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Pull-Down assay between CTCF- N, ZF and C-terminal domains and YB-1 – N, CSD and C terminal domains in RGBM cell line.

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ABSTRACT

CTCF is an 11-Zn finger transcription factor, involved in the regulation of transcription, insulator function, and the X-chromosome inactivation. CTCF is ubiquitously expressed and it is highly conserved between the species. Identification of proteins interacting with CTCF can help to understand the biological function of CTCF in the cell. Previously reported studies have identified numerous CTCF protein interacting partners and two of the protein partners were chosen for this study. The first protein was the transcription factor YB-1, a member of the Y-box family and the second protein was the Large Subunit of RNA Polymerase II (LS Pol II), the principal enzyme for transcription. The interaction of CTCF and YB-1 was characterized in the Glioma-RGBM cell line. The interaction between these two proteins was characterized through *in vitro* pull-down assay. From the results obtained, CTCF-ZF was the only domain binds with YB-1 CSD. The rest of domains did not show any interaction. Next, the significant of functional interaction between these two transcriptional factors was determined via mammalian two-hybrid system. The results showed a strong interaction between CTCF and YB-1 when both were co-transfected into RGBM cell line which proved the biological significant of these two proteins interaction in the cell.Thus the findings demonstrate, for the first time, the biological relevance of the CTCF/YB-1 interaction in the RGBM cell line.

Keywords: *Immunoprecipitation; CTCF/BORIS/YB-1; Transcription factor*

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INTRODUCTION

Within the rapidly evolving field of proteomic, the detection of protein-protein interaction among transcriptional regulators is fundamental to understanding the mechanism of gene regulation. *CTCF* gene was discovered in 1991 by Lobanenkov *et al.* (1). CTCF is an 11-Zn-finger transcription factor with highly versatile functions (2) and it is localized to the nucleus, ubiquitously expressed and highly conserved protein (3). Theoretically, CTCF is a 130 kDa protein consists of three main domains which are the N-terminal domain, the Zinc Finger Domain (ZF) and the C-terminal domain.

YB-1 is a member of the family of Y-box binding factors with each member containing some degree of homology to the cold shock domain. YB-1 is believed to participate in regulation of such diverse functions as transcription, replication, and RNA turnover. In addition, YB-1 family members appear to be involved in mediating effects of UV irradiation, drug and interleukin-2 treatments, and DNA damage (4). Given these properties, we predict that interactions between CTCF and YB-1 are likely to play multiple roles in regulation of major cellular processes.

This study is aimed at determining the interaction between CTCF and YB-1 in the brain cancer cell line, which was developed previously in our lab and was named as RGBM (Recurrent Glioblastoma Multiforme) (5). Previously reported study has shown the interaction between CTCF and YB-1 occurred at ZF and CSD respectively (6). Glioma is one of the most common types of brain tumors in adult and it was reported that the mean survival of patients is less than a year. It is highly invasive and it constitutes more than 90% of all primary malignant central nervous system (CNS) tumors (7). Even though glioma is the major tumor in the primary nervous systems, their etiology is still less understood. Hence, this study was carried out to elucidate the protein-protein interaction of CTCF and YB-1 in glioma cell line.

To determine the ability of these two protein interaction, a reliable and simple affinity chromatography method utilizing matrix conjugated with purified YB-1 protein expressed in a bacteria system was developed. In addition, the significance of CTCF and YB-1 functional interaction was elucidated using Mammalian two-hybrid system. Mammalian two hybrid system consists of two reporter genes which are *Firefly luciferase* reporter gene and *Renilla luciferase* reporter gene. The *luciferase* reporter gene encodes a monomeric luciferase enzyme with the molecular weight of 61 kDa, that catalyzed a two steps oxidation reaction in the presence of ATP, oxygen, Mg^{2+} and D-luciferin substrate to yield light usually in the green-yellow region (550-570 nm) (8). In this experiment, the internal control used for the normalization was the *Renilla luciferase*.

The *Renilla luciferase* uses a substrate distinct from the substrate of *Firefly luciferase* and it is not utilizing ATP which makes it a good choice as an internal control in the dual reporter system. To determine the functional significance of CTCF and YB-1 interaction, the Mammalian two-hybrid (M2H) system was performed in the transiently transfected mammalian cell. In this system, the genes of interest are either fused to pACT VP16 activation domain (AD) or pBIND GAL4 binding domain (BD) vectors. This system provides two positive control vectors that expressed two known proteins that were reported to have interaction in the cell. To the best of the knowledge, this is the first study reporting on the protein-protein interaction between CTCF and YB-1 in the RGBM cancer cell line.

MATERIALS AND METHODS

Plasmid Constructs **-** PCR was carried out to amplify CTCF ZF and YB-1 CSD using the 5' and 3' primers as described in Table 2.1. All the primers were incorporated with restriction sites *Sgf1* and *Pmel1* and sub-cloned into pFN10A (ACT) and pFN11A (BIND) vectors. All the constructs were verified by sequencing and the sequencing results showed 100 % accuracy. The formation of pACT-CTCF ZF and pBIND-YB1 CSD were used for luciferase assay using mammalian two-hybrid system. All CTCF and YB-1 truncated constructs in pET-16b were given as a gift by Prof. Shaharum Shamsuddin and Mr. Tee Chee Wei, USM, Malaysia.

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Table 1: List of oligonucleotides used in this study

Oligonucleotides	Sequences (5'→3')
Cloning Primers	
CTCF ZF Forward	AAGACATTCCAGTATACTGCGATCGCCCTTTGC
CTCF ZF Reverse	CTCTACGCCATGTGCCGTTTAAACCAGC
YB-1 CSD Forward	AGAAGTTTAAACACCTGTAACATTTG
YB-1 CSD Reverse	AGAAGTTTAAACACCTGTAACATTTG

Expression of CTCF and YB-1 Truncated Proteins in the Bacteria System -Transformants carrying the plasmids expressing the three CTCF and YB-1 truncated regions were grown in LB media supplemented with ampicillin (50 mg/ml) for 3 h at 37 °C. Protein expression was induced with an addition of 1 mM isopropyl b-D-thiogalactoside (IPTG) and further incubated for 3 h at 37 °C. Then, the bacterial cells were pelleted, washed twice with 0.1 volume of cold phosphate-buffered saline followed by lysis in 0.1 volume of the original culture in the cold fresh lysis buffer containing 8 M urea, 0.1 M NaH2PO4, 0.01 M Tris-HCl, pH 8.0. The lysates were subjected to immobilized metal ion affinity chromatography for further purification. Total bacterial lysates containing His-tag expressed proteins were supplemented with 20 mM imidazole and loaded onto the nickel-charged His-Bind resin (R&D Systems, Europe Ltd. These complex was washed with one bed volume of the washing buffer containing 8 M urea, 0.1 M NaH2PO4, 0.01 M Tris-HCl, pH 8.0, and 20 mM imidazole and finally, the His-tagged proteins were eluted by10 ml of the buffer with 8 M urea, 0.1 M NaH2PO4, 0.01 M Tris-HCl, pH 8.0, and 0.5 M imidazole.

All the expressed proteins were verified with 10 % SDS-PAGE followed by Western blot assays probed with anti-His-tag antibodies (Abcam, UK).

Western Blot Analysis - Proteins resolved on the 10% SDS-PAGE gels were transferred to Immobilon P polyvinylidene difluoride filters (PVDF) membrane (Millipore) by electroblotting. Membranes were incubated with the primary antibody at 1:1000 for an hour at room temperature. After washing, the membranes were incubated with anti-rabbit-peroxidase-conjugated antibodies (1:2000 dilutions) for an hour at room temperature and the band of interest was detected with enhanced chemiluminescence detection kit (ECL kit, Amersham Pharmacia Biotech) according to the manufacturer's instructions.

Immobilization of the Bacterially Expressed Amino-terminal, Zinc Finger, and Carboxyl-terminal Domains of CTCF on the Matrix - Cystamine-coupled Sepharose 4B was used to bind to the bacterially produced CTCF and YB-1 truncated proteins. Cystamine was first converted into aminoethylthiol by a reducing reaction with 50 mM dithiothreitol in TE buffer (50 mM Tris-EDTA, pH 8.3) for 30 min at room temperature and treated with 5 mM 2,2-dipyridyldisulfide for 2 h. The activated matrix was washed with TE buffer. Each protein was reduced by incubation with 5 mM dithiothreitol (DTT) for an hour at room temperature, passed through a G50 column equilibrated with TE to desalt the proteins, and then incubated with the activated matrix overnight at 4 °C. The amounts of the proteins retained on the matrix were monitored by protein assay (Bio-Rad) according to the manufacturer's instruction.

The protein-Sepharose conjugated were finally washed with the TE buffer to remove non-incorporated materials and stored in the buffer containing 20% glycerol, 50 mM KH2PO4, pH 7.0, and 0.2% Na3N until further use.

Interaction Assay – Pull-down assay was carried out to determine the interaction between CTCF and YB-1 proteins. For this assay, bacterially expressed YB-1 truncated proteins- N, CSD and C domains were conjugated to the CNBr activated sepharose.

The CNBr- N, CNBr- CSD and CNBr-C conjugated proteins were incubated with RGBM total cell lysate. The next day, the complex was recovered and the presence of the interacting partners was detected via Western blot using anti-His tag antibody.

Mammalian Two-Hybrid System **-** The mammalian two-hybrid system was carried out to quantify the functional interaction of CTCF-ZF and YB-1-CSD in the RGBM cell line. Three types of vectors were supplied in the kit namely pACT, pBIND and pGL4.31 vectors. The constructed pACT-CTCF ZF and pBIND-YB1 CSD as well as pGL4.31 vector were transfected into RGBM cell line using jetPRIME transfection kit performed according to the manufacturer's instructions. The positive control was supplied with the kit and labelled as pACT *MyoD* and pBIND *Id.* In this assay, four types of combination constructs were transfected into RGBM cell line which were (pACT *ZF* + pBIND *CSD*), (pACT + pBIND *CSD*), (pACT *ZF* + pBIND), (pACT *MyoD* + pBIND *Id*). Upon transfection, the relative ratio of *Firefly* to *Renilla luciferases* was recorded using dual-luciferase reporter assay system. All the values obtained from this assay were subtracted with the values from the wells added only with the media that served as a background.

RESULTS

This study is aimed to determine the interaction between CTCF and YB-1 and domains that are responsible for the interaction. For this purpose, an affinity chromatography approach was developed that utilizes matrix coupled with CTCF and YB-1 proteins produced in the bacteria expression system.

In addition to high specificity, another advantage of this approach is that the same CTCF proteins conjugated matrix can be reused for isolation of other CTCF protein partners from different cell extracts.

Expression and Purification of the Full-length CTCF Protein — The bacteria expression system was employed to generate the CTCF (Figure 3.1) and YB-1 (Figure 3.3) truncated proteins in quantities sufficient for immobilization on the matrix. Approximately 5 mg of 90% pure His-tagged proteins was obtained. All the expressed proteins migrated abnormally to which the apparent proteins size is different compared to the calculated proteins size. This phenomenon is explained in details in the discussion section.

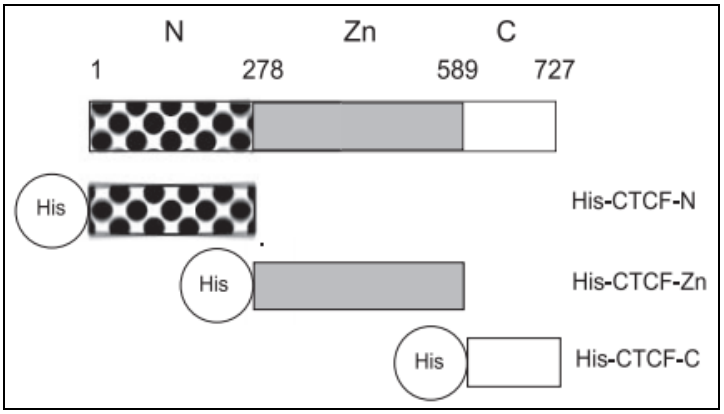

Figure 3.1 CTCF truncated protein. It consist of N terminal domain (1-278 amino acids), Zn domain (278-589 amino acids) and C terminal domain (589 to 727 amino acids).

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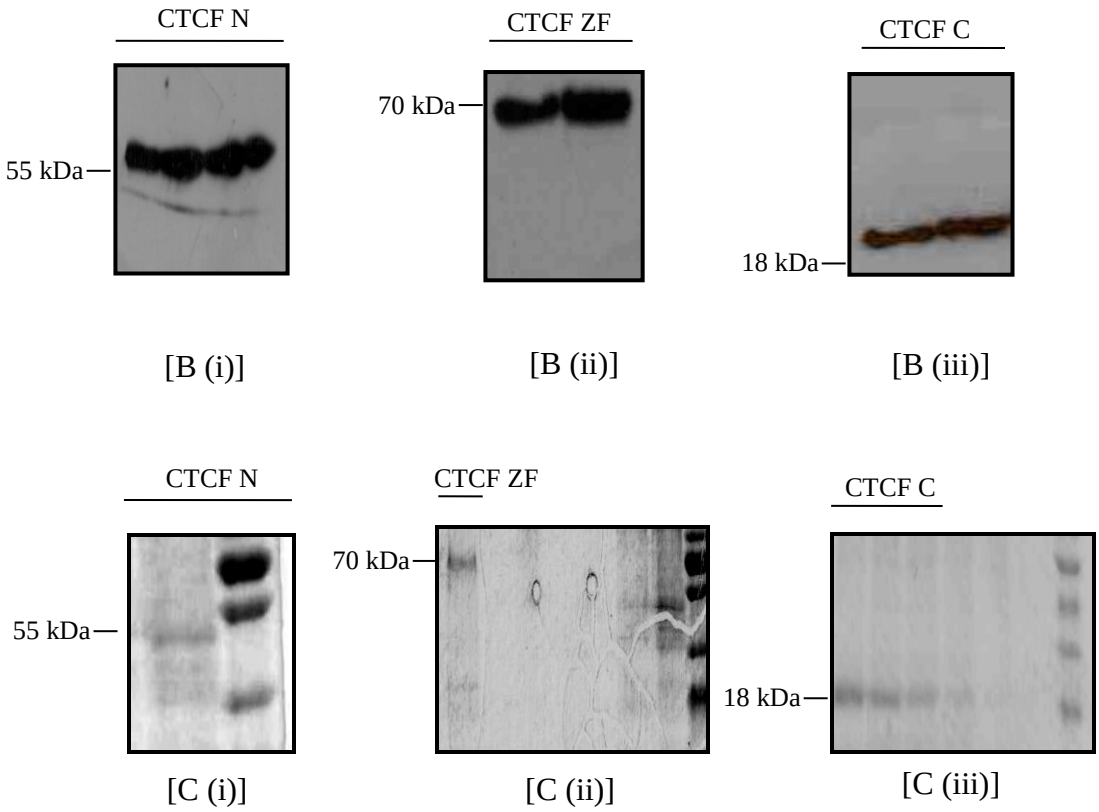

Figure 3.2: *A*, Three-domain structure of CTCF: N, N-terminal domain; Zn, 11-Zn-finger domain; and C, C-terminal domain. *B*, CTCF truncated proteins N domain (i), ZF domain (ii) and C domain (iii) were loaded on the 10% SDS-PAGE and the bands were selectively recognized by anti-His tag antibody. *C*, the same protein N domain (i), ZF domain (ii) and C domain (iii) were purified and loaded on the 10% SDS PAGE and were stained with coomasie blue.

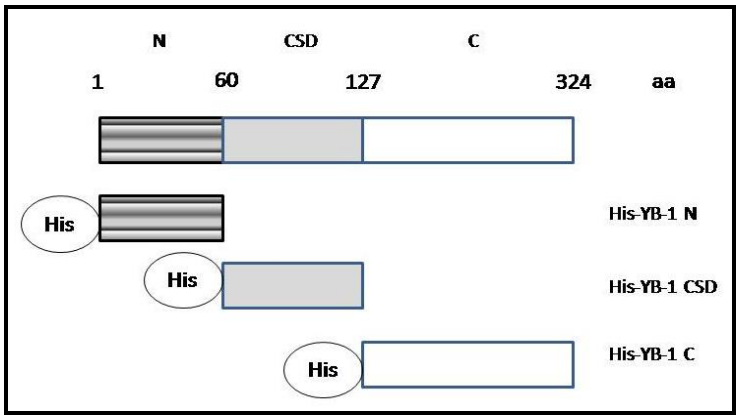

Figure 3.3 YB-1 truncated protein. It consist of N terminal domain (1-60 amino acids), Zn domain (60-127 amino acids) and C terminal domain (127 to 324 amino acids).

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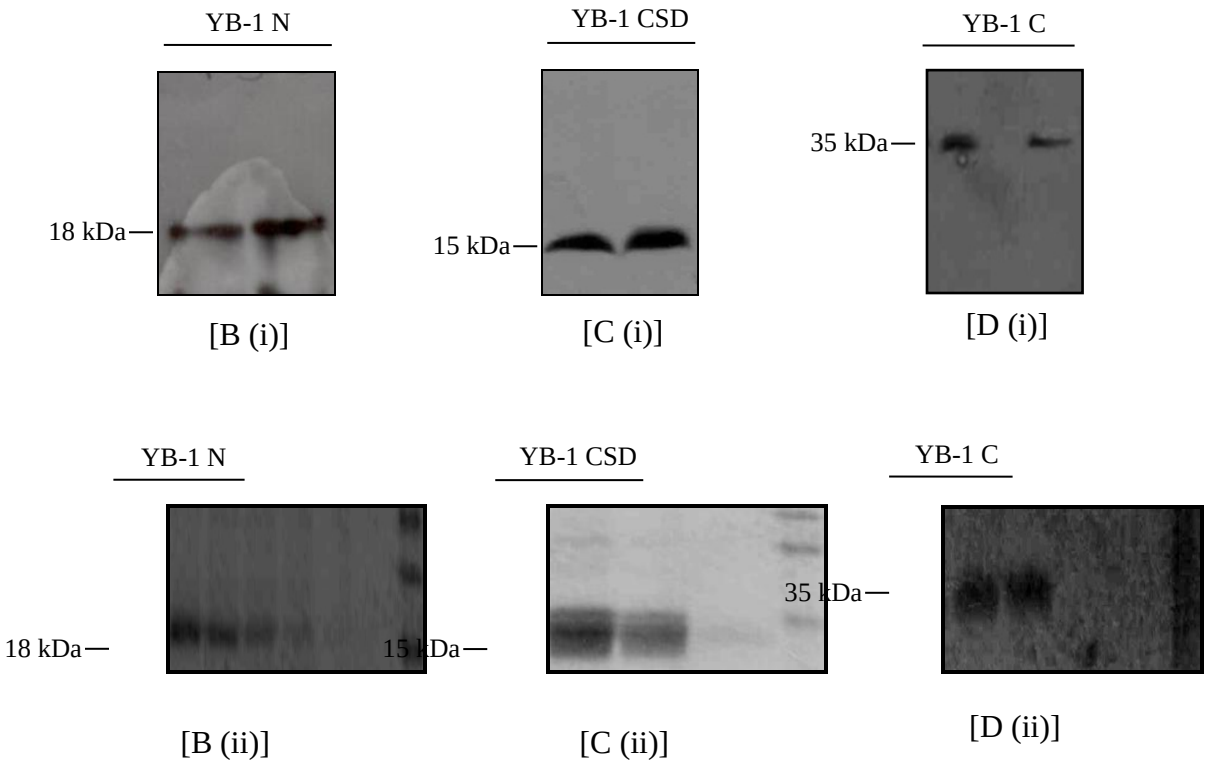

Figure 3.4: *A*, Three-domain structure of YB-1: N, N-terminal domain; CSD, Cold Shock Domain; and C, C-terminal domain. *B*, YB-1 truncated proteins N domain (i), CSD domain (ii) and C domain (iii) were loaded on the 10% SDS-PAGE and the bands were selectively recognized by anti-His tag antibody. *C*, the same protein N domain (i), CSD domain (ii) and C domain (iii) were purified and loaded on the 10% SDS PAGE and were stained with coomassie blue.

YB-1 Interacts with CTCF through the CTCF Zinc Finger Domain — Previous study by Kernain *et al*. (2016) has shown CTCF formed complex *in vivo* with YB-1 in the RGBM cell line. Although the finding demonstrates positive *in vivo* interaction between CTCF and YB-1, further investigation is required to determine whether the interaction is direct or mediated by the third protein partner. Moreover, it is necessary to elucidate the domains responsible for the interaction. For this purpose, *in vitro* pull-down assay was carried out to map the interaction of these proteins. From the result obtained only YB-1 CSD was able to interact with CTCF and the interaction occurred at the ZF domain with the molecular weight detected at 70 kDa (Figure 3.3 B). The rest of domains failed to show any interaction hence from the results obtained it can be deduce that the interaction between CTCF and YB-1 occurred at ZF and CSD domains respectively.

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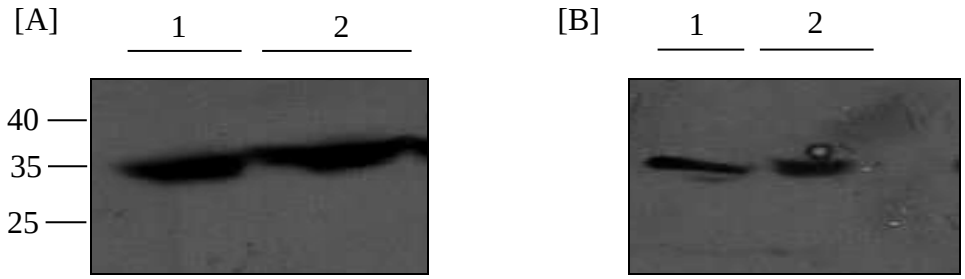

Figure 3.3: Pull-down assay of CTCF and YB-1 in RGBM total cell lysate. [A] Lane 1 and 2: Input of RGBM total cell lysate tagged with anti-YB-1 antibody run in duplicate. [B[lane 1 and 2: CTCF ZF protein coupled to CNBr activated sepharose and incubated with total RGBM cell lysate. The membrane was detected with anti-YB-1 antibody.

Mammalian two-hybrid system — The interaction between CTCF and YB-1 was found to occurred at CTCF-ZF and YB1- CSD, however it is important to determine the functional significance of these two interaction. The assay was carried out using the combination of four different constructs. The assay was run in triplicate and was repeated three times to ensure the reproducibility of the results obtained. The relative *firefly luciferases* to *renilla luciferases* ratio were measured using dual luciferase reporter assay system. The five combination of different constructs pACT + pBIND, pACT *ZF* + pBIND *CSD*, pACT + pBIND *CSD*, pACT *ZF* + pBIND, pACT *MyoD* + pBIND *Id* were transfected into RGBM cell line. Figure 3.5 summarizes the results gained from the functional assay. From the results obtained there was a strong interaction observed in the cell transfected with pACT *ZF* + pBIND *CSD* constructs.

The relative ratio of *firefly* to *renilla luciferases* obtained was 4.5 which was 3.75 fold higher than the value obtained from the cell transfected with pACT *ZF* + pBIND constructs. The second highest value obtained from the functional assay was from the cell transfected with pACT *MyoD* + pBIND *Id* constructs. The relative ratio of *firefly* to *renilla luciferases* recorded was 2.1 which was 1.2 fold higher than the value obtained from the cell transfected with pACT *ZF* + pBIND constructs. The pACT MyoD + pBIND Id constructs were supplied in the kit and served as a positive control for this assay since previously reported study had shown that the interaction of these two proteins was tested positive.

The value obtained between pACT ZF + pBIND CSD transfected cell was higher than the positive control value and this could be due to the interaction between CTCF and YB-1 in the normal biological system is stronger than the interaction between MyoD and Id proteins. The third highest value was obtained from the cell transfected with pACT + pBIND CSD constructs. The relative ratio of *firefly* to *renilla luciferases* obtained was 1.4 which was 1.2 fold higher than the value obtained from the cell transfected with pACT ZF + pBIND constructs. From the results obtained, the interaction between CTCF-ZF and YB1-CSD shown a strong interaction in the RGBM cell line.

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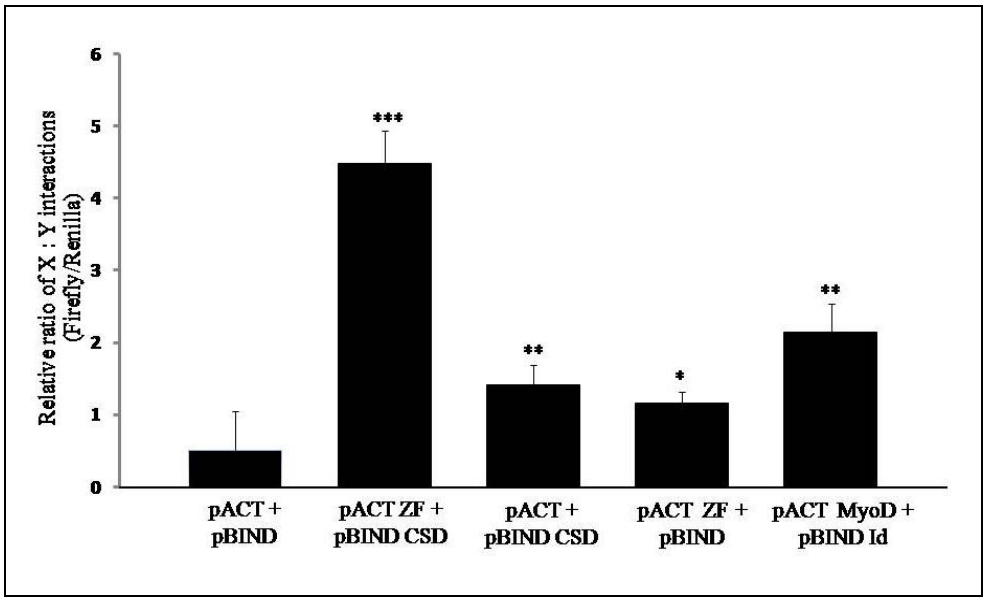

Figure 3.4: Functional interaction of CTCF ZF with YB-1 CSD using dual-luciferase reporter assay system. Each bar represents the mean ± SEM of triplicate samples for three independent experiments. Statistical analysis was performed using one- way ANOVA test with the Turkey HSD *post-hoc* test (*$p < 0.001$; **$p < 0.01$; *$p < 0.05$ vs control vector (pACT + pBIND).**

DISCUSSION

Protein-protein interactions are important to every cellular process. It is reported that over 80% of proteins do not operate alone but in a complex. The main objective of this study is to determine the interaction of CTCF and YB-1 truncated protein in RGBM cell line. Firstly, this study began by describing the production of CTCF and YB-1 truncated protein in the bacterial expression host. The molecular weight of each expressed protein was determined by comparing their mobility with a pre-stained molecular weight marker in the SDS-PAGE and Western blot. All the recombinant proteins expressed in this study were found to migrate differently as compared to their actual theoretical size. The discrepancy between the calculated and apparent molecular weights of truncated protein could be due the specific amino acid composition that may also lead to anomalous electrophoretic migration (9). The same situation of anomalous electrophoretic migration was also reported from the previous studies (10) as well as migration of C-terminal domain of CTCF (11).

Classically, cancer has been viewed as a set of diseases that are driven by progressive genetic abnormalities in the gene sequences. A number of mutations in the CTCF- ZF domain have been found in various tumours, including Wilm's tumours that alter DNA-binding specificity (12). Point mutations in CTCF ZF domain were identified in 21 out of 772 tumors, which indicate the mutation is selective in the cancer cell line. Since the occurrences of mutation are selective in certain types of cancer cell lines, therefore, it is important to elucidate the binding of these two proteins in the RGBM to ensure the ability of these two proteins to bind to each other *in vivo* and *in vitro*.

This study has successfully discovered a positive interaction between CTCF and YB-1 through pull-down assay in the RGBM total cell lysate. Earlier, a protein which is a paralogue of CTCF was discovered in 2002 and was termed as BORIS (Brother of the Regulator of Imprinted Sites; approved symbol CTCFL (12). BORIS expressed only in the male germ cell predominantly in late spermatogonia but silenced in most somatic cells (13). However, BORIS expression re-appeared in the some cancer cell lines. CTCF appeared to be homology to BORIS ZF domains however they are divergent in the N and C-terminal domains.

The previous study has reported BORIS expression in the RGBM cell line with a molecular weight detected at 76 kDa. The expression of BORIS in the RGBM cell line could lead to the possibility of competition between CTCF and BORIS to bind to the same DNA targets sequences and in this case, binding to YB-1. The previous study has disclosed the presence of clustered CTCF binding motifs termed as 2xCTSes which were occupied by both BORIS and CTCF located predominantly at the active promoters and enhancers in the germ and cancer cells (14).

The differences of CTCF and BORIS in the N and C-terminal sequences were utilized in the pull-down assay to which the antibody used in Western blot was raised against the N-terminal domain. Since CTCF and BORIS appear to be similar only in the ZF region hence, utilizing the antibody, which was raised against the other regions rule out the possibility of BORIS binds to YB-1. Having said this, another question raised whether there is a possibility of BORIS to compete with CTCF to binds to YB-1 protein in the RGBM cell line to which the binding of BORIS instead of CTCF could lead to an abnormal activation of YB-1 function and this can cause abnormality in the cell.

Reports show that CTCF available in the two forms generated from the alternative splicing. On the other hand, BORIS was reported to have 23 forms which were also reported to be originated from the alternative splicing. Interestingly, BORIS alternative splicing leads to the loss of zinc fingers in several isoforms (15).

It was also reported that BORIS distribution is uniquely expressed in specific cancer cell line. Having said this, therefore it is important in the future study to analyse BORIS variant in the RGBM cell line to determine the isoform expressed in this cell consist of similar ZF sequences as CTCF ZF sequences. By analyzing, the ZF domains of CTCF as well as BORIS one can interpret the binding ability of these two proteins to the YB-1 domain.

CONCLUSION

This chapter has successfully described the *in vitro* interaction of CTCF and YB-1 in the RGBM cell line. From the result obtained, it shows that CTCF was able to retained YB-1 *in vitro*. Secondly, from the in vitro interaction CTCF was found to interact with YB-1 through the ZF and CSD respectively. Finally, from the functional interaction assay, CTCF ZF was found to have a significant functional interaction with YB-1 CSD in the RGBM cell line. The results of this study provide the first evidence for physical and functional interactions of CTCF and YB-1 in the RGBM cell line.

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