UNIVERSITI SAINS MALAYSIA
GERAN PENYELIDIKAN UNIVERSITI PENYELIDIKAN
LAPORAN AKHIR

MAPPING OF THE INTERRACTION BETWEEN MULTIVALENT TRANSCRIPTION FACTOR, CTCF AND Y BOX BINDING PROTEIN 1 (YB-1) IN GLIOMA AND OSTEOSARCOMA CELL LINES

PENYELIDIK

PROFESOR SHAHARUM SHAMSUDDIN

PENYELIDIK BERSAMA

ASSOC. PROF. DR. SEE TOO WEI CUN
DR. TAN SUAT CHENG

2016

Project Code :
(for RCMO use only)

UNIVERSITI SAINS MALAYSIA

RU GRANT
FINAL REPORT FORM

Please email a softcopy of this report to rcmo@usm.my

A	**PROJECT DETAILS**
i	**Title of Research:** Mapping of the Interaction Between Multivalent Transcription Factor, CTCF and Y-Box-Binding Protein-1 (YB-1) in Glioma and Osteosarcoma Cell Lines
ii	
iii	**Name of Research Leader:** Prof. Shaharum Shamsuddin
iv	**Name of Co-Researcher:** 1. Prof. Madya Dr. See Too Wei Cun 2. Dr. Tan Suat Cheng
v	**Duration of this research:** a) **Start Date** : 15/12/2012 b) **Completion Date** : 14/12/2015 c) **Duration** : 3 Years d) **Revised Date** (*if any*) : -
B	**ABSTRACT OF RESEARCH**
	(An abstract of between 100 and 200 words must be prepared in Bahasa Malaysia and in English. This abstract will be included in the Report of the Research and Innovation Section at a later date as a means of presenting the project findings of the researcher/s to the University and the community at large) Please refer to the attachment.

C BUDGET & EXPENDITURE

i

ii **Equipment Purchased Under Vot 35000**

No.	Name of Equipment	Amount (RM)	Location	Status
1	pH Meter	1,620.00	Molecular Bilogy Lab.	Boleh digunakan

 # Please attach the Asset/Inventory Return Form (Borang Penyerahan Aset/Inventori) – Appendix 1

D RESEARCH ACHIEVEMENTS

i **Project Objectives** *(as stated/approved in the project proposal)*

No.	Project Objectives	Achievement
1	Primary cell lines preparation (Osteosarcoma & Glioma)	Yes
2	Preparation of cell lysates	Yes
3	Expression & purification of truncated CTCF	Yes
4	Expression & purification of truncated YB-1	Yes
5	Pull Down *in vitro* interaction assay	Yes
6	Discussion and conclusion	Yes

ii **Research Output**

a) Publications in ISI Web of Science/Scopus

No.	Publication (authors,title,journal,year,volume,pages,etc.)	Status of Publication (published/accepted/ under review)

b) Publications in Other Journals

No.	Publication (authors,title,journal,year,volume,pages,etc.)	Status of Publication (published/accepted/ under review)
1.	Daruliza Kernain, Shaharum Shamsuddin, Tan Suat Cheng. Immunoprecipitation of 11 ZN fingers Domains, CTCF and BORIS to Multifunctional Y-box DNA/RNA-binding factor, YB-1 in Glioma-RGBM.Journal of Biomedical and Pharmaceutical Research. 2016;5: 46-51.	accepted

c) Other Publications
(book,chapters in book,monograph,magazine,etc.)

No.	Publication (authors,title,journal,year,volume,pages,etc.)	Status of Publication (published/accepted/ under review)

d) Conference Proceeding

No.	Conference (conference name,date,place)	Title of Abstract/Article	Level (International/National)
1.	International conference on Molecular Medicine ; 30-31 March 2014 (Singapore)	In vivo association of multivalent 11 zinc finggers transcriptional factors CTCF and BORIS to YB-1 in multiforme Glioma RGBMM cell line	International Conference
2.	Pan Asian Biomedical Conferences ; 11-12 December 2014 (Hong Kong)	Physical mapping between Y-Box DNA/RNA –binding factor YB-1 truncated proteins & multivalent 11 ZN Fingers transcriptional factors CTCF and BORIS in multiforme RGBM cell line	International Conference

 # Please attach a full copy of the publication/proceeding listed above

iii	**Other Research Ouput/Impact From This Project** *(patent, products, awards, copyright, external grant, networking, etc.)* **none**

E HUMAN CAPITAL DEVELOPMENT

a) Graduated Human Capital

Student	Nationality (No.)		Name
	National	International	
PhD	✓		1. Daruliza Kernain 2.
MSc			1. 2.
Undergraduate			1. 2.

b) On-going Human Capital

Student	Nationality (No.)		Name
	National	International	
PhD			1. 2.
MSc			1. 2.
Undergraduate			1. 2.

c) Others Human Capital

Student	Nationality (No.)		Name
	National	International	
Post Doctoral Fellow			1. 2.
Research Officer			1. 2.
Research Assistant	✓		1. Nurul Aini Samsuddin 2.
Others (.....)			1. 2.

F	COMPREHENSIVE TECHNICAL REPORT
	Applicants are required to prepare a comprehensive technical report explaining the project. The following format should be used (this report must be attached separately): • Introduction • Objectives • Methods • Results • Discussion • Conclusion and Suggestion • Acknowledgements • References Please refer to the attachment.

G	PROBLEMS/CONSTRAINTS/CHALLENGES IF ANY
	Some of the truncated proteins could not be expressed. Few optimization was carried out to solved the problems.

H	RECOMMENDATION
	(Please provide recommendations that can be used to improve the delivery of information, grant management, guidelines and policy, etc.) **None**

I	COMMENTS, IF ANY/ENDORSEMENT BY PTJ'S RESEARCH COMMITTEE

The project leader has successfully supervised a PhD student whom PPSK is proud of.

ABSTRAK

CTCF adalah faktor transkripsi jejari 11-Zn, yang terlibat dalam pengawalan transkripsi, penebat, kawalan cetakan, dan pentakaktifan kromosom X. CTCF merupakan gen yang sentiasa diekspres dan terpelihara antara spesies. Fungsi biologi CTCF dalam sel boleh dikenal pasti melalui interaksi protein dengan protein CTCF yang diekspres. Berdasarkan kajian-kajian yang terdahulu, terdapat pelbagai protein yang boleh berinteraksi dengan protein CTCF. Penggunaan dua daripada protein tersebut telah dipilih untuk kajian ini. Protein yang pertama ialah faktor transkripsi YB-1 yang terdapat dalam kalangan Y-kotak, dan protein yang kedua ialah Subunit Besar RNA polimerase II (LS Pol II) yang merupakan enzim utama untuk transkripsi. Interaksi CTCF dan YB-1 dicirikan dalam titisan sel Glioma-RGBM. Hasil cerakin co-IP menunjukkan CTCF dapat membentuk kompleks dengan YB-1 dalam keseluruhan lisis sel RGBM. Interaksi antara kedua-dua protein tersebut turut digambarkan melalui cerakin *in vitro pull-down*. Berdasarkan hasil kajian, CTCF-ZF adalah satu-satunya domain yang berjaya diikat dengan YB-1 CSD, manakala domain yang selebihnya tidak menunjukkan sebarang interaksi. Seterusnya, fungsi interaksi antara kedua-dua faktor tanskripsi telah ditentukan melalui sistem dua hibrid mamalia. Hasil kajian menunjukkan interaksi yang kukuh antara CTCF dan YB-1 apabila kedua-duanya diperkenalkan ke dalam titisan sel RGBM yang membuktikan kepentingan biologi interaksi dua protein ini dalam sel.

ABSTRACT

CTCF is an 11-Zn finger transcription factor, involved in the regulation of transcription, insulator function, control of imprinting 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 co -IP results showed that CTCF was able to form a complex with YB-1 in the RGBM total cell lysate. The interaction between these two proteins was further 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.

UNIVERSITI SAINS MALAYSIA
JABATAN BENDAHARI
KUMPULAN WANG UNIVERSITI PENYELIDIKAN (RU)
PENYATA PERBELANJAAN SEHINGGA 29 FEBRUARI 2016

	RM		
Jumlah Geran :	228,844.89	Ketua Projek :	PROF. MADYA SHAHARUM SHAMSUDDIN
Peruntukan DIS. 2012 : (Tahun 1)	153,158.29	Tajuk Projek:	MAPPING OF THE INTERACTION BETWEEN MULTIVALENT TRANSCRIPTION FACTOR, CTCF AND Y BOX BINDING PROTEIN 1 (YB-1) IN GLIOMA AND OSTEOSARCOMA CELL LINES
Peruntukan DIS. 2013 : (Tahun 2)	65,974.60	Tempoh :	3 Tahun (15/12/2012-14/12/2015)
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Kwgan	Akaun	PTJ	Projek	Peruntukan Projek	Perbelanjaan Terkumpul sehingga Tahun lalu	Peruntukan Semasa	Tanggungan Semasa	Bayaran Tahun Semasa	Belanja Tahun Semasa	Baki Projek
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1001	25000	PPSK	813074	-	-	-	-	-	-	-
1001	26000	PPSK	813074	-	-	-	-	-	-	-
1001	27000	PPSK	813074	97,705.29	143,083.43	(45,378.14)	-	-	-	(45,378.14)
1001	28000	PPSK	813074	-	500.00	(500.00)	-	-	-	(500.00)
1001	29000	PPSK	813074	5,000.00	9,813.40	(4,813.40)	-	-	-	(4,813.40)
1001	32000	PPSK	813074	-	-	-	-	-	-	-
1001	35000	PPSK	813074	77,650.00	1,620.00	76,030.00	-	-	-	76,030.00
				228,844.89	228,531.50	313.39	-	-	-	313.39

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ReviewArticle

Immunoprecipitation of 11 ZN fingers Domains, CTCF and BORIS to Multifunctional Y-box DNA/RNA-binding factor, YB-1 in Glioma-RGBM

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ABSTRACT

CCCTC binding factor (CTCF) is a unique highly conserved and ubiquitously expressed 11 zinc finger (ZF) transcriptional factor with multiple target site. It is able to bind to various target sequences to perform different regulatory roles and the binding is through the combination of different ZF domains. On the other hand, BORIS (Brother of the Regulator of Imprinted Sites), which is expressed only in the testis and certain cancer cell lines is homology to CTCF 11 ZF domains. Since both transcriptional factors share the same ZF domains, hence there is a possibility for both to bind to the same target sequences. Hence, the aim of this study is to determine the *in vivo* interaction of CTCF and BORIS to YB-1 in the laboratory established Glioma-RGBM cell line. The protein-protein interaction between CTCF/YB-1 and BORIS/YB-1 were discovered using Co-immunoprecipitation (CO-IP) technique through reciprocal experiment by using RGBM total cell lysate. Results showed that both CTCF and BORIS were able to interact with YB-1 in RGBM cell line. To the best of our knowledge, this is the first finding demonstrating the ability of BORIS and YB-1 to form a complex *in vivo*

Key words: Immunoprecipitation; CTCF/BORIS/YB-1; Transcription factor

1.0 INTRODUCTION

CCCTC binding factor (CTCF) is an 11 Zinc finger transcriptional factor with multiple DNA site specificities. It was initially identified as a transcriptional regulator (Klenova *et al.*, 1993). It is able to bind to different target sequences to perform various regulatory roles such as promoter activation or repression, silencing and constitutive and methylation dependent chromatin insulation (Maston, *et al.*, 2006). On the other hand, BORIS (Brother of the regulated of imprinted sites) is a paralog of CTCF. It was classified as a cancer testis antigen (CTA) and human BORIS gene spans over 29 kb at chromosome 20q13 which comprises of 11 exons, 10 of which contain coding sequences (Pugacheva *et al.*, 2010). According to the literature, the ZF domains of both CTCF and BORIS were reported to have 93% homology however the N and C terminal domains were described to be different (Loukinov *et al.*, 2002). This suggests that ZF regions may interact with same binding partners, but altering the gene expression probably via different mechanisms.

CTCF binds to the DNA targets either through N, ZF or C-terminal domains. Study carried out by Chernukhin *et al.* (2007) showed that CTCF binds to the larger subunit of RNA polymerase II (Pol II) via C terminal domain. There is another study carried out by the same group in 2000 reported CTCF formed a complex both *in vivo* and *in vitro* with YB-1 via ZF domains. In this study, CTCF was found to bind to YB-1 via the combination of different ZF domains. Since different sets of ZFs are utilized to recognize different CTCF target DNA sites, each of the diverse DNA-CTCF complexes might engage different essential protein partners to define distinct functional readouts (Mahaley *et al.*, 1986).

YB-1 is known to participate in transcription, replication, RNA processing and DNA repair (Marshall *et al.*, 2014). Given this properties, the interaction between CTCF/YB-1 may have functional significance roles in regulation of major cellular processes. One of the functional significance between CTCF/YB-1 identified by Chernukhin *et al.* (2000) is from the study of c-myc oncogene promoter (a target of CTCF) in the co-transfection study. From this study, the observation was made in which, the expression of YB-1 alone had no effects on the c-myc promoter activity, and however the co-expression of CTCF/YB-1 resulted in marked enhancement of

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CTCF-driven c-myc transcriptional repression.

CTCF and BORIS expression in normal tissue are by mutually exclusive manner (Shamsi *et al.*, 2011). Previously reported studies show that, the expression of BORIS and CTCF both could be detected in cancer cell lines. However, BORIS expression could only be detected in spermatocyte and not in the somatic cells. This is contrary to CTCF expression in which it could only be detected in somatic cell but not in spermatocyte (Figure 1.1). The switch in expression between BORIS and CTCF coincide with the re-establishment of site specific methylation patterns during male germ cell line development (Allegrucci *et al.*, 2005). The detection of BORIS in cancer cell lines and the absence in post-meiotic germ cell line provide an opportunity to be utilized as a biomarker in cancer development.

Malignant gliomas are the most common types of brain tumours in adult and it was reported that the mean survival time of patients is less than a year (Mahaley *et al.*, 1989). It is highly invasive and it constitutes more than 90% of all primary malignant central nervous system (CNS) tumours. Even though glioma is the major tumour in primary nervous systems, their etiology is still less understood. Hence, this study was carried out to elucidate the protein-protein interaction between CTCF and BORIS to YB-1 in glioma cell line. Previously, our group has successfully developed our in-house primary glioma cell line from local clinical tumour sample through explants technique and the cell line was named as RGBM (Recurrent Glioblastoma Multiforme). BORIS expression in this cell line was characterized and it was reported to be expressed at 76 KDa (Siti Zawani *et al.*, 2011). To the best of our knowledge, this is the first study reporting on the *in vivo* protein-protein interaction between BORIS and YB-1 in RGBM-Glioma cell line. In brief, this report is describing the association of CTFC and BORIS to YB-1. YB-1 has been identified as a protein interacting partners with CTCF. We demonstrated that, CTCF is associated *in vivo* with YB-1 through CO-IP (Co-Immunoprecipitation) technique. Furthermore, we show that BORIS (a paralog of CTCFL) was also able to form an *in vivo* complex with YB-1 via the similar technique. Hence, these findings may provide a basic link to the functional role of BORIS in the brain cancer machinery pathway.

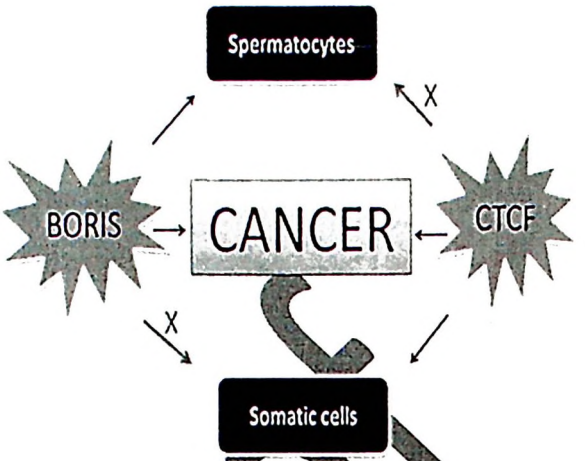

Figure 1.1: BORIS and CTCF both can be detected in cancer cell line. However BORIS expression could only be detected in spermatocytes and not in somatic cells. On the other hand, CTCF expression could only be detected in somatic cell and not in spermatocyte.

2.0 MATERIALS & METHODS

2.1 Cell growth and harvesting:

For CO-IP experiment, RGBM-glioma cells were grown as monolayer in a tissue culture flask as previously described (Klenova et al., 2002). Once the desire cell density (10^7-10^8 cells) was obtained, cells were trypsinized and harvested. Cell pellet was then lysed in sucrose lysis buffer.

2.2 Cell Lysis:

Sucrose lysis buffer (10 mM Tris–HCl (pH 8.0), 10 mM NaCl, 0.3 M sucrose, 3 mM MgCl ,0.5% NP-40) was used to total lysed the cells. Total cell lysate was incubated at 4°C overnight with gentle mixing. The supernatant was recovered the next day by centrifugation at 10, 000 rpm for 10 minutes.

2.3 Co-Immunopercipitation:

2.3.1 Pre-clearing:

For pre-clearing process, 50 µl of protein G sepharose (Calbiochem) was added into the supernatant and incubated at 4°C for 1 hr with gentle mixing on a rotating mixer. The supernatant was recovered after an hour by centrifugation at 1200g for 5 min.

2.3.2 Precipitation with antibody:

The antibodies used to precipitate the proteins of

interest are listed in Figure 2.1. The exact input of antibody required to give a moderate excess over specific antigen should be determined empirically by trial experiment. Five µl of respective antibodies were added into the pre-cleared cell lysate, incubated overnight at 4°C with gentle mixing. The following day, immune complex was recovered by centrifugation at 1200 g for 5 min. The immune complex was washed 3 times by gently resuspending in lysis buffer followed by centrifugation at 1200 g. Immune complex was then analysed by SDS-PAGE.

2.3.3 Western Blot analysis:

Immune complex was resuspended in 20µl SDS lysis buffer (60 mM Tris– HCl pH 6.8, 2% SDS, 20% glycerol, 100mM DTT and 0.02% bromphenol blue) heated at 95°C for 5 mins and analyzed on a 12% SDS-PAGE. The electrophoresed proteins were transferred onto nitrocellulose membrane using mini trans-blot apparatus (Bio-Rad, USA) according to the manufacturer's recommendations. The blotted membrane was blocked with 5% skim milk (Sun Lac), washed three times in Tris-buffered saline (TBS) with 0.05% of Tween-20 (TBS-T) for 10 min each time and incubated with respective antibodies (Figure 2.1) in 5% blocking solution) respectively for an hour at room temperature. The membrane was washed 3 times with TBS-T followed by incubation with respective secondary antibodies (Figure 2.1) for an hour at room temperature. The membrane again washed three times in TBST followed by addition of chemiluminescent substrate (Amersham) according to the suggested procedure by the manufacturer.

Primary antibody	Dilution	Manufacture	Secondary antibody	Dilution	Manufacture
Anti-YB1 monoclonal antibody (59Q)	1:1000	Santa Cruz	Goat anti-mouse	1:2000	Santa Cruz
Anti-CTCF monoclonal antibody (G-8)	1:1000	Santa Cruz	Goat anti-mouse	1:2000	Santa Cruz
Anti-Boris polyclonal antibody (Ab126766)	1:1000	Abcam	Goat anti-rabbit	1:2000	Santa Cruz

Figure 2.1: The list of primary and secondary antibodies used in this study.

3.0 RESULTS

This study was carried out to determine the *in vivo* protein-protein interaction between CTCF and BORIS to YB-1 in RGBM-Glioma cell line. To achieve this objective series of reciprocal CO-IP assays with RGBM-Glioma cell lysate were carried out. The total cell lysate was first precipitated with acetone to obtain a more concentrated protein before preceded with CO-IP experiment. Figure 1 shows SDS-PAGE results of RGBM total cell lysate before (lane 1) and after (lane 2) acetone precipitation.

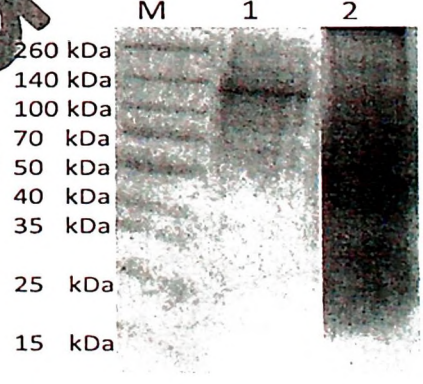

Figure 3.1: Coomassie stained of RGBM total cell lysate was resolved in 10% SDS-PAGE. Lane M: Protein ladder; Lane 1: RGBM total cell lysate before acetone precipitation. Lane 2: RGBM total cell lysate after acetone precipitation.

Figure 2 shows CO-IP results of CTCF and YB-1 from RGBM total protein. Figure 2A (lane 1) shows the presence of CTCF protein from RGBM cell after subjected to western blot and probed with anti-CTCF antibody. Figure 2A (lane 2) shows the immunoprecipitated result of YB-1 in RGBM cell and co-migrating with YB-1 protein. The complex was resolved in SDS-PAGE and probed with anti-CTFC antibody. Result obtained shows that CTCF protein migrated at 50 KDa which coincide with the position of CTCF in unfractionated RGBM cell line. Moreover, on the reciprocal experiment, Figure 2B (lane 1) shows the presence of YB-1 protein in RGBM cell line, which migrated at 30-70 KDa after subjected to western blot and probed with anti-YB-1 antibody. Figure 2B (lane 2) shows the result of immunoprecipitated CTCF in RGBM cell co-migrating with CTCF protein. The complex was resolved on SDS-PAGE and probed with anti-YB1 antibody. The result obtained shows that, YB-1 protein migrated at 50-90 KDa which corresponded to the position of YB-1 protein in the unfractionated RGBM cell line.

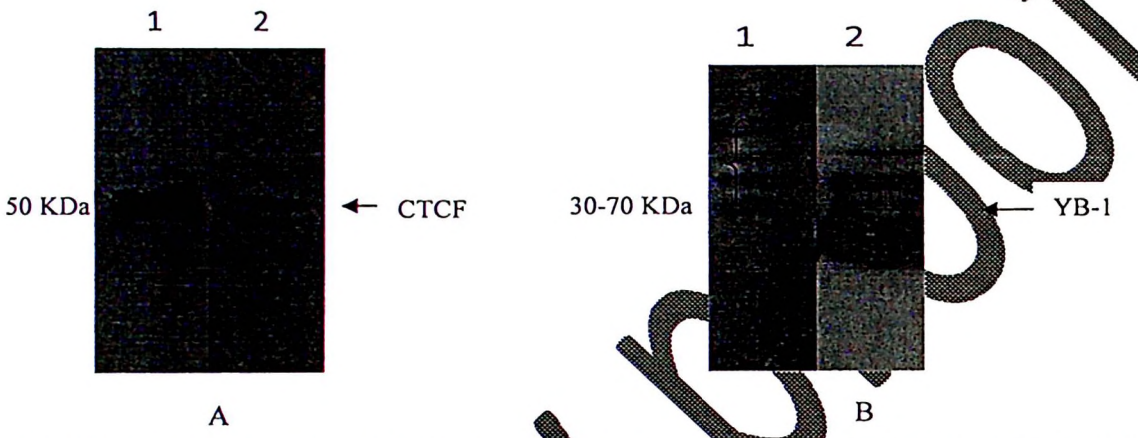

Figure 3.2: Reciprocal experiment on *in vivo* interaction assay of CTCF/YB-1 in RGBM cell line. *In vivo* interaction assay was investigated by CO-IP followed by western blot techniques (A) Lane 1 shows the result of RGBM total protein probed with anti-CTCF antibodies whereas Lane 2 shows the result of total proteins immunoprecipitated with anti-YB1 and probed with anti-CTCF in western blot. (B) Lane 1 shows the result of RGBM total protein probed with anti-YB-1 antibodies whereas Lane 2 shows the result of total proteins immunoprecipitated with anti-CTCF and probed with anti-YB1 in western blot.

Furthermore, the interaction between BORIS and YB-1 in RGBM cell line was also determined in this study. Anti-YB-1 antibody and anti-BORIS antibody were used to precipitate the corresponding interacting proteins in the cells. Figure 3A (lane 1) shows the presence of YB-1 protein from RGBM cell after subjected to western blot and probed with anti-YB1 antibody. Whereas Figure 3A (lane 2) shows the immunoprecipitated result of BORIS in RGBM cell and co-migrating with BORIS protein. The complex was resolved in SDS-PAGE and probed with anti-YB1 antibody. Result obtained shows that, four bands of YB-1 protein could be detected and the protein migrated at 30-70 KDa. Furthermore, on the reciprocal experiment, Figure 3B (lane 1) shows the presence of BORIS protein from RGBM cell line after subjected to western blot and probed with anti-BORIS antibody. Figure 3B (lane 2) shows the immunoprecipitated result of YB-1 in RGBM co-migrating with YB-1 protein. The complex was resolved in SDS-PAGE and probed with anti-BORIS antibody in which the protein migrated at 35 KDa and the size was coincide with BORIS protein from the unfractionated RGBM cell lysate.

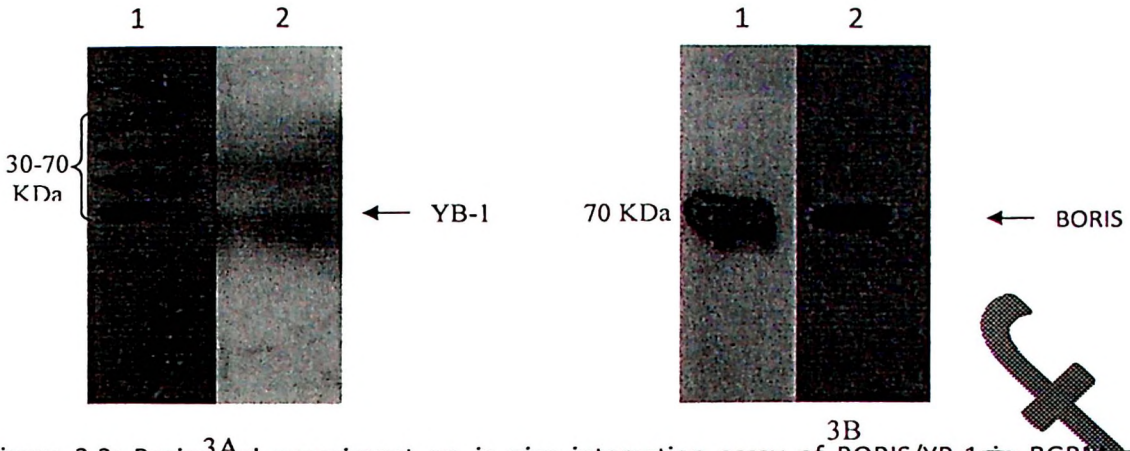

Figure 3.3: Reciprocal experiment on *in vivo* interaction assay of BORIS/YB-1 in RGBM cell line. *In vivo* interaction was investigated by CO-IP followed by western blot techniques using protein from RGBM cells. (A) Lane 1 shows the result of RGBM total protein probed with anti-YB1 antibody whereas Lane 2 shows the result of total proteins immunoprecipitated with anti-BORIS antibody and probed with anti-YB1 antibody in western blot. (B) Lane 1 shows the result of RGBM total protein probed with anti-BORIS antibody whereas Lane 2 shows the result of total proteins immunoprecipitated with anti-YB1 antibody and probed with anti-BORIS antibody in western blot.

4.0 DISCUSSION

The aim of this study is to elucidate the potential binding of CTCF and BORIS to YB-1. To identify such factor, we employed CO-IP technique. CO-IP is probably the most widely employed method for detecting *in-vivo* protein-protein interaction, particularly involving transcriptional factor complex. The advantage of this technique is that the endogenous protein complexes are studied, therefore the artificial effects of affinity tag or overexpression are avoided. From this study, we have successfully identified an interaction between CTCF and BORIS to YB-1 in RGBM cell line. Previously, Chernukhin *et al.* (2007) carried out a study on CTCF/YB-1 interaction in HeLa cell lysate. However so far there is no reported study on CTCF/YB-1 interaction in brain cancer cell line. Hence this is the first study reporting on CTCF/YB-1 interaction in brain cancer cell line. Chernukhin and his group reported the interaction of CTCF to YB-1 were specific against the ZFs domain and there was no interaction detected in the N and C terminal domains. BORIS on the other hand, shares the similar amino acid with CTCF in the ZFs region. Our study has successfully identified an *in vivo* interaction between BORIS and YB-1 in RGBM. To the best of our knowledge, this is the first study reporting on the interaction of BORIS to YB-1 in the brain cancer cell line.

Previously reported studies showed that, there are numbers of proteins could interact with CTCF. Such protein identified by Chernukhin *et al.* (2007) is a larger subunit pol II. In this study, K562 derived larger subunit Pol II was able to form a complex with CTCF through C domain however there is no interaction detected through ZFs and N domains. Therefore, CTCF was proven to be a diversified protein in which it is able to bind to other protein partners through specific domains. In this current study, we have successfully determined the interaction of CTCF/BORIS to YB-1, however it is important to determine whether these two proteins share the same binding region.

In this study, both CTCF and BORIS show a positive interaction to YB-1. CTCF and BORIS found to have a single band however YB-1 was detected to have few bands. From this study, YB-1 protein was detected to presence as a multi-subunit proteins and it migrated anomalously in the SDS-PAGE (Klenova *et al.*, 2002). In CO-IP procedure, it is important to determine the integrity of the cell line and the optimum protein extraction used. For that, a control, cell lysate without the addition of appropriate antibody was included in this study. According to the research carried out by Filippova *et al.* (1996) BORIS expression is not uniform among the cell lines. From the study reported, breast cancer cell line was reported to have 80-100% BORIS expression whereas prostate cancer

cell line was reported to have 50-60% BORIS expression. Hence it is important to determine BORIS expression in the cancer cell line use in CO-IP procedure and in this study, BORIS expression detected was high in RGBM cancer cell line.

The presence of BORIS and CTCF in the cancer cells may interfere with the normal physiological function of the cell. Both CTCF and BORIS seem to be co-expressed in tumour cells, suggesting a potential competition between these two proteins for the same CTCF target sequences. Marshall *et al.*, 2014 has shown that, almost 64% of BORIS binding sites are overlapping with CTCF binding sites. Therefore, incline BORIS expression, especially in the cancer cell, reduced CTCF occupancy at BORIS binding sites, indicating BORIS can compete with CTCF for the same binding region thus resulting in cellular phenotype permissive for transformation.
The conclusion, to the best of our knowledge this is the first study reported on the *in vivo* interaction of BORIS to YB-1. However, further experiment need to be carried out to confirm the interaction between these two proteins is direct and it is not mediated by the third protein that acts as a scaffold. For this reason *in vitro* interaction assay such as pull down assay can be perform to further map the interaction of these two proteins. In addition, it is also necessary to establish the functional study such as reporter assay to further understand the interaction of these two proteins as strong interactions can be non-specific and biological irrelevant whereas even weak interactions can be specific and have an important biological implication. Since BORIS expression is restricted to several cancer cells and spermatocytes, hence it could present as an immunotherapeutic avenue for the treatment of many cancers.

5.0 ACKNOWLEDGEMENT

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In-vivo Association of Multivalent 11 Zinc Fingers Transcriptional Factors CTCF and Boris to YB-1 in Multiforme Glioma-RGBM Cell Line

Daruliza Kernain, Shaharum Shamsuddin, See Too Wei Cun

***Abstract*—CTCF is a unique, highly conserved and ubiquitously expressed 11 zinc finger (ZF) transcriptional factor with multiple target site. It is able to bind to various target sequences to perform different regulatory roles including promoter activation or repression, creating hormone-responsive gene silencing element, and functional block of enhancer-promoter interactions. The binding of CTCF to the essential binding site is through the combination of different ZF domain. On the other hand, BORIS for Brother of the Regulator of Imprinted Sites, which expressed only in the testis and certain cancer cell line is homology to CTCF 11 ZF domains. Since both transcriptional factors share the same ZF domains hence there is a possibility for both to bind to the same target sequences. In this study, the interaction of these two proteins to multifunctional Y-box DNA/RNA-binding factor, YB-1 was determined. The protein-protein interaction between CTCF/YB-1 and BORIS/YB-1 were discovered by Co-immunoprecipitation (CO-IP) technique through reciprocal experiment from RGBM total cell lysate. The results showed that both CTCF and BORIS were able to interacts with YB-1 in Glioma RGBM cell line. To the best of our knowledge, this is the first findings demonstrating the ability of BORIS and YB-1 to formed a complex *in vivo*.**

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

I. Introduction

CTCF or CCCTC binding factor, is an 11 Zinc finger transcriptional factors with multiple DNA site specificities. It is able to bind to different target sequences to perform various regulatory roles such as promoter activation or repression, silencing and constitutive and methylation dependent chromatin insulation [6]. On the other hand, BORIS or CTCFL (Brother of the regulated of imprinted sites) is a paralog of CTCF. It was classified as a cancer testis antigen (CTA) and human BORIS gene spans over 29 kb at 20q13 which comprises of 11 exons, 10 of which are coding [8].

According to the literature, the ZF domains of both CTCF and BORIS were reported to have 95 % homology however the N and C terminal domains are described to be different [4]. This may suggest that ZF regions may interacts with the same binding partners, altering the gene expression probably by different mechanisms.

Previously reported study shows that, CTCF binds to the DNA targets via a combination of different ZF domains [5]. Study carried out by [1] who carried out the study on matrix-immobilized purified recombinant CTCF on the isolation of CTCF protein partners reported that, one of the protein partner found to interacts with CTCF was YB-1 (multifunctional Y-box DNA/RNA-binding factor). YB-1 known to be involved in transcription, replication and RNA processing. Hence, the interaction between CTCF/YB-1 may have a significance roles in regulation of major cellular processes.

CTCF and BORIS expression in normal tissue are by mutually exclusive manner [9]. BORIS expression was reported to be restricted in testis and several cancer cell lines [3]. The detection of BORIS in cancer cell lines and the absence in post-meiotic germ cell line provides an opportunity to be utilized as a biomarker in cancer development. Both CTCF and BORIS seems to be co-expressed in tumour cells suggesting a potential competition between these two proteins for the same target CTCF DNA binding sequences.

Malignant gliomas are the most common types of brain tumours in adults and it was reported that the mean survival time of patients is less than a year [7]. It is highly invasive and its constitute more than 90 % of all primary malignant central nervous system (CNS) tumours. Previously, our group has successfully developed our very own primary glioma cell line from local clinical tumour sample through explants technique and the cell line was named as RGBM. BORIS expression in this cell line was characterized and it was reported to be expressed at 76 KDa [8]. To the best of our knowledge, this is the first study, reporting on the *in vivo* protein-protein interaction between BORIS and YB-1 in RGBM-Glioma cell line. YB-1 was identified as a protein interacting partners with CTCF. We demonstrated that, CTCF is associated in vivo with YB-1 through CO-IP technique. Furthermore, we show that BORIS (a paralog of CTCF) was also able to formed an *in vivo* complex with YB-1 via the similar technique. Hence, these finding may provide a basic link to the functional role of BORIS in the cancer machinery pathway.

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PS-05

Physical mapping between Y-box DNA/RNA-binding factor, YB-1 truncated proteins and multivalent 11 Zinc Fingers Transcriptional Factors, CTCF and BORIS in Multiforme RGBM cell lines

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CTCF is a unique, highly conserved and ubiquitously expressed 11 zinc finger (ZF) transcriptional factor with multiple target site. It is able to bind to various target sequences to perform different regulatory roles including promoter activation or repression, creating hormone-responsive gene silencing element, and functional block of enhancer-promoter interactions. The binding of CTCF to the essential binding site is through the combination of different ZF domain. On the other hand, BORIS for Brother of the Regulator of Imprinted Sites, which expressed only in the testis and certain cancer cell line is homology to CTCF 11 ZF domains. Since both transcriptional factors share the same ZF domains hence there is a possibility for both to bind to the same target sequences. In this study, both in-vivo and in-vitro interaction of these two proteins to multifunctional Y-box DNA/RNA-binding factor, YB-1 was determined. The protein-protein interaction between CTCF/YB-1 and BORIS/YB-1 were discovered by Co-immunoprecipitation (CO-IP) and pull-down assays through reciprocal experiment from RGBM total cell lysate. The **in vivo** interaction assay showed that CTCF results showed that both CTCF and BORIS were able to interact with YB-1 in Glioma RGBM cell line. In vitro interaction assay showed that the interaction CTCF TO YB-1 occurred through ZF domains and CSD. To the best of our knowledge, this is the first findings demonstrating the ability of BORIS and YB-1 to form a complex *in vivo*.

BIOCHEMICAL AND FUNCTIONAL CHARACTERIZATION OF THE INTERACTIONS BETWEEN A TRUNCATED TRANSCRIPTIONAL FACTOR, CTCF WITH ITS PARTNERS Y-BOX BINDING PROTEIN-1 AND THE CTD OF POL II

By

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MARCH 2016

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FINAL REPORT

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Mapping of the Interaction Between Multivalent Transcription Factor, CTCF and Y-Box-Binding Protein-1 (YB-1) in Glioma and Osteosarcoma Cell Lines

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ABSTRAK

CTCF adalah faktor transkripsi jejari 11-Zn, yang terlibat dalam pengawalan transkripsi, penebat, kawalan cetakan, dan pentakaktifan kromosom X. CTCF merupakan gen yang sentiasa diekspres dan terpelihara antara spesies. Fungsi biologi CTCF dalam sel boleh dikenal pasti melalui interaksi protein dengan protein CTCF yang diekspres. Berdasarkan kajian-kajian yang terdahulu, terdapat pelbagai protein yang boleh berinteraksi dengan protein CTCF. Penggunaan dua daripada protein tersebut telah dipilih untuk kajian ini. Protein yang pertama ialah faktor transkripsi YB-1 yang terdapat dalam kalangan Y-kotak, dan protein yang kedua ialah Subunit Besar RNA polimerase II (LS Pol II) yang merupakan enzim utama untuk transkripsi. Interaksi CTCF dan YB-1 dicirikan dalam titisan sel Glioma-RGBM. Hasil cerakin co-IP menunjukkan CTCF dapat membentuk kompleks dengan YB-1 dalam keseluruhan lisis sel RGBM. Interaksi antara kedua-dua protein tersebut turut digambarkan melalui cerakin *in vitro pull-down*. Berdasarkan hasil kajian, CTCF-ZF adalah satu-satunya domain yang berjaya diikat dengan YB-1 CSD, manakala domain yang selebihnya tidak menunjukkan sebarang interaksi. Seterusnya, fungsi interaksi antara kedua-dua faktor tanskripsi telah ditentukan melalui sistem dua hibrid mamalia. Hasil kajian menunjukkan interaksi yang kukuh antara CTCF dan YB-1 apabila kedua-duanya diperkenalkan ke dalam titisan sel RGBM yang membuktikan kepentingan biologi interaksi dua protein ini dalam sel.

1.1 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. Interactions among transcriptional factor as with other protein can be analyzed and detected by many methods such as *in vivo*, *ex vivo* and *in vitro*. In this study, two methods were chosen to determine the interaction between CTCF and YB-1. This chapter elucidates the interaction of CTCF and YB-1 truncated regions which were prepared previously as described in Table 2.4.

The *YB-1* gene was reported to binds to the Y-box promoter sequences and it is known to involve in diverse biological processes which include transcription, replication, RNA processing, DNA or RNA-dependent events, pre-mRNA transcription and mRNA packaging (Kohno *et al.*, 2003). Theoretically, YB-1 is a 36 kDa protein consists of three main domains which are the N-terminal domain, the Cold Shock Domain (CSD) and the C-terminal domain.

The *CTCF* gene on the other hand, was discovered in 1991 by Lobanenkov *et al.* (1991). The group has found a protein that bound to the three regularly spaced CCCTC repeats at 12-13 bp intervals, thus it was named as a CTCF (CCCTC-binding factor). CTCF is an 11-Zn-finger transcription factor with highly versatile functions (Klenova *et al.*, 2002) and it is localized to the nucleus, ubiquitously expressed and highly conserved protein (Loukinov *et al.*, 2002). 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.

All the CTCF and YB-1 truncated constructs were prepared previously as described in Table 2.4. These genes were cloned into a modified version of pET-16b which was named as pET-16b-SH3(Cys) to which the original vector sequences was modified by adding four cysteine residues coding sequences to the vector before the stop codon (Figure 4.1). These residues were added to improve the binding procedure of bacterially expressed proteins to the matrix for the pull-down assay by supplying the functional thiol group (SH) for the binding process.