCL patient exosomes was detected. Proliferation of DLBCL following CA1 knockdown was investigated both *in vitro* and *in vivo*, along with the effects on nuclear factor κB (NF-κB) and signal transducer and activator of transcription 3 (STAT3) pathways. We identified 54 differentially expressed proteins. We validated that the expression level of exosomal CA1 was higher in chemo-resistant DLBCL cells than in chemo-sensitive counterparts. Knockdown of CA1 inhibited the growth of DLBCL via inhibiting the activation of NF-κB and STAT3 signaling pathways both *in vitro* and *in vivo*. An increased expression level of exosomal CA1 was associated with poorer prognosis, and exosomal CA1 could be used as a biomarker to predict chemotherapeutic efficacy. Our study suggests that exosomal CA1 can promote chemotherapy resistance in DLBCL via the NF-κB and STAT3 pathways, and it can serve as a biomarker for DLBCL prognosis.

INTRODUCTION

Among the most aggressive types of non-Hodgkin's lymphoma (NHL), diffuse large B cell lymphoma (DLBCL) is the most frequently diagnosed. According to the 2016 World Health Organization (WHO) classification, DLBCL is classified based on the cell of origin as germinal center B cell (GCB)-like and activated B cell (ABC)-like subtypes.[1] The standard treatment regimen for DLBCL is chemotherapy, and patient prognosis can be improved by combining chemotherapy with rituximab, a monoclonal antibody of CD20. Despite that more than half of patients can benefit from the combined treatments, up to a third of patients develop relapse/refractory disease caused by chemoresistance.[2,3] Searching for new targets and treatment approaches to overcome chemoresistance in DLBCL is an urgent need.

Exosomes are produced by nearly all cell types, which are small, lipid bilayer vesicles.[4] They are about 40–100 nm in diameter and contain active substances such as proteins, microRNAs (miRNAs), and mRNAs.[5] Exosomes can enhance cell-cell communications by transferring functional substances, such as proteins, RNAs, and DNAs.[6] Previous studies have shown that exosomes are involved in tumor chemotherapy resistance in various ways, such as pumping chemotherapy drugs out of tumor cells[7] and mediating drug resistance by exosomal proteins[8] and miRNAs.[9] For example, exosomal miRNAs or proteins derived from chemo-resistant tumor cells can reduce chemosensitivity of chemo-sensitive tumor cells via bystander effects.[10,11] However, the role of exosomal proteins in DLBCL chemoresistance has not been elucidated.

Carbonic anhydrase 1 (CA1) is a zinc metalloenzyme that belongs to the CA family. It can catalyze the reversible hydration of carbon dioxide to bicarbonate. More and more studies have shown that CA1 is closely related to tumor carcinogenesis; however, it plays different roles in different types of tumors—the expression level of CA1 in liver cancer is low[12] while in breast cancer it is high.[13] However, the role of CA1 in DLBCL has not been well characterized.

In the present study, we identified the proteomic profile of exosomes in serum of patients with DLBCL. Results showed that exosomal CA1 was highly expressed in chemo-resistant DLBCL cells relative to chemo-sensitive counterparts. Knockdown of CA1 not only inhibited the growth of DLBCL both *in vitro* and *in vivo*, but it also inhibited the activation of the nuclear factor κB (NF-κB) and signal transducer and activator of transcription 3 (STAT3) pathways. Moreover, we analyzed the expression level of CA1 in exosomes from DLBCL patient serum and found that

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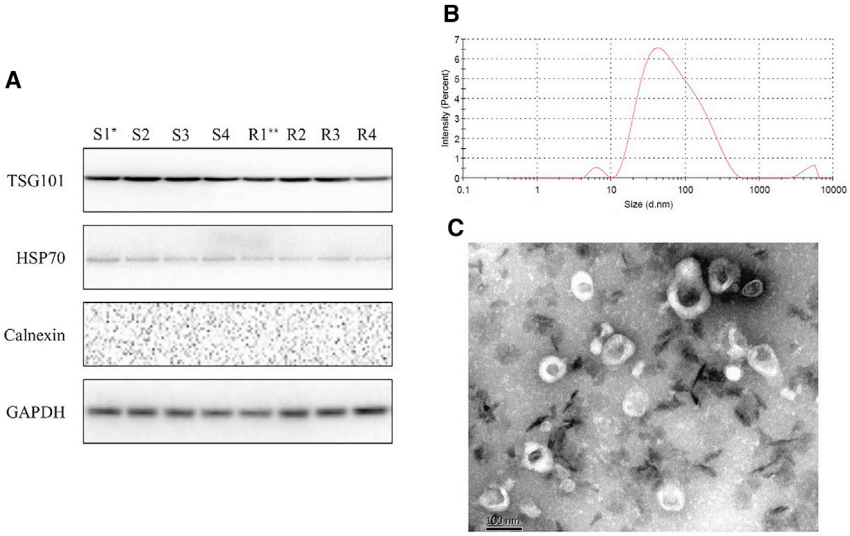

Figure 1. Characterization of Exosomes

(A) Western blot demonstrating the presence of exosomes exhibiting exosome-specific markers TSG101 and HSP70. (B) DLS revealing that the mean size of exosomes was ~70 nm. (C) TEM image of exosomes showing typical cup-shaped morphology. S, chemo-sensitive group; R, chemo-resistant group.

higher expression levels of CA1 were associated with poorer prognosis in the patient. This marker could therefore be used to predict chemotherapeutic efficacy.

RESULTS

Characterization of Exosomes from DLBCL Patient Serum

We successfully isolated exosomes from DLBCL patients' serum, and characterization of exosomes was assessed by western blotting, dynamic light scattering (DLS), and transmission electron microscopy (TEM). Results showed that these isolated exosomes positively expressed the exosome-specific markers tumor susceptibility gene 101 (TSG101) and heat shock protein 70 (HSP70), while they lacked the expression of calnexin (Figure 1A). The mean size of the exosomes was ~70 nm (Figure 1B), and exosomes showed typical cup-shaped morphology (Figure 1C).

Proteomic Profiles of Exosomes from DLBCL Patient Serum

To identify exosomal proteomic profiles from serum of DLBCL patients, we performed tandem mass tag (TMT) labeling quantitative proteomics analysis in chemo-sensitive and chemo-resistant groups. A total of 391 proteins were detected. After comparing the proteomics profiles of the two groups, there were 16 significantly upregulated proteins and 38 significantly downregulated proteins (Figure 2). Fold change ≥ 1.25 (or ≤ 0.8) and p value <0.05 were considered significant, and the top 10 significantly upregulated and downregulated proteins are listed in Table 1.

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Analyses

54 differentially expressed proteins were selected to undergo GO and KEGG pathway analyses. The top 10 most enriched categories in biological process, cellular component, and molecular function are shown in Figure 3. The important signaling pathways are shown in Figure 4.

Protein-Protein Interaction (PPI) Analysis

To analyze the interaction between proteins, we used the STRING database to analyze PPI between 54 differentially expressed proteins. The result showed significantly enriched interactions between differential proteins (Figure 5).

Exosomal CA1 Is Upregulated in DLBCL

According to the proteomics profiling and antibodies available in our experiment, we chose CA1 for further study. Proteomic profiling showed that CA1 was upregulated in the chemo-resistant group. To explore the role of CA1 in DLBCL chemotherapy resistance, we then verified the expression of CA1 in exosomes from DLBCL cell lines. Western blotting showed that the expression level of CA1 in exosomes from SU-DHL-2/R was significantly higher than from SU-DHL-2, and this result was consistent with the proteomics profiling (Figure 6).

Exosomal CA1 Reduces Chemotherapy Sensitivity in DLBCL

We next determined the role of exosomal CA1 in DLBCL chemotherapy resistance. CA1 was knocked down in SU-DHL-2/R cells by small interfering RNA (siRNA) transfection. Western blotting showed that the expression level of CA1 in exosomes obtained from transfected SU-DHL-2/R cells was lower than in the negative control group (Figure 7A). Forty-eight hours after treatment with differing concentrations of R-CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone, combined with rituximab; ranging from 320 to 40,960 ng/mL), we detected the cell viability by MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium). As shown in Figures 7B and 7C, CA1 knockdown increased the drug sensitivity of SU-DHL-2/R cells, and drug sensitivity increased over time.

Exosomal CA1 Mediates Chemotherapy Resistance in DLBCL via NF-κB and STAT3 Signaling Pathways

In DLBCL, the NF-κB signaling pathway is constitutively activated, and in turn it activates the transcription and translation of downstream target genes interleukin 6 (IL-6) and IL-10. High expression of IL-6 and IL-10 activates the STAT3 signaling pathway, and activated STAT3 binds to the NF-κB subunit p65, further activating the NF-κB signaling pathway, thus forming a positive feedback loop between NF-κB and STAT3. These positive feedback pathways synergistically promote DLBCL tumor growth.[14]

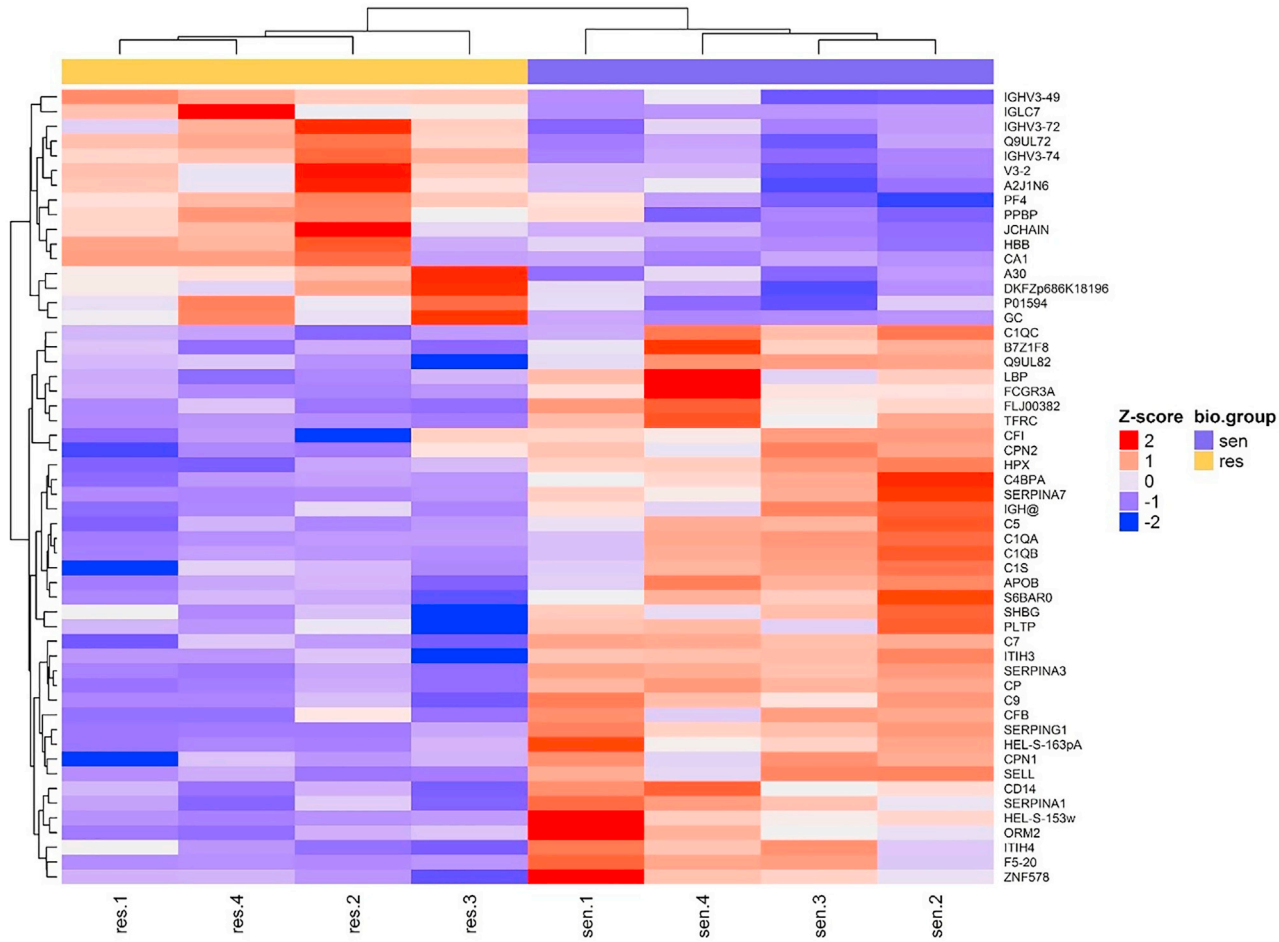

Figure 2. Heatmap Showing Significantly Upregulated and Downregulated Proteins in the Chemo-Sensitive and Chemo-Resistant Groups

To investigate whether exosomal CA1 mediates DLBCL chemotherapy resistance via the NF-κB and STAT3 signaling pathways, we determined the expression levels of NF-κB, STAT3, phosphorylated (p-)NF-κB and p-STAT3 in CA1 knockdown and negative control (NC)-transfected SU-DHL-2/R cells. After treatment with R-CHOP, the expression levels of p-NF-κB/p65 and p-STAT3/Tyr705 in the nuclei of CA1 knockdown SU-DHL-2/R cells were significantly lower than those of the NC group (Figure 8). These results suggest that exosomal CA1 mediates chemotherapy resistance of DLBCL via promotion of the NF-κB and STAT3 signaling pathways.

Exosomal CA1 Reduces Chemotherapy Sensitivity of DLBCL via NF-κB and STAT3 Signaling Pathways *In Vivo*

To determine the effects of exosomal CA1 on the chemosensitivity of DLBCL, we conducted xenograft tumor models in BALB/c nude mice. SU-DHL-2 cells pre-treated with either SU-DHL-2/R-derived exosomes (S2R group), exosomes from NC-siRNA-transfected SU-DHL-2/R (NC-siRNA group), or exosomes from CA1-siRNA-transfected SU-DHL-2/R (CA1-siRNA group) were subcutaneously implanted into nude mice. After 4 weeks of R-CHOP treatment, the tumor weight and volume of the CA1-siRNA group were significantly lower than in the S2R and NC-siRNA groups (Figure 9), indicating that exosomal CA1 reduces chemotherapy sensitivity of DLBCL *in vivo*.

The expression levels of NF-κB and STAT3 in tumor tissues from the CA1-siRNA group were significantly lower than in the S2R and NC-siRNA groups, as determined by immunohistochemistry (IHC) (Figure 10), suggesting that exosomal CA1 may also mediate DLBCL chemoresistance via the NF-κB and STAT3 signaling pathways *in vivo*.

Expression Level of Exosomal CA1 and Its Correlation with Clinicopathological Features in DLBCL Patients

112 DLBCL patients' serum exosomal CA1 levels were detected. According to treatment efficacy, patients were divided into two groups, that is, chemo-sensitive (n = 81) and chemo-resistant (n = 31) groups. It was observed that expression of exosomal CA1 was significantly higher in the chemo-resistant group than in the chemo-sensitive group ($p < 0.001$) (Figure 11A). The expression level of exosomal CA1 was closely associated with the international prognostic index

Table 1. Top 10 Upregulated and Downregulated Proteins

Gene Names	Protein Names	Fold Change	p Value	Description
IGLC7	Ig lambda-7 chain C region	2.496846826	0.041493134	up
HBB	hemoglobin subunit β	2.49600317	0.040489478	up
DKFZp686K18196	DKFZp686K18196	2.350629045	0.036575988	up
CA1	carbonic anhydrase 1	2.160436713	0.04937307	up
V3-2	Ig lambda chain V region 4A	1.742769771	0.017147221	up
IGHV3-74	IGHV3-74	1.665649819	0.001030012	up
JCHAIN	immunoglobulin J chain	1.600505736	0.028608701	up
Q9UL72	Q9UL72	1.59782709	0.000537667	up
GC	GC	1.52163537	0.035582196	up
P01594	Ig kappa chain V-I region AU	1.500488393	0.036537648	up
F5-20	F5-20	0.313401094	0.022315043	down
ORM1	alpha-1-acid glycoprotein	0.452444203	0.025821935	down
IGHD	Ig delta chain C region	0.496599067	0.004960248	down
SERPINA3	alpha-1-antichymotrypsin	0.513617092	3.01E-05	down
TFRC	transferrin receptor protein 1	0.562102074	0.009907065	down
C1QB	complement C1q subcomponent subunit B	0.571194716	0.027755915	down
C1QA	complement C1q subcomponent subunit A	0.577189301	0.02406817	down
SERPINA7	thyroxine-binding globulin	0.596461869	0.012866339	down
S6BAR0	S6BAR0	0.603603997	0.008289204	down
FCGR3A	low affinity immunoglobulin gamma Fc region receptor III-A	0.605685414	0.036492218	down

(IPI) score ($p = 0.004$), but further examination showed that this was not the case for the sex, age, subtype, B symptoms (unexplained weight loss, fever, night sweats), lactate dehydrogenase (LDH), or Ann Arbor stage of the patients ($p > 0.05$) (Table 2).

The Prognostic Prediction Value of Exosomal CA1 and Its Potential to Assess Treatment Efficacy in DLBCL Patients

Further analyses were carried out to assess the prognostic prediction potential of exosomal CA1 and IPI scores for treatment efficacy. Receiver operating characteristic (ROC) curve analysis results showed that the area under the curve (AUC) values of CA1 and IPI scores were 0.8122 ($p < 0.001$, cutoff = 1.085) and 0.6386 ($p < 0.05$), respectively. The AUC value for CA1 combined with the IPI score was 0.8212 ($p < 0.001$) (Figure 11B). A combination of the two tests provided a superior prediction performance than did either of the two tests applied alone.

The median progression-free survival (PFS) time of these 112 DLBCL patients was 28.5 months. Compared to the rest of clinical characteristics of subjects, the Ann Arbor stage strongly predicted PFS (Table 3). We used the median relative expression value for CA1 to stratify the patients into high CA1 (≥ 0.81) or low CA1 (<0.81) groups and found that high expression levels of CA1 correlated with poor prognosis of DLBCL patients ($p < 0.001$) (Figure 11C).

DISCUSSION

Our study presents the proteomic profiling of exosomes from the serum of DLBCL patients for the first time. Furthermore, we have confirmed that exosomal CA1 was upregulated in chemo-resistant DLBCL cells compared with their chemo-sensitive counterparts. Exosomal CA1 not only enhanced the growth of DLBCL both *in vitro* and *in vivo*, but it also enhanced activation of the NF-κB and STAT3 signaling pathways. Moreover, we found that higher expression levels of CA1 were associated with poorer prognosis. In addition, exosomal CA1 can be used as a predictor for chemotherapeutic efficacy.

DLBCL is the most commonly seen NHL, despite the emergence of increasing numbers of treatment regimens, and treatment remains ineffective in some patients due to chemotherapy resistance. Exosomes are vesicles that can be secreted by almost all types of cells, including tumor cells. Exosomes contain various substances such as proteins, miRNAs, and DNAs, and they facilitate tumor growth and metastasis via transport of these functional substances.[15] In the chemoresistance research field, more and more studies are focusing on exosomes. Importantly, exosomes can mediate chemoresistance via transporting functional proteins.[16] Exosomal proteins are carried in body fluids and their stability is not affected, making them ideal biomarkers for predicting the performance of chemotherapies and the risk of developing chemoresistance.[17]

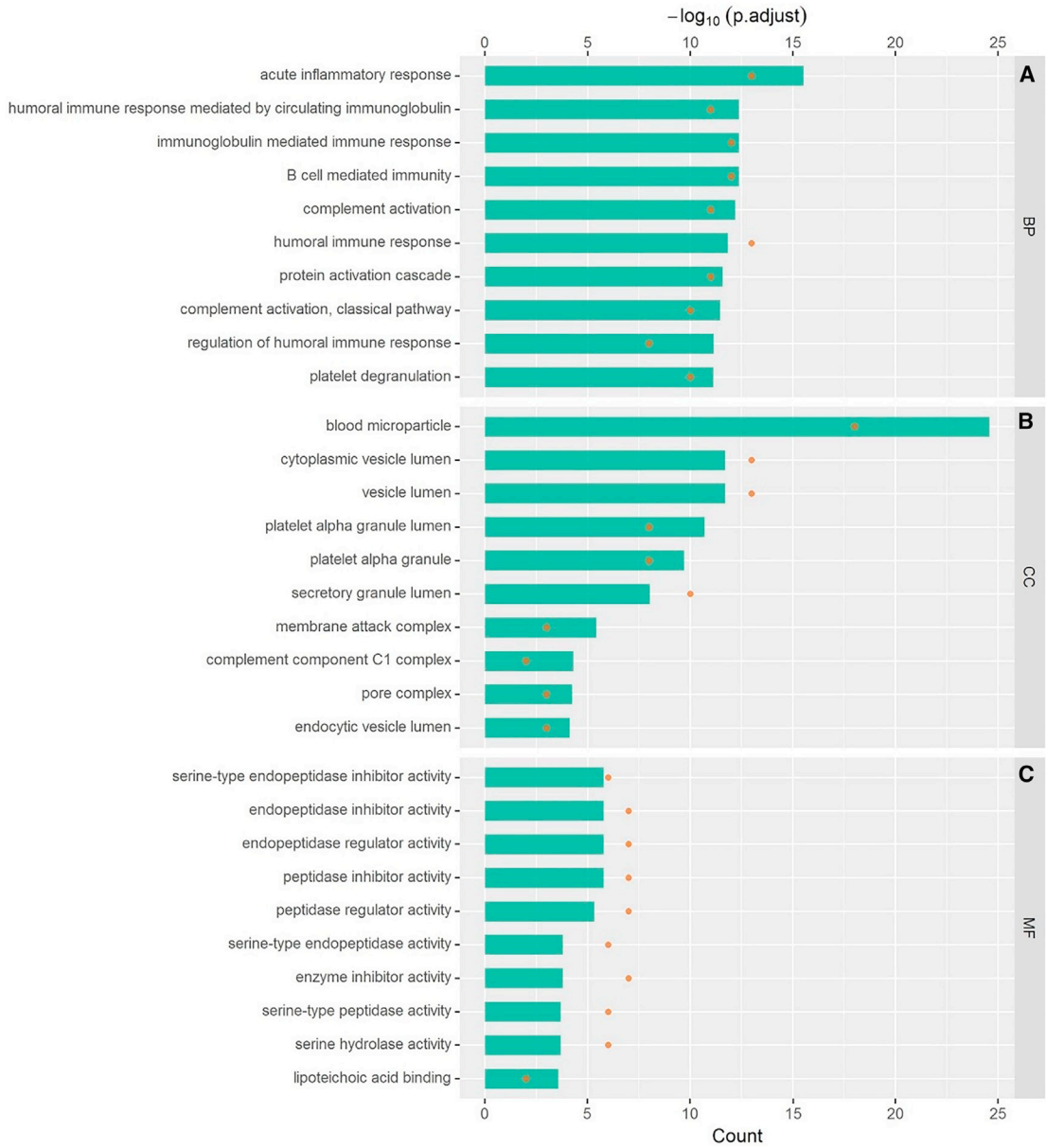

Figure 3. GO Classification of 54 Differentially Expressed Proteins

(A–C) The 10 most enriched categories and the enrichment scores ($-\log_{10}$(p value), $p < 0.05$) in biological process (A), cellular component (B), and molecular function (C) are shown.

In this study, 391 proteins were identified, of which 16 were upregulated proteins and 38 were downregulated proteins in exosomes isolated from DLBCL patient serum. Of the 54 differentially expressed proteins, CA1 was one of the 16 upregulated proteins, while alpha-1 acid glycoprotein (ORM1) was one of the 38 downregulated proteins. CA1 belongs to the CA family and was found to be highly expressed in breast cancer[13] and pancreatic cancer,[18] with a close association with chemoresistance. ORM1 was found highly expressed in cisplatin-resistant ovarian cancer.[19] In KEGG pathway analysis, we found the ferroptosis pathway and other pathways that regulate cancer resistance. In acute myeloid leukemia (AML), inducing ferroptosis by the ferroptosis activator erastin could significantly increase chemotherapy sensitivity of AML cells to cytarabine and doxorubicin.[20] Consistent with this, the results of our proteomic profiling revealed exosomal proteins related to chemoresistance in DLBCL.

Among the 54 differentially expressed proteins, we selected CA1 for validation in DLBCL cell lines. The results showed that the expression level of CA1 in exosomes from SU-SHL-2/R was significantly higher than those from SU-DHL-2, which was consistent with the proteomic

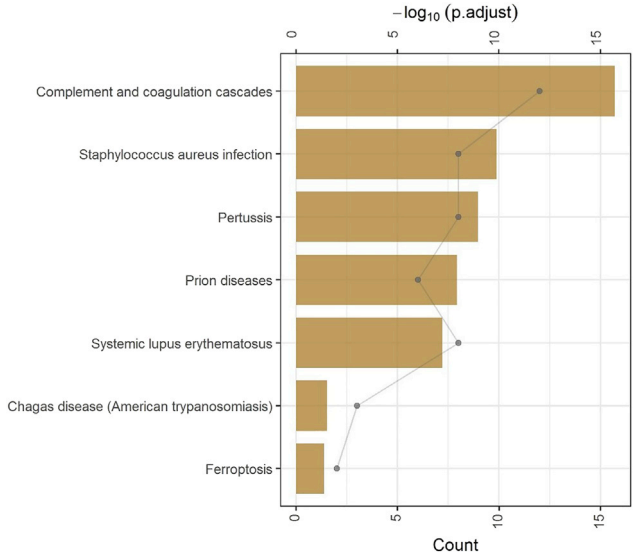

Figure 4. KEGG Analysis of 54 Differentially Expressed Proteins

profiling. A previous study has shown that when interacting with nearby cells, exosomes secreted from chemo-resistant tumor cells can induce chemoresistance in chemo-sensitive tumor cells via bystander effects, and ionizing radiation-treated breast cancer cells could affect the untreated cells by releasing exosomes.[21] Our study showed that CA1 was highly expressed in chemo-resistant cells and was secreted via exosomes, and DLBCL cells secreted exosomes containing CA1 to induce chemoresistance. In a cohort study, we found that the expression level of CA1 was higher in exosomes from R-CHOP chemo-resistant patients compared with chemo-sensitive patients, and exosomal CA1 predicted prognosis and chemotherapeutic efficacy in DLBCL patients. Increasing numbers of reports have shown that CA1 is closely related to tumor carcinogenesis. CA1 has not previously been studied with regard to chemotherapy resistance, but there are many studies on CA1 in cancer. It has been reported that the expression level of CA1 in oral squamous cell carcinoma was higher than that in normal healthy people, and the expression level was closely related to tumor stage and size.[22] In another study, the combined detection of CA1 and prostate-specific antigen (PSA) significantly improved diagnostic sensitivity in prostate cancer

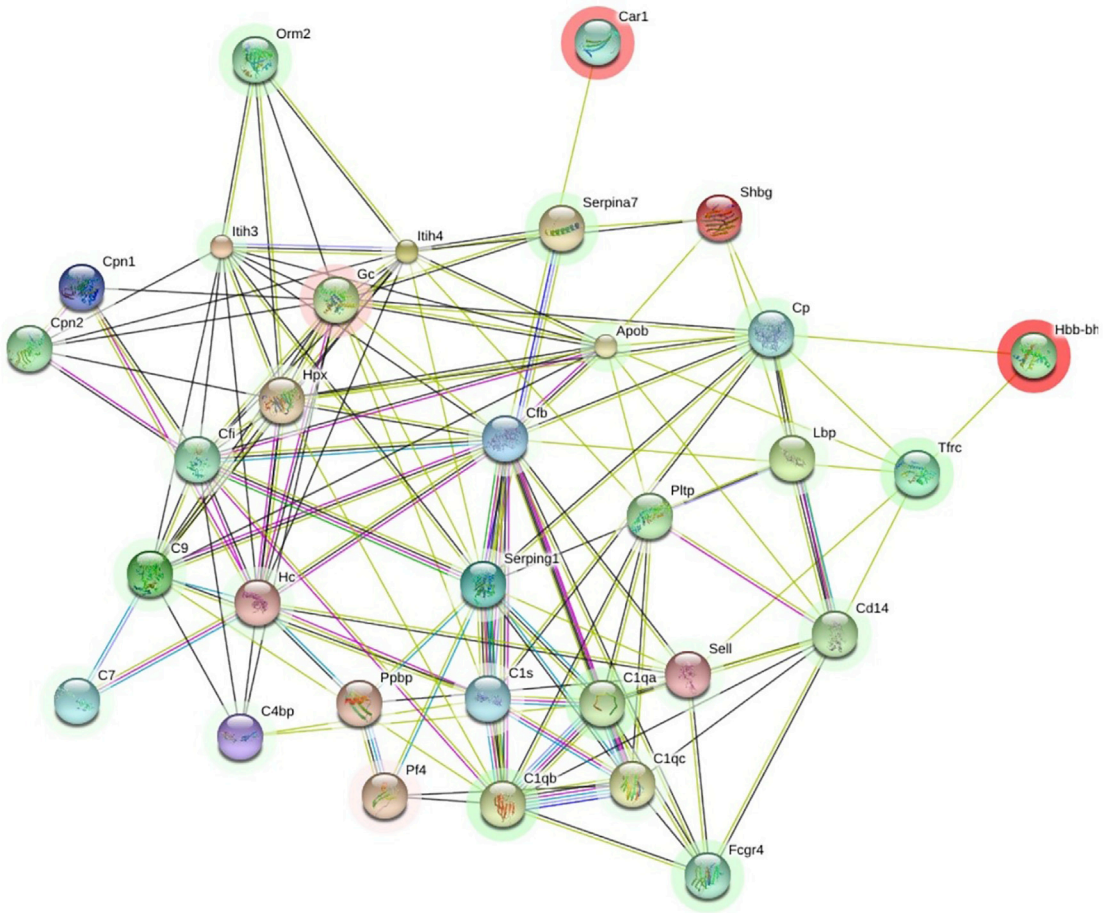

Figure 5. PPI Analysis
Red indicates upregulated proteins; green indicates downregulated proteins.

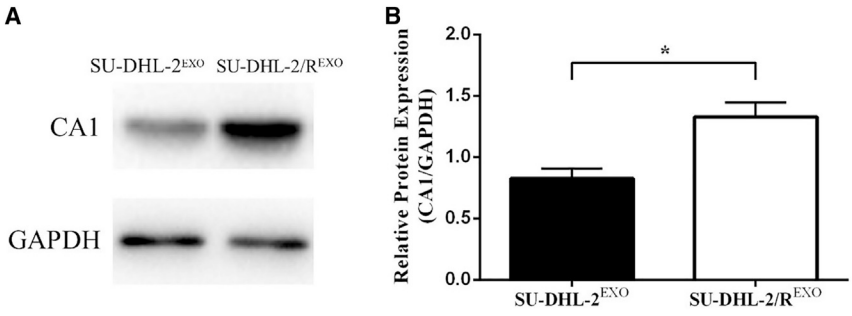

Figure 6. Western Blot Validation of CA1 in exosomes from SU-DHL-2/R Cells and SU-DHL-2 Cells

(A) Expression of CA1 in exosomes from SU-DHL-2/R Cells and SU-DHL-2 Cells. (B) The relative protein expression of CA1.

The graphs are displayed as the mean ± SEM (t test, n = 3). *p < 0.05.

compared with detection of PSA alone.[23] These results were consistent with ours, indicating that CA1 can serve as a predictor for chemoresistance and acts as an inferior prognosis factor for DLBCL patients.

In the present study, we also found that exosomal CA1 reduced chemosensitivity of DLBCL both *in vitro* and *in vivo*, and that knockdown of CA1 could enhance chemosensitivity in DLBCL. While the role of CA1 in chemotherapy resistance has not been previously documented, CA12, which belongs to the same family as CA1, has been reported to be involved in chemoresistance in colorectal cancer. Proteomics profiling of P-glycoprotein (P-gp) high-expressed colorectal cancer cells showed that the expression level of CA12 was significantly increased, and CA12 could enhance the activity of P-gp, indicating that together they co-mediated chemotherapy resistance in colorectal cancer.[24] Consistent with this, our study may provide a new direction for research into chemotherapy resistance in DLBCL.

Our study showed that exosomal CA1 mediates chemotherapy resistance in DLBCL via the NF-κB and STAT3 signaling pathways *in vitro* and *in vivo*. Many signaling pathways are involved in the development of DLBCL disease, and they play various roles in pathogenesis. NF-κB is a transcription factor complex, and the NF-κB signaling pathway is one of the most important pathways in DLBCL, especially in refractory/relapse and ABC subtypes. NF-κB can be activated by canonical and non-canonical pathways, with both being involved in the pathogenesis of DLBCL.[25] STAT3 belongs to the STAT family and plays vital roles in cell proliferation and apoptosis. Activation of STAT3 is predictive of poor prognosis in DLBCL.[26] The NF-κB and STAT3 pathways can form positive feedback pathways and synergistically promote DLBCL tumor growth.[14] A previous study showed that the expression of NF-κB was related to poor prognosis in DLBCL, and knockdown of NF-κB inhibited the proliferation of DLBCL cells.[27] In another study, p-STAT3 was highly expressed in DLBCL cells, and treatment of DLBCL cells with a STAT3 inhibitor could increase the chemosensitivity of DLBCL cells.[3] These results are consistent with our findings, indicating that exosomal CA1 may mediate chemotherapy resistance in DLBCL via the NF-κB and STAT3 pathways.

In summary, we reported specific exosome-derived protein profiles of DLBCL patients. Exosomal CA1 can promote chemoresistance in DLBCL via the NF-κB and STAT3 signaling pathways, and it can serve as a biomarker for DLBCL prognosis.

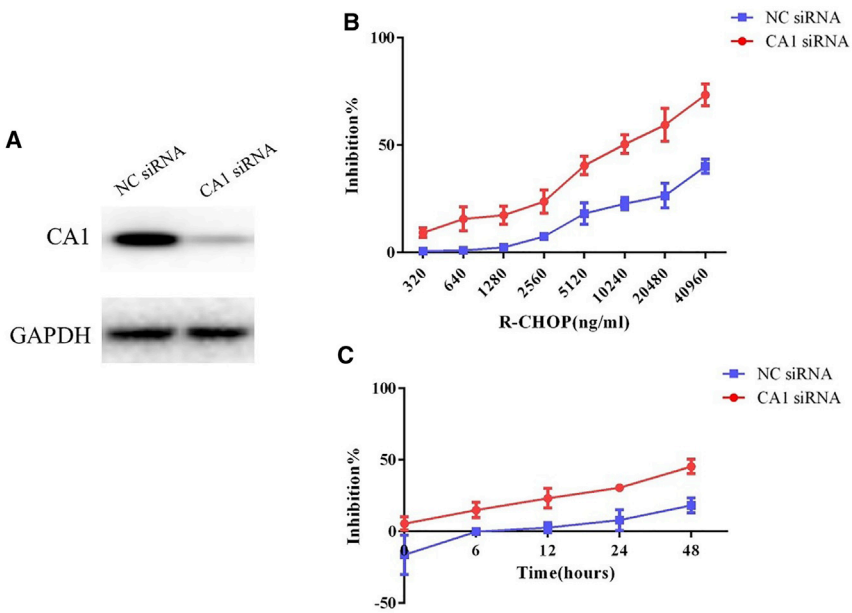

Figure 7. Exosomal CA1 Reduces Chemotherapy Sensitivity of DLBCL

(A) Expression level of CA1 in exosomes obtained from transfected SU-DHL-2/R cells and the NC group. (B) CA1 knockdown increased the drug sensitivity of SU-DHL-2/R cells. (C) Drug sensitivity increased over time.

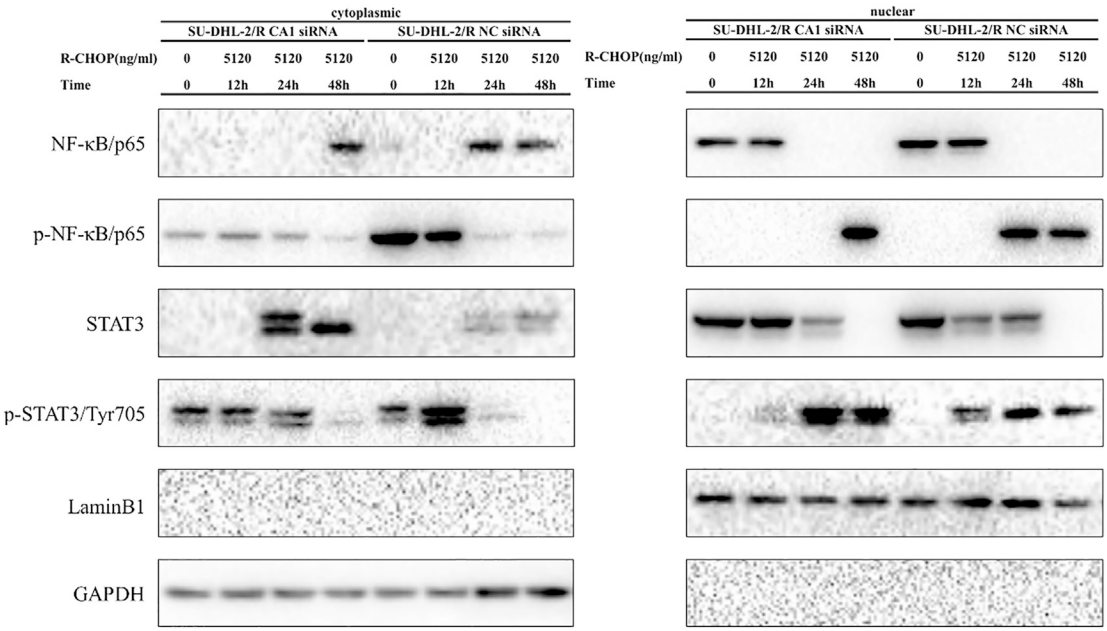

Figure 8. Exosomal CA1 Mediates Chemotherapy Resistance in DLBCL via NF-κB and STAT3 Signaling Pathways

MATERIALS AND METHODS

Patient Samples

Between June 2015 and August 2016, we collected DLBCL patients' serum samples (n = 112; average amount, 250 μL) from Xiangya Hospital, Central South University, and samples were stored at −80°C until used. All subjects had *de novo* DLBCL and received no cancer-related treatments before diagnosis. We collected 112 patients' clinical information, including sex, age, subtype (defined by cell of origin classification, using IHC, according to the Hans algorithm), IPI score, B symptoms, LDH level (elevated was defined as >245 U/L), Ann Arbor stage, and treatment efficacy. All patients' clinical details and follow-up data were completed. We evaluated efficacies after four cycles of R-CHOP regimen chemotherapy by RECIST standard 1.1. According to the chemotherapy efficacies, we divided 112 DLBCL patients into two groups as follows: chemo-sensitive (assessment of efficacy in terms of complete response or partial response, n = 81) and chemo-resistant (assessment of efficacy in terms of progression disease, n = 31) groups. The harvesting of tissues for this study conformed with the national and institutional ethical guidelines by the Committee on Use of Human Samples for Research and with those of Helsinki Declaration of 1975,

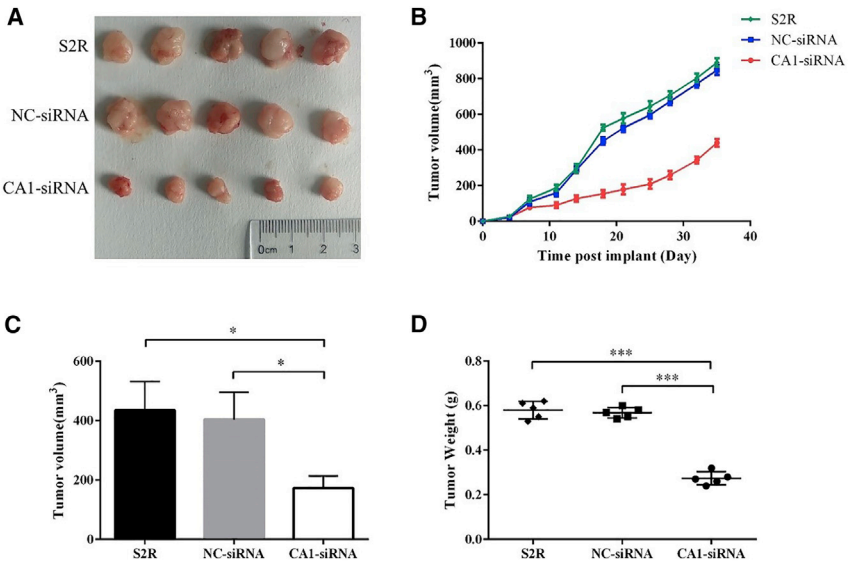

Figure 9. Exosomal CA1 Reduces Chemotherapy Sensitivity in DLBCL *In Vivo*

(A) The effects of exosomal CA1 on tumor size. (B) Growth curves of tumors in S2R, NC-siRNA, and CA1-siRNA groups. (C) Comparison of tumor volumes of S2R, NC-siRNA, and CA1-siRNA groups (t test). (D) Comparison of tumor weights of S2R, NC-siRNA, and CA1-siRNA groups (t test). *$p < 0.05$, ***$p < 0.001$.

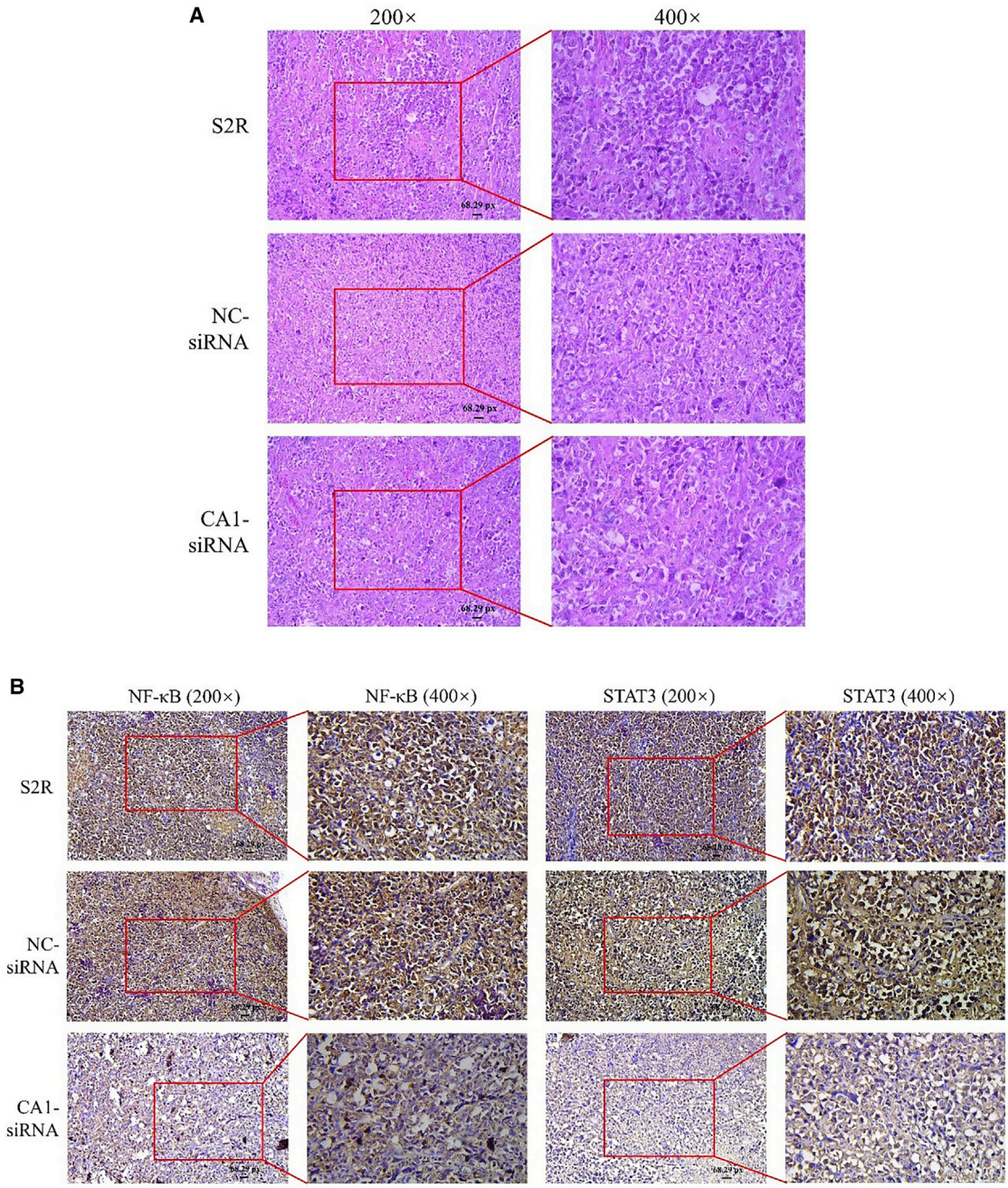

Figure 10. Histopathological Features of Tumor Tissues in Nude Mice and Expression Levels of NF-κB and STAT3 in Tumor Tissues of Nude Mice
(A) Hematoxylin and eosin staining of tumor tissues. (B) Expression levels of NF-κB and STAT3 in tumor tissues from the CA1-siRNA, S2R, and NC-siRNA groups.

updated in 2013. The clinical study was approved by the Ethics Committee of Xiangya Hospital of Central South University (no. 201603172). Follow-up was conducted for all subjects from the day of diagnosis to October 2018, and all cases were successfully followed up.

Among the 112 DLBCL patients, serum from 8 DLBCL patients (4 in each of the chemo-sensitive and chemo-resistant groups) was used to perform exosomal proteomic profiling. Exosomal CA1 expression was detected in the serum of all 112 patients.

Cell Lines

The SU-DHL-2 cells (a human DLBCL cell line) were purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA). This cell line was cultured in RPMI 1640 medium,

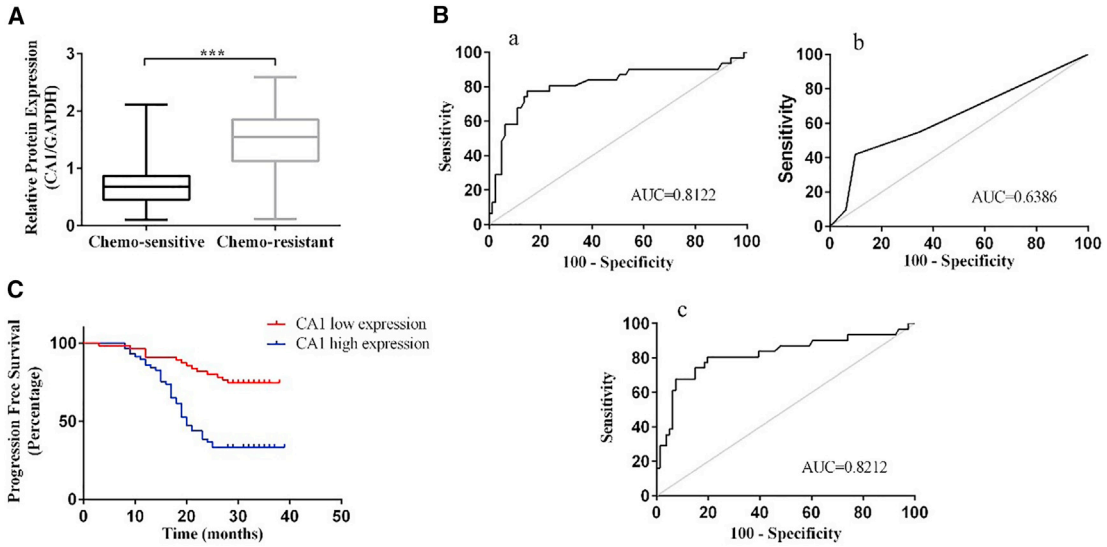

Figure 11. Expression of Serum Exosomal CA1 in the Chemo-Sensitive and Chemo-Resistant DLBCL Groups, and the Value of Exosomal CA1 in Predicting Treatment Efficacy and Prognosis for DLBCL Patients

(A) Expression of serum exosomal CA1 in chemo-sensitive and chemo-resistant DLBCL groups (t test). (B) ROC curves: (a) CA1 ROC curve; (b) IPI score ROC curve; and (c) CA1+IPI score ROC curve. (C) Kaplan-Meier curves of DLBCL patients according to serum levels of exosomal CA1 ($p < 0.001$). ***$p < 0.001$.

which was enriched with 100 U/mL penicillin, 100 mg/mL streptomycin, and 10% fetal bovine serum (FBS) in an incubator with 5% CO_2 at 37°C. SU-DHL-2 cells were repeatedly exposed to R-CHOP, as we have previously described to isolate R-CHOP-resistant DLBCL cells.[28] R-CHOP-resistant DLBCL cells were termed SU-DHL-2/R.

Isolation and Identification of Exosomes

After culturing in FBS-free media for 48 h (to eliminate interference from FBS-secreted exosomes), an ExoQuick-TC kit (EXOTC50A-1, EXOQ20A-1, System Biosciences, Palo Alto, CA, USA) was used to extract exosomes following the protocol provided by the manufacturer. This was followed by characterization of the isolated exosomes using the following biomarkers: HSP70 (Abcam, Cambridge, UK, ab133586, 1:2,000), TSG101 (Abcam, Cambridge, UK, ab133586, 1:1,000), and calnexin (Abcam, Cambridge, UK, ab22595, 1:1,000). The shapes and sizes of exosomes were analyzed using TEM (FEI, Hillsboro, OR, USA) and DLS (Malvern Instruments, Worcestershire, UK), respectively.

Western Blotting

Protein quantification was performed by using the bicinchoninic acid (BCA) method according to the manufacturer's instructions. 30 μg of protein samples was resolved on a 10% SDS-PAGE gel followed by electro-transfer to a polyvinylidene fluoride (PVDF) membrane. The membrane was blocked using 5% fat-free milk and then treated with primary antibodies at 4°C for 12 h. Horseradish peroxidase-conjugated secondary antibody (Sigma-Aldrich) was then applied and the blots were developed with enhanced chemiluminescence reagents.

TMT Labeling Quantitative Proteomics Analysis

Total proteins were extracted from exosomes of eight DLBCL patients' serum. An equal amount of protein from each sample was subjected to trypsin digestion to obtain polypeptide samples. Eight samples were labeled with different TMT labels and then mixed into a single sample, which was subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. The LC-MS/MS proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the iProX partner repository with the dataset identifier PXD019780.[29] In order to compare the differentially expressed protein profiles between two groups, we calculated and used fold change and p value to identify significant differentially expressed proteins. Differentially expressed proteins were further characterized with GO, KEGG pathway, and PPI analyses.

Cell Line Chemosensitivity Assay

The MTS-based CellTiter 96 AQueous One solution cell proliferation assay (Promega, Madison, WI, USA, G3580) was used to assess the chemosensitivity of cells. Briefly, tumor cells were treated with 20 μL of MTS solution in 96-well plates for 4 h. This was performed for cells at a density of 4.44×10^4 cells/well. The absorbance results were read at 490 nm and normalized to the absorbance of a blank (filled with MTS reagent only). 50% inhibitory concentration (IC_{50}) was determined with probit regression.

siRNA Transfection

The NC siRNA and specific siRNA were constructed by GenePharma (Shanghai, China). CA1-targeted siRNA (sense, 5′-GCCUCAAAGG CUGAUGGUUTT-3′; antisense, 5′-AACCAUCAGCCUUUGAGG

Table 2. Association between Exosomal CA1 Expression Level and Clinicopathological Features of DLBCL Patients

Characteristics	No.	Low	High	p Value
Sex				>0.05
Male	66	30	36	
Female	46	25	21	
Age (years)				>0.05
<60	71	34	37	
≥60	41	21	20	
Subtype				>0.05
ABC	59	27	32	
GCB	53	28	25	
B symptoms				>0.05
Absent	96	46	50	
Present	16	9	7	
LDH				>0.05
Normal	74	34	40	
Elevated	38	21	17	
Ann Arbor stage				>0.05
I+II	58	26	32	
III+IV	54	29	25	
IPI score				0.004
Low-risk group	67	39	28	
Low- to medium-risk group	24	13	11	
High- to medium-risk group	13	1	12	
High-risk group	8	2	6	

ABC, activated B cell subtype; DLBCL, diffuse large B cell lymphoma; GCB, germinal center B cell subtype; IPI, international prognostic index; LDH, lactate dehydrogenase.

Table 3. Association between PFS and Clinicopathological Features of DLBCL Patients

Characteristics	PFS (Months)	p Value
Sex		>0.05
Male	not reached	
Female	25	
Age (years)		>0.05
<60	not reached	
≥60	not reached	
Subtype		>0.05
ABC	28	
GCB	not reached	
B symptoms		>0.05
Absent	not reached	
Present	not reached	
LDH		>0.05
Normal	not reached	
Elevated	24	
Ann Arbor stage		0.031
I+II	23	
III+IV	not reached	
IPI score		>0.05
Low-risk group	not reached	
Low- to medium-risk group	not reached	
High- to medium-risk group	23	
High-risk group	16	

ABC, activated B cell subtype; DLCBL, diffuse large B cell lymphoma; GCB, germinal center B cell; IPI, international prognostic index; LDH, lactate dehydrogenase; PFS, progression-free survival.

CTT-3′) was chosen from four individual siRNAs. The siRNAs were transfected into cells using DharmaFECT transfection reagent (Dharmacon, USA) following the protocols given by the manufacturer.

Animal Model

Four-week-old female BALB/c nude mice were obtained from the SJA Laboratory Animal Company (Hunan, China). They were randomly divided into three groups (n = 5) according to the different types of cells used in formation of subcutaneous tumors as follows: SU-DHL-2 cells pre-treated with 50 μg of SU-DHL-2/R-derived exosomes for 48 h (S2R group); SU-DHL-2 cells pretreated with 50 μg of exosomes from NC-siRNA-transfected SU-DHL-2/R for 48 h (NC-siRNA group); and SU-DHL-2 cells pretreated with 50 μg of exosomes from CA1-siRNA transfected SU-DHL-2/R for 48 h (CA1-siRNA group). All mice were injected with R-CHOP (rituximab at 10 mg/kg, cyclophosphamide at 40 mg/kg, doxorubicin at 1.6 mg/kg, vincristine at 0.5 mg/kg, and prednisone at 0.2 mg/kg) regimen chemotherapy once a week, for a total of four treatments. The volume of the tumors was determined as (length × width2)/2, whereas the size was assessed by sliding calipers. At 4 weeks after injection, the tumor specimen was obtained and then fixed in 10% formalin overnight or stored at −80°C. The animal model study was approved by the Ethics Committee of Xiangya Hospital of Central South University (no. 201603173).

IHC

The expression levels of NF-κB and STAT3 of subcutaneous tumor tissues from BALB/c nude mice were detected by IHC using the streptavidin-peroxidase method according to the manufacturer's instructions. Tissues were incubated with primary antibodies, including anti-NF-κB rabbit antibody (working dilution of 1:300; #8242, CST, Beverly, MA, USA) and anti-STAT3 mouse antibody (working dilution of 1:300; #9139, CST, Beverly, MA, USA). The results of the staining were assessed in five randomly selected slices at ×400 magnification, and these slides were evaluated in double-blind fashion by two experienced pathologists. Expressions of NF-κB and STAT3 were evaluated by a semiquantitative immunoreactivity score.[30]

Statistical Analysis

All data are shown as mean ± SEM and were processed and analyzed with GraphPad Prism 6 and SPSS 17.0 software. p values <0.05 were

considered significant. A Student's t test was used when comparing two groups of quantitative data, whereas a one-way ANOVA was used when comparing three or more groups. Categorical data were assessed with the chi-square test. Kaplan-Meier curves were plotted to analyze the survival rates. The AUC values were calculated by GraphPad Prism 6 software to assess the therapy efficacies.

AUTHOR CONTRIBUTIONS

H.Z. designed the research study. Y.F. performed the research and drafted the manuscript. M.Z., Y.T., and X.L. participated in the literature search. Y.L. analyzed the data. L.W. collected the clinical data. All authors read and approved the final version of the manuscript.

CONFLICTS OF INTEREST

The authors declare no competing interests.

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REFERENCES

1. Swerdlow, S.H., Campo, E., Pileri, S.A., Harris, N.L., Stein, H., Siebert, R., Advani, R., Ghielmini, M., Salles, G.A., Zelenetz, A.D., and Jaffe, E.S. (2016). The 2016 revision of the World Health Organization classification of lymphoid neoplasms. Blood *127*, 2375–2390.
2. Roschewski, M., Staudt, L.M., and Wilson, W.H. (2014). Diffuse large B-cell lymphoma—treatment approaches in the molecular era. Nat. Rev. Clin. Oncol. *11*, 12–23.
3. Jiang, J., Liu, Y., Tang, Y., Li, L., Zeng, R., Zeng, S., and Zhong, M. (2016). ALDH1A1 induces resistance to CHOP in diffuse large B-cell lymphoma through activation of the JAK2/STAT3 pathway. OncoTargets Ther. *9*, 5349–5360.
4. Zhang, X., Yuan, X., Shi, H., Wu, L., Qian, H., and Xu, W. (2015). Exosomes in cancer: small particle, big player. J. Hematol. Oncol. *8*, 83.
5. Isola, A.L., and Chen, S. (2017). Exosomes: the messengers of health and disease. Curr. Neuropharmacol. *15*, 157–165.
6. Colombo, M., Raposo, G., and Théry, C. (2014). Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. Annu. Rev. Cell Dev. Biol. *30*, 255–289.
7. Koch, R., Aung, T., Vogel, D., Chapuy, B., Wenzel, D., Becker, S., Sinzig, U., Venkataramani, V., von Mach, T., Jacob, R., et al. (2016). Nuclear trapping through inhibition of exosomal export by indomethacin increases cytostatic efficacy of doxorubicin and pixantrone. Clin. Cancer Res. *22*, 395–404.
8. Kawakami, K., Fujita, Y., Kato, T., Mizutani, K., Kameyama, K., Tsumoto, H., Miura, Y., Deguchi, T., and Ito, M. (2015). Integrin β4 and vinculin contained in exosomes are potential markers for progression of prostate cancer associated with taxane-resistance. Int. J. Oncol. *47*, 384–390.
9. Yang, Y.C., Liu, G.J., Yuan, D.F., Li, C.Q., Xue, M., and Chen, L.J. (2019). Influence of exosome-derived miR-21 on chemotherapy resistance of esophageal cancer. Eur. Rev. Med. Pharmacol. Sci. *23*, 1513–1519.
10. Nawaz, M., Fatima, F., Nazarenko, I., Ekström, K., Murtaza, I., Anees, M., Sultan, A., Neder, L., Camussi, G., Valadi, H., et al. (2016). Extracellular vesicles in ovarian cancer: applications to tumor biology, immunotherapy and biomarker discovery. Expert Rev. Proteomics *13*, 395–409.
11. Fatima, F., and Nawaz, M. (2017). Vesiculated long non-coding RNAs: offshore packages deciphering trans-regulation between cells, cancer progression and resistance to therapies. Noncoding RNA *3*, 10.
12. Kuo, W.H., Chiang, W.L., Yang, S.F., Yeh, K.T., Yeh, C.M., Hsieh, Y.S., and Chu, S.C. (2003). The differential expression of cytosolic carbonic anhydrase in human hepatocellular carcinoma. Life Sci. *73*, 2211–2223.
13. Thongwatchara, P., Promwikorn, W., Srisomsap, C., Chokchaichamnankit, D., Boonyaphiphat, P., and Thongsuksai, P. (2011). Differential protein expression in primary breast cancer and matched axillary node metastasis. Oncol. Rep. *26*, 185–191.
14. Lam, L.T., Wright, G., Davis, R.E., Lenz, G., Farinha, P., Dang, L., Chan, J.W., Rosenwald, A., Gascoyne, R.D., and Staudt, L.M. (2008). Cooperative signaling through the signal transducer and activator of transcription 3 and nuclear factor-κB pathways in subtypes of diffuse large B-cell lymphoma. Blood *111*, 3701–3713.
15. Azmi, A.S., Bao, B., and Sarkar, F.H. (2013). Exosomes in cancer development, metastasis, and drug resistance: a comprehensive review. Cancer Metastasis Rev. *32*, 623–642.
16. Lv, M.M., Zhu, X.Y., Chen, W.X., Zhong, S.L., Hu, Q., Ma, T.F., Zhang, J., Chen, L., Tang, J.H., and Zhao, J.H. (2014). Exosomes mediate drug resistance transfer in MCF-7 breast cancer cells and a probable mechanism is delivery of P-glycoprotein. Tumour Biol. *35*, 10773–10779.
17. Sharma, A. (2017). Chemoresistance in cancer cells: exosomes as potential regulators of therapeutic tumor heterogeneity. Nanomedicine (Lond.) *12*, 2137–2148.
18. Parkkila, S., Parkkila, A.K., Juvonen, T., Lehto, V.P., and Rajaniemi, H. (1995). Immunohistochemical demonstration of the carbonic anhydrase isoenzymes I and II in pancreatic tumours. Histochem. J. *27*, 133–138.
19. Wu, W., Wang, Q., Yin, F., Yang, Z., Zhang, W., Gabra, H., and Li, L. (2016). Identification of proteomic and metabolic signatures associated with chemoresistance of human epithelial ovarian cancer. Int. J. Oncol. *49*, 1651–1665.
20. Kazan, H.H., Urfali-Mamatoglu, C., and Gunduz, U. (2017). Iron metabolism and drug resistance in cancer. Biometals *30*, 629–641.
21. Jabbari, N., Nawaz, M., and Rezaie, J. (2019). Ionizing radiation increases the activity of exosomal secretory pathway in MCF-7 human breast cancer cells: a possible way to communicate resistance against radiotherapy. Int. J. Mol. Sci. *20*, 3649.
22. Liu, C.M., Lin, Y.M., Yeh, K.T., Chen, M.K., Chang, J.H., Chen, C.J., Chou, M.Y., Yang, S.F., and Chien, M.H. (2012). Expression of carbonic anhydrases I/II and the correlation to clinical aspects of oral squamous cell carcinoma analyzed using tissue microarray. J. Oral Pathol. Med. *41*, 533–539.
23. Takakura, M., Yokomizo, A., Tanaka, Y., Kobayashi, M., Jung, G., Banno, M., Sakuma, T., Imada, K., Oda, Y., Kamita, M., et al. (2012). Carbonic anhydrase I as a new plasma biomarker for prostate cancer. ISRN Oncol. *2012*, 768190.
24. Kopecka, J., Campia, I., Jacobs, A., Frei, A.P., Ghigo, D., Wollscheid, B., and Riganti, C. (2015). Carbonic anhydrase XII is a new therapeutic target to overcome chemoresistance in cancer cells. Oncotarget *6*, 6776–6793.
25. Pasqualucci, L., and Zhang, B. (2016). Genetic drivers of NF-κB deregulation in diffuse large B-cell lymphoma. Semin. Cancer Biol. *39*, 26–31.
26. Huang, X., Meng, B., Iqbal, J., Ding, B.B., Perry, A.M., Cao, W., Smith, L.M., Bi, C., Jiang, C., Greiner, T.C., et al. (2013). Activation of the STAT3 signaling pathway is associated with poor survival in diffuse large B-cell lymphoma treated with R-CHOP. J. Clin. Oncol. *31*, 4520–4528.
27. Zhang, M., Xu-Monette, Z.Y., Li, L., Manyam, G.C., Visco, C., Tzankov, A., Wang, J., Montes-Moreno, S., Dybkaer, K., Chiu, A., et al. (2016). RelA NF-κB subunit activation as a therapeutic target in diffuse large B-cell lymphoma. Aging (Albany NY) *8*, 3321–3340.
28. Feng, Y., Zhong, M., Zeng, S., Wang, L., Liu, P., Xiao, X., and Liu, Y. (2019). Exosome-derived miRNAs as predictive biomarkers for diffuse large B-cell lymphoma chemotherapy resistance. Epigenomics *11*, 35–51.
29. Ma, J., Chen, T., Wu, S., Yang, C., Bai, M., Shu, K., Li, K., Zhang, G., Jin, Z., He, F., et al. (2019). iProX: an integrated proteome resource. Nucleic Acids Res. *47* (D1), D1211–D1217.
30. Feng, Y., Lin, J., Liu, Y., Tang, Y., Zhou, Y., and Zhong, M. (2019). Investigation of expressions of PDK1, PLK1 and c-Myc in diffuse large B-cell lymphoma. Int. J. Exp. Pathol. *100*, 32–40.