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Interaction between Two Transcriptional Factors CTCF and YB-1 – Truncated domains in Brain Cancer Cell line

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ABSTRACT

CTCF is a protein involved in the regulation of transcription, insulator function, and the X-chromosome inactivation. It is an 11 ZF transcriptional factor which is highly conserved between the species. Identification of proteins interacting with CTCF can help to elucidate the function in the cell. Previously reported studies had identified numerous CTCF protein interacting partners, and one of the interacting partners chosen in this study is YB-1. Brain cancer cell –RGBM was selected as a model to study the interaction between CTCF and YB-1. Firstly, proteins were transformed and expressed in the bacterial expression system, and these proteins were chosen to further map the interaction via pull-down assay. Results showed CTCF-ZF was the only domain able to binds to YB-1 CSD. Other truncated areas did not show any interaction hence demonstrating the interaction between these two proteins took place at the ZF for CTCF and CSD for YB-1. Next, the significant of the interaction was further characterized using the mammalian two-hybrid system. Results show strong interaction when both we co-transfected into RGBM cells. Thus, this study shows a significant binding between CTCF/YB-1 interaction in the brain cell line.

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INTRODUCTION

Within the rapidly evolving field of proteomic, the detection of protein interaction is a building block to understanding the mechanism of gene activation. *CTCF* was discovered in 1991 by Lobanenkov *et al.* (1991). CTCF is localized in the nucleus besides ubiquitously expressed in the cells (Thabrew, 1995). This protein consists of 130 kDa protein. It made up of N, Zinc Finger Domain (ZF), and the C-terminal domains.

On the other hand, YB-1 involved in the regulation of transcription and RNA turnover. Moreover, it was also reported to mediate the effects of UV irradiation and DNA damage (Klenova *et al.*, 2002). Thus previous studies show these two interactions contribute to multiple roles in the regulation of major cellular processes.

This study focuses on elucidating CTCF and YB-1 RGBM (Recurrent Glioblastoma Multiforme). Glioma is a brain tumor in an adult. The mean survival of patients diagnosed with this cancer is less than a year (Ramli and Shamsuddin, 2011). It covers almost 90% of the tumorFigure 1 's central nervous system (CNS) in the human being (Kuwano *et al.*, 2004). However, the etiology of this glioma

cancer is still fairly understood. Hence, this study aimed at elucidating these two proteins at the mentioned cell line.

To determine the ability of these two proteins interaction, a reliable and simple affinity chromatography method utilizing matrix conjugated with purified YB-1 protein expressed in a bacteria system was developed. The Mammalian two-hybrid system was used to elucidate the significance of these two protein interactions in the cancer cell line. This system employed reporter systems. There are two reporter systems, namely *Firefly luciferase* and *Renilla luciferase* reporter genes (Rosenberg *et al.*, 1993). The first reporter system encodes a monomeric luciferase enzyme. Therefore, the reaction will yield a significant light in the green-yellow region (550-570 nm) (Chernukhin *et al.*, 2000). *Renilla luciferase* was used as an internal control for this experiment.

A mammalian two-hybrid (M2H) system was performed to determine the functional significance of these two proteins. Gene of interest was fused into the respective vectors, and two internal controls were used in this system to determine the effectiveness of the whole experimental procedure. To our understanding, this is the first study reporting on 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. 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- ZF and pBIND- CSD were transformed into mammalian cells. pET-16b expression constructs were given as a gift by Prof. Shaharum Shamsuddin and Mr. Tee Chee Wei, USM, Malaysia.

Expression of CTCF and YB-1 Truncated Proteins in the Bacteria System Expression constructs were grown in LB media, and following incubation, one mM of IPTG was included in the media for protein induction. The cell lysate was lysed using a cold fresh lysis buffer containing 8 M urea. After lysis, the lysate was further purified using the affinity chromatography method. Obtained lysates were incubated with 20 mM imidazole, and nickel-charged His-Bind resin was then mixed into the lysate. Then complex was subjected to the washing step. Lastly, the protein was eluted using elution buffer. All proteins were analyzed via western blot.

Western Blot Analysis

Analysis of proteins was carried out using 10% SDS-PAGE gels. Firstly, the gel was transferred to PVDF membrane (Millipore). The membrane was incubated with the primary antibody at 1:1000, followed with secondary antibody incubation at dilution 1:2000 at room temperature. Lastly, the band of interest was detected with a chemiluminescence detection kit, ECL kit, Amersham Pharmacia Biotech.

Immobilization of the Bacterially proteins to the Matrix

Cystamine-coupled Sepharose 4B was used for pull-down assay. Firstly, the beads were converted into amino ethyl thiol, and the mixture was treated with five mM 2,2-dipyridyl disulfide for two h. Then each of the proteins is reduced with five mM DTT and passed through a G50 column. The mixture was then left at 4 °C overnight. Finally, the mixture subjected to the washing step before being stored for further use.

Interaction Assay

To determine the interaction between two proteins of interest. Truncated proteins were conjugated to the CNBr activated sepharose and incubated with RGBM total cell lysate for 24 h. Following day, centrifugation takes place to recovered the mixture and subjected to Western blot to detect the presence of interacting protein partners.

Mammalian Two-Hybrid System

This assay was used to determine the functional significance of the interaction between two proteins of interest in the RGBM cell line. Four types of constructs were transfected into RGBM cell line which were (pACT *ZF* + pBIND *CSD*), (pACT + pBIND *CSD*), (pACT *ZF* + pBIND), (pACT *MyoD* + pBIND *Id*). The ratio between *Firefly* to *Renilla luciferases* was taken, and the value obtained was subtracted with the value obtained from the negative control that served as a background.

RESULTS AND DISCUSSION

Affinity chromatography was developed to determine the interaction efficiency between the two proteins. Bacteria expression system was used to generate the proteins of interest Figure 1 demonstrated results for CTCF truncated protein expression, whereas Figure 2 showed the results for YB-1 truncated protein expression. All the proteins were expressed in BL 21 cell. All the expressed proteins migrated abnormally to which the size of the apparent proteins is different compared to the size of the

calculated proteins. This phenomenon is explained in detail in the discussion section.

YB-1 Interacts with CTCF through the CTCF Zinc Finger Domain

The Previous study reported by Kernain *et al*. (2016) demonstrates CTCF formed a complex *in vivo* with YB-1 in the RGBM cell line. Although this finding demonstrates positive interaction between CTCF and YB-1, a details study is needed to understand if the interaction is intermediated by the third protein partner. Moreover, it is necessary to elucidate the domains responsible for the interaction. For this purpose, *an* in-vitro pull-down assay was used to study the interaction of these two proteins (Figure 3). 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 [B]). The rest of the domains failed to show any interaction; hence, from the results obtained, it can be deduced that the interaction between the protein of interest occurred at ZF and CSD domains, respectively.

Mammalian two-hybrid system

The relative *firefly luciferases* to *renilla luciferases* ratio was determined using a dual-luciferase reporter system. Five combinations of constructs were transfected into the RGBM cell line, respectively. Figure 4 summarizes the results obtained. A high value obtained from the wells transfected with pACT *ZF* + pBIND *CSD* constructs.

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 (Surawicz *et al.*, 1999). Hence this study aims to determine the interaction of two proteins of interest in the RGBM cell line. Cancer has been viewed as a set of diseases with genetic abnormalities in the gene sequences (Spencer *et al.*, 2012). A number of mutations in the CTCF- ZF domain has been found in various tumors, that alter DNA-binding specificity (Thorne *et al.*, 2010). Point mutations in the CTCF ZF domain were identified in 21 out of 772 tumors, which indicate the mutation is selective in the cancer cell line (Klenova, 2004). 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 the two proteins of interest through pull-down assay. 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 (Klenova, 2004). BORIS expressed only in the male germ cell predominantly in late spermatogonia but silenced in most somatic cells (Novy *et al.*, 2001). However, BORIS expression re-appeared in some cancer cell lines. CTCF appeared to be homology to BORIS ZF (Thanaraj and Argos, 1996).

The previous study has reported BORIS expression in the RGBM cell line with a 76 kDa molecular weight. Its expression could lead to the possibility of competition between CTCF and BORIS to attach to the same targets region in the genome sequences and in this case, binding to YB-1 (Yu *et al.*, 2015). 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 (Ohlsson *et al.*, 2010).

The differences between 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 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 (Ohlsson *et al.*, 2010).

It was also reported that BORIS distribution is uniquely expressed in specific cancer cell lines. Having said this, therefore, it is important in the future study to analyses the 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.

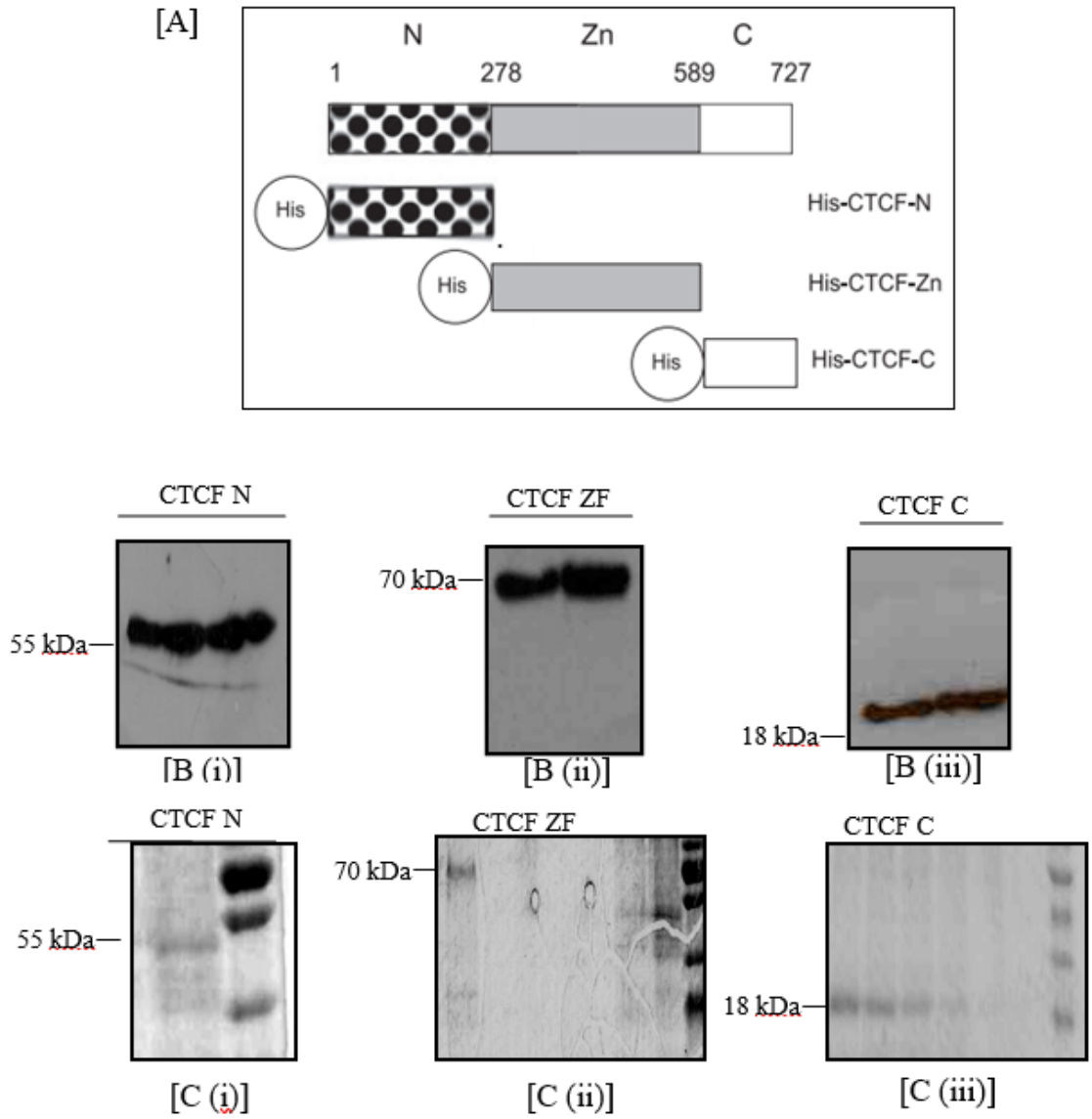

Figure 1: A, Three-domain structure of CTCF: N, N-terminal domain; Zn, 11-Zn-finger domain; and C, C-terminal domain. B, CTCF truncated proteins N domain (i), ZF domain (ii), and C domain (iii) were loaded on the 10% SDS-PAGE, and the bands were selectively recognized by anti-His taganti body. C, the 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 coomassie blue

CONCLUSION

This study shows two proteins interact in the RGBM cell line. Results shows, the interaction between CTCF and YB-1 occurred at the ZF and CSD domains. The functional assay shows significant functional interaction between the two proteins. This experiment has provided new knowledge on the interaction ability between two proteins CTCF and YB-1 in the RGBM cell line.

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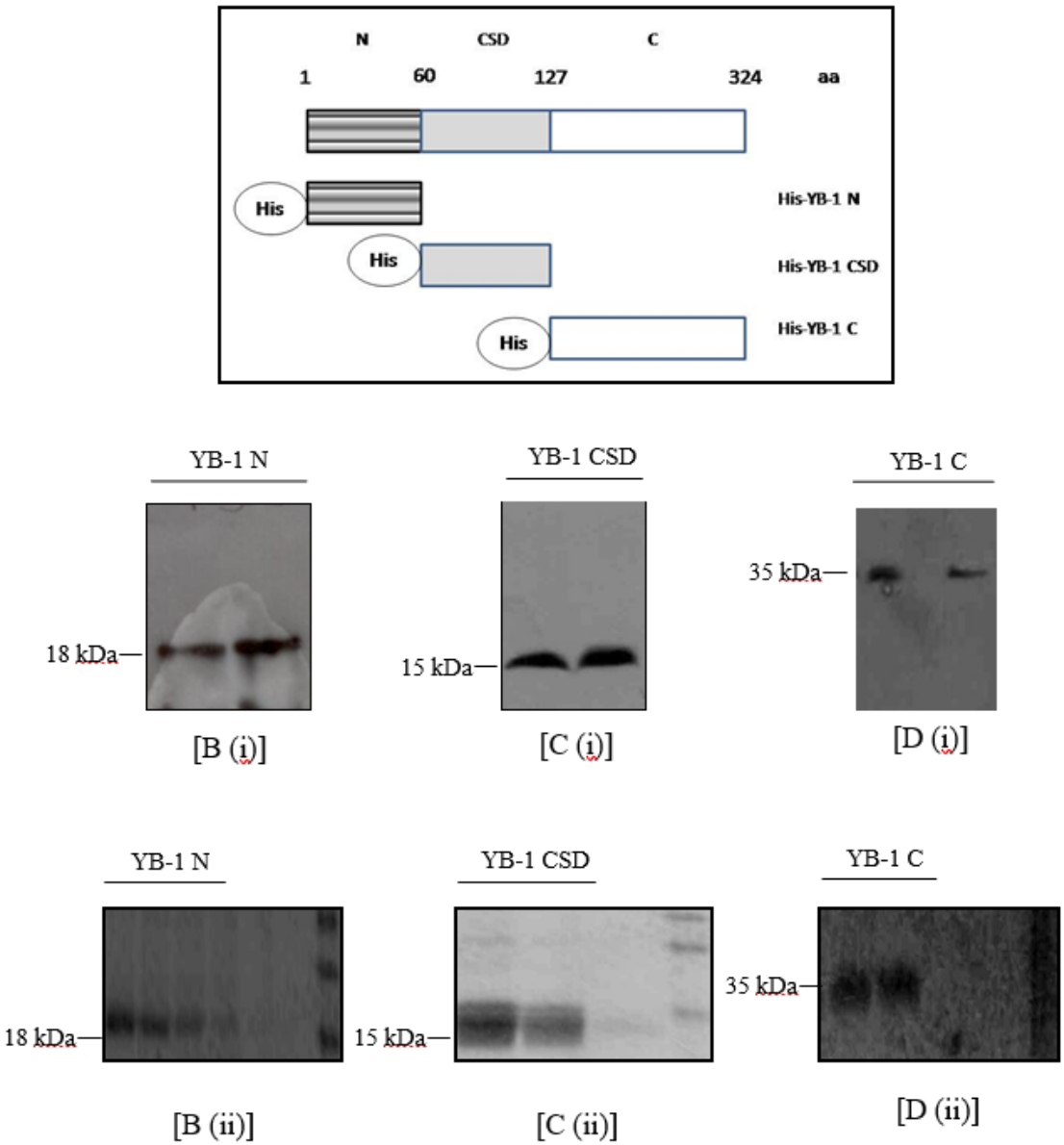

Figure 2: A, Three-domain structure of YB-1: N, N-terminal domain; CSD, Cold Shock Domain; and C, C-terminal domain. B, YB-1truncated proteins N domain (i), CSD domain (ii), and C domain (iii) were loaded on the 10% SDS-PAGE, and the bands were selectively recognized by anti-Histag antibody. C, the same 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

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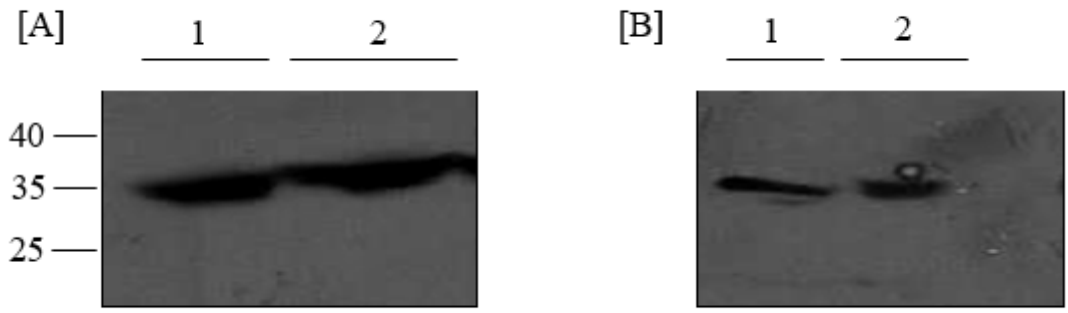

Figure 3: Interaction of CTCF and YB-1 in RGBM total cell lysate. [A] Lane 1 and 2: RGBM total cell lysate tagged with an anti-YB-1 antibody. [B[lane 1 and 2: CTCF ZF protein-coupled to CNBr activated sepharose and incubated with total RGBM cell lysate. The membrane was detected with an anti-YB-1 antibody

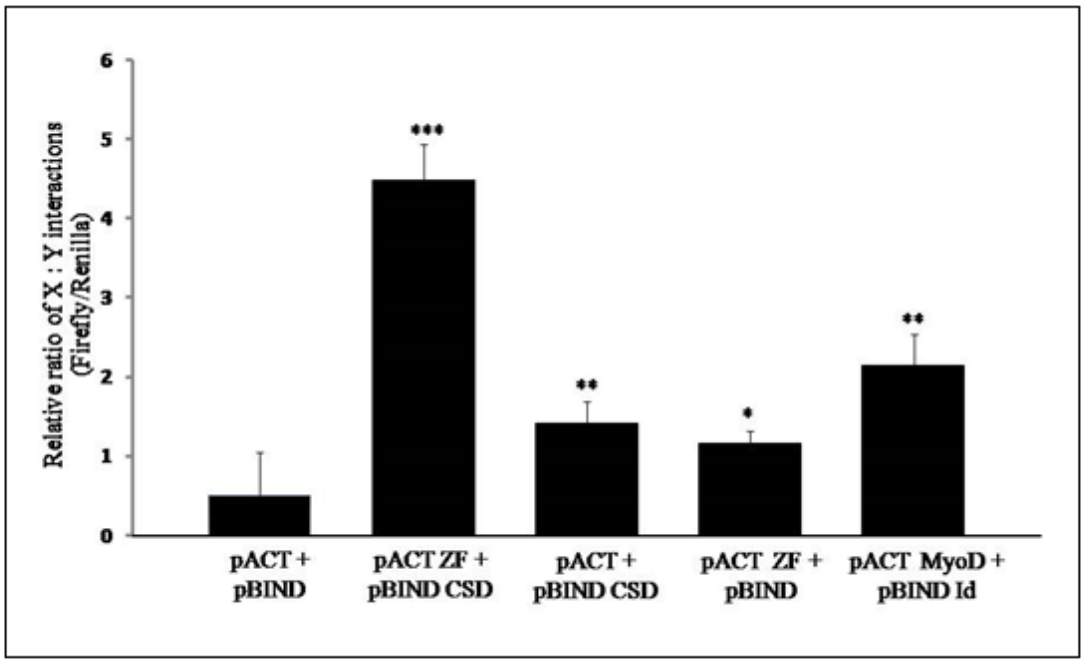

Figure 4: Functional interaction of CTCF ZF with YB-1 CSD using a dual-luciferase reporter assay system. Each bar represents the mean ± SEM of triplicate samples for three independent experiments. Statistical analysis was performed using one- way ANOVA test with the Turkey HSD *posthoc* test (**p < 0.001; **p<0.01; *p < 0.05* vs control vector (pACT + pBIND)**

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