

Full Paper

Molecular cloning and DNA sequencing of Duffy antigen receptor for chemokine (DARC) from MDA-MB-231 breast cancer cells

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Abstract: Duffy antigen receptor for chemokine (DARC) is a promiscuous chemokine receptor and also a receptor for malaria parasite. It has been studied extensively in malaria research and started to gain considerable attention in the field of cancer studies in recent years. In this study the presence of DARC in invasive MDA-MB-231 breast cancer cells was investigated. The gene was amplified with two different sets of primers and different types of DNA polymerases, cloned into bacterial cells and sent for DNA sequencing. After the clones had been sequenced several times, the sequence analysis showed a single base substitution (G→A) at the position of 131 bp, based on nucleotide number of reference sequence which yielded an amino acid change from glycine to aspartic acid. The findings confirm the presence of DARC in MDA-MB-231 cells. The single change of amino acid suggests the identity of DARC in MDA-MB-231 cells as polymorphism which represents different allelic forms of the Duffy blood group.

Keywords: DARC, DNA sequencing, MDA-MB-231, molecular cloning

INTRODUCTION

Duffy antigen receptor for chemokine (DARC) is well known and intensively studied in malaria research. It was found to be the receptor of malaria parasites *Plasmodium vivax* and *Plasmodium knowlesi*. DARC was required for plasmodium infection of erythrocytes [1]. Human red blood cells which are Duffy-negative were reported to be resistant to malaria parasite infection [2-3].

Similar results were reported that the infection of malaria parasites on Duffy-positive individuals happened occasionally but Duffy-negative individuals were not prone to the infection [4-5].

Although DARC is always popular in malaria studies, Yadav et al. [6] reported that it was found to be expressed not only by red blood cells but also by other types of cells including fibroblasts. Meanwhile, DARC study has been extended to cancer research in recent years. DARC was known to be an atypical chemokine receptor that regulates pro-inflammatory cytokines by working together with an array of CC and CXC chemokines [7-8]. These decoy chemokine receptors silent the respective chemokines activities; no signalling is observed upon the binding of DARC with respective chemokine receptors [6-11]. This is because the motif which is associated with G-protein coupling does not exist in the second intracellular loop of DARC [9, 12]. DARC has been found to be an atypical chemokine receptor and the binding of this receptor with its ligand has been found to show negative regulation on tumour progression [13].

In the study of pancreatic ductal adenocarcinoma, DARC was observed to suppress CXCR-2 mediated STAT3 activation and thus inhibited cell proliferation, angiogenesis and invasiveness of human pancreatic ductal adenocarcinoma [14]. The study of association between DARC and epithelial ovarian cancer (EOC) demonstrated that DARC inhibited EOC viability *in vitro* and also inhibited EOC development *in vivo*, thus suggesting DARC as a negative regulator in EOC [9]. Addison et al. [15] reported that the overexpression of DARC by non-small cell lung carcinoma tumour cells was found to increase tumour necrosis significantly. A reduction in tumour cellularity was observed, as compared to the control. Shen et al. [16] reported that the growth of prostate tumours was observed to be greater in DARC-deficient mice compared to the wild-type mice, which carried the DARC genes. Wang et al. [17] also reported that the spontaneous pulmonary metastasis has been successfully suppressed by the overexpression of DARC in breast cancer cells. The involvement of DARC in breast cancer has started to draw the attention of researchers [17-19].

In the present study the invasive breast cancer cell line MDA-MB-231 was selected and the existence of DARC in the cell line was investigated. The gene was cloned, sequenced and compared with the previously published DARC sequence in Genbank (accession number: NM_001122951.2).

MATERIALS AND METHODS

Maintenance of MDA-MB-231 Cell Line

A highly invasive human breast cancer cell line, MDA-MB-231 (ATCC, USA), was routinely maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/mL of penicillin and 100 µg/mL of streptomycin in a humidified 5% carbon dioxide incubator. The cells were then used for total RNA extraction or maintained in liquid nitrogen for long-term storage after addition of a freezing medium.

Total RNA Extraction

Commercially available RNA extraction kit (AxyPrep Multisource Total RNA Miniprep kit, Axygen, USA) was used to purify total RNA from the cultured cells. Standard protocol provided by

the manufacturer was followed without any modification. The 80% confluent cells were trypsinised and pelleted with a table-top centrifuge prior to total RNA purification steps.

Synthesis of cDNA

Total RNA (200 ng/ μ L) was reverse transcribed into cDNA using cDNA synthesis kit (Fermentas, USA). Standard protocol provided by the manufacturer was strictly followed without any modification. The total cDNA was used in polymerase chain reaction (PCR) for the generation of full-length DARC sequence using specific primers.

Design of Specific Primers

The published sequence of DARC with accession number of NM_001122951.2 was used as a reference gene. Primers were designed based on the reference sequence by Primer-Blast from National Center for Biotechnology Information (NCBI). The length of the published sequence is 1258 bp. Forward primer was designed to hook from 176 bp to 198 bp nucleotides of the reference sequence, flanking the coding region. The forward primer was also designed to consist of start codon to enable the initiation of coding region in PCR. The presence of ATG codon was to make sure the amplicon consists of initiation codon, which serves as part of the yeast consensus sequence.

For reverse primer, it was designed to hook from 1168 bp to 1189 bp on the same sequence but stop codon was not included in the reverse primer. This is to enable the fusion of gene of interest with myc epitope and His-tag sequence at the C terminal in the expression vector later on. Moreover, restriction enzyme sequences were incorporated in the primer pairs for cloning purpose. For the addition of different restriction sites, two sets of primer pairs with different restriction sequences, different melting temperatures and guanine-cytosine contents were designed. The gene-specific primers are shown in Table 1 (restriction sequences are in bold).

Table 1. Specific primer sets used in PCR

Set	Primers and sequences	Melting temperature
A	DARC_Forward_SacII 5'-CGCC GCGG ATGGCCTCCTCTGGGTATGTCCT-3'	76.5
	DARC_Reverse_ApaI 5'-GCGGGCCCGGATTTGCTTCCAAGGGTGTCCA-3'	69.6
B	DARC_Forward_PstI 5'-GCCTG CAGT AATGGCCTCCTCTGGGTATGTCCT-3'	73.4
	DARC_Reverse_XbaI 5'-GCT CTAGAT AGGATTTGCTTCCAAGGGTGTCCA-3'	69.6

One-step Reverse-transcription (RT) PCR with Pfu DNA Polymerase

Maxime RT-PCR premix kit (iNtRON Biotechnology, Korea) was used to convert total RNA to cDNA and the cDNA was then used as a template for exponential amplification using PCR, continuously in a single reaction tube. In the premix reaction mix, total RNA purified from MDA-MB-231 cells were used as template. Pfu DNA polymerase (BioAtlas, Estonia) was added to proofread the PCR products. Two different sets of gene-specific primers (Set A and Set B) as mentioned in Table 1 were used in the PCR reaction. PCR product amplified by primer set A was termed “DARC” while that by primer set B was termed “DARC α ”. The hot start at 45°C for 30 min. was performed on 20 μ L of one-step RT-PCR mixture initially, followed by initial denaturation of DNA at 94°C for 5 min. The reaction was then cycled 35 times at 94°C for 30 sec., annealed at 62°C for 30 sec. and elongated at 72°C for 2 min. The reaction was extended at 72°C for 10 min. towards the end of PCR. Negative control reaction consisted of everything as mentioned in the sample reaction except the sample of total RNA.

PCR with Phusion DNA Polymerase

PCR with Phusion DNA polymerase was carried out by using Phusion high-fidelity PCR master mix (Thermo Fisher Scientific, USA). In this experiment synthesised total cDNA (as mentioned above) was used as template and specific primers mentioned in Table 1 were used to generate full-length DARC sequence. Both of the primer sets were high melting temperature primer pairs so instead of conventional three-step cycling, a two-step cycling profile was chosen. This was recommended in the master mix user manual. Besides, as mentioned in the user manual, omitting primer annealing step by choosing a two-step protocol reduced the chance of getting non-specific products.

In this PCR a final volume of 20 μ L PCR mixture was used. The ready-made PCR master mix was added with 0.4 μ L of total cDNA (800 ng/ μ L), 1 μ L each of forward and reverse primers respectively, and topped up with sterile distilled water to the final volume. Two-step cycling consisted of an initiation at 98°C for 30 sec., denaturation at 98°C for 5 sec., elongation at 72°C for 40 sec. (repeated for 30 cycles) and a final extension at 72°C for 5 min. Negative control was done by replacing template cDNA with sterile distilled water in the 20 μ L reaction mixture.

Recombinant Plasmid Construction

PCR products amplified with Pfu DNA polymerase and Phusion DNA polymerase were gel-electrophoresed through 1% agarose gel respectively. Gel electrophoresis was carried out at 100 V, 60 min. A100-bp Plus DNA ladder (Fermentas, USA) was used as a marker. After gel purification (with gel purification kit, Fermentas, USA), samples amplified with Pfu DNA polymerase were ligated into TA cloning (rapid cloning or T cloning) vector, pTZ57R/T (Fermentas, USA). Ligation was performed at 20°C by using T4 ligase (Fermentas, USA). After 2 hr, the reaction was heat-inactivated at 65°C for 10 min. prior to further experiments. The recombinant TA vectors were termed TA-DARC and TA-DARC α for intracellular and extracellular expression respectively.

On the other hand, as PCR with Phusion DNA polymerase generated blunt end products, the gel-purified samples were ligated into yeast expression vectors, pPICZ A and pPICZ B alpha (Invitrogen, USA) directly. The ligation mix was incubated for 2 hr at 20°C and heat-inactivated at 65°C for 10 min. The recombinant yeast expression vectors were termed pPICZ A-DARC and pPICZB α -DARC α .

Electro-transformation of *E. Coli*

Upon ligation, recombinant vectors were cloned into bacterial cells. Electrocompetent TOP 10 cells (Invitrogen, USA) were used for the transformation process. Around 2 μ L of samples were added to 50 μ L of electrocompetent bacterial cells. The cells were pulsed in an electroporation cuvette (0.2 cm) using an electroporator (Bio-Rad, USA). Cells were recovered by addition of 1 mL Luria-Bertani (LB) broth immediately after pulsing. After that, the cells were agitated for 30 min. at 37°C, 750 rpm using a thermoblock shaker. The transformed cells were plated on low-salt LB agar containing Zeocin (Life Technologies, USA) and incubated at 37°C overnight. Positive clones were then selected from the agar plates and further propagated overnight in low-salt LB broth with Zeocin. Plasmid purification was carried out by using plasmid extraction kit (Fermentas, USA). Restriction analysis with respective restriction enzymes was carried out on the purified clones to check the presence of the insert. Positive clones with insert were sent for DNA sequencing (First Base Laboratories Sdn. Bhd., Singapore).

Sequencing of DARC cDNA

Two sequencing reactions were carried out for each of the clones to confirm the sequencing results. More than 3 clones were sequenced for each of the clones generated either by one-step RT PCR with Pfu DNA polymerase or PCR by Phusion DNA polymerase. For recombinant TA vector, M13F_20 was used as forward sequencing primer while M13R_pUC_26 was used as reverse sequencing primer. On the other hand, for recombinant yeast expression vector, 5'AOX1 and 3'AOX1 sequencing primers (Invitrogen, USA) were used. Nucleotide sequence from Genbank (NCBI, NIH, USA) with accession number NM_001122951.2 was used as a reference. The sequences of inserts were analysed by BioEdit Sequence Alignment Editor (Ibis Therapeutics, USA). The sequences were also aligned with the coding region of reference sequence and analysed with ClustalW.

RESULTS AND DISCUSSION

DARC, which is one of the candidates of atypical chemokine receptors, is also known as CD234 or atypical chemokine receptor-1 (ACKR1). Besides being described as a blood group antigen with its role being studied extensively in red blood cells, it was also reported that DARC modulates the bioavailability of serum chemokines and at the same time serves as an entry receptor for *P. vivax* and *P. knowlesi*, enabling infection of red blood cells by these pathogens [20].

Variation of DNA polymerase in PCR

Instead of common PCR, current study demonstrated that DARC can be easily amplified by one-step RT PCR (with Pfu DNA polymerase and I-star *Taq* polymerase in the premix reaction) with a simple manipulation by initiating the PCR with a hot start at 45°C for 30 min. The purpose of having hot start was to reduce non-specific target amplification [21]. As reported by Melissis et al. [22], Pfu DNA polymerase is a high-fidelity proofread enzyme which may minimise the errors of amplified products. It is a member of DNA polymerase II, proofreads with 3'·5' exonuclease activity and corrects errors during the polymerisation step (5'·3' activity) [23-24]. I-star *Taq* is a *Taq* polymerase which catalyses the polymerisation reaction of DNA bases during PCR cycles, and at the same time the tendency of adding polyA tail to the C terminal of DNA sequence allows the cloning of PCR products into TA vector. These two polymerases complement each other to generate a PCR product with polyA tail at a high-accuracy rate.

Apart from that, another PCR using Phusion DNA polymerase was carried out as well by using total cDNA from MDA-MB-231 cells as template. Different pairs of primers (Table 1) were used in PCR reactions including one-step RT PCR and PCR using Phusion DNA polymerase to amplify DARC from MDA-MB-231 cells. Phusion DNA polymerase, which generates blunt end PCR products, contains a novel *Pyrococcus*-like enzyme with processivity enhancing domain. It was claimed to possess an error rate sixfold lower than Pfu DNA polymerase.

One-step RT PCR with Pfu DNA Polymerase

According to the position of gene-specific primer pairs on reference sequence from Genbank, which flanked the coding region of DARC, the size of amplicons from one-step RT-PCR was expected to be in the range of 1000-1200 bp, considering the addition of restriction sequences on 5' and 3' of the sequence. The size of PCR products of one-step RT-PCR with Pfu DNA polymerase was 1032 bp for DARC and 1034 bp for DARC α . The amplicons were thus observed to be in the range of 1000-1200 bp on the ethidium bromide-stained agarose gel, with 100 bp Plus DNA ladder (Fermentas, USA) as a reference (Figure 1). The size of the PCR product matched the estimated length of DARC sequence and suggested the presence of DARC in MDA-MB-231 cells.

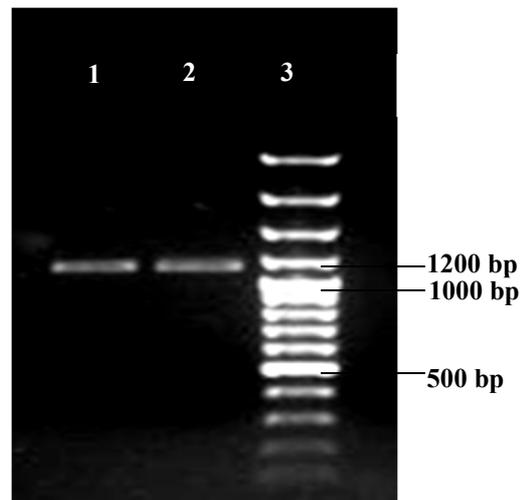
PCR with Phusion DNA Polymerase

Apart from one-step RT-PCR, another PCR with Phusion DNA polymerase was carried out by using total cDNA from MDA-MB-231 cells as template. The two-step cycling thermoprofile showed that the PCR product ranged between 1000-1200 bp, corresponding to the expected size of DARC sequence, which was 1032 bp for DARC and 1034 bp for DARC α (Figure 2). Moreover, the PCR result suggested the presence of DARC in MDA-MB-231 cells.

Recombinant Plasmid Construction

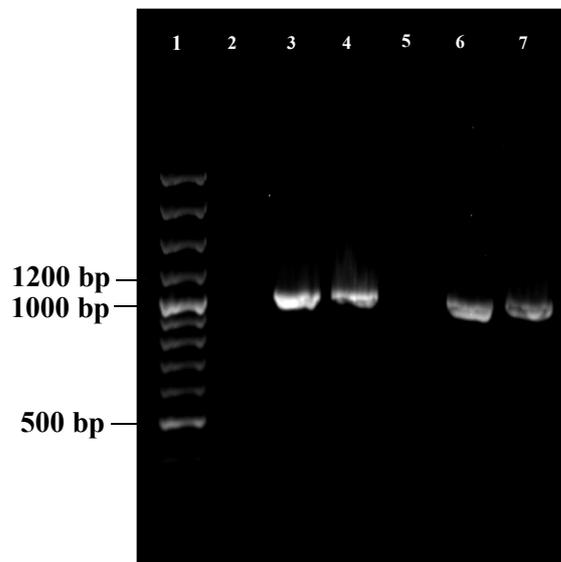
Figure 3 shows the result of restriction analysis for recombinant TA clones. Restriction enzymes *Sac*II and *Apa*I were used to digest TA-DARC, whereas restriction enzymes *Pst*I and *Xba*I were used on TA-DARC α . The double digestions gave two intense bands in a single lane on 1%

agarose gel. The bands correspond to the empty TA vector (2886 bp) and estimated sizes of amplified DARC sequence (1032 bp for DARC and 1034 bp for DARC α).



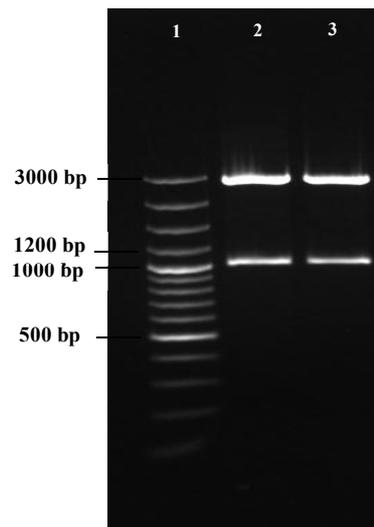
Lane 1: DARC amplified by primer Set A
 Lane 2: DARC α amplified by primer Set B
 Lane 3: 100 bp Plus DNA ladder (Fermentas, USA)

Figure 1. DARC sequence amplified with Pfu DNA polymerase



Lane 1: 100 bp Plus DNA ladder (Fermentas, USA)
 Lane 2: Empty lane
 Lane 3: DARC amplified by primer Set A (1st replication)
 Lane 4: DARC amplified by primer Set A (2nd replication)
 Lane 5: Empty lane
 Lane 6: DARC α amplified by primer Set B (1st replication)
 Lane 7: DARC α amplified by primer Set B (2nd replication)

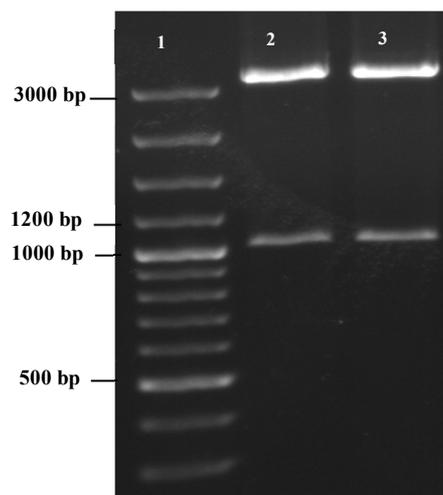
Figure 2. DARC sequence amplified with Phusion DNA polymerase



Lane 1: 100 bp Plus DNA ladder (Fermentas, USA)
 Lane 2: Products of double digestion on TA-DARC
 Lane 3: Products of double digestion on TA-DARC α

Figure 3. Restriction analysis of TA-DARC and TA-DARC α

On the other hand, double digestion performed on pPICZA-DARC and pPICZB α -DARC α also gave two intense bands on ethidium bromide-stained 1% agarose gel. pPICZA-DARC was digested by SacII and ApaI whereas pPICZB α -DARC α was digested by PstI and XbaI. The two bands observed (Figure 4) correspond to empty yeast expression vector and estimated size of amplified DARC sequence. The sizes of pPICZ A and pPICZ B α were 3329 bp and 3597 bp respectively. The sizes of amplified DARC and DARC α were 1032 bp and 1034 bp respectively.



Lane 1: 100 bp Plus DNA ladder (Fermentas, USA)
 Lane 2: Products of double digestion on pPICZA-DARC
 Lane 3: Products of double digestion on pPICZB α -DARC α

Figure 4. Restriction analysis of pPICZA-DARC and pPICZB α -DARC α

The results of restriction analysis for recombinant TA vectors and recombinant yeast expression vectors (Figure 3 and Figure 4) showed the presence of inserts in the vector plasmids. In other words, the cloning of gene of interest into the respective plasmids was a success as double digestion of pPICZA-DARC and pPICZB α -DARC α showed the presence of inserts in the plasmids.

Sequencing of DARC cDNA

As mentioned in the methodology, the published sequence of DARC with accession number NM_001122951.2 (Genbank) was used as a reference for the analysis of DNA sequencing results. The reference sequence was DARC transcript variant 1 from erythrocytes and encoded for an acidic glycoprotein. It is a linear sequence with a total of 1258 bp of nucleotides. The length of coding DNA sequence is 1017 bp with the open reading frame led by ATG (methionine) at position 176 bp and closed by a stop codon TAG at position 1192 bp. The open reading frame codes for 338 amino acids.

Sanger sequencing, which is a DNA sequencing method with the use of chain terminating inhibitors [25] is known to be accurate in DNA sequencing compared to other methods such as Maxam Gilbert method [26]. Two reactions (forward and reverse sequencing) were performed to completely sequence the full-length of sample DNA. DNA sequencing results of TA-DARC, TA-DARC α , pPICZA-DARC and pPICZB α -DARC α analysed by BioEdit Sequence Alignment Editor (Ibis Therapeutics, USA) are shown in Table 2. Different lengths of inserts were observed from different recombinant vectors due to the incorporation of different restriction sites on the N and C terminals of the respective sequences. Besides, inserts cloned into yeast expression vectors (pPICZ A and pPICZ B α) were designed to fuse with myc-epitope and polyhistidine tag at the C terminal. Regardless of the size of insert, all of the inserts were shown to cover the 1017 nucleotides of open reading frame of DARC sequence, based on the published DARC sequence (NM_001122951.2) as a reference.

Table 2. Lengths of inserts in different vectors

Recombinant vector	Length of insert
TA-DARC	1032 bp
TA-DARC α	1034 bp
pPICZA-DARC	1032 bp
pPICZB α -DARC α	1034 bp

ClustalW analysis showed that all of the four clones had a perfect nucleotide sequence match with the coding region of the reference sequence except at position 131 bp where a base substitution yielded a different amino acid prediction. The analysis showed a base substitution of adenine (A) for guanine (G) at the position of 131 bp, based on the nucleotide number of the reference coding region sequence. Figure 5 shows that the substitution of adenine for guanine in the nucleotide changes the amino acid from glycine to aspartic acid.

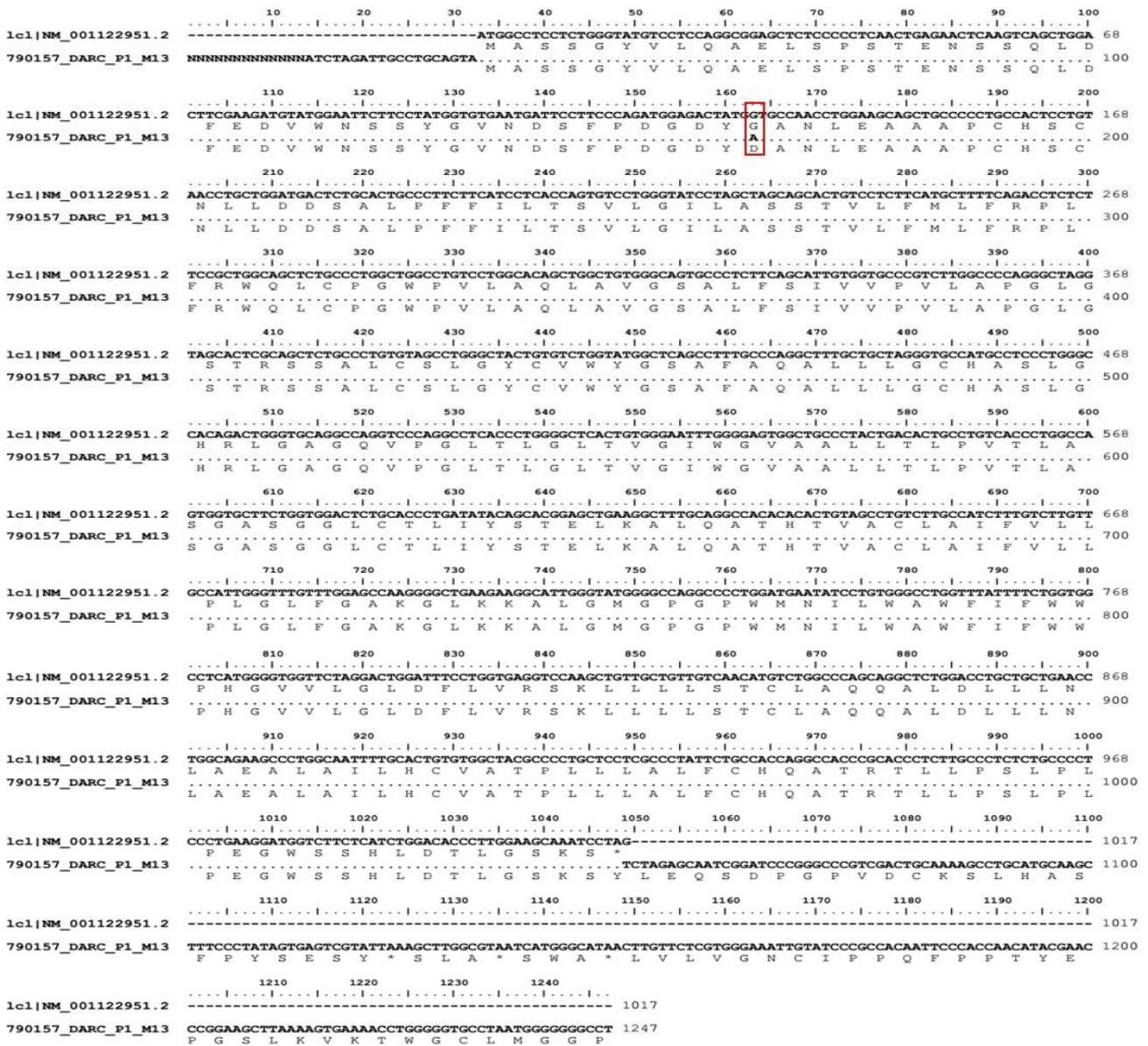


Figure 5. ClustalW analysis shows the alignment result of DNA sequence of one of the clones with the coding region of reference sequence (NM_001122951.2). Nucleotides and the deduced amino acid sequences are shown. DNA sequence of the clone whose nucleotide was identical with the nucleotide of the reference sequence is shown as a dotted line and the changed nucleotide is labelled out (bolded). The changed nucleotide leads to the change in amino acid from glycine to aspartic acid. The stop codon is indicated by an asterisk.

It was observed that the use of different primer pairs with different melting temperature values and guanine-cytosine contents did not influence the outcome of DNA sequencing analysis. Moreover, despite the emphasis on PCR fidelity, neither Pfu nor Phusion DNA polymerases could resolve the base substitution (G→A). The discrepancy was not a sequencing error as different clones had been sequenced and the DNA strands were sequenced several times. With this, it is suggested that the DARC sequence from invasive MDA-MB-231 breast cancer cells, possesses a different nucleotide on 131 bp (nucleotide number corresponds to reference coding region sequence), as compared with DARC sequence from erythrocytes (NM_001122951.2). The single amino acid

difference observed on the amplified DARC sequence could be polymorphism which corresponds to a different allelic form of the Duffy blood group. Neote et al. [12] also reported on a base substitution in cloned DARC antigen from placental RNA at position 479 based on its reference nucleotide sequence (U01839), changing Ala to Thr. Similar amino acid differences near the N-terminus were also reported by Chaudhuri et al [27].

Pogo and Chaudhuri [2] reported that Duffy blood group system consists of four alleles: FYA, FYB, FYBES and FYBWK. The two principal antigens, Fya and Fyb, are produced by FYA and FYB, the codominant alleles. Single amino acid substitution (G→A) was observed at position 42 on the amino acid sequence of the codominant alleles, where in FYA guanine (G) is the base and in FYB adenine (A) is the base. This single amino acid substitution at the amino terminal domain of the protein defines antigen Fya and Fyb. The Fy glycoprotein which carries these Duffy antigenic determinants is biologically significant as it is demonstrated to be the receptor for certain malarial parasites and chemokines. Another characteristic feature of the Duffy system is the wide divergence in distribution of Duffy antigenic determinants among racial groups [28]. For example, Fya is prevalent in Chinese, Japanese and Melanesians but is infrequent in African blacks [29] while Fyb is more abundant in the white population than in Asian and African blacks.

CONCLUSIONS

The study shows that DARC is present in invasive MDA-MB-231 breast cancer cells. DARC sequence amplified by PCR with different types of DNA polymerases and different sets of primers is in perfect matching with DARC sequence from erythrocyte NM_001122951.2 except that a base substitution (G→A) at position 131 bp yields an amino acid change from glycine to aspartic acid. The amplified nucleotide sequence of DARC from MDA-MB-231 cells has been deposited in Genbank with accession number JX081310. This single change of amino acid on the amplified sequence suggests polymorphism which symbolises a different allelic form of Duffy blood group. Further investigation of polymorphism can be carried out in a future study. FY-genotyping test based on the use of allele-specific primers can be applied to detect the major Duffy alleles in clinical practice using PCR amplification to locate mutations in the promoter and the coding sequence of FY.

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