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The Expression of BORIS Protein in a Newly Established Primary Glioma Cell Culture Line

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1. Introduction

1.1 Glioma

Malignant gliomas are the most common types of brain tumors in adults. These tumors are highly invasive and despite multi- modality treatment, the mean survival time of patients is still less than a year (Machado et al., 2005). Gliomas, a cancer of glial cells constitute more than 90% of all primary malignant central nervous system (CNS) tumors. Although gliomas are the most common major subgroup of primary nervous systems tumors, their etiology is less well understood than is the etiology of the other two major subgroups: meningiomas and neuromas. The development of glioma is a complex process with the involvement of both oncogenes and tumor suppressor genes in the accumulation of genetic lesions (Zhang et al., 2005). Brain tumors arise as a result of gradual accumulation of several genetic aberrations in precursor cells which can occur either at the chromosomal level or at the gene expression level (Furnari et al. 1995). Despite considerable efforts to unravel the etiologic basis for this cancer and attempts to find a cure, gliomas largely remain refractory to treatment. Except for a small percentage of cases, the tumors continue to show high morbidity and mortality (Wrensch et al. 2005). The identification and evaluation of the cellular sites in which such genes and their encoded proteins are expressed, could help us to determine how those cellular expression sites are linked to the convoluted circuits of neurons that control brain function. To date, there is major technological challenge in the capability to detect and map genes whose expression is altered either by the disease's process or by the cells adaptations to resist the disease's process (Bloom et al., 2004).

1.2 Primary culture

Primary culture is the initial culture established from an individual which represents the environment most closely related to the original cell. The availability of unlimited number of cells and the possibility of performing multiple, repetitive experiments over long period of time renders cultured tumor cells a valuable mean for preliminary assessments of new therapeutic agents (Bakir et al., 1998). Development of biotechnology methods including primary culture technique gives us the opportunity to study the malignant glioma in depth

in order to understand the etiology of the brain tumor especially on gene regulations and cellular pathway mechanisms.

1.2.1 Primary culture: explant technique

Primary explant technique is one of the primary culture techniques. It was the original method developed by Harrison (1907) and Carrel (1912), and others for initiating a tissue culture. As originally performed, a fragment of tissue was embedded in blood plasma or lymph, mixed with heterologous serum and embryo extract, and placed on a coverslip that was inverted over a concative slide. The clotted plasma held the tissue in place, and the explant could be examined with a conventional microscope. The heterologous serum induced clotting of the plasma, the embryo extract and serum, together with the plasma, supplied nutrients and growth factors will stimulate cell migration from the explant. This technique is still being used but has been largely replaced by the simplified method. First of all, the tissue is chopped finely and rinsed, the pieces are seeded onto the surface of a culture flask or petri dish in a small volume of medium with a high concentration of serum, such that surface tension holds the pieces in place until they adhere spontaneously to the surface. Once this is archieved, outgrowth of cells usually follows.

1.3 Glioma primary culture

The established glioma primary culture gives us permanent material to examine the expression of proteins of interest and their functional characterization *in vivo* and *in vitro*. For that reason, we developed our very own primary culture of the cancerous glioma from local clinical tumor sample so as to understand better its genetic background and cellular development. Establishing primary glioma cell line in culture, the malignant cells will represent an excellent permanent representative material for molecular and cellular characterization of tumor supported with specific antigenic identification and biochemical characterization of cellular proliferation (Tomaso et al., 2000; Halfter et al., 1998; Pohl et al., 1999; Grippo et al., 2001; Machado et al., 2005). In this study, we used four well known antigens such as GFAP (Glial Fibrillary Acidic Protein), which has been shown to be a marker of glial cell origin (Zhang et al., 2005), S-100 protein which was originally identified as a brain specific marker (Machado et al., 2005), Vimentin which has been detected in a few primary cultured glioma (Bakir et al., 1998) and positive expression of BORIS protein in glioma (D'Arcy et al., 2006).

1.4 Transcription factor in cancer development

Cancer is known to be a pathophysiological condition with many different genes involved including transcription factor as a main. Transcription factor is a molecule that participates, alone or as part of a complex, in the binding to a gene's enhancer response element or promoter. Transcription factors also known as regulatory proteins which have an ability to bind to specific DNA sequences (Nebert, 2002). Transcription factor have two separate protein domains called a DNA binding domain and a regulatory domain. DNA binding domain binds to their specific DNA binding sites and the regulatory domain modulates transcription. The DNA binding domain interacts with the promoters and the regulatory domain allows interaction with other transcription factors. The binding of the transcription factors activates (or in some cases, to inhibits) transcription of the specific DNA with the ultimate outcome being the up- or down-regulation of expression of that gene (Nebert, 2002, Theresa Phillips, 2008) which leads to the production of the gene's end product; mainly in protein form or in certain circumstances without production of functional end product.

1.4.1 BORIS as a transcription factor and cancer testis antigen (CTA) family

BORIS is one of the transcription factors which believed to play role in cancer development. Human BORIS (Brother of the Regulator of Imprinted Sites) gene maps to chromosome 20q13.2 (Loukinov et al, 2002). This chromosome region is often amplified in many cancers and is believed to contain a dominant immortalising or transforming gene(s).

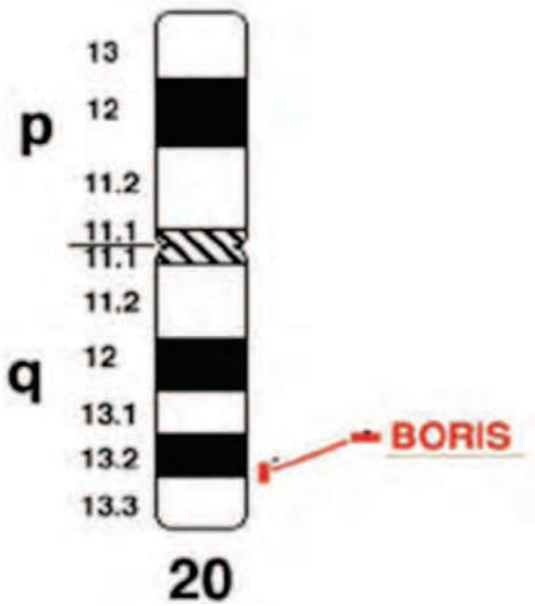


Fig. 1. BORIS map to cancer-associated 'hot spots' on HUMAN CHROMOSOME 20q13. (Modified from Klenova et al., 2002)

BORIS is a conserved, ubiquitous, and highly versatile 11-zinc-finger transcription factor involved in various aspects of gene regulation, forms methylation-sensitive insulators that regulate X chromosome inactivation and expression of imprinted genes. It is a multivalent Zinc Finger transcription factor which present only in the testis and expressed in a mutually exclusive manner with CTCF (CCCTC- binding factor) Zinc Finger transcription factor during spermatogenesis. BORIS is paralogous to CTCF and thus has the same DNA-binding potential, but with distinct amino and carboxy termini (Loukinov et al., 2002). Under normal physiologic conditions, BORIS is predominantly expressed during embryonic male germ cell development. However, BORIS is also expressed in tumors and cancer cell lines including glioma and it has been classified as a cancer-germ line or cancer-testis gene (Nguyen et al., 2008). Since BORIS bears the same DNA-binding domain that CTCF employs for recognition of methylation marks in the soma, it was identified as a candidate protein for the elusive epigenetic reprogramming factor acting in the male germ line (Loukinov et al., 2002). Other than glioma, recent studies reported that BORIS is aberrantly expressed in various cancer cell lines including uterine cancer and breast cancer (Klenova et al. 2002; Loukinov et al. 2002;Hoffmann et al., 2006; Risinger et al.; 2007, D'Arcy et al., 2008).

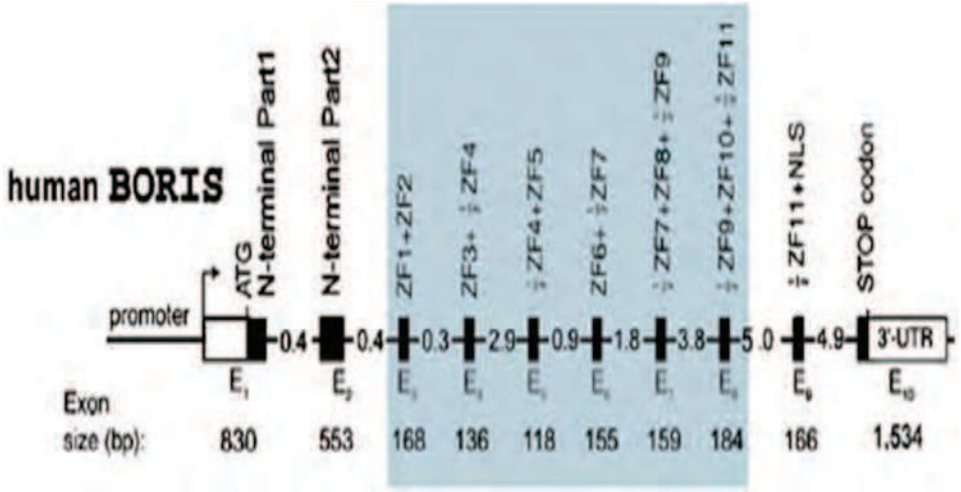


Fig. 2. Exon- Intron structure of BORIS is shown. The 11 Zn fingers are positioned in exons E3 through E8, with several individual fingers being separated between neighbouring exons. Exon 10 harbours the stop codon and a long 3'- UTR. (Klenova et al., 2002)

BORIS has been implicated in the initiation of a series of methylation events at the imprinting control regions, in the vicinity of the CTCF-/BORIS-binding sites which may be significant for cancer development (Jelinic and Shaw, 2007). However, recent study by Renaud et al. (2007) reveal that the regulation of BORIS activity is complex and being both promoter- and cell type-dependent. Varying promoter usage driving multiple BORIS transcripts influence many aspects of epigenetic reprogramming in normal development and also in tumorigenesis.

BORIS belongs to the cancer testis antigen (CTA) family which its genes products exhibit highly tissue-restricted expression and are immunogenic in cancer patients (Vatolin et al., 2005, Risinger et al., 2007). The function of the majority CTAs is still unknown; however, some CTAs may play role in a regulation of gene expression and others in gametogenesis (Old, 2001; Kalejs & Erenpreisa, 2005). The CTAs are attractive targets for developing cancer-specific therapy due to their highly restricted expression in normal tissues and broad expression in a wide range of tumors (Risinger et al., 2007). Studies by Hong et al., (2005) and Vatolin et al. (2005) reveal that the expression of BORIS correlated with and induced the expression of other cancer testis genes MAGE-A1 and NY-ESO-1.

The main focus of this study was to reveal the expression of BORIS protein as well as morphological characteristics of this newly established clinical derived glioma primary cell line. BORIS has been selected in this study due to its physical and functional related to CTCF, multifunctional transcription factor. BORIS which also known as CTCFL (CTCF-like), is the mammalian paralog of a highly conserved (Pugacheva et al., 2006), multi-functional chromatin factor encoded by a candidate tumor suppressor gene, CTCF; CCCTC- binding factor (Ohlsson et al., 2001). Loukinov et al., (2002) showed that, both BORIS and CTCF encode polypeptides of similar size that share a centrally positioned nearly identical DNA-binding domain (DBD). The DBD is composed of 11 Zn-fingers (11ZF), including ten of the classic DNA-binding C2H2-class and one (ZF 11) of the C2HC-class capable of binding both

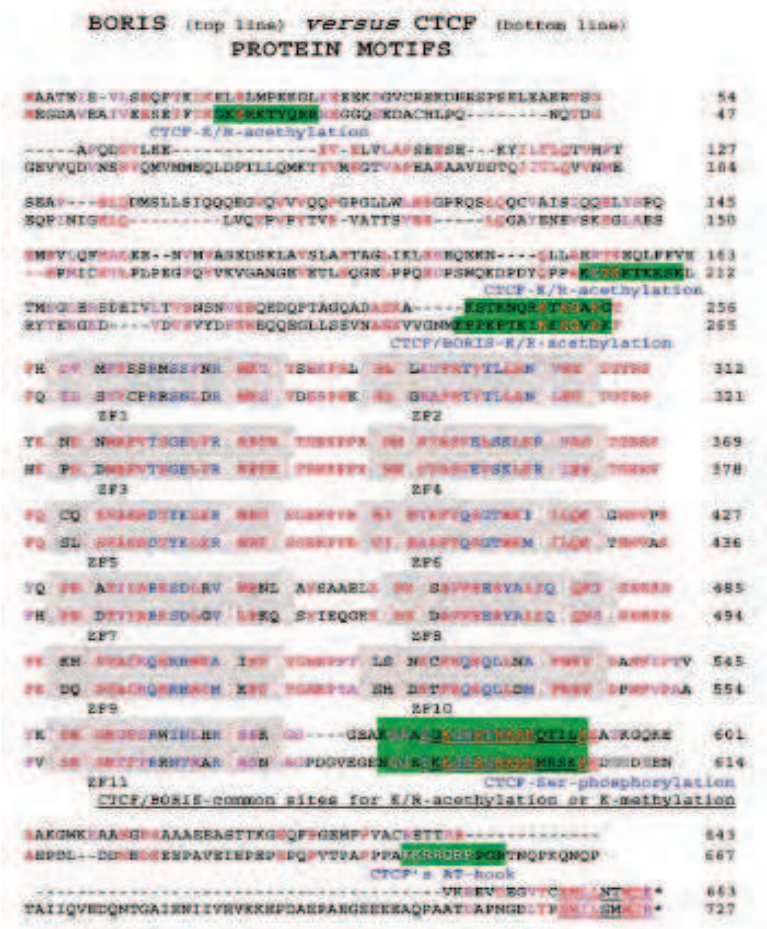


Fig. 3. Comparison of protein motifs in BORIS and CTCF. The best-fit alignment of the human CTCF and BORIS polypeptides produced by the GCG-package of programs with zero-penalty for the gap extension. Identical and similar amino acids are shown by red letters. The CxxC and HxxxH invariant residues involved in Zn-coordinated formation of the C2H2-ZF structures are shown by outlined letters in a bigger font. The near-identical ZFs, numbered 1–11, are highlighted by a gray background, and the critical base-recognizing residues at positions−1, 2, 3, and 6 inside of each ZF are shown by blue letters. Dashed lines show the gaps introduced to reveal minor similarities outside of the ZF-domains. The major Ser-phosphorylation sites and the putative additional DNA-binding 'AT-hook' motif, conserved in vertebrate CTCF proteins but absent in BORIS proteins, are shown by outlined letters. The most likely sites for K/R-acetylation and K-methylation, similar to those in the H3 and H4 histones, are highlighted in green. The experimentally determined acetylation site in the K/R-rich CTCF region downstream of the 11th ZF, and a homologous site in BORIS, are underlined in addition to green background that shows major H3-like putative K-methylation sites in both proteins. (Klenova et al., 2002)

single-strand DNA and RNA (Ladomery & Dellaire, 2002). It is remarkable that the 11 ZF DBD regions in BORIS and CTCF are encoded by genomic sequences, which present an accurate duplication of a region containing all ZF-coding exons of the CTCF gene from an early mammal. The accuracy of this duplication in the human genome was maintained to the extent that genomic nucleotide similarities between CTCF and BORIS at individual intron–exon junctions approach 100% identity at the single nucleotide level (Loukinov et al. 2002). Due to this shared DNA-recognition domain, BORIS can bind specifically to the same DNA target sequences that interact with CTCF. However, the consequences of BORIS or CTCF bound to the same site would be different because the amino- and carboxy-termini of BORIS and CTCF are totally unrelated (Klenova et al, 2002; Loukinov et al, 2002; Campbell et al., 2010). And the different functional outcomes depending on which one of the two paralogs occupies a CTCF/BORIS-11ZF binding site (Vatolin et al., 2005).

2. Materials and methods

2.1 Patient and tumor

Malignant glioma was obtained directly from the operation theater , Hospital Universiti Sains Malaysia Kubang Kerian, Malaysia with the permission from the Human Ethical Committee, Universiti Sains Malaysia (Reference number: USM/ PPSP/Ethics Com/ 2006 (179.5(2). The sample was derived from an adult male patient with recurrent glioblastoma multiforme. After surgery, the sample was immediately transferred into sterile falcon tube and transported in ice to the laboratory for subsequent culture. The cell line has been successfully cryopreserved and passaged up to 32 times.

2.2 Primary culture work

2.2.1 Preparation of sterile complete media RPMI 1640 (50mL)

10mL pre- warmed Fetal Bovine Serum (Sigma, USA) was poured into a sterile falcon tube (50mL). 1mL of penicillin-streptomycin and 100µL antibiotic cocktail (kanamycin, gentamycin, streptomycin, ampicillin, penicillin and chloramphenicol with total working concentration 50µg/ mL) (Sigma, USA) were added into the same falcon tube. Finally, RPMI 1640 with L-Glutamine and $NaHCO_3$ (HyClone, A Perbio Science Company) was added until the mixture volume became 50mL. Then, the completed media was dispensed into another sterile 50mL falcon tubes after filtering it through 0.22 micron (µm) membrane filter. The media was kept in 4^0C refrigerator until use.

2.2.2 Glioma culture

As for the primary culture technique, we chose the explant method developed by Harrison (1907) and Carrel (1912) for initiating the culture of clinically derived glioma tissue since it was a type of soft tissue and in most cases, we only managed to take out a very small amount. Based on local adaptation to our operation theatre (OT), we added a few extra steps to modify the standard explant method to reduce or to eliminate possible contamination at the early stage. For that purpose, the addition of antibiotic cocktail in the complete culture media and the pre-rinsing of the collected tissue with 70% ethanol before culturing in the flasks help us in achieving that goal. In addition, full cooperation from the neurosurgeons and OT staffs in avoiding cross contamination between surgical procedure, careful retrieval of the clinical sample from the patient's brain and careful transportation of collected sample from the OT to our culture laboratory also helped to reduce and eliminate contamination.

The tumor was given a quick rinse in 70% ethanol to disinfect all the possible microorganisms, followed by a brief rinse in sterile 1x PBS (Sigma, USA) to wash out the ethanol and also the blood. The tumor was then placed in a sterile petri dish filled with complete culture media and minced into fragments sized 1-2 mm by using a sterile blade in sterile complete medium RPMI 1640 with L-Glutamine and $NaHCO_3$ (refer to 2.2.1). The fragments were then layered on the bottom of plastic flasks (25 cm^2) and 3mL of the complete medium was poured gently into the flask to avoid detachment of the tissue fragments. The culture was incubated at 37°C in a humidified atmosphere of 5% CO_2 for about 5-7 days (Figure 1). Once the cells had migrated out and attached to the bottom of the flasks, medium was changed after intervals of 48- 72 hrs.

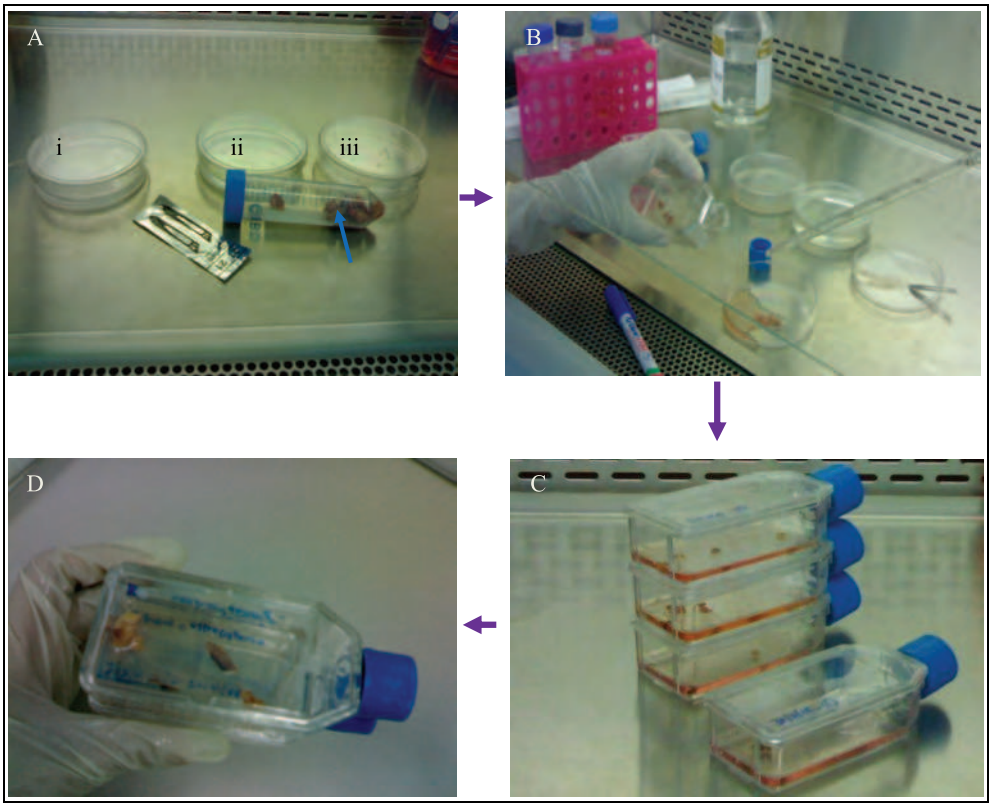


Fig. 4. Primary cell culture; steps in processing the clinical samples. (A) Materials used to culture clinical tissues (⟶); i- 70% ethanol to disinfect the tissues; ii- sterile PBS to rinse the tissues; iii- complete media RPMI 1640 wherein tissues been minced to small pieces. (B) Minced tissues transferred into cell culture flask with complete media RPMI 1640. (C) Volume of the complete media RPMI 1640 sufficient for the minced tissue contact to the bottom of the inner flask surface. (D) Labeled flask is ready to be placed in humid CO_2 incubator

When the primary culture reached confluence the cells were sub-cultured by treatment with 0.05% trypsin and 0.02% EDTA and maintained in the same medium. Subsequently the cultures were serially sub-cultured using the same procedure. Large quantities of this glioma cells were routinely frozen at various passage levels in 95% fetal bovine serum plus 5% DMSO (v/v) for future use.

2.3 Image analysis work: phase contrast microscopy

The cellular glioma cultured cells at various passages were grown in sterile cell culture flasks. The cultured cells were observed and photographed using an inverted microscope fitted with a camera (Axiovert 25 ZEISS with 0.5 X magnifier software evolution LC Kit and Image Pro- Express software).

2.4 Confirmation of cell line establishment based on marker antigen

2.4.1 ProteinExpression: SDS-PAGE & western blotting

The human malignant glioma cells were cultured in 75cm^2 flasks. Subconfluent tumor cells were collected after incubation with 0.25% trypsin and 0.02% EDTA, and washed once with pre- cooled PBS. The neutralization of the trypsin was done by the addition of pre-cooled complete medium. The detached cells suspension was spun down by centrifugation at 1500rpm for 5 minutes. The supernatant was discarded and the pellet was washed with pre-cooled sterile 1x PBS. Following centrifugation, the supernatant was discarded and the pellet was lyzed with pre- cooled radioimmunoprecipitation assay (RIPA) buffer (0.4 M NaCl, 50 mM Tris pH 7.5 (by HEPES), 1% NP- 40, 0.1% SDS, 0.1% Na deoxycholate, 1mM EDTA, 1mM PMSF) overnight at 4^{o}C . The next day, the suspension was spun at 10, 000 rpm for 20 minutes at 4^{o}C and the supernatant was then extracted. Protein samples with concentration of approximately 0.72 mg/mL were mixed with an equal volume of 2x loading dye/ treatment buffer and then heated at 95^{0}C for 5 minutes. Samples were electrophoresed in gradient (12.5% and 10%) SDS-PAGE. Once electrophoresis was completed, the proteins were visualized either by staining with commasie blue or by western blotting. SDS- PAGE used in this study was a modified of Laemmli system (Laemmli, 1970).

After electrophoresis, gels were electroblotted onto the PVDF membrane (MILLIPORE Immobilon P- Transfer membrane). The membrane was treated with 5% skim milk for 1 hour. After washing with the washing buffer, the membrane was then incubated with primary antibody; GFAP (abcam), Vimentin (abcam) or BORIS (Santa Cruz) for 1 hour at room temperature. Following three times (5 minutes each) washes in washing buffer, the membrane was treated with anti- mouse or anti- rabbit immunoglobulin antibody conjugated with Horse-Radish Peroxidase (HRP) for 1 hour at room temperature. A final wash was performed, and the membrane was equilibrated with detection buffer ECL solution (Immobilon Western Chemiluminescent HRP Substrate) for about 3 minutes and the membrane was placed in developing folder on the hypercassette (Amersham Life Science). In the dark room, the film (Kodak X- Omat LS Film Sigma- Aldrich) was exposed to the membrane for about 10 minutes before it was developed accordingly.

2.4.2 Immunocytochemistry

Immunocytochemical analysis of neuron- glial markers (Beta III- Tubulin, MAP2, Oligodendrocytes and GFAP) were performed using specific antibodies purchased ; neuron-glial cell marker sampler kit from Millipore, Chemicon whereas the other glial markers (S-

100 and vimentin) and BORIS were performed using specific antibodies purchased from Abcam and Santa Cruz respectively. IHC Select HRP/ DAB (Millipore, Chemicon) kit was used as the secondary antibody system in this analysis.
Briefly, cultured glioma cells were harvested at a specific passage, trypsinized and cultured in new chambered slides. When the cells in each chamber reached semi confluent, the medium was discarded, washed with sterile 1X PBS plus 0.05% tween 20. The slides were then fixed in cold methanol for 20 minutes at 4^{o} C. After a thorough wash with 0.05% tween 20 in 1X PBS, the cells were treated with all of the above primary antibodies according to the manufacture's instructions. The bound primary antibody was detected using peroxidase labeled polymer conjugated to either mouse or rabbit secondary antibodies. Subsequently, the slides were incubated with a substrate mixture of 3, 3- diaminobenzidine (DAB) and 0.02% H_2O_2. Cells were then counterstained with haematoxylin. Negative control slide was also included by omission of the primary antibody.

2.5 Growth curve

Glioma cells were collected from the 16th passage for determination of growth curves. Briefly, semi confluent cultures were trypsinized and cells resuspended in complete medium for counting. Cells (1×10^4) were plated into each well of a 12-well plate and counts from triplicate wells were made daily for 8 days. Trypsinized cells were counted in haemocytometer chamber and numbers were averaged for each time interval. Cell population doubling time was calculated from the linear phase of the growth curve, and the saturation density was the plateau point on the growth curve after the linear growth phase.

2.6 Real-time PCR analysis

Total RNA from cell lines (~$1x10^7$ cells) were extracted using RNeasy Mini Kit (Qiagen, Germany) and checked for concentration, integrity and purity. Two microgram of total RNA was reverse transcribed into cDNA using Revert Aid H Minus First Strand cDNA Synthesis Kit (Fermentas, USA). The BORIS primers and probe were the same as previously published by D'Arcy et al., (2006); the sequences (64bp) were as follows, forward: 5'-CCCATTGTGCCACCATCA-3', reverse: AGCATGCAAGTTGCGCATAT--3', and probe: 6FAM-ACGGAAAAGCGACCTAC-MGB; they were purchased from Applied Biosystems, USA. Real-time PCR analysis with optimal condition was performed using Taqman probe Master Mix (ABI, USA) via Applied Biosystems 7500 Real-Time PCR System (USA). The Real-time PCR products were also subsequently subjected to 2% agarose gel electrophoresis for the confirmation of PCR amplicons.

3. Results and discussion

Working in a high humidity and tropical condition exposed our clinical sample to bacterial and fungal contamination. Indeed, it is one of the constraints in the establishments of any primary tumor culture taken from a clinical source. We established an antibiotic cocktail that we used in our initial complete media which consists of amphotericin B (fungizone), gentamycin, chloramphenicol, kanamycin and ampicilin. The concentration of the cocktail used in this study varies depending on the severity of contamination in the initial condition of our clinical samples. A referral to a comprehensive pharmacology guide for antibiotic-

cellular incompatibilities information was very important when we were using two or more antibiotics in the same culture system because combined antibiotics frequently exert cytotoxic effects at lower concentrations than those indicated as appropriate for the individual antibiotics (Sigma-Aldrich.inc). In this case, we have started the cocktail addition at the lowest concentration possible.

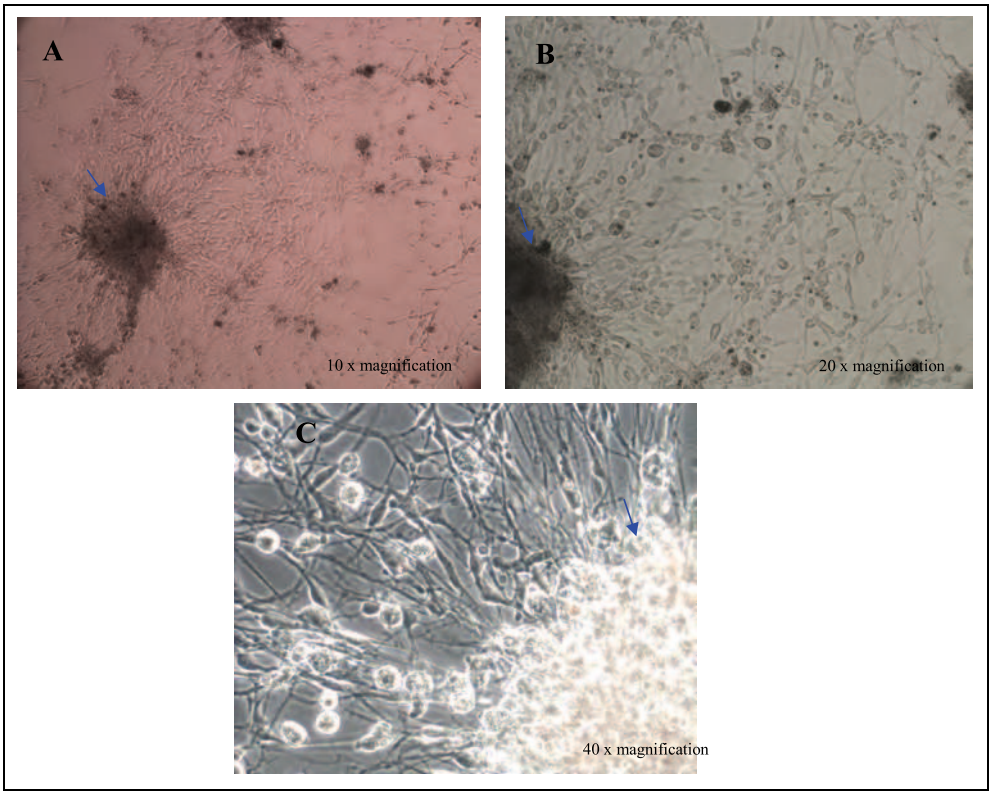


Fig. 5. Morphology of the clinical derived glioblastoma multiforme primary cell line at the initial stage; Attached tissue fragment (⟶). (A) 10 X magnification; (B) 20 X magnification; and (C) 40 X magnification.

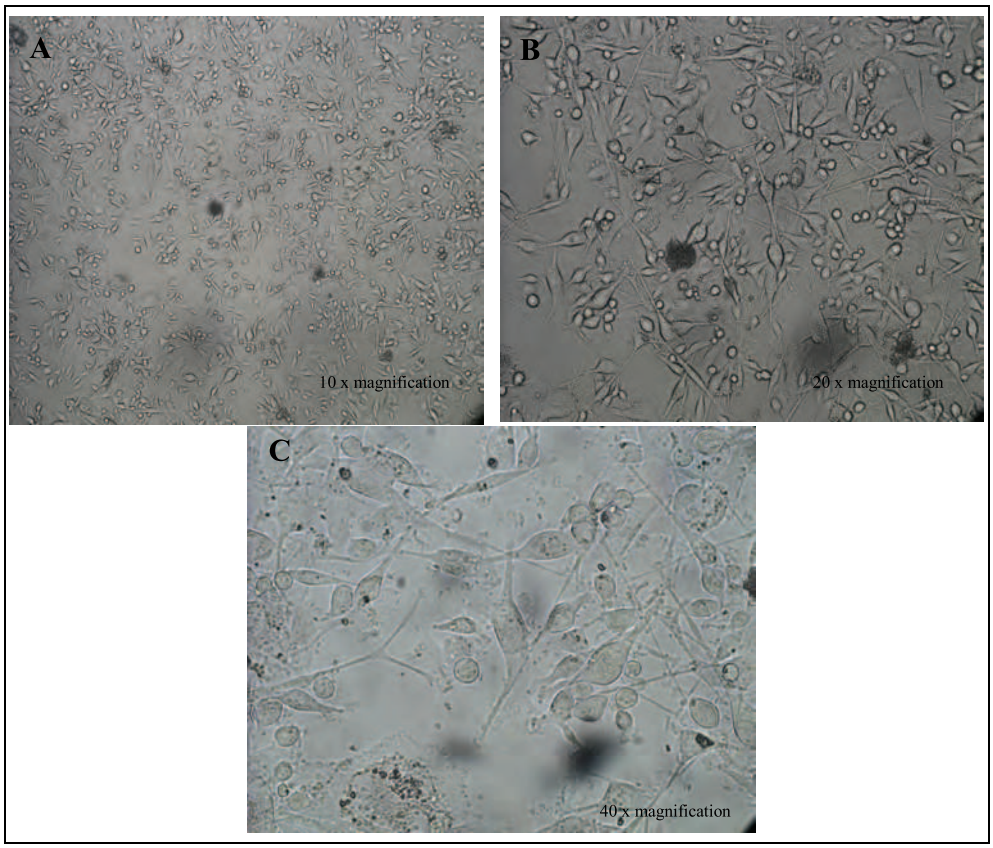


Fig. 6. Morphology of the clinical derived glioblastoma multiforme primary cell line at passage 1; rounded cells and dendritic-like cells with extensive cytoplasmatic prolongations (→).
(A) 10 X magnification; (B) 20 X magnification; and (C) 40 X magnification.

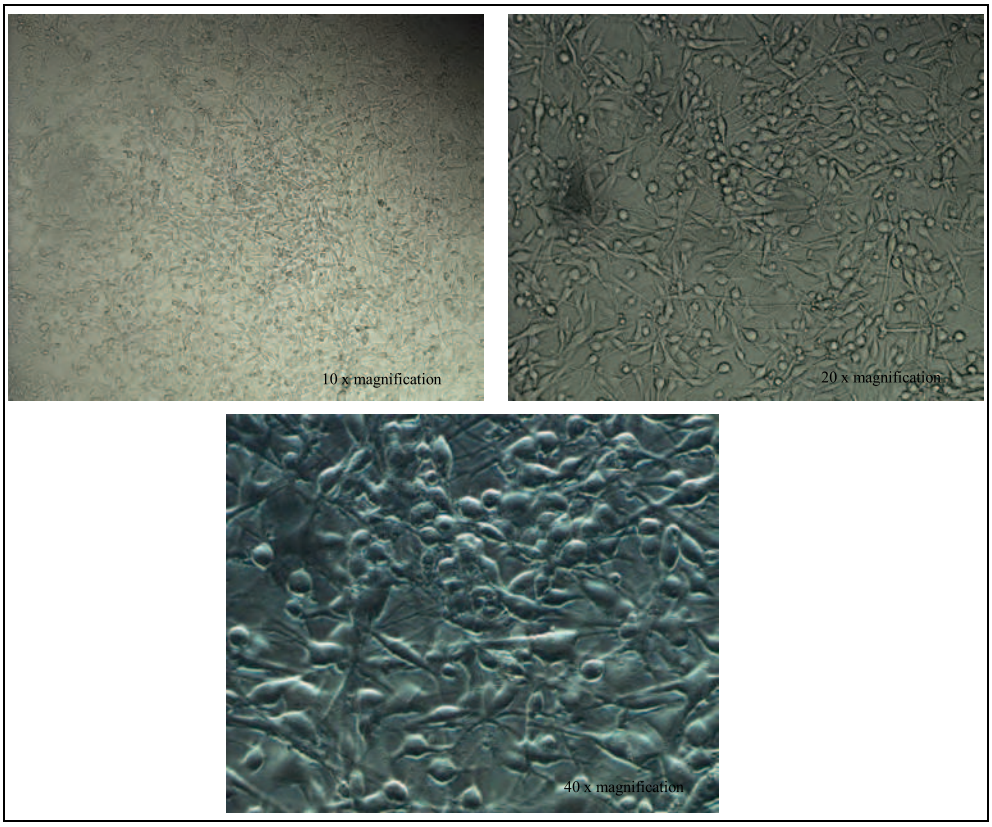


Fig. 7. Confluent monolayer of the clinical derived glioblastoma multiforme primary cell line with rounded and dendritic-like cells. (A) 10 X magnification; (B) 20 X magnification; and (C) 40 X magnification.

Variable morphological features could be found in the glioblastoma multiforme cells i.e small rounded cells and dendritic- like cells with extensive cytoplasmatic prolongations (figure 2, figure 3 and figure 4). Our finding was in agreement with other works in literature that showed evidences suggesting that some types of cancers, notably solid tumors,were not homogeneous in their cell composition but are composed of heterogeneous cell types (Bigner et al.,1979, 1981; Perzelova et al., 1998; Grippo et al., 2001; Machado et al., 2005). Similar morphological features were observed throughout serial passages, as described. This tumor cell line presented features of malignant glioma characterized by cell pleomorphism, necrosis and aggressive growth (Machado et al. 2005; Grippo MC et al. 2001). Low passage cultures grew slower than high passage cultures. This fact could indicated a perfect adaptation of cells to the culture conditions. This cell line would be useful for testing potential anticancer agents either *in vitro* or *in vivo*.

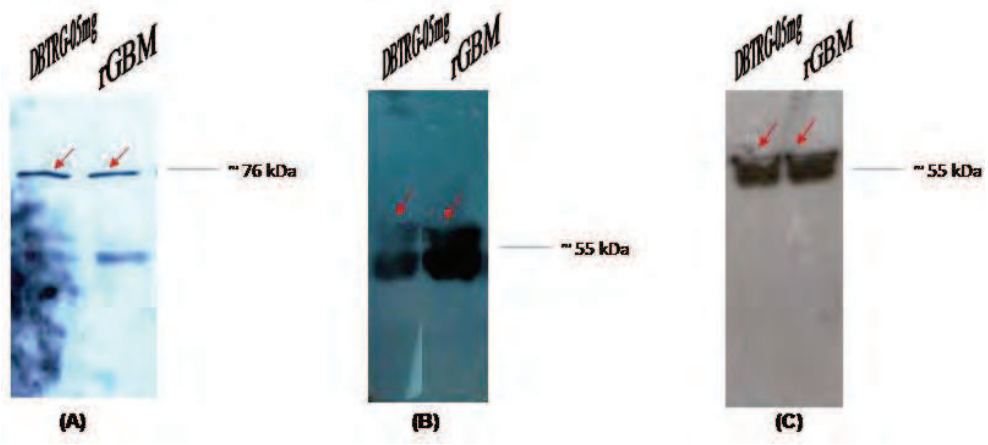


Fig. 8. Western Blotting of the clinical derived glioblastoma multiforme (rGBM) primary cell line.

Western blotting of total lysate of DBTRG-05MG (ATCC derived glioma) and primary culture cell glioblastoma multiforme (rGBM) separated in SDS-PAGE and probed with:

(A) α-BORIS polyclonal (dilution 1:500). The presence of BORIS is indicated by red arrow at ~76 kDa for both DBTRG-05MG and rGBM.

(B) α-GFAP monoclonal (dilution 1:500). The presence of GFAP is indicated by red arrow at ~50 kDa to ~57kDa for both DBTRG-05MG and rGBM.

(C) α-Vimentin monoclonal (dilution 1:500). The presence of vimentin is indicated by red arrow at ~50 to ~57 kDa for both DBTRG-05MG and rGBM.

Western blot analyses showed strong expression for BORIS protein (figure 8, A). BORIS is a CTCF paralog, which contains all eleven zinc fingers of CTCF, and has been shown to promote cell growth leading to transformation (Loukinov et al., 2002). One mechanism of action by which BORIS is thought to cause cancer through interference is the maintenance of an appropriate methylation pattern in the genome (Klenova et al., 2002). The detection of BORIS protein in glioblastoma multiforme suggested its potential as an early marker of this type of glioma since BORIS has been reported as an early tumor marker in breast cancer (D'Arcy et al., 2006). Western blot analysis also showed that the glioblastoma multiforme cell line retains the expression of GFAP (figure 5, B) which is a reliable marker of astrocytic cells (Moore 1965; Machado et al., 2005). However,literature has shown a negative correlation between the degree of malignancy and the expression of GFAP protein in the majority of human gliomas (Machado et al., 2005). We also showed that vimentin which has been identified in some human glioma cell lines (Roessmann et al., 1983; Rutka et al., 1998) was also detectable in this glioma cell line (figure 8, C). Machado et al., 2005 claimed that the effect of vimentin filaments on the invaginations or folding in the nucleus is not absolute and raised the possibility that this nuclear configuration could be an indirect effect of a metabolic difference between cells that contain or lack organized vimentin filaments. To all appearances, ours and previous study results indicated that the absence or presence of an organized vimentin filament network do not affect the shape of the cell. However,

interestingly S100 protein that was originally identified as brain specific (Moore, 1965) and believed to be expressed in a few types of gliomas was not identified in this cell line. But still, literature has shown a negative correlation between the degree of malignancy and the expression of GFAP and S100 protein in the majority of human gliomas (Jacques et al., 1981; Duffy et al., 1982).

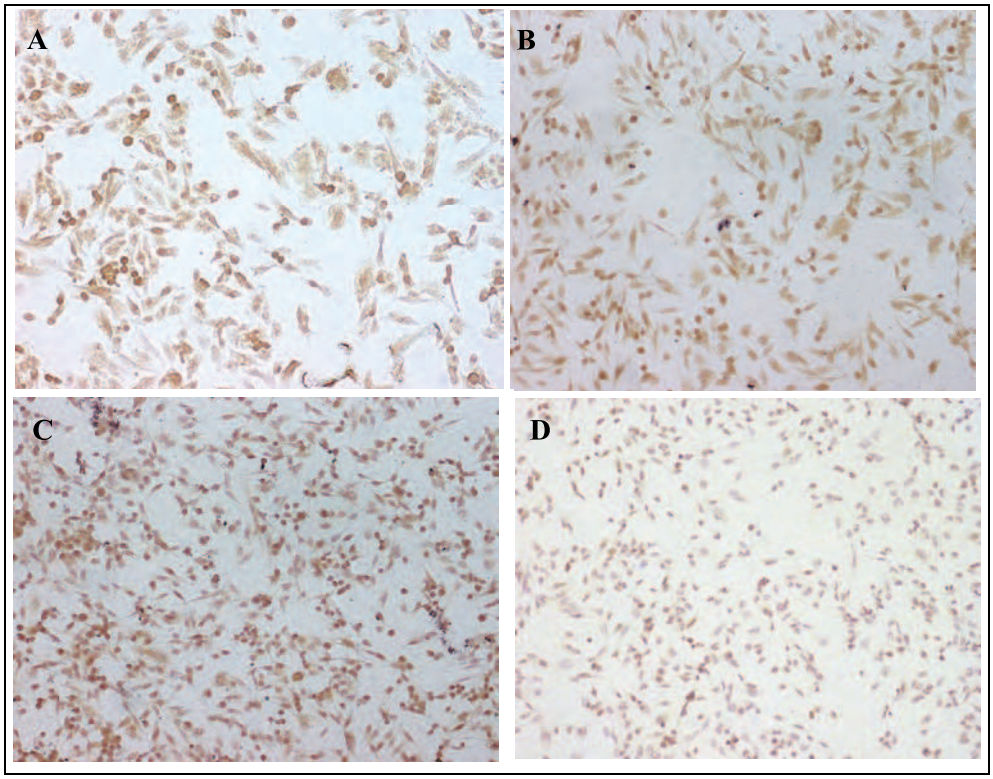


Fig. 9. Immunocytochemical staining in glioma cell line (glioblastoma multiforme) at passage 6 to passage 17 at 5 x magnification. Note the immunopositive staining for (A) BORIS, (B) GFAP and (C) B- tubulin. On the other hand, cells were S-100- negative stained (D). The negative control is the slide that stained with secondary antibody only.

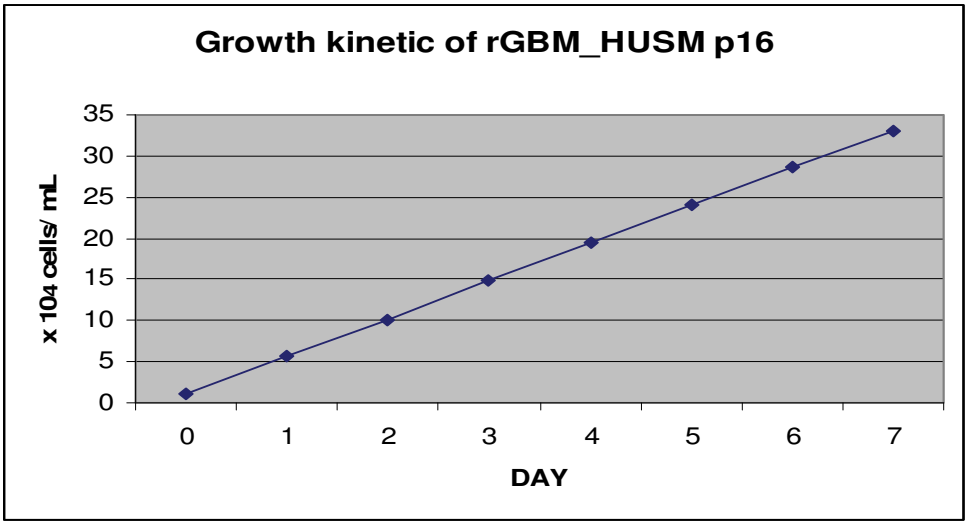
Immunopositive (figure 9) and western blotting results for BORIS indicates that the tumor-antigenic polypeptide is expressed in the glioma cell lines. These results suggest that BORIS might play an important role in regulating the tumorigenesis pathway. And if that persists, BORIS can be manipulated as a biomarker in detecting the presence of tumoric condition in glial cells in an uninvasive way and at the very early stage. Deeper understanding of the place and the role of BORIS in that exact molecular pathway, gave us a good chance in preventing or disrupting the tumorigenesis pathway. This could lead to the application of genetic therapy in treating glioma patients. Understanding and moving towards that goal, require us to study deeper and not just stop at this preliminary study. This cell line showed negative expression of MAP-2, a neuron-marker which suggests that the tumor cell line is

not originated from neuron cell and the negative expression of oligodendrocytes suggested that the cells absolutely not oligodendrocytes.

The newly established cells entered into an exponential growth phase at the 16th passage, when the cells grew aggressively in the culture. The high growth rate was observed for these successive passages when the population doubling time of the glioblastoma multiforme cell line was about 6 hrs at 37°C and the saturation cell density was reached at 3.3×10^5 cells/cm^2 (graph 1). The cells showed continuous progressive growth until day 7 without showing any sign of senescence. On the other hand, the growth kinetic of the DBTRG-05MG cells, used as a reference cells in this study, achieved population doubling time of approximately 34 to 41 hours. The saturation density of the cells reached 8.3 X 10^5 cells/ well (Kruise et al., 1992).

In comparison, DBTRG-05MG the well known established glioma cell line, introduced by Kruse et al. in 1992, showed rounded and spindle shape cells with fibroblast-like cells (figure 7).This is exactly different with our newly established clinically derived glioma but the heterogeneity of the morphology was in parallel with most studies done in glioma cells.

The study of characterization of the ATCC derived cell line also revealed positive expression of BORIS, GFAP, Vimentin (figure 8) and S100 proteins (Kruise et al., 1992). This profiling was in parallel with our newly established glioma cells with the exclusion of S100 protein. Figure 9 (D) showed our newly established cells immunonegative for S100 protein. The comparison of protein expression in both DBTRG-05MG the ATCC derived glioma cell line and our newly established glioma is shown and summarized in Figure 11 and Table 1.



Graph 1. Growth kinetic of glioblastoma multiforme cell line for passage 16 (linear graph)

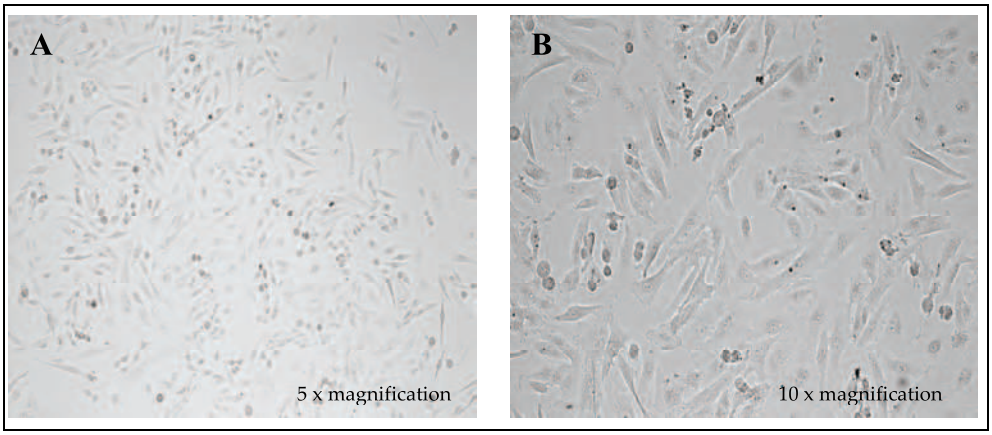


Fig. 10. Morphology of DBTRG-05MG (ATCC derived glioma cell line) under phase contrast microscopy. Rounded cell with fibroblastic-like cells.

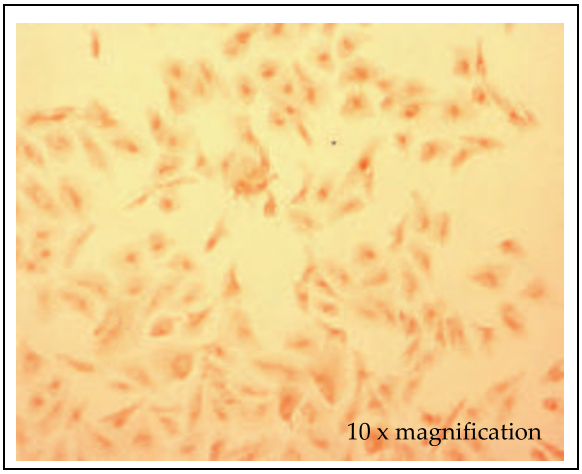


Fig. 11. Immunopositive of BORIS in DBTRG-05MG ATCC derived cell line. The negative control is the slide that stained with secondary antibody only (not shown).

Specificity	Cells	
	Clinically derived glioma (this study)	ATCC derived glioma (DBTRG-05MG)
BORIS	+	+ (Kruise et al., 1992)
GFAP	+	+ (this study)
Vimentin	+	+ (Kruise et al., 1992)
S100	-	+ (Kruise et al., 1992)

Table 1. The summary of protein studies based on immunocytochemistry and western blotting assay for established glioma cell line in comparison with the ATCC derived glioma cell line (adapted from Kruise et al., 1992).

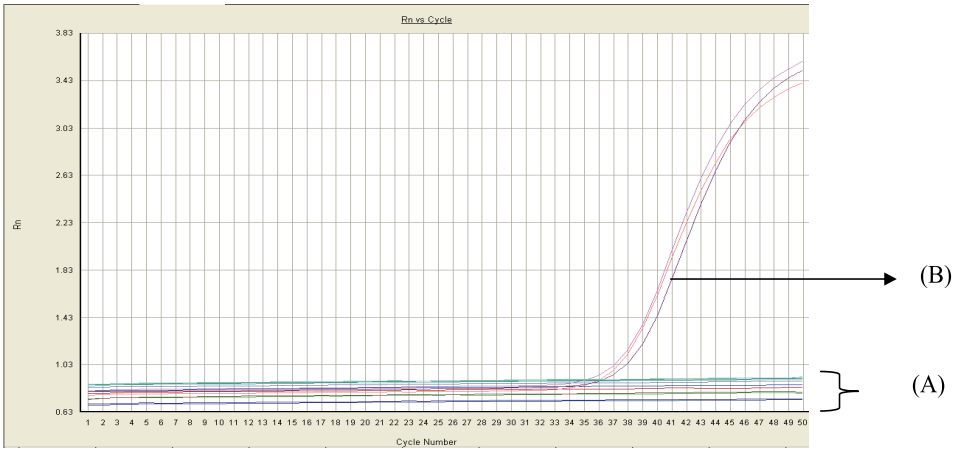


Fig. 12. Amplification plots of BORIS mRNA expression using Taqman probe master mix. The result showed that BORIS mRNA was unexpressed in DBTRG-05MG (ATCC derived glioma), ATCC derived normal osteoblast, ATCC derived normal glial cell lines and in no template controls (A). BORIS mRNA was expressed in positive control osteosarcoma U2OS cells (ATCC derived) detected at ~38 cycles (B).

The expression of BORIS mRNA was not found in DBTRG-05MG ATCC derived glioma cell line (Figure 12) although we observed the positive expression of BORIS protein in the same sample. Recently Hines et al., 2010 has reported that BORIS mRNA was not expressed in most human breast cancer cell lines and tumors. Furthermore expression of BORIS mRNA showed no significant difference between normal and prostate cancer tissues overall and no relationship was seen to clinical parameters (D'Arcy et al., 2006). This strengthened the study done by Renaud et al., in 2007 that BORIS involved in complex mechanism and it is promoter dependent gene. Therefore, at this stage we expected the undetected expression of BORIS mRNA in our newly established clinically derived glioma primary cell line with positive expression of BORIS protein in the same clinical derived sample.

4. Conclusion

We established our glioblastoma multiforme cell line with the basic characteristics including the morphology study, surface antigen profile, protein study and also the growth analysis. This cell line which was derived from our clinical sample would be useful for studies on the molecular basis of malignancy and progression of glioma as well as in studies of glioma treatment. BORIS has been shown to be expressed in our glioma primary cell line. This study has strengthened the suggestion of a previous study (D' Arcy et al., 2001) where BORIS could play an important role in the early diagnosis of glioma which requires further study with consideration of its complex behaviour in cancer development as well as the splice variants in promoter dependent gene.

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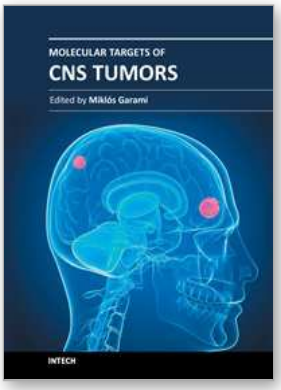

Molecular Targets of CNS Tumors

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Molecular Targets of CNS Tumors is a selected review of Central Nervous System (CNS) tumors with particular emphasis on signaling pathway of the most common CNS tumor types. To develop drugs which specifically attack the cancer cells requires an understanding of the distinct characteristics of those cells. Additional detailed information is provided on selected signal pathways in CNS tumors.

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