INVESTIGATION OF BORIS AND CTCF GENES EXPRESSION BY SYBR-GREEN BASED REAL-TIME RT-PCR IN HUMAN BRAIN TUMORS

by

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CERTIFICATE

This is to certify that the dissertation entitled "INVESTIGATION OF BORIS AND CTCF GENES EXPRESSION BY SYBR-GREEN BASED REAL-TIME PCR IN HUMAN BRAIN TUMORS" is the bonafide record of research work done by MR. FAIZUL RAHMAN BIN SJAFRI during the period from July 2008 to October 2008 under my supervision.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

µL	Microlitre
A	Absorbance
BORIS	Brother of the Regulator of Imprinted Sites
bp	Base pair
cDNA	Complementary deoxyribonucleic acid
CNS	Central nervous system
C_T	Threshold cycle
CTA	Cancer testis antigen
CTCF	CTCF
CTS	CTCF target sequences
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
EtBr	Ethidium Bromide
EDTA	Ethylenediaminetetraacetic
GBM	Glioblastoma multiforme
hr	Hour
HUSM	Hospital Universiti Sains Malaysia
L	Litre
LOH	Loss of heterozygosity
MAGE-A1	Monoclonal and Polyclonal Antibodies from Abnova
mg	Milligrams
min	minutes
mL	Millilitre
mM	Millimolar
M-MuLV	Moloney-Murine Leukemia Virus
MRC	Molecular Research Centre

mRNA	Messenger ribonucleic acid
MW	Molecular weight
NCBI	National Center for Biotechnology Information
nm	Nanometre
OD	Optical density
°C	Degree celcius
PCR	Polymerase Chain Reaction
pg	Picograms
Rn	Normalized reporter
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RT-PCR	Reverse Transcriptase – Polymerase Chain Reaction
SDS	Sodium dodecyl-sulfate
TAE	Tris-Acetic-EDTA
USA	United States of America
UV	Ultraviolet
WHO	World Health Organization
w/v	Weight per volume
x g	Gravity
ZF	Zinc Finger
ΔRn	Delta Rn
λ	Lambda

ABSTRACT

The major aim of this study is to study the BORIS and CTCF genes expression in brain tumors by determines the relative quantification of expression by Real-Time RT–PCR analyses. The result in this study is vital as one of other new approaches for the diagnostic or prognostic brain tumour purposes. To investigate the expression of these genes' mRNA, the samples of total RNA was prepared for each tumour sample. Intact total RNA with purity between 1.8 – 2.0 was used for synthesis of cDNA. In order to confirm the expression level of BORIS and CTCF gene, gene-specific primer were designed using sequence obtained from GenBank (Primer Express Software version 2.0 Apllied Biosystem). Real-Time RT-PCR analysis was conducted by measuring the given level of fluorescence as the number of cycle threshold (C_T). These C_T values serve as indirect indicators of gene expression so that samples with high expression of a given gene will exhibit lower C_T value than samples showing low level of gene expression. A gene is considered differentially expressed if its relative expression is two-fold or greater. In this study, the results of Real-Time RT-PCR demonstrated that the expression level of the CTCF was significantly higher in tumor than in normal in 2.8 fold. However, there was no BORIS expression was detected either in tumor or normal sample. Even though this is only preliminary study due to restricted number of samples and also limited time given, however, we believe that by further this study will guide us to appreciate better the role of BORIS and CTCF genes in formation of brain tumor.

ABSTRAK

Tujuan utama kajian ini adalah untuk mengkaji pengekspresan BORIS dan CTCF di dalam tumor otak dengan cara menentukan kuantiti relatifnya berdasarkan analisis oleh 'Real-Time RT-PCR'. Keputusan kajian ini penting kerana ia merupakan salah satu pendekatan terbaru dalam diagnostik dan prognostik penyakit tumor otak. Dalam menyiasat pengekspresan gen mRNA, sampel dari RNA total disediakan dari setiap sampel tumor. Hanya RNA total yang memiliki $A_{260/280}$ di dalam julat 1.8 – 2.0 akan digunakan untuk menghasilkan cDNA. Untuk menentukan peringkat pengekspresan gen, primer spesifik-gen akan direka menggunakan jujukan yang diperolehi dari GenBank (Primer Express Software version 2.0 Apllied Biosystem). Analisis 'Real-Time RT-PCR' ini telah dijalankan dengan mengukur aras fluorescence yang ditunjukkan sebagai 'cycle threshold' (C_T). Nilai C_T ini berperanan sebagai petunjuk tidak langsung untuk pengekspresan gen. Oleh sebab itu, sampel yang memiliki pengekspresan gen yang tinggi akan mempamerkan nilai C_T yang lebih rendah berbanding sampel yang memiliki aras ekspressi gen yang rendah. Di dalam kajian ini, keputusan 'Real-Time PCR' menunjukkan bahawa aras pengekspresan untuk gen CTCF adalah lebih tinggi di dalam sampel tumor berbanding sampel normal sebanyak 2.8 kali. Walaupun kajian ini hanya sebagai saringan disebabkan oleh bilangan sampel yang agak terhad serta kekangan masa yang dialami, namun, kami percaya bahawa dengan meneruskan kajian ini dapat membawa kita untuk lebih memahami peranan BORIS dan CTCF di dalam pembentukan tumor otak.

CHAPTER 1 INTRODUCTION

1.1 Introduction to Brain Tumour

Tumors of the central nervous system (CNS) are the second commonest form of cancer in children and the sixth commonest form in adult (Ironside *et al.*, 2002) while glioblastoma multiforme type is (GBM) being the most malignant form of this disease (Tsuchiya *et al.*, 2005). The biological behaviour of gliomas varies from slow-growing well-distinct tumors that are curable by excision to malignant invasive tumors that are uniformly fatal. While pathology has a major role to play in the management of patients with gliomas by providing a histological diagnosis and tumour grade, which are of major prognostic significance, however, molecular genetic studies have found loss of genetic material in many gliomas, with progressive losses identified with increasing tumour grade. For instance in oligodendrogliomas, loss of heterozygosity (LOH) on chromosomes 1p and 19q is of therapeutic significance as a predictor of tumour response to chemotherapy (Colin *et al.*, 2006). Besides, glioblastomas are highly malignant brain tumors and they have been described as one of the most deadly human cancers (Carter *et al.*, 2008).

There are two theoretical classifications of the condition exist: primary (de novo), which does not exhibit prior disease and secondary glioblastoma, which develops from a pre-existing glioma (Carter *et al.*, 2008). Glioma can arise either spontaneously (primary glioma) or can progress from a lower to a higher grade of tumour (secondary glioma) (Colin *et al.*, 2006). The complex accumulation of genetic mutations leading to each type of glioma (astrocytoma, oligodendroglioma, mixed oligoastrocytoma and glioblastomas) and to primary and secondary glioma of the same tissue type, are known to be very different. These include increased expression of genes, reduced expression of genes and a LOH on chromosome arms (Carter *et al.*, 2008).

However, the classifications of brain tumor can now be enhanced with new techniques for comprehensive molecular characterization. Advanced in diagnosis/prognosis by improved molecular profiling of brain tumors and identifying targets for novel and rational therapeutic approaches is indispensable nowadays. In order that, neurosurgeons and neuro-oncologists should be aware of these new developments so they can better advise and treat their patients (Boudreau *et al.*, 2005).

1.2 Statistic and prevalence of brain tumors in Malaysia 2003

Based on the report release by National Cancer Registry (2003), there are 420 cases of brain tumor were documented, comprising 2.4 % of male cancer and 1.6% of female cancer. The male: female ratio in terms of incidence for Peninsular Malaysia was 1.6: 1. Rates of brain tumour rose progressively with age for males and female but slightly decrease at 70 years old people. Based on the table 1.1, there was a steeper rise in incidence after the age 45 years for males and 55 years for females, with a progressive divergence of the curves for the two sexes.

Table 1.1 Brain and Other Nervous System Age specific Cancer Incidence per 100,000 populations (CR), by sex, Peninsular Malaysia 2003

		Male		Female		
Age, year	*No.*	%	CR	No.	%	CR
0-9	33	13.3	1.5	32	14.6	1.5
10-19	38	15.3	1.9	31	14.2	1.6
20-29	34	13.7	2.2	26	11.9	1.7
30-39	43	17.3	3.2	36	16.4	2.7
40-49	33	13.3	2.9	26	11.9	2.3
50-59	26	10.4	3.5	36	16.4	5
60-69	29	11.6	7.3	22	10.0	5.7
70+	13	5.2	6.0	10	4.6	3.7

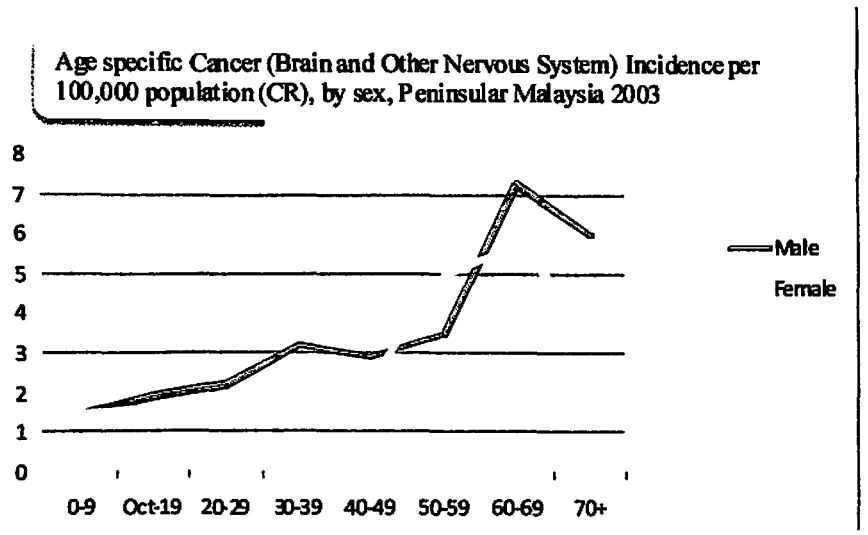

Figure 1.1 Age specific Cancer (Brain and Other Nervous System) Incidence per 100,000 populations (CR), by sex, Peninsular Malaysia 2003.
(Second Report of the National Cancer Registry Cancer Incidence in Malaysia 2003)

1.2.1 Incidence of brain tumor (HUSM)

The retrospective study of the epidemiology and prognostic factors of brain tumors was carried out at Hospital Universiti Sains Malaysia (HUSM) in Kelantan. HUSM serve as a regional referral for both Kelantan and Terengganu states. The crude incidence of brain tumor in this study was 0.44 per 100.000 population per year. Yusoff *et. al.*, (2005) have reported there are slightly significant difference of brain tumor incidence between the sexes. The incidence of brain tumors in general was more common in males than females. This finding was similar to that of Institute of Neurology in Kuala Lumpur (ratio 2:1) and mainly studies on primary neoplasms of the brain. Seventy patients with brain tumors confirmed by CT scan were admitted to HUSM during the study period. 93% of patients were Malays and 71% belonged to the low socio-economic group. The distribution of brain tumors is shown in Table 1.2. Out the 70 patients admitted over the study period, 49 had histopathological confirmation of type of brain tumour (Table 1.2). 96% were primary brain tumors and the remainder (4%) were metastatic cancer. Neuroglial tumors were the most common (35%), followed by meningioma (33%), medulloblastoma (12%) and schwannomas (6%). In children younger than 15 years of age, medulloblastoma was the most common variety encountered (41.7%), followed by other specific tumors (18.3%), astrocytoma (8.3%), and meningioma (8.3%).

Table 1.2 Distribution of brain tumors based on type of tumor and age in HUSM

Type of tumor	Age			
	Number	< 15 years	15 – 44 years	> 45 years
Astrocytoma	7 (10%)	4 (5.7%)	2 (2.9%)	1 (1.4%)
Oligodendro- glioma	2 (2.9%)	0 (0%)	2 (2.9%)	0 (0%)
Glioblastoma multiforme	8 (11.4%)	1 (1.4%)	6 (8.6%)	1 (1.4%)
Meningioma	16 (22.9%)	1 (1.4%)	6 (8.6%)	1 (1.4%)
Medulloblastoma	16 (22.9%)	1 (1.4%)	9 (12.9%)	6 (8.6%)
Nerve sheat tumour	3 (4.3%)	0 (0%)	2 (2.9%)	1 (1.4%)
Other specified tumour	7 (10%)	1 (1.4%)	5 (7.1%)	1 (1.4%)
Not Microscopically confirmed	21 (30%)	4 (5.7%)	10 (14.3%)	7 (10%)
Total	70 (100%)	16 (22.6%)	37 (52.9%)	17 (24.2%)

1.3 The Classification of Brain Tumour

1.3.1 Primary Malignant Brain Tumors

A primary malignant brain tumor is one that originates in the brain itself. Although primary brain tumors often shed cancerous cells to other sites in the central nervous system (the brain or spine), they rarely spread to other parts of the body.

The degree of malignancy in brain glioma (Bredel and Pollack, 1999) decides the treatment, because if it is grade I or II according to Kernohan, the success rate of operation is satisfactory; otherwise, if it is grade III or IV, there will be high surgical risk and poor life quality after surgery which must be taken into account before any further decision (Wang *et al.*, 2000).

Brain tumors are generally named and classified according to the following:

- The normal brain cells from which they originate, or
- The location in which the cancer develops

The biologic diversity of these tumors, however, makes classification difficult, and some experts believe that categories that are more specific are needed.

The World Health Organization (WHO) classification (2000), groups these CNS tumors into five main types (Kleihues and Sobin, 2000): tumors of neuroepithelial tissue, tumors of peripheral nerve, tumors of the meninges, lymphomas and haematopoietic neoplasms, and germ cell tumors. Of these, the commonest group is the tumors of neuroepithelial tissue, which are dominated by the group of tumors known as gliomas.

1.3.2 Categories of Primary Glioma Brain Tumors by Cell Types

About half of all primary brain tumors are known collectively as *gliomas*. They are cancerous forms of *glial* cells, the building-block cells of the connective, or supportive, tissue in the central nervous system. There are several glial cells types from which gliomas form. Their names are:

Astrocytomas represent the most common type of glioma. They develop from the supporting cells of the brain, which are star-shaped glial cells called astrocytes. Functions of normal astrocytes are to provide nutrients, support, and insulation for nerve cells and are one of the primary neurologic cells in the body. The malignant astrocytomas called glioblastomas account for 23% of brain tumors and are the most common ones (Terada *et al.*, 2002).

Oligodendrogliomas represent 4% of all primary brain tumors (McDonald *et al.*, 2005). They develop from glial cells called oligodendroglia, which are the cells that form the myelin sheath (covering insulation) of the nerve fibers within the brain. They occur most often in young and middle-aged adults but are also seen in children. The most common site of oligodendrogliomas is in the brain's cerebral hemisphere.

Ependymoma is a type of glioma that arises from the ependymal cells lining the ventricles within the brain and the central canal of the spinal cord. Ependymomas most often affect children, representing 10% of all childhood brain tumors. In adults, they occur most commonly in the spinal cord (Iwadate *et al.*, 2004).

1.3.3 Categories of Brain Tumors by Location

Some brain tumors are categorized by their location in the brain. Such tumors often contain gliomas but are also frequently a mixture of different cell types.

Meningiomas develop from meninges, the thin, protective membranes that cover the brain and spinal cord. They account for approximately 25% of all brain tumors (Marosi *et al.*, 2008). They occur more often in women than in men. Most grow very slowly, and the majority of people who have them never know they are present. Since this tends to be a slow growing tumor, it can be quite large before being diagnosed.

Medulloblastoma are primitive neuroectodermal tumors (PNET) that arise in the cerebellum. They are the most common malignant brain tumor among children, representing more than 25% of all childhood brain tumors (Maher *et al.*, 2001). This is an aggressive and invasive tumor, which frequently spreads throughout the central nervous system by the spinal fluid. Although medulloblastomas usually occur in children, with boys more often being affected than girls, they also occur in adults.

Brain stem gliomas develop in the lowest portion of the brain. The brain stem connects the *cerebrum* (the higher centers of the brain) to the spinal cord. The *brain stem* is thought to be the primitive brain because it controls the most basic functions. A stroke affecting the brain stem is potentially life threatening since this area of the brain controls functions such as breathing and instructing the heart to beat. Besides, brain stem stroke may also cause double vision, nausea and loss of coordination.

1.4 Involvement of BORIS and CTCF Gene in the Epigenetic of Normal Biology and Cancer

CTCF is a ubiquitous, preserved and highly versatile 11 Zn finger (ZF) factor. There are identified more than 20 genes of CTCF target sequences (CTSs). The distinct feature of CTCF is its ability to bind different CTCF-target sites by utilization of different ZFs (Klenova *et al.*, 2002). Recent identified of CTCF paralogue, BORIS (for brother of the regulator of imprinted sites) represent a unique pair of protein that share the same DNA-binding domain. While CTCF is ubiquitous, highly versatile and has features of a tumor suppressor gene, BORIS is expressed only in male germ cell differentiation, but expression in BORIS-negative cell promote cell growth that lead to transformation. Thus, it is activated high proportion in cancer cell and has a feature of oncogene.

1.5 The Structure and Function of the CTCF Protein

CTCF is a ubiquitous 11-zinc-finger protein that plays a role in gene silencing or activation, chromatin insulation and genomic imprinting. The CTCF gene has been mapped to the chromosome band 16q22.1 that shows frequent of LOH in breast cancer (Frengen *et al.*, 2000). CTCF encodes a DNA-binding 11-zinc-finger protein that shows a highly versatile function and multiple DNA sequence specificity (Filippova *et al.*, 1998). CTCF is a widely expressed transcription factor that is involved in different aspects of gene regulation including promoter activation (Vostrov and Quitschke, 1997) and repression (Filippova *et al.*, 1996), hormone-responsive gene silencing (Burcin *et al.*, 1997), methylation-dependent chromatin insulation and genomic imprinting (Filippova *et al.*, 2002). In addition, it has been demonstrated that CTCF can inhibit cell growth and induce cell cycle arrest at multiple stages (Rasko *et al.*, 2001). The tumour suppressor role of CTCF was suspected because of its involvement in regulating the

expression of some genes that are directly implicated in cancer (i.e., MYC, IGF2, p53, P27, p19/ARF and BRCA1) (Klenova *et al.*, 2002), its cell growth inhibitory effect and its genetic mapping to 16q22.1. In addition, some tumour-specific mutations have been detected in some tumors including breast cancer (Filippova *et al.*, 2002).

1.6 The Structure and Function of the BORIS Protein

BORIS is a paralogue of the 11 zinc-finger transcription factor, CTCF. Although the zinc-finger domain of BORIS protein has remarkable homology to the CTCF zinc-finger domain; but the N- and C-terminal domains of BORIS are different from these domains of CTCF (Ghochikyan *et al.*, 2007). BORIS is normally expressed only in spermatocytes in the testis; however, it is abnormally expressed in various tumors and cancer cell lines.

Scanlan *et al.*, 2004, BORIS has been reported to be classified as a protein belonging to the cancer testis antigen (CTA) family. The CTA gene products exhibit highly tissue-restricted expression and are immunogenic in cancer patients (Scanlan *et al.*, 2004). Though the function of the majority of the CTAs is still unknown; yet, some CTAs are thought to be implicated in the regulation of gene expression and others may control gametogenesis (Old, 2001). On the other hand, The CTAs are attractive targets for developing cancer-specific immunotherapy because of their highly restricted expression in normal tissues and broad expression in a wide range of tumors (Chitale *et al.*, 2005).

1.7 Comparison Structure of CTCF and BORIS Gene

Since BORIS is a paralogue of CTCF, thus, it shares the same exon encoding the 11 ZF-domain as mammalian CTCF genes, and therefore interacts with similar *cis* elements, but encodes amino and carboxy termini distinct from those in CTCF (Loukinov *et al.*, 2002). For that reason, the antagonistic features of these two gene siblings are underscored by showing that while CTCF over expression blocks cell proliferation; expression of BORIS in normally BORIS-negative cells promotes cell growth that can lead to transformation (Klenova *et al.*, 2002). Consequently, the sibling competition occasioned by aberrant expression of BORIS in cancer may interfere with normal functions of CTCF including growth suppression, and contribute to epigenetic dysregulation, which is a common feature in human cancer (Klenova *et al.*, 2002).

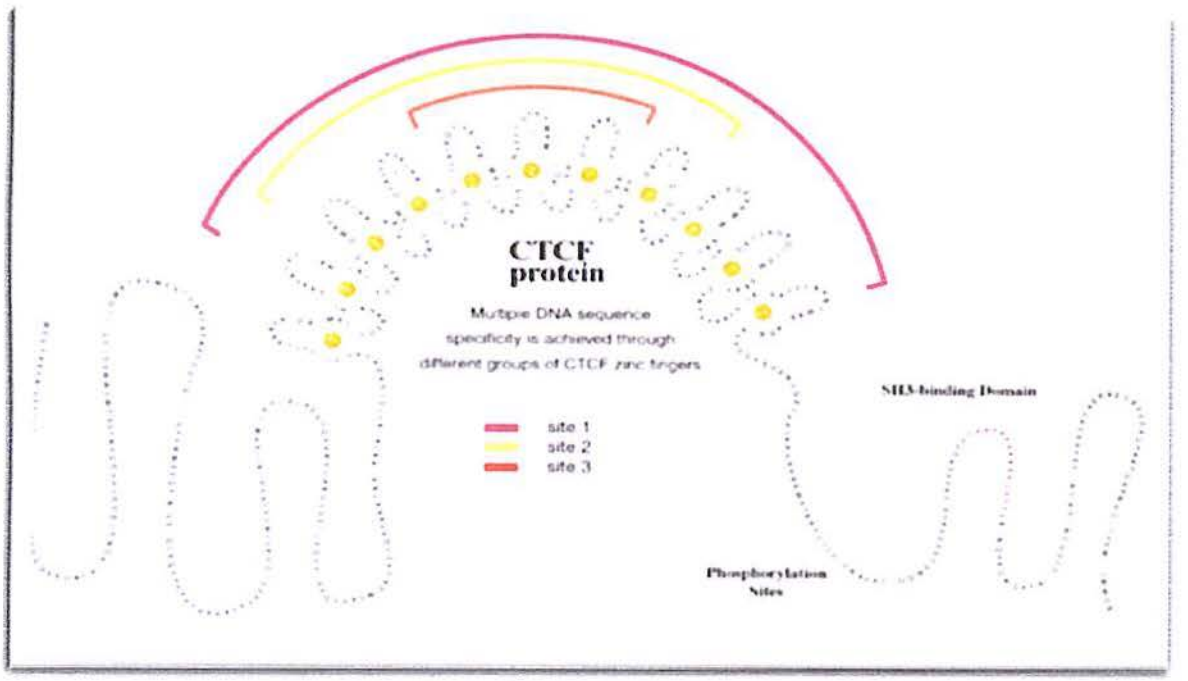

Figure 1.2 Structure of CTCF. CTCF is the first example of a true 'multivalent' transcriptional factor with multiple DNA-binding sequence specificity.

Multiple sequence specificity is achieved through combination utilization of different group of CTCF zinc fingers

1.8 Role of BORIS Gene in Tumor Development

The human BORIS gene maps to chromosome 20q13.2 (Loukinov *et al.*, 2002). However, this chromosome region is often amplified in many cancers and is believed to contain a dominant immortalising or transforming gene(s) (Tanner *et al.*, 1994). So far, recent reports show that BORIS is a downstream regulator of cancer–testis genes: expression of BORIS in normal cells leads to derepression of cancer–testis genes MAGE-A1, NY-ESO-1 and others (Hong *et al.*, 2005). As a results of CTCF and BORIS are expressed in a mutually exclusive manner during male germ-line development (Loukinov *et al.*, 2002); thus, suggesting that BORIS may be important for epigenetic reprogramming occurring during development in these cells. Definitely, BORIS has been implicated in the initiation of a series of methylation events at the imprinting control regions, in the area of the CTCF-/BORIS-binding sites, which may be significant for cancer development (Jelinic *et al.*, 2006). CTCF and BORIS thus present a uniquely paired set of genes with dysfunction contributing to the pathogenesis of multiple tumor types.

An interesting question is why and how BORIS itself is activated. Recent reports reveal that both genetic and epigenetic mechanisms are likely to be implicated in this process. Thus, DNA methylation, functional p53 and CTCF play an important role in the negative regulation of the promoters of the BORIS gene (Renaud *et al.*, 2007). Because of demethylation of DNA, knockout of CTCF and absence of functional p53, they lead to strong activation of BORIS. To date, the research efforts of several laboratories are currently focused on uncovering the details of the molecular mechanisms of activation and regulation of BORIS.