Original Article Impact of TNFRSF6B/DcR3 neutralization antibody and SiRNA on biological function of MCF-7 breast cancer cells

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Abstract: Background and Aim: Tumor necrosis factor receptor superfamily member 6b (TNFRSF6B), also identified as DcR3, has been stated to play a crucial role in the tumorigenesis and progression of several cancers. However, the biological function and clinical role of DcR3 in breast cancer (BRCA) has not been fully clarified. Hence, the aim of the study was to explore the impact of DcR3 on biological function of BRCA cells with in vitro experiments, as well as to assess the clinical significance of DcR3 bases on a meta-analysis and public data. Material and Methods: DcR3 neutralization antibody and DcR3-siRNA were transfected into BRCA MCF-7 cells. Three approaches including MTS assay, viability assay and Hoechst 33342/propidium iodide (PI) double staining assay were employed to examine the cell growth. Furthermore, the apoptosis was monitored by fluorescent caspase-3/7 assay and the Hoechst 33342/PI assay. Information of the association of DcR3 expression and some clinicopathological parameters including tumor differentiation, lymph node metastasis, clinical TNM stage, etc. were extracted in BRCA related literature, and pooled odds ratios (ORs) and 95% confidence intervals (CIs) were evaluated by Forest plot in the current meta-analysis. Furthermore, data mining was performed in The Cancer Genome Atlas (TCGA) public database to verify the clinical significance of DcR3 in BRCA. Results: Both DcR3 neutralization antibody and siRNA showed obvious cell growth suppression effect on BRCA MCF-7 cells as detected by three methods. Consistently, DcR3 neutralization antibody and siRNA also vielded remarkable impact on inducing caspase-3/7 activity and apoptosis. DcR3-siRNA led to a much more potent influence on biological function than DcR3 neutralization antibody in BRCA cells. In the meta-analysis, over-expression of DcR3 was strongly interrelated with the occurrence of BRCA, also differentiation, lymph node metastasis and TNM stage. Moreover, 9% genetic alteration was noted based on TCGA database in 1105 BRCA samples. Conclusions: The present discoveries propose that the over-expression of DcR3 might be associated with the malignant aggressiveness and progression of BRCA, via influencing cell growth and apoptosis. DcR3 may become a probable notable target for the molecular therapy of BRCA. But the specific pathway and precise molecular mechanisms of DcR3 in BRCA cells remain to be further explored.

Keywords: TNFRSF6B/DcR3, neutralization antibody, SiRNA, breast cancer, proliferation, apoptosis

Introduction

Tumor necrosis factor receptor superfamily member 6b (TNFRSF6B), also identified as DcR3; M68; TR6; M68E; DJ583P15.1.1 (location: 20q13.3) belongs to the TNF receptor superfamily. DcR3 is a soluble receptor which is able to neutralize the natural impacts of three other TNFSF family members, including Fas ligand (FasL/TNFSF6/CD95L), LIGHT (TN-FSF14) and TNF-like molecule 1A (TL1A/TN- FSF15). Because FasL is a strong inducing factor for apoptosis and inflammation, LIGHT has been confirmed to be involved in the process of apoptosis and inflammation, and TL1A is a T cell co-stimulator and reported to be required in gut inflammation, DcR3 thus can be interpreted as an immuno-modulator on the grounds of its neutralizing impacts on FasL, LIGHT, and TL1A [1-11]. Initial studies have recently revealed that DcR3 over-expression has been noted in several malignancies, such as esophageal, gas-



Figure 1. *In Vitro* experiments to investigate the impact of DcR3 on breast cancer (BRCA) cells. A: Cell proliferation detected by tetrazolium MTS assay; B: Cell viability assessed by fluorimetric resorufin viability assay; C: Cell viability examined by microscopic Hoechst 33342/PI double fluorescent chromatin staining; D: Caspase-3/7 activity monitored by fluorescent caspase-3/7 assay. *P<0.05, **P<0.01, ***P<0.001.



Figure 2. Microscopic Hoechst 33342/PI double fluorescent chromatin staining assay to examine the impact of DcR3 on breast cancer (BRCA) cells. A: hlgG1 as negative control for antibody; B: DcR3 neutralization antibody; C: Scrambled siRNA as negative control for siRNA; D: DcR3-siRNA; E: 3% DMS0 as positive control × 400.

tric, colon, liver, pancreatic, bladder urothelial, lung cancer, glioma and also female reproductive cancers, including cervical, ovarian and breast cancer [12, 21]. These reports have suggested an oncogenic role of DcR3 on various malignancies.

Breast cancer (BRCA) is one of the commonest diagnosed cancers around the world, and it is the main cause of cancer-related death among female. When searching in PubMed, we only found two relevant papers concerning the relationship between DcR3 and BRCA. Wu Q et al [22] investigated the DcR3 expression, as well as the relationship between DcR3 expression and lymphatic micro-vessel density (LMVD) in BRCA with real-time PCR and immunohistochemistry. They found that DcR3 was upregulated in BRCA tissue of 58 cases (92.1%) among 63 patients. Moreover, the over-expression of DcR3 was positively associated with lymphatic micro-vessel density (LMVD) in both BRCA tissue and metastatic lymph nodes. However, the diagnostic role of DcR3 expression has not yet been verified. And the association between DcR3 expression and disease development of BRCA has not been clarified in English literature either. Ge et al [23] examined DcR3 expression in BRCA MCF7 and MDA-MB-231 cell lines using immunocytochemistry and RT-PCR and found that DcR3 protein was over-expressed. They also constructed anti-DcR3 hammerhead ribozyme transgenes and transfected them into BRCA MCF7 and MDA-MB-231 cells to silence DcR3. Further, DcR3 knock-down in BRCA cells could suppress invasiveness and migration. However, no study has been available using traditional gene silencing tool, e.g. RNA interference with siRNAs or neutralization antibody. In the study, we firstly explored the potential role of DcR3 on the biological function of BRCA cells in vitro with both DcR3 neutralization antibody and siRNA. Secondly, we performed a comprehensive meta-analysis to sum-

NO	Year	Author	Country	Organ	Number	Expression Cancer (+/-) Control (+/-)	Age Old (+/-) Young (+/-)	TNM 3, 4 (+/-) 1, 2 (+/-)	Differentia- tion Low (+/-) High (+/-)	Lymph node metas- tasis Metastasis (+/-) Non-metastasis (+/-)	Overall sur- vival time (month)
1	2011	Gao-HH [29]	China	Breast	82	24/17	NA	7/1	NA	17/4	NA
						10/31		17/16		7/13	
2	2005	Wang-LP [30]	China	Breast	53	18/11	NA	NA	NA	14/6	NA
						0/4				4/5	
3	2007	Shan-GP [31]	China	Breast	74	25/16	11/8	6/2	11/5	20/7	NA
						0/33	13/8	19/14	14/11	5/9	
4	2010	Chen-G [32]	China	Breast	136	58/21	32/13	51/6	29/1	32/1	NA
						0/57	26/8	7/15	29/20	26/20	
5	2014	Wu-QW [22]	China	Breast	126	58/5	17/1	5/1	30/2	28/2	NA
						34/29	41/4	53/4	23/3	30/3	

Table 1. Characteristics of studies included in the meta-analysis

marize the clinical significance of DcR3 in BRCA based on literatures with no limitation to publication languages. Lastly, we also displayed the genomic alterations of DcR3 in BRCA based on data from The Cancer Genome Atlas (TCGA).

Materials and methods

Cell culture and different treatments

Human BRCA cell line MCF-7 (HTB-22) from the American Type Culture Collection (ATCC) was cultured in Dulbecco's modified essential medium (DMEM, GIBCOL BRL, USA), accompanied with 10% heat-inactivated fetal bovine serum (FBS, GIBCOL BRL, USA), 2 mm glutamine, gentamicin at 37°C in a humidified incubator with 5% CO2. Previously, we found that DcR3 protein level was over-expressed both in the supernatants and cells of MCF-7. Thus, this BRCA cell line was cultured and treated with different agents for the current in vitro experiments. The MCF-7cells were transferred to serum-free medium for 24 h before all experiments. Next, the MCF-7cells were supplemented with serumfree medium containing anti-DcR3 neutralization antibody or hlgG1 (0.8 mg/L, R&D Systems, Inc. Minneapolis, MN USA) for 48 h, which was previously found to achieve the strongest cell proliferation inhibitory effect (data not shown). In parallel, the MCF-7 cells were also transfected by combiMAGnetofection method with DcR3-specific-siRNAs or scramble siRNAs (200 nmol/L), which were synthesized by Gene-Pharma in Shanghai as previously reported [24]. Other control groups were also set up in the current study, including black control, mock control and negative siRNA control [24]. All experiments were completed three times in exactly the same way in the present study.

Western blot analysis

After the cells were treated alterably according to the experimental design for 48 h in 6-wellplate, the western blot was performed as previously reported [15, 25-28]. The primary antibodies of the current study included DcR3 (ab11930, Abcam), and β -actin (AC-15, Sigma-Aldrich).

Cell proliferation

Cell proliferation was evaluated with a colorimetric tetrazolium (MTS) assay (CellTiter96 Aqueous One Solution Cell Proliferation Assay, USA) as previously reported [15, 25-28]. The proliferation rates were the average value of six wells and displayed as the rate of the absorbance of experimental wells/absorbance of mock control × 100.

Cell viability

To verify the finding from the above MTS method, cell viability was assessed with fluorimetric detection of resorufin (CellTiter-Blue Cell Viability Assay, USA) as reported [15, 25-28]. Fluorescence data were shown as the fluorescence of experimental groups/mock control × 100.

Caspase-3/7 activity evaluation

Caspase-3/7 activity was evaluated with a synthetic rhodamine labeled caspase-3/7 substrate (Apo-ONE[®] Homogeneous Caspase-3/7



Figure 3. Flow chart of literature screening and selection in the current metaanalysis.

Assay, USA) as previously reported [15, 25-28]. Caspase-3/7 activity was displayed as fluorescence of experimental groups/mock control × 100.

Fluorescent assessment of cellular apoptosis

The impact of anti-DcR3 antibody and DcR3 siRNAs on apoptosis was assessed with Hoechst 33342 (Sigma-Aldrich N.V. Belgium) and PI double fluorescent chromatin staining as we reported formerly [15, 25-28]. The apoptotic rate was from the comparison of the number of apoptotic cells from distinct experimental groups/the number of viable cells in the same well.

Statistical analysis

For the data achieved from *in vitro* study, statistical analysis was employed with SPSS 22.0. Data were exhibited as mean \pm Standard Deviation (SD). Differences inter-group was analyzed with analysis of variance (ANOVA) or Student's *t*-test. *P* value of less than 0.05 was regarded as to be statistically significant.

Meta-analysis to investigate the clinical significance of DcR3 in BRCA

Literatures searching were conducted with both English and Chinese databases. The En-

glish databases were Wiley Online Library, PubMed, Science Direct, Web of Science, EMBASE, Cochrane Central Register of Controlled Trials. Ovid, Google Scholar and LILACS. And the Chinese databases included Chong Qing VIP, CNKI, Wan Fang and China Biology Medicine Disc. Literature searching was performed up to 19th September 2016 with a combination of the following terms: 'TR6 or DcR3 or M68 or TNFRSF6B'. 'breast or mammary or mastocarcinoma' and 'cancer or tumor or carcinoma or neoplasm* or malignancy*'. All of the related sources including review papers and references in the text were also examined for additional pertinent stud-

ies. Papers were sought and screened by three authors individualistically (Rui Zhang, Mengtong Jiang and Gang Chen). In the meta-analysis, studies should meet the following criteria: (1) Patients were evidently diagnosed as BRCA: (2) Studies were case-control studies and these studies assessed the association between DcR3 expression and clinical aspects or prognosis in BRCA; (3) The characterization of being DcR3-positive was confirmed by immunohistochemistry (IHC) staining; (4) Adequate information of the association of DcR3 expression with clinicopathological aspects or overall survival (OS) time was supplied to evaluate odds ratio (OR) and hazard ratio (HR). Letters, conference reports, conference abstract, reviews, and duplicated studies were excluded. Three reviewers (Rui Zhang, Meng-Tong Jiang and Gang Chen) screened all relevant information separately to eliminate the inappropriate studies. Discrepancies on each paper were determined by a fourth reviewer (Min-Hua Rong). Then three independent reviewers (Rui Zhang, Meng-Tong Jiang and Gang Chen) scrutinized the whole paper of all appropriate studies and derived related information including name of the authors, year of publishing, country, cancer type, number of patient in the study, test method, clinical parameters like age, gender, grade of differentiation, lymph node metastasis and TNM stage), as well as the OS. Stata 12.0 was



Figure 4. Immunohistochemistry of DcR3 in breast tissues. Non-cancerous breast tissues (A: \times 100, B: \times 400), Breast cancer (BRCA) tissues (C: \times 100, D: \times 400). The immunohistochemistry was performed by our group and the results were published in Reference [32] (These figures have not been published before).

applied in the current meta-analysis. ORs with 95% confidence intervals (CIs) were assessed with Forest plot. The heterogeneity was examined by I² test. Potential sources of statistical heterogeneity were found out by subgroup analysis. The software Engauge Digitizer 4.1 was applied to collect the survival data from a Kaplan-Meier (K-M) curve if necessary. The publication bias was analyzed by Begg's funnel plot test. P<0.05 was regarded statistically significant.

DcR3 data from the cancer genome atlas (TCGA)

Furthermore, data mining was also employed with The Cancer Genome Atlas (TCGA) public database to explore the clinical significance of DcR3 genomic alterations in BRCA via cBioPortal (www.cbioportal.org).

Results

In vitro experiments

DcR3 protein level was down-regulated to 60%-80% by DcR3 neutralization antibody or siRNA, which indicated that the treatment and transfection efficiency was optimal (data not shown). The impact of DcR3 neutralization antibody and siRNAs on the cell growth of BRCA cells was evaluated by using three individual approaches, including tetrazolium MTS assay, fluorimetric resorufin viability assay and microscopic Hoechst 33342/PI double fluorescent chromatin staining, respectively. All the three assays yielded consistent inhibitory effect of DcR3 neutralization antibody and siRNA on the cell growth in BRCA MCF-7 cells (Figures 1A-C, 2). DcR3 neutralization antibody presented a moderate impact on suppressing the cell prolif-





Figure 5. Meta-analysis investigating the clinical role of DcR3 in breast cancer (BRCA). A: Forest plot of odds ratios (ORs) with corresponding 95% Cls for the association of DcR3 overexpression with the occurrence of BRCA. B: Begg's publication bias plot of publication bias for studies regarding overexpressed DcR3 and the occurrence of DcR3 overexpression with level and grade of differentiation of BRCA. D: Begg's publication bias plot of publication bias for studies regarding overexpression bias plot of publication bias for studies regarding overexpression bias plot of publication bias for studies regarding overexpression bias plot of publication bias for studies regarding overexpressed DcR3 and the level and grade of differentiation of BRCA. D: Begg's publication bias plot of publication bias for studies regarding overexpression with corresponding 95% Cls for the association of DcR3 overexpression with lymph node metastasis of BRCA. F: Begg's publication bias plot of publication bias for studies regarding overexpression with TNM stage of BRCA. H: Begg's publication bias plot of publication bias for studies regarding overexpression with TNM stage of BRCA. H: Begg's publication bias plot of publication bias plot of publication bias for studies regarding overexpression with TNM stage of BRCA. H: Begg's publication bias plot of publication bias plot of publication bias for studies regarding overexpression with TNM stage of BRCA. H: Begg's publication bias plot of publication bias for studies regarding overexpressed DcR3 and TNM stage of BRCA in the meta-analysis.

eration and viability, while DcR3 siRNA showed a much stronger effect. Thus, DcR3 could inhibit the cell growth of BRCA cells, as proved by both neutralization antibody and siRNA detected by three methods.

We were also curious about the impact of DcR3 on the apoptosis of BRCA cells. The fluorescent caspase-3/7 assay revealed that the caspase-3/7 activity was increased a little with the treatment of DcR3 neutralization antibody. And paralleled to the different effect of cell growth inhibition by neutralization antibody and siRNA, DcR3-siRNA led to much more potent enhancing impact on caspase-3/7 activity in BRCA cells (**Figure 1D**). Furthermore, the impact on cell apoptosis was verified under microscope as observed with Hoechst 33342 and PI double fluorescent staining (**Figure 2**).

Results from meta-analysis

Totally, 469 articles were retrieved primarily. 363 of those articles were omitted after three investigators reviewing the titles and abstracts, since these studies were based on other disease but not BRCA, or they were non-human experiments. Subsequently, another 71 articles were omitted because of duplication or they did not include relevant consequences. Ultimately, five eligible studies [22, 29-32], including the study performed by our own group [32], were included in this meta-analysis with 471 participants. The major information of all five articles was outlined in **Table 1** and **Figure 3**. IHC was the only test method for all included studies (**Figure 4**).

In the meta-analysis, we evaluated the association between DcR3 expression and the risk, as well as clinicopathological parameters representing disease progression of BRCA. Overexpression of DcR3 was strongly correlated with the occurrence of BRCA (OR=19.35, 95% CI: 4.46-84.03, Figure 5A) with z=-0.24 (continuity corrected), Pr>|z|=1.000 (continuity corrected) from Begg's funnel plot (P=1.000, Figure 5B). As shown in Figure 5C and 5D, when concerning the relationship between DcR3 expression level and grade of differentiation, higher expression of DcR3 tended to occur in poorly differentiated BRCA (OR=3.56, 95%



Figure 6. Clinical significance of DcR3 genomic alteration from TCGA in patients with breast cancer (BRCA). A: The OncoPrint of genetic alterations of DcR3, including amplification, mRNA upregulation, and missense mutation, was shown in BRCA by cBioPortal (www.cbioportal.org). B: Overall survival (OS, P=0.523). C: Disease free survival (DFS, P=0.838). Kaplan-Meier Estimate was used for the survival analysis.

CI: 0.79-16.00) and Begg's funnel plot revealed z=1.04 (continuity corrected), Pr>|z|=0.296 (continuity corrected, P=0.296). Next, we also found that upregulation of DcR3 was remarkably associated with lymph node metastasis of BRCA (OR=5.10, 95% CI: 2.25-11.59, Figure **5E**) with z=0.24 (continuity corrected), Pr>|z| =0.806 (continuity corrected) as calculated by Begg's funnel plot (P=0.806, Figure **5F**). Finally, a potent correlation was observed between overexpression of DcR3 and clinical TNM stage in BRCA (OR=3.73, 95% CI: 0.73-19.09, Figure **5G**) and Begg's funnel plot showed z=-0.34 (continuity corrected), Pr>|z|=1.000 (continuity corrected, P=0.296, Figure **5H**).

Results from TCGA

Besides the protein expression level, other genomic alterations of DcR3 could also affect 11042

the clinical progression of BRCA. Thus, we further analyzed the genomic alterations of DcR3 from TCGA data. Genetic alteration of 9% was noted based on TCGA database among 1105 cases of BRCA via cBioPortal, including amplification, mRNA upregulation and missense mutation (**Figure 6A**). However, no difference of DFS or OS existed between patients with and without genomic alterations (both P>0.05, **Figure 6B, 6C**).

Discussion

TNFRSF6B/DcR3 belongs to the TNFR superfamily, which has been reported to act as the decoy receptor for FasL, LIGHT and TL1A [1-11]. Since no transmembrane structure exists in amino acid sequence of DcR3, DcR3 is a secretory protein. DcR3 has been revealed that it is capable of sheltering cancer cells from immune surveillance because it plays a vital part in the inhibition of the host anti-tumor immunity, hence DcR3 becomes an anti-inflammatory and anti-apoptotic factor. For instance, treatments of DcR3 protein pronouncedly enhanced survival in septic mice in

vivo, as well as remarkably decreased the inflammatory response and reduced lymphocyte apoptosis in the thymus and spleen of septic mice. Therefore, DcR3 protein might be valuable in the clinical treatment of sepsis [6]. Besides the impact on inflammation, overexpression of DcR3 immunoreactivity was observed to be closely related to a higher ratio of the pathological concordance of acute T cellmediated rejection in renal tubular epithelial cells (RTECs). And DcR3 expression in kidneys is pronouncedly related to allograft survival after kidney transplant rejection [33]. Moreover, the clinical significance and therapeutic potential of DcR3 in cancers attracted the attention of scientists, including in BRCA. Ge et al [23] explored the functional effects of DcR3 on BRCA MCF-7 and MDA-MB-231 cell lines in vitro. In their study, anti-DcR3 hammerhead Int J Clin Exp Pathol 2016;9(11):11035-11045

ribozyme transgenes were constructed and transfected into BRCA MCF-7 and MDA-MB-231 cells to set up DcR3 knock-down cell sublines. The invasive capacity of the DcR3 knock-down MCF-7 cells was markedly inhibited when compared with pEF6 control cell lines. Moreover, the migration capacity in MCF7DcR3KO was strongly suppressed than MCF7pEF6. The study performed by Ge et al [23] indicated that DcR3 could be an oncogene in BRCA, which influence the invasion and migration of tumor cells. In the present study, we focused on the impact of DcR3 on the cell growth and apoptosis of BRCA cells. A noticeable inhibitory effect of both DcR3 neutralization antibody and siRNA was observed. Similarly, both DcR3 neutralization antibody and siRNA could yield caspase-3/7 activation and apoptosis induction in BRCA cells in vitro, which further confirmed the oncogenic role of DcR3 in BRCA and suggested the therapeutic potential of DcR3 as a molecular tool in the future treatment of BRCA patients.

When concerning the clinicopathological significance of DcR3 in BRCA, only Wu et al published one paper in PubMed and they found that aberrant expression of DcR3 in human BRCA was relevant to lymphangiogenesis [22]. Unfortunately, they did not investigate the relationship between DcR3 expression and the disease deterioration of BRCA. Meanwhile, some Chinese articles published the data on the clinical role of DcR3 in BRCA. To obtain a more comprehensive overview of the clinicopathological significance of DcR3 in BRCA, we performed a meta-analysis with five papers being involved [22, 29-32], including one study of our own group [32]. The meta-analysis showed that upregulation of DcR3 was closely related to the occurrence of BRCA. Moreover, overexpression of DcR3 in BRCA was also strongly associated with tumor cell differentiation, lymph node metastasis and clinical TNM stage, which demonstrates that DcR3 may play a pivotal part not only in the tumorigenesis but also in the progression of BRCA.

Interestingly, TCGA data with RNA-seq revealed that several genomic alterations of DcR3 were detected in BRCA tissues, including amplification, mRNA upregulation and missense mutation. However, the relationship between these genomic alterations and the mechanism of BRCA tumorigenesis and progression remains largely unknown and thus it is required to be further studied in the future. Previously, we also found that in hepatocellular carcinoma tissues, DcR3 level was negatively related to a microR-NA, miR-152, which also gains the complementary sequence of 3'-untranslated region of DcR3 [25]. This suggests a possibility that DcR3 could also act as the target gene of some microRNAs in BRCA tissues, which also needs further investigation.

Conclusions

Taken together, the current observations confirm that DcR3 neutralization antibody and DcR3-siRNA can directly suppress cell growth and induce cell apoptosis *in vitro*. The overexpression of DcR3 was also detected in BRCA, which suggests that the abnormal expression of DcR3 might be associated with the malignant aggressiveness and progression of BRCA. DcR3 may become a prospective remarkable target for the molecular therapeutic strategies of BRCA. But the specific pathway and precise molecular mechanism of DcR3 in BRCA carcinogenesis and deterioration remains to be further explored.

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Disclosure of conflict of interest

None.

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