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 parental, chemo-resistant DLBCL 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 chemoresistance in various ways, such as pumping chemotherapy drugs out of tumor cells7 and mediating drug resistance by exosomal proteins’ and miRNAs.8 For example, exosomal miRNAs or proteins derived from chemo-resistant tumor cells can reduce chemosensitivity of chemo-sensitive tumor cells via bystander effects.9,10 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 low11 while in breast cancer it is high.12 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...
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.

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.

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)-2H-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.
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, chemosensitive (n = 81) and chemoresistant (n = 31) groups. It was observed that expression of exosomal CA1 was significantly higher in the chemoresistant group than in the chemosensitive group (p < 0.001) (Figure 11A). The expression level of exosomal CA1 was closely associated with the international prognostic index.
(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 \((\geq 0.81)\) 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-kB 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 chemotherapy 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. In the chemoresistance research field, more and more studies are focusing on exosomes. Importantly, exosomes can mediate chemoresistance via transporting functional proteins. 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.

**Table 2** 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 \(\beta\) | 2.49600317  | 0.040489478 | up          |
| DKFz686K18196 | DKFz686K18196 | 2.350629045 | 0.036579588 | 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         | 1.66549819  | 0.001030012 | up          |
| JCHAIN     | immunoglobulin J chain | 1.600505736 | 0.028608701 | up          |
| Q9UL72     | Q9UL72           | 1.59782709  | 0.000537667 | up          |
| GC         | GC               | 1.52163537 | 0.03582196 | up          |
| P01594     | Ig kappa chain V-I region AU | 1.508483939 | 0.036537648 | up          |
| F5-20      | F5-20            | 0.513401094 | 0.022315043 | down        |
| ORM1       | alpha-1-acid glycoprotein | 0.452444203 | 0.025821935 | down        |
| IGHD       | Ig delta chain C region | 0.496599067 | 0.00490248 | down        |
| SERPINA3   | alpha-1-antichymotrypsin | 0.513617092 | 3.01E-05 | down        |
| TFRC       | transferrin receptor protein 1 | 0.562102074 | 0.009907065 | down        |
| CIQB       | complement CIq subcomponent subunit B | 0.571194716 | 0.027755915 | down        |
| CIQA       | complement CIq subcomponent subunit A | 0.577189301 | 0.02406817 | down        |
| SERPINA7   | thyroxine-binding globulin | 0.59641869 | 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        |
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 and pancreatic cancer, with a close association with chemoresistance. ORM1 was found highly expressed in cisplatin-resistant ovarian cancer. 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. 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
profi ling. 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.\textsuperscript{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.\textsuperscript{22} In another study, the combined detection of CA1 and prostate-specific antigen (PSA) significantly improved diagnostic sensitivity in prostate cancer.

Figure 4. KEGG Analysis of 54 Differentially Expressed Proteins

Figure 5. PPI Analysis
Red indicates upregulated proteins; green indicates downregulated proteins.
compared with detection of PSA alone. 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. 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 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.
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 8. Exosomal CA1 Mediates Chemotherapy Resistance in DLBCL via NF-κB and STAT3 Signaling Pathways

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.
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,
which was enriched with 100 U/mL penicillin, 100 mg/mL streptomycin, and 10% fetal bovine serum (FBS) in an incubator with 5% CO\textsubscript{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.\textsuperscript{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.\textsuperscript{29} In order to compare the differentially expressed protein profiles between two groups, we calculated and used fold change and p value to identify significantly 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 \times 10\textsuperscript{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\textsubscript{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’-GCCUCAAAAGG CUGAUGGUUTT-3’; antisense, 5’-AACCAUCAGCCUUUGAGG
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 $(\text{length} \times \text{width})^2/2$, 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.

**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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