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Mutagenesis Analysis of *ABCB4* Gene Promoter of *Danio rerio*

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ABSTRACT

Zebrafish *abcb4* gene (ortholog to human *ABCB1* gene) serves primarily in multidrug resistance (MDR) mechanism by effluxing chemotherapeutic agents, chemicals, xenobiotics, and numerous anti-cancer drugs out of the cells. This study aims to identify the specific transcription factor binding sites (TFBS) within the promoter region of zebrafish *abcb4* gene and determine the functional roles of these factors in *abcb4* gene expression regulation via mutagenesis analysis. First, primers were designed to target and amplify the promoter region of zebrafish *abcb4* gene through gradient PCR. The zebrafish *abcb4* gene promoter was then cloned into pGL3.0 vector and sent for sequencing. The sequencing results revealed high similarity to zebrafish DNA sequence from clone DKEY-24I24 in linkage group 16, indicating a successful cloning of targeted gene. Thereafter, consensus sequence of zebrafish *abcb4* gene promoter was generated with the length of 1,392 bp which was close to its expected size during primer design (1,500 bp). Using MATCH tool, 155 TFBSs were found within zebrafish *abcb4* gene promoter region. Activator protein 1 (AP-1) TFBS at 1,255 bp was chosen to be mutated through site-directed mutagenesis. Mutagenic primers (forward primer: 5' GGG CAA GGC AGT ATA AAC GTG 3' and reverse primer: 5' TTA TGT TTC TAG GGA TTA CGT CAC 3') were designed to substitute AGT with GGG to remove the AP-1 TFBS. By mutating the zebrafish *abcb4* gene promoter, the MDR phenomenon driven by zebrafish *abcb4* gene can be elucidated and this might provide clues to the development of tumor and malignancy in human. The results from this study may enrich the knowledge in chemotherapy and cancer treatments.

Keywords: ABC transporters gene family, multidrug resistance, site-directed mutagenesis, promoter, xenobiotics, transcription factor

INTRODUCTION

Tumor occurs very commonly in humans, some mammals and even in plants. Generally, tumor can be classified into three main types, namely benign, premalignant and malignant tumors. Azizah *et al.* (2019) reported that the mortality rate among the cancer (malignancy) patients is relatively high in Malaysia despite the vast availability of modern health facilities and medical chemotherapy treatments. This is probably due to the resistance of cancer cells to the chemotherapeutic agents over time.

The resistance to chemotherapeutic agents and some other anti-cancer drugs is driven by the ATP-binding cassette (ABC) transporters in multidrug resistance mechanism. Ferreira, Costa, and Reis-Henriques (2014) discovered the role of ABC proteins in multidrug resistance (MDR) for chemotherapeutic treatments. According to Annilo *et al.* (2006), all the ABC protein subfamilies (except for ABCH subfamily) found in zebrafish correspond to their respective human counterparts. For instance, the zebrafish *abcb4* gene is orthologous to that of the human *ABCB1* gene. Ergo, zebrafish *abcb4* gene is functionally similar to human *ABCB1* gene and they are important in cellular resistance to chemicals (Fischer *et al.*, 2013).

Previously, the *abcb4* gene of zebrafish and Sarawak rasbora (a close family member to zebrafish) has been functionally characterized by Fisher *et al.* (2013) as well as Lim *et al.* (2018a), respectively, and they had come to the same consensus that this gene has significant expressions in the intestinal tract of both species. In addition, the transcriptional analysis performed by Hamli (2019) as well as Lim and Chung (in press) on *abcb4* promoter of zebrafish showed that the zebrafish *abcb4* regulatory region is mainly controlled by SORY, HOMF, BRNF and HOXF. However, available knowledge and findings about zebrafish *abcb4* gene regulation is still

limited at current stage despite the fact that gene regulatory elements like the promoter and enhancer have been proven imperative in orchestrating the activation and deactivation of genes (Hernandez-Garcia & Finer, 2014; Lim, Chung, Chong, & Lee, 2018b; 2019a; 2019b; Liu & States, 2002; Mishra, Dhanda, Siwach, Aggarwal, & Jayaram, 2020; Wang, Cheng, Li, Wu, & Zhao, 2018). Therefore, this study is carried out with the aims to determine the specific transcription factor binding site (TFBS) within the promoter region of zebrafish *abcb4* gene and to conduct mutagenesis analysis on the promoter. Thus, it is hypothesized that zebrafish *abcb4* gene expression and regulation will be affected after mutagenesis.

MATERIAL & METHOD

Total DNA extraction

Fish maintenance, fish harvesting and total DNA extraction (using CTAB method as described by Chung (2018)) were conducted with acquiescence to the permission and guidelines drawn out by the Universiti Malaysia Sarawak Animal Ethics Committee (UNIMAS/TNC(PI)-04.01/06-09(17)). A healthy zebrafish was first euthanized by immersing it in ice-cold water and then the zebrafish was sliced finely on ice. Afterwards, the sliced zebrafish in conical tube was added with 9.5 ml of CTAB lysis buffer before incubation at 65ºC water bath for about 30 min. Next, 10 µl of proteinase K was added and the tube was incubated at 65ºC water bath for another 30 min. Throughout the incubation, the tube was shaken and vortexed intermittently to ensure all the minced zebrafish tissues dissolved completely. Upon the completion of incubation, the lysate was equally aliquoted into several 2 ml microcentrifuge tubes before the addition of an equal volume of Phenol:Chloroform:Isoamyl Alcohol (25:24:1) in fume hood. The tubes were then inverted gently to mix the PCI and lysate, followed by incubation at room temperature for 10 min to allow precipitation of proteins and nucleic acids.

After that, the lysate was centrifuged at 13,000 rpm for 10 min. The supernatant was equally transferred to several new microcentrifuge tubes and equal volume of isopropanol was added to each tube, respectively. The tubes were incubated at room temperature for 30 min to allow DNA precipitation, followed by centrifugation at 13,500 rpm for 10 min. Upon centrifugation, the supernatant was discharged gently, and the DNA pellet was washed with 1 ml of pre-chilled 70% ethanol and centrifuged at 12,500 rpm for 5 min. Then, the DNA pellet was air-dried for 30 min in fume hood. Lastly, the DNA pellet was dissolved in 20 µl of ultrapure water and kept in -20ºC until use.

Gradient polymerase chain reaction (PCR)

Gradient PCR was carried out, emulating that of Lim, Chung, and Hasnain (2020) with several modifications, to determine the optimum annealing temperature for the designed primer pair (forward primer: 5' ATG GTA CCT TTA TTT GAG TGA GTG GCC C 3' and reverse primer: 5' ATG AGC TCT GAA TGA AAG ATA CCT ACC GC 3', designed based on GenBank entry: NC_007127.7) via T100^{TM} Thermal Cycler (BIO-RAD, USA). To perform gradient PCR, PCR master mixture was first prepared as indicated in Table 1.

Table 1. Components of PCR master mixture for zebrafish *abcb4* gene promoter.

Components	Final concentration	Volume (1X)	Volume (4.5X)
Nuclease-free water (ddH_2O)	N/A	14.4 µl	64.8 µl
10X *EasyTaq* buffer (with Mg^{2+})	1X	2.0 µl	9.0 µl
2.5 mM of dNTPs	0.2 mM	1.6 µl	7.2 µl
10 µM of *abcb4* forward primer	0.2 µM	0.4 µl	1.8 µl
10 µM of *abcb4* reverse primer	0.2 µM	0.4 µl	1.8 µl
EasyTaq DNA polymerase	2.5 units	0.2 µl	0.9 µl
Genomic DNA of zebrafish (DNA template)	As required	1.0 µl	4.5 µl
Final volume	**-**	**20.0 µl**	**90.0 µl**

The prepared PCR master mixture was kept on ice to prevent the enzymes and dNTPs from degrading. Next, the PCR master mixture was aliquoted into four pre-labeled PCR tubes (20 µl each) before subjected to thermal cycling for 35 cycles. The thermal cycling conditions were initial denaturation at 95ºC for 3 min, followed by 35 cycles of denaturation at 95ºC for 30 sec, primers annealing between 59ºC and 65ºC for 30 sec as well as extension at 72ºC for 1 min 30 sec. The PCR products were then quality checked through agarose gel electrophoresis (AGE).

Cloning into pGL3.0 Vector

Cloning process (as performed by Jee et al. (2017)) was started with double RE digestion. Wherewith, *Kpn*I and *Sac*I restriction enzymes were used to cut both the zebrafish *abcb4* gene promoter and the pGL3.0 vector (Promega, USA). The restriction digestion mixtures were prepared and then incubated at 37ºC water bath for 16 hr. Upon incubation, the restriction enzymes were inactivated through the incubation at 80ºC water bath for 20 min. Followed by, the inactivated digestion mixtures were analyzed through AGE. Depending on the number of band yields, both the cut zebrafish *abcb4* gene promoter and pGL3.0 vector were subjected to either gel extraction or purification process.

The purified zebrafish *abcb4* gene promoter was then ligated into cut pGL3.0 vector by using 2X Rapid Ligation Buffer. The ligation reaction mixture was prepared by mixing 10 µl of 2X Rapid Ligation Buffer, 2 µl of pGL3.0 vector, 6 µl of purified zebrafish *abcb4* gene promoter and 2 µl of T4 DNA Ligase. Then, the ligation reaction mixture was incubated overnight at 4ºC to ensure maximum number of transformants. On the next day, the ligated pGL3.0 vector with zebrafish *abcb4* gene promoter was transformed into *E. coli* XLI-Blue strain. The transformants were then plated and transformation efficiency was calculated by using the formula shown below. The colonies grown were then screened and verified through colony PCR.

$$\text{Transformation efficiency (transformants per µg)} = \frac{\text{colonies per plate}}{\text{ng of plasmid DNA plated}} \text{ x 1000 ng/µg}$$

Analysis of DNA sequencing result

Upon dsDNA quantification, 15 µl of isolated plasmid DNA (pGL3.0 vector with zebrafish *abcb4* gene promoter) was sent for forward and reverse sequencing. The sequencing results were then confirmed by performing NCBI nucleotide BLAST (BLASTn). The outcomes (gaps, identities, score and coverage) were studied and recorded for data analysis. Subsequently, the sequencing results were compared and pairwise alignment was carried out. Then, the incorrect base calls were edited. Eventually, the consensus sequence of zebrafish *abcb4* gene promoter was generated and subjected to downstream analysis.

Transcription factor binding site analysis

Transcription factor binding site **(**TFBS) analysis was performed using MATCH tool. Firstly, the consensus sequence of zebrafish *abcb4* gene promoter was inputted into MATCH tool. Then, specific matrix and profile were selected. Wherewith, vertebrate was selected as the group of matrices and liver specific profile was set. From the MATCH outcomes, a specific TFBS was then chosen to be mutated. In this study, the TFBS for activator protein 1 (AP-1) was selected and subjected to the downstream mutagenesis analysis.

Mutagenic primer design

Mutagenic primers were designed based on the consensus sequence of zebrafish *abcb4* gene promoter. Since TFBS of AP-1 was chosen to be mutated in this study, thus the AP-1 TFBS located nearest to TATA-box was selected. Then, the mutagenic primers were designed by substituting three bases in the AP-1 TFBS sequence to another bases, thus creating mutation. Consequently, this mutation was expected to lead to the deletion of AP-1 TFBS in the specific region of zebrafish *abcb4* gene promoter. The mutagenic primers (forward and reverse primers) were then sent to Apical Scientific Sdn. Bhd. for primer synthesis.

Site-directed mutagenesis

Firstly, the plasmid template DNA (pGL3.0 vector with zebrafish *abcb4* gene promoter) was subjected to inverse PCR. Wherewith, the master mixture was prepared as depicted in Table 2.

Table 2. Components of inverse PCR master mixture for zebrafish *abcb4* gene promoter.

Components	Volume (1X)	Volume (3.5X)
ddH_2O	14.4 µl	50.4 µl
10X buffer for iPCR	2.0 µl	7.0 µl
2 mM dNTPs	2.0 µl	7.0 µl
Mutagenic forward primer for *abcb4* (10 µmol/µl)	0.4 µl	1.4 µl
Mutagenic reverse primer for *abcb4* (10 µmol/µl)	0.4 µl	1.4 µl
Plasmid template DNA (50 ng/µl)	0.4 µl	1.4 µl
KOD-Plus-DNA Polymerase	0.4 µl	1.4 µl
Total volume	**20.0 µl**	**70.0 µl**

The prepared master mixture was then aliquoted into three pre-labeled PCR tubes (20 µl each). Afterwards, the PCR tubes were subjected to thermal cycling for 7 cycles. The thermal cycling conditions were initial denaturation at 94ºC for 2 min, followed by 7 cycles of denaturation at 98ºC for 10 sec, primers annealing between 47.3ºC and 56.2ºC for 30 sec as well as extension at 68ºC for 7 min. Next, the PCR products were checked through AGE before subjected to *Dpn*I digestion.

For *Dpn*I digestion, 1 µl of *Dpn*I restriction enzyme (10 U/µl) was added to 20 µl of the PCR product. Then, the reaction mixture was mixed gently, spun down and incubated at 37ºC water bath for 1 hr. Then, the *Dpn*I-treated PCR product was subjected to self-ligation process. Wherewith, the ligation reaction mixture was prepared by mixing 2 µl of *Dpn*I-treated PCR product, 7 µl of ddH_2O, 5 µl of ligation high and 1 µl of T4 Polynucleotide Kinase. Then, the ligation reaction mixture was incubated at 16ºC for 1 hr. Next, the self-ligated PCR product was then transformed into *E. coli* XLI-Blue strains and the transformants were then plated. Lastly, the transformants (mutants) were analyzed through DNA sequencing and further verified through TFBS analysis.

RESULTS

Analysis of DNA Sequencing Results

The forward sequencing result showed 94% homology to the zebrafish DNA sequence from clone DKEY-24I24 in linkage group 16 (GenBank accession number: CR388365.10) (Supplementary Figure 1). The E-value obtained is 0.0, which indicates that the amount of alignments with the query sequence that would be expected to occur by chance in the database is zero (Supplementary Table 1). The identities obtained is 717/762 with 1% of gaps (14/762) found. On the other hand, the reverse sequencing result revealed 95% homology to zebrafish DNA sequence from clone DKEY-24I24 in linkage group 16 (Accession number: CR388365.10) (Supplementary Figure 2). The E-value obtained is 0.0 which indicates that the amount of alignments with the query sequence that would be expected to occur by chance in the database is zero. The identities obtained is 561/590 with approximately 0% of gaps (2/590) found (Supplementary Table 2).

TFBS Analysis

The MATCH outcomes revealed 155 transcription factor binding sites within zebrafish *abcb4* gene promoter sequence based on liver-specific profile. In addition, the frequency of sites per nucleotide in liver-specific profile depicted the value of 0.111351. Besides, there were 11 types of TFBS detected within the zebrafish *abcb4* gene promoter sequence under the liver-specific profile, namely HNF-3beta, C/EBPbeta, HNF-1, AP-1, TATA, CHOP-C/EBPalpha, GATA-3, NF-1, USF, CREB and GR (Figure 1). The distribution pattern of TFBS detected within zebrafish *abcb4* gene promoter region using liver-specific profile is shown in Figure 2.

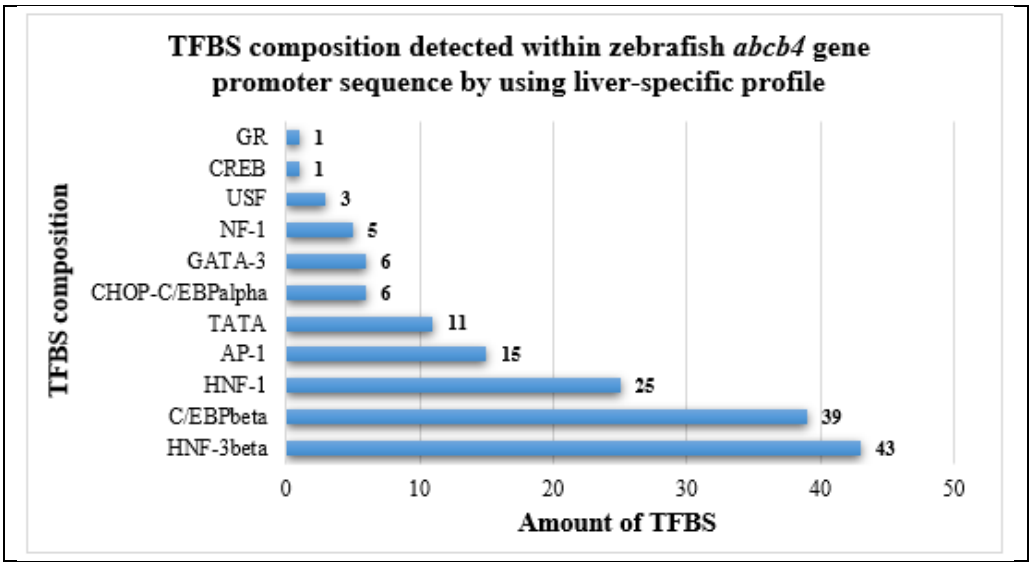

Figure 1. The amount of TFBS and TFBS composition detected within zebrafish *abcb4* gene promoter sequence by using liver-specific profile.

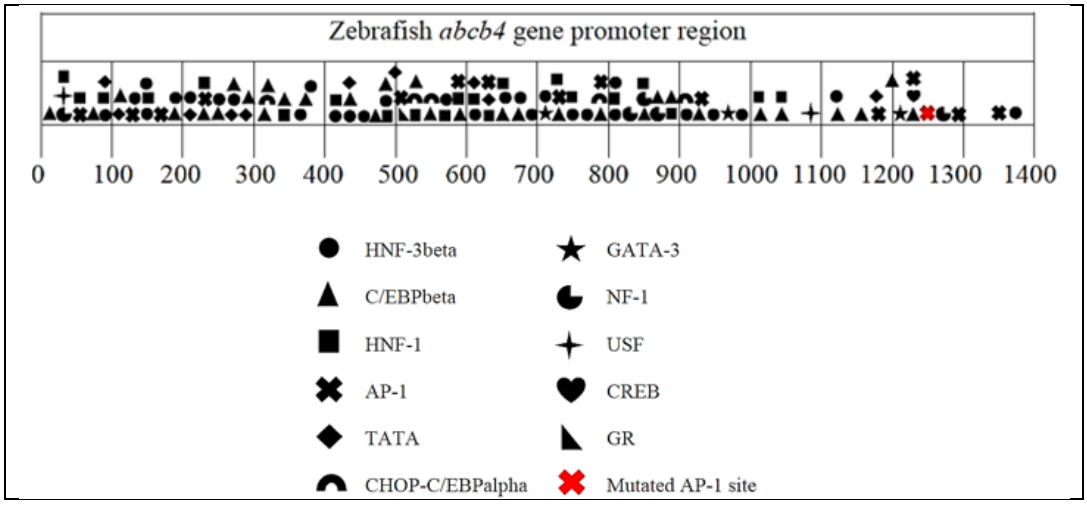

Figure 2. The TFBS distribution pattern per 100 bp interval. Stacking TFBS represents overlapping TFBS sites. Mutated AP-1 site was located at 1,255 bp.

Mutagenic primer designing

The mutagenic primers were designed by substituting three bases (AGT) in the AP-1 TFBS with another three bases (GGG), to remove the AP-1 TFBS, creating a mutation within the promoter. The parameters of the mutagenic primers designed for zebrafish *abcb4* gene promoter are shown below (Table 3).

Site-Directed Mutagenesis

Mutants were successfully generated via site-directed mutagenesis approach. The reverse sequencing result of mutated zebrafish *abcb4* gene promoter showed 98% homology to zebrafish DNA sequence from clone DKEY-24I24 in linkage group 16 (Accession number: CR388365.10) (Supplementary Figure 3 & Supplementary Table 3). Besides, the mutation site was created successfully and can be found in the correct region within zebrafish *abcb4* gene promoter (Figure 3).

Table 3. Parameters of the mutagenic primers designed for zebrafish *abcb4* gene promoter. The mutation induced site is indicated in red fond color.

Primer	Forward primer	Reverse primer
Sequence (5'-3')	GGG CAA GGC AGT ATA AAC GTG	TTA TGT TTC TAG GGA TTA CGT CAC
Length	21 mer	24 mer
Melting temperature (T_m)	56.3ºC	52.3ºC
GC content	52.4%	37.5%
Oligo amount	23.9 nmoles	25.3 nmoles

Figure 3. BLASTn analysis of the reverse sequencing result (partial sequence). Wherewith, the query sequence is the reverse-complement sequence of mutated zebrafish *abcb4* gene promoter that obtained from Apical Scientifc Sdn. Bhd. The sequence that framed in red colored box indicates the mutation site, at which the bases AGT had been substituted into bases GGG, thus creating mutation in the AP-1 TFBS.

In addition, the MATCH outcomes showed that AP-1 TFBS presents at 1,255 bp position within the consensus sequence of zebrafish *abcb4* gene promoter before mutagenesis (Figure 4). When the bases AGT within targeted AP-1 TFBS were substituted with bases GGG, the targeted AP-1 TFBS at 1,255 bp position was deleted (Figure 5), indicating the mutation was successful.

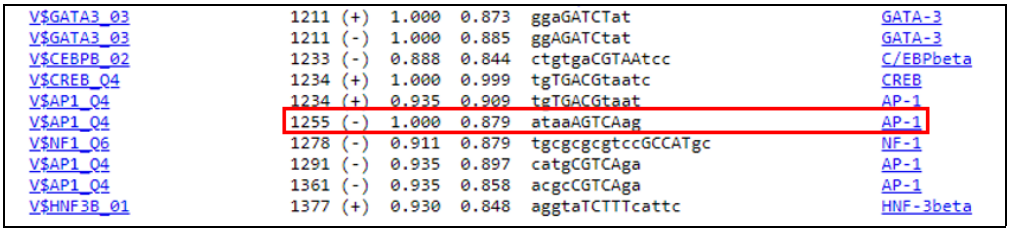

Figure 4. Part of TFBS list before mutation. The AP-1 TFBS presents at 1,255 bp within the consensus sequence of zebrafish *abcb4* gene promoter.

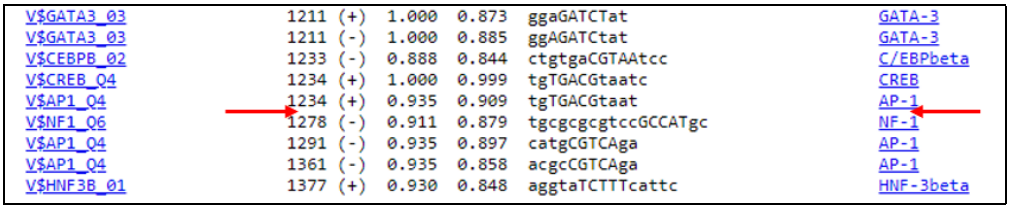

Figure 5. Part of TFBS list after mutation. The targeted AP-1 TFBS at 1,255 bp was deleted successfully and mutation was induced.

DISCUSSION

In this study, the potential TFBS within zebrafish *abcb4* gene promoter sequence was identified via MATCH tool. The human *ABCB1* gene is expressed on the apical surface of hepatocytes in liver. Both the zebrafish *abcb4* and human *ABCB1* genes are associated with liver disease, thus the search was specified to liver-specific profile (Abanda, Riches, & Collier, 2017; Zebrafish Information Network, 2019). According to Kel *et al.* (2003), liver-specific profile is designed to search for the potential binding sites within regulatory regions of liver-enriched genes. In addition, the most abundant TFBS found within the zebrafish *abcb4* gene promoter in the present study is the hepatocyte nuclear factor 3 beta (HNF-3beta) TFBS with a total of 43 binding sites found. This echoed the study by Yu, Guo, Jing, Dong, and Wei (2015) on HNF predominate expression in the liver and their role in regulating the hepatocytes differentiation and liver development.

Among the TFBS detected within zebrafish *abcb4* gene promoter region under liver-specific profile, only AP-1 TFBS located at 1,255 bp position was chosen to be mutated via site-directed mutagenesis approach. According to Gustems *et al.* (2014), AP-1 is a family of transcription factors that involved primarily in cell survival, proliferation and transformation, apoptosis as well as oncogenesis. Generally, AP-1 consists of a heterodimer between c-Fos and c-Jun that play a major role in transcriptional regulation of viral oncogene expression (Foppoli, Coccia, & Perluigi, 2014). Therefore, AP-1 TFBS was chosen to be mutated because AP-1 is closely linked to the proliferation and transformation of tumor cells (Foppoli *et al.*, 2014). In order to proliferate and transform, tumor cell population uses a protective mechanism known as the multidrug resistance (MDR) mechanism, to fight against numerous drugs (Stavrovskaya, 2000). The MDR mechanism is regulated by zebrafish *abcb4* gene and human *ABCB1* gene respectively via the efflux of the xenobiotic components, chemotherapeutic agents, chemicals and some other anti-cancer drugs out of the cells (Dermauw & Van Leeuwen, 2014; Fischer *et al.*, 2013; Liu *et al.*, 2016). Therefore, AP-1 TFBS mutation may provide insights on the elucidation of tumor development from benign to malignant stage.

Additionally, the study by Daschner, Ciolino, Plouzek, and Yeh (1999) had unearthed the close relationship between AP-1 and MDR mechanism in human cancer cells. The study revealed that when there is an elevation in the amount of c-Fos and c-Jun mRNA (AP-1 heterodimer) in cells, the cells are found to possess 12-fold, 65-fold or even 200-fold higher resistance towards the Adriamycin as compared to drug-sensitive MCF-7 (human breast cancer cells) wild type cells (Daschner *et al.*, 1999). This statement had been supported by Foppoli *et al.* (2014) as their studies showed that there was absence or very low level of AP-1 binding in normal cell as well as the premalignant lesions, but the AP-1 binding and transcriptional activity were found to be skyrocketed in malignant tissues. Ergo, by mutating AP-1 TFBS within the promoter and transfecting the mutant into cancer cell lines, the comprehension on the MDR phenomenon can be improved.

Mutation in TFBS can alter the conformation of binding site and disrupt the binding affinity of the TF, causing downstream transcriptional process to be hampered. Hence, the function and post-translational modification of proteins and DNA elements such as promoter region can be verified via this method (Chauhan, 2019; Hsieh & Vaisvila, 2013). The AP-1 TFBS mutation in the present study is the first imperative step in elucidating the transcriptional roles of the cis elements like AP-1 in orchestrating zebrafish *abcb4* gene promoter expression. This is relatively important because the transcriptional activation of zebrafish *abcb4* gene is closely associated with the MDR mechanism and cancer progression.

CONCLUSION

In conclusion, the objectives of this study had been achieved with the successful identification of specific TFBSs within zebrafish *abcb4* gene promoter as well as mutagenesis analysis. Using liver-specific profile in MATCH tool, 155 binding sites were found within the consensus sequence of zebrafish *abcb4* gene promoter and the TFBSs detected are HNF-3beta, C/EBPbeta, HNF-1, AP-1, TATA, CHOP-C/EBPalpha, GATA-3, NF-1, USF, CREB and GR. Mutation in AP-1 TFBS can therefore provide insights to tumor development up till malignant stage, which can be linked to the MDR mechanism that driven by zebrafish *abcb4* gene. It is recommended to do

functional characterization on the mutant via cancer cell line transfection and zebrafish embryo microinjection to further reveal the spatiotemporal expression patterns. Furthermore, mutation can be introduced to other TFBSs or a specific cluster of TFBS to unearth the functional cooperation of these TFs in the zebrafish *abcb4* gene promoter expression regulation.

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