

Expression of a soluble decoy receptor 3 in patients with diffuse large B-cell lymphoma predicts clinical outcome

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Abstract. The soluble decoy receptor 3 (DcR3) is a member of the TNF receptor superfamily. It is regarded as a decoy receptor released from tumor cells to escape host immune response by neutralizing the cytotoxic and immunomodulatory effects of FasL, LIGHT and TL1A. Overexpression of DcR3 has been observed in several human malignancies; however, only limited information exists on the role of DcR3 in non-Hodgkin lymphoma especially for B-cell origin. In the current study, the expression profile of DcR3 was analyzed by RT-PCR and immunohistochemistry (IHC) in a set of lymphoma cell lines including T-cell and B-cell lymphomas. The result demonstrated that overexpression of DcR3 was detected in most T-cell lymphoma cells, which was consistent with previous reports. Interestingly, overexpression of DcR3 was also detected both in the B-cell lymphoma cell lines and diffuse large B cell lymphoma (DLBCL) patients. DcR3 overexpression was associated with a worse prognosis in DLBCL patients ($p=0.05$). An *in vitro* study showed that neutralization of DcR3 increased the percentage of doxorubicin-mediated apoptosis in two B-cell lymphoma cell lines, which indicated the possibility of DcR3 mediated chemo-resistance in B-cell lymphomas. We suggest that overexpression of DcR3 is associated with a worse prognosis in DLBCL and the possible mechanism may act through the increase of chemo-resistance of lymphoma cells.

Introduction

Non-Hodgkin lymphomas (NHLs) are one of the leading causes of death from cancer in the world including Taiwan

(1). NHLs can be divided into B-cell and T-cell origin, in which B-cell NHLs account for 64% of all cases (2). Diffuse large B cell lymphoma (DLBCL) is the most common pathologic subtype of lymphoma and nearly 40% of newly diagnosed NHLs are DLBCL (2). The CHOP regimen (cyclophosphamide, doxorubicin, vincristine and prednisone) is the standard treatment for both younger and elder DLBCL patients, with a complete response rate about 44% (3). Recent study demonstrates the efficacy of incorporating rituximab into CHOP regimen in treating DLBCLs (4). Although DLBCL is one of the most chemo-sensitive malignancies, many newly diagnosed DLBCL patients failed to be cured by standard anthracycline-based chemotherapy. The variability in response to a homogeneous treatment suggests underlying heterogeneity in this disease (5). Recently several genetic abnormalities including Fas mutations have been identified in subsets of DLBCLs (6), which may predict the response to chemotherapy and clinical outcome.

Decoy receptor 3 (DcR3), also known as TR6 or M68, is mapped to chromosome 20q13.3. It is regarded as a novel decoy receptor which interacts with other members of the TNF superfamily (7), such as FasL (8,9), LIGHT (10) and TL1A (11) by neutralizing their cytotoxic and immunomodulatory effects, and help tumor cells to escape host immune response. For example, Fas ligand (FasL) and its receptor Fas (CD95/APO-1) are a set of components mediating the cytotoxicity of T lymphocytes and regulating immune responses, tissue development and homeostasis (12). The cell surface Fas binds with FasL and results in cell apoptosis (13,14). Attenuation of Fas pathway may cause lymphoproliferative disorders and may accelerate autoimmune diseases (12). Many studies have suggested that suppression of Fas/FasL binding may play an important role in the pathogenesis of several malignancies (15,16). Expression of DcR3 have been identified to influence the FasL-induced apoptosis and associated with development and prognosis in certain cancers including cancers originating from esophagus, stomach, glioma, lung, colon, liver and pancreas (17-21). Regarding the role of DcR3 in non-Hodgkin lymphomas, only one study has reported on the expression of DcR3 in

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Table I. Baseline characteristics of DLBCL patients (n=42).

Characteristics	No. (%)
Age (years)	
Range	15-86
Median	57
Gender	
Male	32 (76.2)
Female	10 (23.8)
Ann Arbor stage	
I	4 (9.5)
II	10 (23.8)
III	19 (45.3)
IV	9 (21.4)
IPI score at diagnosis	
0-2	12 (28.6)
3-5	30 (71.4)
Complete remission after CHOP therapy	
Yes	23 (54.8)
No	19 (45.2)
Progression/Recurrence after CHOP therapy	
Yes	20 (47.6)
No	22 (52.4)
Increased DcR3 expression	
Yes (++)	10 (23.8)
No (0 to +)	32 (76.2)

virus-associated and T-cell lymphomas (22). However, the role of DcR3 in B-cell NHL is still unclear.

The aim of this study is to delineate the role of DcR3 in aggressive B-cell lymphomas. Firstly we evaluated the expression profile of DcR3 in a set of lymphoma cell lines including T-cell and B-cell origin. We subsequently focused on the role of DcR3 expression in B-cell lymphomas, especially in DLBCLs, and the clinical significance of DcR3 expression in DLBCL patients was evaluated. Finally, we evaluated the relationship between DcR3 and chemoresistance of B-cell lymphomas by clinical correlation and *in vitro* analysis to clarify the possible mechanism of DcR3-induced poor prognosis of DLBCL.

Materials and methods

Eligibility of patients. From January 1993 to December 2002, 42 patients with histologically documented DLBCL treated with first-line CHOP chemotherapy, with at least one measurable lesion (>2 cm) for response evaluation and available tissues for DcR3 mRNA and protein detection before treatment, were enrolled in this study. Tumor responses were evaluated every 3 months and the response was based on RECIST criteria as previously described (23). In case of disease progression or residual tumors after 6 cycles of first-line chemotherapy, salvage chemotherapy would be given according to the choice of the physicians. All

patients signed informed consent for the tissue sampling. The clinical data of 42 DLBCL cases are given in Table I.

Cell lines and tissue samples. Human B-cell lymphoma cell lines (Daudi, Raji and Ramos) and T-cell lymphoma cell lines (CCRF-CEM, RPMI-8402, MOLT-3, Jurkat, Sup-T1, J45.01) were cultured in RPMI-1640 medium with 2 mM L-glutamine adjusted to contain 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 10 mM HEPES, 1.0 mM sodium pyruvate and 10% fetal bovine serum at 37°C. Specimens of lymph node biopsy were obtained from 42 patients with DLBCL as previously described.

Isolation of CD34⁺ hematopoietic stem cells. The CD34⁺ stem cells were used as a control for DcR3 and related signal expression in this study. Ten healthy donors received peripheral blood stem cell collection after signing of informed consent. Stem cell purging was performed using the cell isolation device CliniMACS[®] on the day of stem cell harvest and for three consecutive days. During the stem cell purging procedure with CliniMACS, superparamagnetic iron-dextran particles specifically stick to the cells, while the iron-dextran particles covalently conjugated to mouse anti-human CD34 antibody. After magnetizing, the CliniMACS system automatically passed the cells through a separation column with a strong permanent magnetism. The magnetically labeled cells were retained in the column and separated from the unlabeled cells, which flowed through. The CliniMACS eluted the retained cells by removing the column from the magnetic field, washing the cells out, and collecting them. The purged stem cells were sent for counting the CD34⁺ subpopulation by flow cytometry.

Immunohistochemistry. Immunohistochemistry was performed for lymph node specimens and lymphoma cell lines to detect the surface DcR3 expression. Briefly, 6- μ m thick sections of tumor tissue were cut from the frozen specimens for immunohistochemistry (IHC) analysis, and the cell line samples were prepared by cytospin method. The samples were fixed in acetone, air-dried, and subsequently bathed in Tris-buffered saline (TBS) solution (pH 7.6). The endogenous peroxidase activity was blocked with 3% hydrogen peroxide. For DcR3 IHC staining, a rabbit polyclonal anti-DcR3 antibody (sc-25464, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used at the dilution of 1:100 and incubated at 4°C overnight. After reacting with a biotinylated secondary antibody for 30 min, antigen-antibody reactions were visualized using streptavidin-horseradish peroxidase conjugate (Dako LSAB kit; Dako, Los Angeles, CA), with AEC as the chromogen. All slides were counterstained with hematoxylin. For interpretation of IHC results of DcR3, we counted 100 cells from 5 representative areas in each lesion and the degree of staining was subdivided as follows: 0, no staining; +, focal or fine granular weak staining; ++, diffuse intense staining. Overexpression of DcR3 was defined as staining of ++.

RNA preparation and RT-PCR. For suspension cells, we harvested cells by centrifugation and added 1 ml of RNApure[™] solution per 10⁶-10⁷ cells. For tissues, we added 1 ml of RNApure solution per 100 mg tissue and homogenized

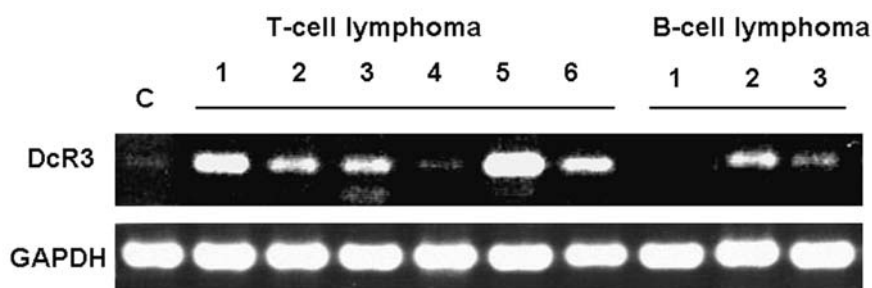


Figure 1. RT-PCR analysis of DcR3 mRNA expression in lymphoma cell lines. GAPDH was used as an internal control of experiment. T-cell lymphoma cell lines: 1, RPMI-8402; 2, CCRF-CEM; 3, MOLT-3; 4, Jurket; 5, Sup-T1; 6, J45.01. B-cell lymphoma cell lines: 1, Daudi; 2, Raji; 3, Ramos.

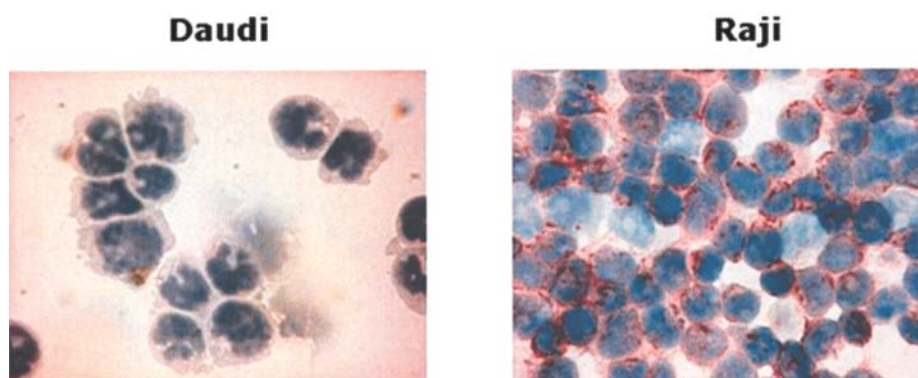


Figure 2. Immunohistochemistry analysis of DcR3 expression in Daudi and Raji cell. Increased expression of DcR3 can be identified in Raji cells.

the tissue at low temperature (ice bath) with a polytron homogenizer. Total-RNA was extracted by the RNApure kit according to the manufacturer's instructions. For DcR3 mRNA detection, reverse transcriptions were performed using 5 μ g of total-RNA with SuperScript II kit (Invitrogen). cDNA (1 μ l) was used to amplify the DcR3 transcripts with primer pair: 5'-GCTTTCCAGGACATCTCCATCAAG-3' for sense and 5'-AAGCCTCTTTTCAGTGCAAGTGG-3' for antisense by the PCR program: 35 cycles of 94°C for 40 sec, 61°C for 30 sec and 72°C for 45 sec. Finally, the PCR products were detected by agarose gel electrophoresis.

MTT assay. MTT assays were performed using the MTT kit (Roche Diagnostics GmbH, Mannheim, Germany). Cells (5×10^3) were seeded in 96-well microtiter plates and cultured for 24 and 72 h. MTT solution (30 μ l, 5 mg/ml) was added and incubated for 4 h. Me₂SO (100 μ l) was added to dissolve formazan crystals under vigorous shaking for 30 min to detect the absorption using an enzyme-linked immunosorbent assay reader.

Statistical analysis. Statistical Package of Social Sciences software (version 14.0; SPSS, Inc., Chicago, IL) was used for statistical analysis. The Kaplan-Meier estimate was used for survival analysis, and the log-rank test was selected to compare the cumulative survival durations in different patient groups. Overall survival (OS) was defined as time elapsed between diagnosis and date of death or the date last seen. Time to progression (TTP) was defined as time between diagnosis and date of progression/recurrence or last follow-up. Then the response analysis of each clinical factor, using χ^2 or

Fisher's exact test for category variables, was performed. Two-sided p-values <0.05 were considered statistically significant.

Results

DcR3 expression profile in lymphoma cell lines. To investigate the expression profile of DcR3 in lymphoma cells, we screened nine lymphoma cell lines including six T-cell lymphoma cell lines and three B-cell lymphoma cell lines as described in Materials and methods. Increased DcR3 expression was observed both in the B-cell and T-cell lymphoma cell lines compared with the CD34⁺ cells (Fig. 1). Variable expressions of DcR3 mRNA level were noted between different lymphoma cell lines, and T lymphoma cells seemed to have a higher DcR3 mRNA level compared with B lymphoma cells, which was consistent with previous results. Among them, RPMI-8402 and Sup-T1 carried the highest DcR3 level of the studied lymphoma cell lines. For B lymphoma cells, Raji and Ramos seemed to have a higher DcR3 expression level (Fig. 1). The result indicated that increased DcR3 mRNA expression, was not limited to T lymphoma cells, but was also identified in B lymphoma cells.

In order to confirm the increased DcR3 expression in B-cell lymphomas, immunocytochemistry was performed in representative B lymphoma cell lines with different expression levels of DcR3 (Daudi and Raji). Increased expression of DcR3 was noted in Raji cells, whereas only scanty expression was found in Daudi cells (Fig. 2). This result was consistent with the RT-PCR finding. All these results indicated that DcR3 overexpression was identified in

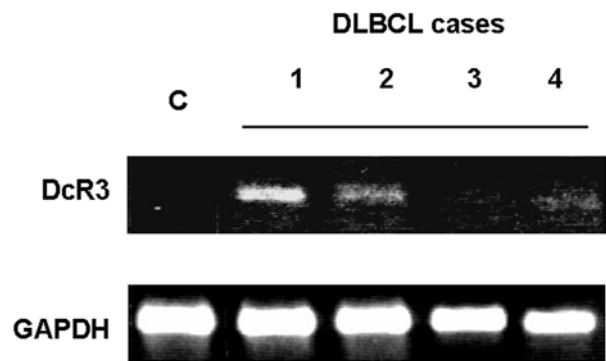


Figure 3. Increased DcR3 mRNA expression in representative DLBCL patients. GAPDH was used as an internal control.

certain B-lymphoma cell lines, which might indicate the possible role of DcR3 expression in the B-lymphomagenesis.

Prognostic significance of DcR3 overexpression in DLBCL patients. To identify the clinical significance of DcR3 overexpression in B-cell lymphoma, we selected DLBCL as the population to be tested in order to homogenize the patient characteristics. The mRNA levels of DcR3 were tested in representative DLBCL patients and increased DcR3 mRNA expression was noted in DLBCL samples compared with the CD34⁺ cells (Fig. 3). To further confirm the result, IHC analysis of DcR3 expression was performed in all 42 DLBCL cases, and DcR3 overexpression (IHC ++) was noted in 10 (23.8%) cases (Fig. 4; Table I). Kaplan-Meier survival analysis was performed in 42 DLBCL patients, and the results demonstrated a significantly worse overall survival in DcR3 (++) patients ($p=0.05$; Fig. 5). The prognostic effect of DcR3 overexpression can be identified both in the International Prognostic Index (IPI) low- and intermediate to high-risk group of patients ($p=0.05$ and 0.05 , respectively; data not shown). These results indicated that overexpression of DcR3 could be identified in a certain proportion of DLBCL patients, and was associated with a worse clinical outcome.

Significance of DcR3 expression in the chemo-sensitivity of DLBCL. Because anthracycline (e.g., doxorubicin) is the

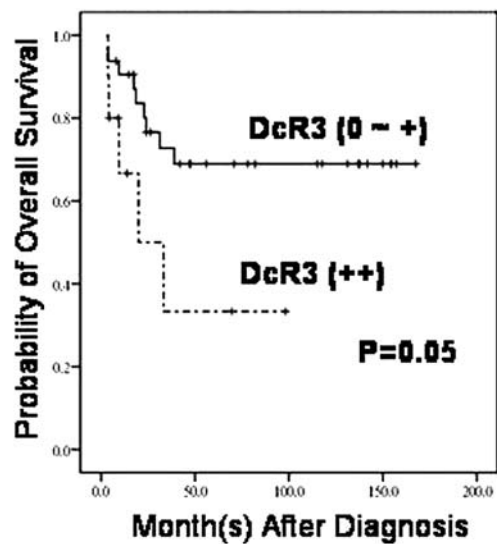


Figure 5. Kaplan-Meier overall survival analysis of 42 DLBCL patients with increased DcR3 expression (IHC ++) or not (0 to +).

mainstay of chemotherapy in DLBCL, we investigated the correlation between DcR3 overexpression and response to first line CHOP chemotherapy in our 42 DLBCL cases. The result showed that there was a trend of poorer response to first line CHOP chemotherapy in DcR3 overexpressed (i.e., IHC ++) patients (complete remission rate: 62.5% in DcR3 ++ vs. 30% in DcR3 0 to +, $p=0.07$). This result indicated that overexpression of DcR3 may be associated with anthracycline resistance of B-cell lymphomas. To confirm the DcR3 related chemo-sensitivity change in B-cell lymphoma, MTT assay was performed in two representative B-cell lymphoma cell lines (Raji and Ramos) with/without doxorubicin treatment, and DcR3 neutralizing antibody was added to observe the effect on cell apoptosis. Neutralization of DcR3 increased the percentage of doxorubicin mediated apoptosis in both cell lines (Fig. 6). The results from clinical analysis and *in vitro* experiments indicates the potential role of DcR3 mediated chemo-resistance in DLBCL, which leads to worse prognosis of DcR3 overexpressed DLBCL cases.

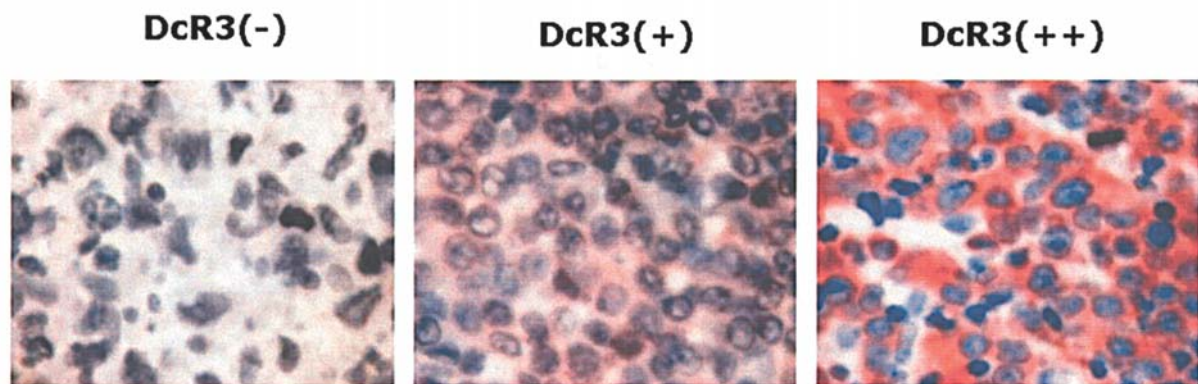


Figure 4. Immunohistochemistry analysis of DcR3 expression in DLBCL tumor samples. DcR3 (-), no DcR3 expression; DcR3 (+), weak cytoplasmic expression; DcR3 (++) , strong cytoplasmic expression.

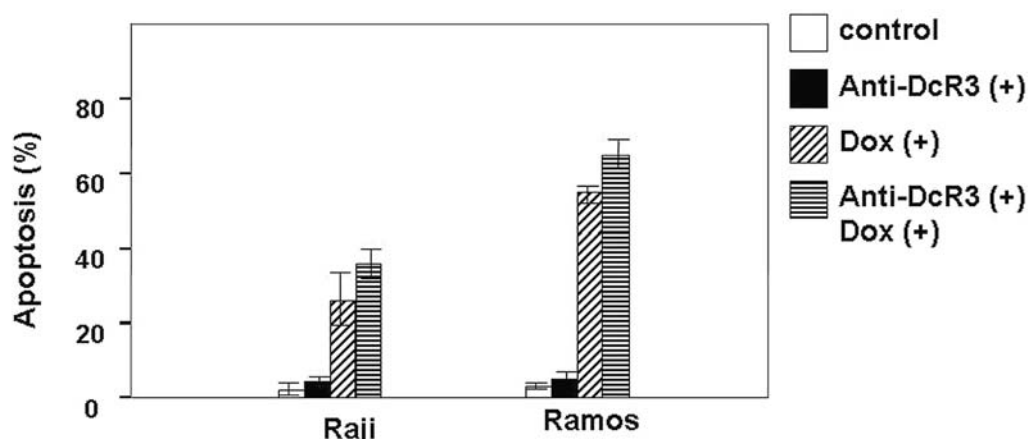


Figure 6. Results of MTT assays of Raji and Ramos cells after anti-DcR3 neutralizing antibodies, doxorubicin or both treatments.

Discussion

In this study, we demonstrated that overexpression of DcR3 could be identified in B-cell lymphoma cell lines as well as in T-cell lymphoma cell lines. Increased DcR3 expression was also observed in a section (10 in total 42, ratio 23.8%) of DLBCL patients, and was associated with a worse prognosis. As far as we know, this is the first study that clearly identifies the DcR3 expression in aggressive B-cell lymphomas both *in vitro* and *in vivo*, and further attributes the association with clinical outcome in DLBCL patients. In a previous study on virus-associated lymphomas, Ohshima *et al* reported that DcR3 expression was confined to the neoplastic cells rather than in the reactive cells. They suggested that DcR3 was important in the virus-associated lymphomagenesis probably due to its escape from immune system (22). Our current study showed that the non-virus-associated B-cell lymphomas also had DcR3 overexpression. Further study to confirm the speculation is warranted.

The prognostic factors of DLBCL patients have been studied for decades. In addition to previous standard risk stratification using the international prognostic index (IPI) score, which is based upon the assessment of clinical variable (24,25), recent studies have focused on the difference between germinal center B-cells like lymphoma (GCB DLBCL) and activated B-lymphocytes like lymphoma (ABC DLBCL). For example, Alizadeh *et al* used the mRNA profiles to identify the distinct subtypes of DLBCL, and Rosenwald *et al* further used molecular profiling to predict survival after chemotherapy for DLBCL (26,27). In the protein level by immunohistochemistry, Hans *et al* proposed an algorithm using the expression of CD10, Bcl-6, and MUM1 to discriminate GCB DLBCL, which was more indolent and had better prognosis, with ABC DLBCL (28). In the current study, we found that overexpression of DcR3 in DLBCL patients seemed to be a poor prognostic factor in overall survival. The possible explanation might be the immunomodulation, anti-apoptosis or chemoresistant effect mediated by DcR3, which require more studies to confirm. There were actually many other co-factors that would influence the clinical outcome of DLBCL. In addition, we did not further analyze the relationship

between DcR3 and other clinical factors due to the small sample size of our study. However, our study suggests that DcR3 may be considered as one of the important prognostic factors in DLBCL patients.

Doxorubicin, a chemotherapeutic agent of anthracycline group, is considered as the cornerstone of DLBCL treatment (3). Resistance to anthracycline has been reported to be associated with the inhibition of FasL-induced apoptosis (29,30). Because overexpression of DcR3 is associated with a worse prognosis of DLBCL, we speculated that DcR3 may increase chemo-resistance in DLBCL cells that leads to the poor prognosis of patients. In the current study, we found a trend of poor response to first-line CHOP therapy in DcR3-overexpressed DLBCL patients. The *in vitro* study confirmed that neutralization of DcR3 increased the doxorubicin-induced apoptosis both in the Raji and Ramos cells (Fig. 6). Previous studies showed that resistance to anthracycline is associated with the inhibition of FasL-induced apoptosis (32). DcR3 has been reported to inhibit Fas-induced apoptosis (8), therefore we considered that overexpression of DcR3 in B-cell lymphoma cells may promote anthracycline resistance through inhibition of anthracycline induced Fas-mediated apoptosis. According to the presented results, our experiments support this point of view; however, further study is warranted to confirm this speculation.

In conclusion, our results showed that overexpression of DcR3 could be identified in certain B-cell lymphoma cell lines and DLBCL patients, and overexpression of DcR3 was associated with a worse prognosis in DLBCL patients. Increased DcR3 expression was associated with anthracycline resistance possibly through inhibition of Fas-mediated apoptosis. These results could be important in the pathogenesis, diagnosis, prognosis and treatment of DLBCL cases; however, further studies on the mechanistic clarification of DcR3 mediated B-lymphomagenesis, chemo-sensitivity as well as clinical outcome is required.

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