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DETECTION AND IDENTIFICATION OF D6 AND DUFFY ANTIGEN/RECEPTOR FOR CHEMOKINES (DARC) BY ANTIBODY BASED ASSAYS

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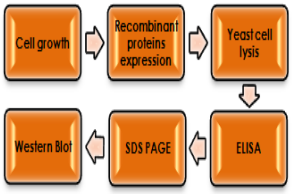

Abstract

D6 and DARC are decoy chemokine binding receptors. They had been studied extensively in recent years for their role in breast cancer studies. The purpose of this study was to improve some critical steps in Western Blot in order to detect and identify recombinant D6 and DARC which has been expressed intracellularly by *Pichia*-GS115. Total proteins of *Pichia*-GS115 were obtained by breaking the yeast cells using acid-washed glass beads. Then, the lysates were treated with treatment buffer before electrophoresed on SDS-polyacrylamide gel. Western Blot was carried out after SDS PAGE to detect and identify the presence of recombinant D6 and DARC. Several parameters of Western Blot were studied and improved to enhance the specificity of results obtained. It was found that blocking of nitrocellulose membrane with 3% (w/v) skimmed milk at room temperature for 1 hour, incubation of primary antibody at the dilution of 1: 300 at room temperature for 16 hours and incubation of secondary antibody at the dilution of 1: 1000 at room temperature for 1 hour were able to enhance the sensitivity and specificity of Western Blot results. With the aid of prestained protein ladder as a marker, recombinant D6 and also DARC were shown as clear and precise bands at the range of molecular weight of 50-60 kDa on developed films using ECL substrate. The film exposure time was found to be 5 minutes for D6 and 10 minutes for DARC. As a result, the studies showed that improved Western Blot condition detected and identified the presence of recombinant D6 and DARC in the cell pellets of *Pichia*-GS115.

Keywords: D6, DARC, Western Blot, Pichia-GS115

Abstrak

D6 dan DARC adalah penarik reseptor pengikat kemokina. Mereka telah dikaji secara meluas dalam tahun-tahun kebelakangan ini atas peranan mereka dalam kajian kanser payudara. Tujuan kajian ini adalah untuk memperbaiki beberapa langkah penting dalam pemblotan Western untuk mengesan dan mengenalpasti D6 dan DARC rekombinan yang telah dihasilkan dalam sel Pichia-GS115. Protein dalam sel Pichia-GS115 diperolehi dengan memecahkan sel-sel yis menggunakan manik kaca yang telah dibasuh dengan asid. Kemudian, lisat sel yis dirawat dengan penimbal rawatan sebelum dielektroforesiskan pada gel SDS-poliakrilamida. Pemblotan Western dijalankan selepas SDS PAGE untuk mengesan dan mengenalpasti kehadiran D6 dan DARC rekombinan. Beberapa parameter pemblotan Western telah dikaji dan diperbaiki untuk meningkatkan spesifisiti keputusan yang diperolehi. Didapati bahawa menyekat membran nitrocellulosa dengan 3% (w / v) susu skim pada suhu bilik selama 1 jam, pengeraman antibodi pertama pada pencairan 1: 300 pada suhu bilik selama 16 jam dan inkubasi antibodi skunder pada pencairan 1: 1000 pada suhu bilik selama 1 jam dapat meningkatkan sensitiviti dan spesifisiti keputusan pemblotan Western. Dengan bantuan pembaris protein berwarna sebagai penanda, D6 dan juga DARC rekombinan ditunjukkan sebagai jalur yang jelas

dan tepat pada julat berat molekul 50-60 kDa pada filem dengan menggunakan substrat ECL. Masa pendedahan filem yang diperolehi ialah 5 minit untuk D6 dan 10 minit untuk DARC. Hasilnya, kajian menunjukkan bahawa keadaan pemblotan Western yang diperbaiki mampu mengesan dan mengenalpasti kehadiran D6 dan DARC rekombinan dalam pelet sel Pichia-GS115.

Kata kunci: D6, DARC, Pemblotan Western, Pichia-GS115

1.0 INTRODUCTION

In tumor microenvironment, the presence of chemokines and chemokine receptors affects tumor development in various different ways. The binding of a chemokine receptor to the respective chemokine will activate typical signaling pathway. Thus, the complex network of chemokines and chemokine receptors in tumor microenvironment are implicated in the process of cancer cell survival, proliferation and metastasis [1, 2, 3, 4, 5].

However, there are certain chemokine receptors which act atypically. These receptors do not activate typical signaling pathway upon binding with specific ligands, but intercept the pathway and neutralize the action of the respective chemokines. This kind of chemokine receptors are known as scavenger proteins. D6 and DARC are the examples of these decoy chemokine receptors [4, 5, 6, 7, 8].

D6 binds to most of the CC chemokines whereas DARC binds to CC and CXC chemokines [4]. To date, the overexpression of D6 and DARC in human breast cancer cells had been reported to downregulate CCL2 level and subsequently inhibit breast cancer cell proliferation and metastasis *in vivo* and *in vitro* [6, 7, 8, 9, 10].

In this study, several critical steps of Western Blot were studied and improved to detect and identify the expression of recombinant D6 and DARC in *Pichia pastoris* GS115.

2.0 METHODOLOGY

2.1 Cell Growth

The inoculum was prepared by growing single colony of recombinant *Pichia*-GS115 in 5 ml of YPD medium overnight at 30°C. The overnight culture was inoculated to 25 ml of buffered glycerol-complex medium, BMGY (1% (w/v) yeast extract, 2% (w/v) peptone, 100 mM potassium phosphate (pH 6.0), 1.34% (w/v) YNB, 4 × 10-5% (w/v) biotin and 1% (v/v) glycerol) to generate biomass. Then BMGY culture was inoculated to 50 ml of buffered methanol-complex medium, BMMY (1% (w/v) yeast extract, 2% (w/v) peptone, 100 mM potassium phosphate (pH 6.0), 1.34% (w/v) YNB, 4 × 10-5% (w/v) biotin and 0.5% (v/v) methanol). The BMMY culture was incubated at 16°C in a 500 ml Erlenmeyer flask, agitated at 250 rpm for 120 hours. Induction with methanol was carried out every 24 hours. This was done by adding filter sterilized absolute methanol to the culture medium to the final concentration of 0.5% (v/v) to maintain induction.

2.2 Preparation of Protein Extracts

Pichia GS115-D6 and GS115-DARC were harvested by centrifuging the cultures at 4400 rpm, 4°C for 10 minutes. The cell pellet was suspended in breaking buffer (50 mM sodium phosphate (pH 7.4), 1 mM PMSF (phenylmethylsulfonyl fluoride), 1 mM EDTA and 5% glycerol) and added with equal volume of sterile glass beads (size 0.5 mm). The cell suspension was vortexed at high speed for 30 seconds and then incubated on ice for 30 seconds. It was repeated for 10 cycles. After that, the cell suspension was centrifuged at 12,000 rpm for 5 minutes to pellet the cell debris. Collected supernatant was spun again at 12,000 rpm at 4°C for 30 minutes to spin down insoluble proteins. Again, the supernatant was collected and used for intracellular protein analysis.

2.3 ELISA

Intracellular proteins were subjected to ELISA analysis for D6 and DARC quantification. For ELISA, about 100 µl of samples in coating buffer (0.53% Na_2CO_3, 0.42% (w/v) $NaHCO_3$) were coated on ELISA plate (NUNC Immnuno ™ Maxisorp). The plate was incubated at 4°C without shaking. After an overnight incubation, the plate was washed with PBS-T (Phosphate buffer saline with 0.05% (v/v) Tween 20, pH 7.2) for three times. Bovine serum albumin at 3% (w/v) was used as blocking reagent. The plate was blocked at room temperature for 1 hour. After washing the blocked wells with PBS-T for three times, anti-DARC (mice IgG, Abnova) was diluted in blocking buffer at 1:500, 100ul of the diluted antibody was added to the wells and incubated in the dark for 1 hour at static condition in room temperature. Wells were washed with PBS-T for 6 times after incubation with primary antibody. Secondary antibody, goat anti-mice IgG (KPL), diluted in blocking buffer (1: 5000) was added to the wells and incubated in the dark for 30 minutes, without shaking at room temperature. Again, the wells were washed

for 6 times with PBS-T after the incubation with secondary antibody. Then, 100 µl of TMB substrate was added to each well. The plate was agitated in the dark at 180 rpm for 10 minutes at room temperature. The reaction was terminated by adding 100 µl of 1M H_3PO_4. The absorbance was measured at 450 nm. In this experiment, triplicates were carried out; average of the triplicate readings was reported.

2.4 Western Blot

ELISA quantified protein sample was electrophoresed on SDS PAGE [11]. The gel was blotted onto nitrocellulose paper (0.45 µm) under a semi-dry condition by Trans-blot Turbo (Bio-Rad). Blocking was performed at room temperature with gentle rocking. Then, washing of nitrocellulose membrane was carried out for 5 minutes for 3 times with Tris-buffered saline with 0.05% (v/v) Tween 20 (TBS-T). The nitrocellulose membrane was incubated with primary antibody (anti-DARC, IgG or anti-D6, IgG) diluted in blocking buffer. After 3 times of 5 minutes washing, incubation with horseradish peroxidase (HRP) conjugated secondary antibody (goat anti-mice IgG, diluted in blocking buffer) was performed at room temperature with gentle rocking. After the final washing (10 minutes for 3 times), the nitrocellulose membrane was exposed to film in a dark room with the aid of enhanced chemiluminescence (ECL) substrate.

2.4.1 Types of Blocking Reagent

Bovine serum albumin and skimmed milk at 3% (w/v) were used as blocking reagent. Tris-buffered saline with Tween 20 (0.05% v/v) was used to prepare these reagents. Nitrocellulose membrane was immersed in blocking reagent and rocked gently on a benchtop rocker.

2.4.2 Blocking Duration

Different blocking durations were studied. The nitrocellulose membrane was blocked for 30 minutes, 1 hour and 2 hours with blocking reagent. The blocking was carried out on a benchtop rocker, at room temperature.

2.4.3 Dilution of Primary Antibody

Primary antibody used in the Western Blot was specific antibody which binds to the recombinant protein. Mouse anti-D6, IgG (Abnova) was used as primary antibody to bind recombinant D6, and mouse anti-DARC, IgG (Abnova) was used as primary antibody to bind recombinant DARC. Different dilutions of primary antibody had been studied. The antibody had been diluted at 1:100, 1: 200, 1: 300, 1: 400, 1: 500 and 1:1000. Blocking reagent was used to dilute the antibody to desired concentration.

2.4.4 Incubation Duration Of Primary Antibody

Nitrocellulose membrane was immersed in primary antibody solution for different duration, namely 4 hours, 16 hours and 24 hours. The membrane was rocked on a benchtop rocker, at room temperature.

2.4.5 Dilution of Secondary Antibody

Goat anti-mouse IgG (KPL) was used as a secondary antibody in the Western Blotting of recombinant protein D6 and DARC. This horseradish peroxidase labeled antibody was diluted with blocking reagent to different concentrations: 1:500, 1: 1000, 1: 2000, 1: 3000, 1:4000 and 1:5000.

2.4.6 Incubation Duration Of Secondary Antibody

The incubation of nitrocellulose membrane with secondary antibody was carried out for different duration, which is 30 minutes, 45 minutes, 1 hour and 1.5 hours. The incubation was carried out at room temperature, using a benchtop rocker.

2.4.7 Film Exposure Time

Chemiluminescence method was used to detect the signal of enzymatic reaction. Different film exposure time had been studied, namely 2 minutes, 5 minutes, 10 minutes and 20 minutes. The film exposure was carried out in a dark room with the aid of film developer and fixer solution.

3.0 RESULTS AND DISCUSSION

Expression of intracellular recombinant D6 was found to be maximal at 48 hours of incubation time based on the growth profile of *Pichia*-GS115 which was induced with methanol (Figure 1). ELISA analysis showed that the highest yield of recombinant D6 was 121.30 ng/ ml at 48 hours. On the other hand, intracellular expression of recombinant DARC by *Pichia*-GS115 was observed to be highest at 72 hours of incubation time based on expression profile of recombinant DARC in *Pichia*-GS115 (Figure 2). The yield of recombinant DARC was found to be 15.38 ng/ ml. Thus, recombinant D6 expressed for 48 hours and recombinant DARC expressed for 72 hours were collected and used in Western Blot analysis.

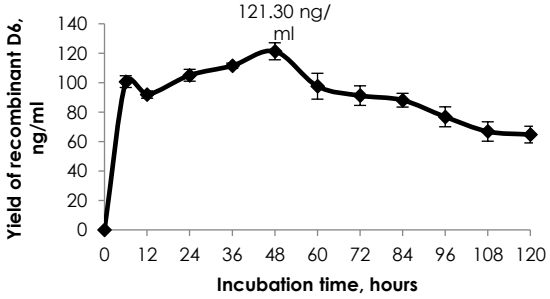

Figure 1 The expression profile of recombinant D6 in *Pichia*-GS115.

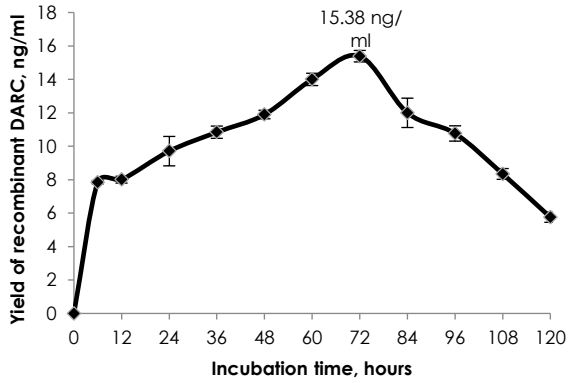

Figure 2 The expression profile of recombinant DARC in *Pichia*-GS115.

Several critical steps of Western Blot were studied and improved in the study. All the improved conditions were pooled and tabulated. Table 1 demonstrates that improved Western Blot conditions for both recombinant D6 and DARC are similar. 3% (w/v) skimmed milk was found to be a more compatible blocking reagent for both DARC and D6 compared to bovine serum albumin. Dankwa *et al*. [12] and Huang *et al*. [13] also reported on the use of milk as blocking reagent in Western Blot analysis of DARC. One hour was found to be the optimum blocking time for recombinant D6 and DARC. 30 minutes of blocking time was too short and did not manage to reduce the background interference while 2 hours of blocking time was found to be too long as a weak signal was observed at the end of Western Blot (result not shown). It is suggested that too long of blocking duration, the antigen on nitrocellulose membrane might have been masked by blocking reagent. Primary antibody at the dilution of 1:300 and secondary antibody at the dilution of 1:1000 was found to be appropriate concentrations for the Western blotting of D6 and DARC. Too low of antibody concentration resulted in a failed blot while too much of antibody produced non-specific bands as a result. Nguyen *et al.* [14] stated in their report that primary antibody was diluted to 500 times while secondary antibody was diluted to 1000 to 5000 times. Cho *et al*. [15] reported that primary antibody used in their Western Blot of D6 was diluted at 1: 1000 while the secondary antibody used was diluted at 1:2000. Huang *et al*. [13] reported that primary antibody was diluted at 1:200 and secondary antibody was diluted at 1:5000 for their Western Blot on DARC. However, Dankwa *et al*. [12] reported primary antibody dilution of 1:1000 and secondary antibody dilution of 1: 10,000 were used in their Western Blot of DARC. Besides, incubation time of primary antibody for Western blot on D6 and DARC was found to be 16 hours. Incubation time of HRP-conjugated secondary antibody for Western blot on D6 and DARC was shorter compared to the primary antibody. Secondary antibody was optimally incubated for an hour prior to the imaging step in the dark room. Last but not least, in the dark room, 5 minutes of film exposure time was found to be sufficient for Western Blot on D6 but 10 minutes was needed for DARC in order to get a strong signal.

Table 1 The summary of improved condition for Western Blot on recombinant D6 and DARC.

Parameters	Improved condition	
	D6	DARC
Blocking reagents	3% (w/v) skimmed milk	3% (w/v) skimmed milk
Blocking duration	1 hour	1 hour
Dilution of primary antibody	1:300	1:300
Incubation duration of primary antibody	16 hours	16 hours
Dilution of secondary antibody	1:1000	1:1000
Incubation duration of scondary antibody	1 hour	1 hour
Film exposure time	5 minutes	10 minutes

Figure 3 showed Western Blot result of recombinant DARC. DARC expressed in *Pichia*-GS115 was observed to have molecular weight at the range of 50-60 kDa. The expressed recombinant protein was detected and identified as DARC by Western Blot using specific antibody, the anti-DARC antibody. The molecular weight of the band shown, matched the calculated molecular weight of amino acids of constructed DARC clone. Protein properties of DARC expressed in *Pichia*-GS115 are to be investigated in further studies.

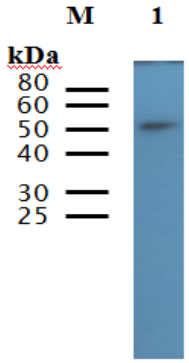

Figure 3 Western Blot result for DARC expressed in *Pichia*-GS115.
Lane M refers to Prestained Protein Ladder (New England BioLabs).
Lane 1 refers to Western Blot result using specific antibody, anti-DARC antibody.

Figure 4 showed Western Blot result of recombinant D6. D6 expressed in *Pichia*-GS115 was also observed to have molecular weight at the range of of 50-60

kDa. The expressed recombinant protein was identified and detected by Western Blot by using specific antibody, the anti-D6 antibody. Like recombinant DARC (Figure 1), the molecular weight of the single band of Figure 2 was also found to be matching with molecular weight calculated from amino acids sequence of the constructed D6 clone. Protein properties of D6 expressed in *Pichia*-GS115 will be investigated in further studies.

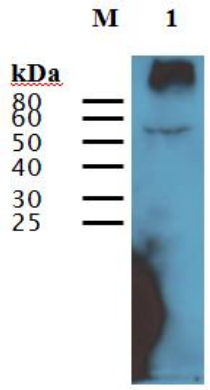

Figure 4. Western Blot result for D6 expressed in *Pichia*-GS115.
Lane M refers to Prestained Protein Ladder (New England BioLabs).
Lane 1 refers to Western Blot result using specific antibody, anti-D6 antibody.

4.0 CONCLUSION

In the present study, upon improving several critical steps of Western Blot, recombinant D6 and DARC were shown as clear and precise bands on the developed films. The bands appeared at the range of molecular weight of 50-60 kDa on developed films using ECL substrate. This matched the molecular weight calculated from the amino acid sequence of the constructed D6 and DARC clones respectively. The use of specific antibody, anti-DARC antibody and anti-D6 antibody in the improved Western Blot, further clarified the identity of expressed proteins as recombinant D6 and DARC.

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