Short paper

Molecular signatures of cell cycle transcripts in the pathogenesis of Glial tumors

Anitha A Pillai[1], Rabindra Narayan Bhattacharya[2], Vishnampet V Radhakrishnan[3] and Moinak Banerjee*[1]

Address: [1]Rajiv Gandhi Centre for Biotechnology, Thycaud P.O., Thiruvananthapuram, Kerala, India, [2]Dept. of Neurosurgery, Sree Chitra Tirunal Institute for Medical Sciences & Technology, Thiruvananthapuram, Kerala, India and [3]Dept. of Pathology, Sree Chitra Tirunal Institute for Medical Sciences & Technology, Thiruvananthapuram, Kerala, India

Email: Anitha A Pillai - ani_pillai@yahoo.com; Rabindra Narayan Bhattacharya - rnb@sctimst.ker.nic.in; Vishnampet V Radhakrishnan - vvr@sctimst.ker.nic.in; Moinak Banerjee* - moinak_ban@hotmail.com

* Corresponding author

Abstract

Background: Astrocytic brain tumors are among the most lethal and morbid tumors of adults, often occurring during the prime of life. These tumors form an interesting group of cancer to understand the molecular mechanism of pathogenesis. Histological grading of Astrocytoma based on WHO classification does not provide complete information on the proliferation potential and biological behavior of the tumors. It is known that cancer results from the disruption of the orderly regulated cycle of replication and division. In the present study, we made an attempt to identify the cell cycle signatures and their involvement in the clinical aggressiveness of gliomas.

Methods: The variation in expression of various cell cycle genes was studied in different stages of glial tumor progression (low and high grades), and the results were compared with their corresponding expression levels in the normal brain tissue. Macroarray analysis was used for the purpose.

Results: Macroarray analysis of 114 cell cycle genes in different grades of glioma indicated differential expression pattern in 34% of the gene transcripts, when compared to the normal tissue. Majority of the transcripts belong to the intracellular kinase networks, cell cycle regulating kinases, transcription factors and transcription activators.

Conclusion: Based on the observation in the expression pattern in low grade and high grade gliomas, it can be suggested that the upregulation of cell cycle activators are seen as an early event in glioma; however, in malignancy it is not the cell cycle activators alone, which are involved in tumorigenesis. Understanding the molecular details of cell cycle regulation and checkpoint abnormalities in cancer could offer an insight into potential therapeutic strategies.

Background

Gliomas are the most common primary brain tumors. These ectodermal primary brain tumors form ~50% of all intracranial tumors. They can occur at any age, especially

young to middle age, and are comparatively more common in men. Gliomas can be subtyped as astrocytic, oligodendroglial, or ependymal. They may be classified as low-grade (WHO grade II), anaplastic (WHO grade III), or glioblastoma multiforme (WHO grade IV), in the order of increasing malignancy [1]. Currently, these classifications are based on the observed histopathological characteristics of the tumor such as nuclear atypia, mitotic activity, cellularity, vascular proliferation and necrosis [2]. Malignancy grades are pathologically determined and are sometimes subjective and inconsistent. Several molecular genetic and cell biological data have helped to identify some of the genes involved in the oncogenesis and progression of gliomas [3-5].

The classification of malignant gliomas remains controversial, and effective therapies have been elusive; hence, primary brain tumors have come under intense scientific scrutiny in the recent years [6,7]. It is known that cancers have very complex patterns of differential gene expression, and are confounded by multiple environmental variables of carcinogenesis [8]. Therefore, the study of single gene at single time point in the progression of a tumor may provide only limited information towards our understanding of the bigger picture of cancer. Most of the cancers result from the disruption of the orderly regulated cycle of cell replication and division. Different stages of cell cycle are programmed by the cell cycle clock via a variety of gene products [9]. It is stimulated by the products of proto-oncogenes and is under the negative control of tumor suppressor genes. Malignancies in cancer result from imbalance in control caused by increased effect of proliferation promoting factors i.e. oncogenes or decrease in inhibition, namely tumor suppressor genes [10]. A mutation in one of these genes can lead to cancerous condition.

Recently, a more comprehensive pathway-specific approach has been developed, which includes analysis of differential gene expression, reflecting regulatory differences [12]. In general gliomas are believed to have a linear grade of progression towards malignancy. In the present study, we seek to identify differentially expressed cell cycle genes relevant for tumor development and malignant transformation, in order to gain insights into the molecular mechanisms of Glial tumor progression and uncover new therapeutic targets.

Methods
Sample Collection
Fresh biopsy samples of glioma were collected in sterilized tubes containing RNAse inhibitor, from the Dept. of Neurosurgery, SCTIMST. Three samples in each grade, with similar age and pathological features, were considered in the present study in order to minimize tissue het-

erogeneity and the influence of other variables for the cause of the disease. Normal glial tissue was obtained from the autopsy of a head injury patient. All the samples were collected as per the institutional ethical committee guidelines. The gliomas were pathologically diagnosed and the regions of pleomorphism were identified and surgically scraped to avail maximum tumor cell population. The variation in expression of the various cell cycle genes was studied in different stages of glial tumor progression (low and high grades), and the results were compared with their corresponding expression levels in the normal brain tissue. The macroarray differential display method, which is known for its excellent throughput and outstanding expression analysis capability, was used for the purpose (Clontech Atlas Cell Cycle Array kit 7748).

RNA extraction
Total RNA was extracted from the tissue sample by the standard TRIzol method (Invitrogen). The RNA sample was digested with DNAse I (10 U/μg) to remove DNA contamination, which might lead to false positivity during hybridization. After digestion, DNAse was removed from the sample by phenol-chloroform extraction, followed by ethanol precipitation. The RNA sample was stored in -70°C till use. The quantity and quality of the purified total RNA was estimated in a UV-spectrophotometer.

Poly A+ enrichment
Poly A+ enrichment was done using CLONTECH mRNA extraction kit (7748), as per the manufacturer's protocol. The first strand of cDNA was synthesized using biotinylated oligo (dT). Poly A+ RNA (mRNA) was separated from the total RNA pool using streptavidin-coated magnetic beads.

cDNA probe synthesis
cDNAs were synthesized using the CDS primer mix (CLONTECH Atlas Human Cell Cycle CDS primer mix 7748-CDS). α-^{32}P dATP was included in the cDNA synthesis reaction to facilitate probe labeling. The labeled cDNAs were column-purified using the Atlas NucleoSpin Extraction Kit. The purified labeled probes were stored in -20°C till use.

Hybridization
The labeled cDNA probes were hybridized to the macroarray nylon membrane (Atlas Human Cell Cycle Array 7748-1) according to the manufacturer's protocol. ExpressHyb solution was used for hybridization, with sheared salmon testes DNA as the blocking agent. Along with the probe, Cot-1 DNA was added to block hybridization to repetitive DNA, which might be present in the array. Hybridization was carried out at 68°C for 18 hrs. Following hybridization, the membrane was washed thrice in wash solution I (2 × SSC, 1% SDS), and once in
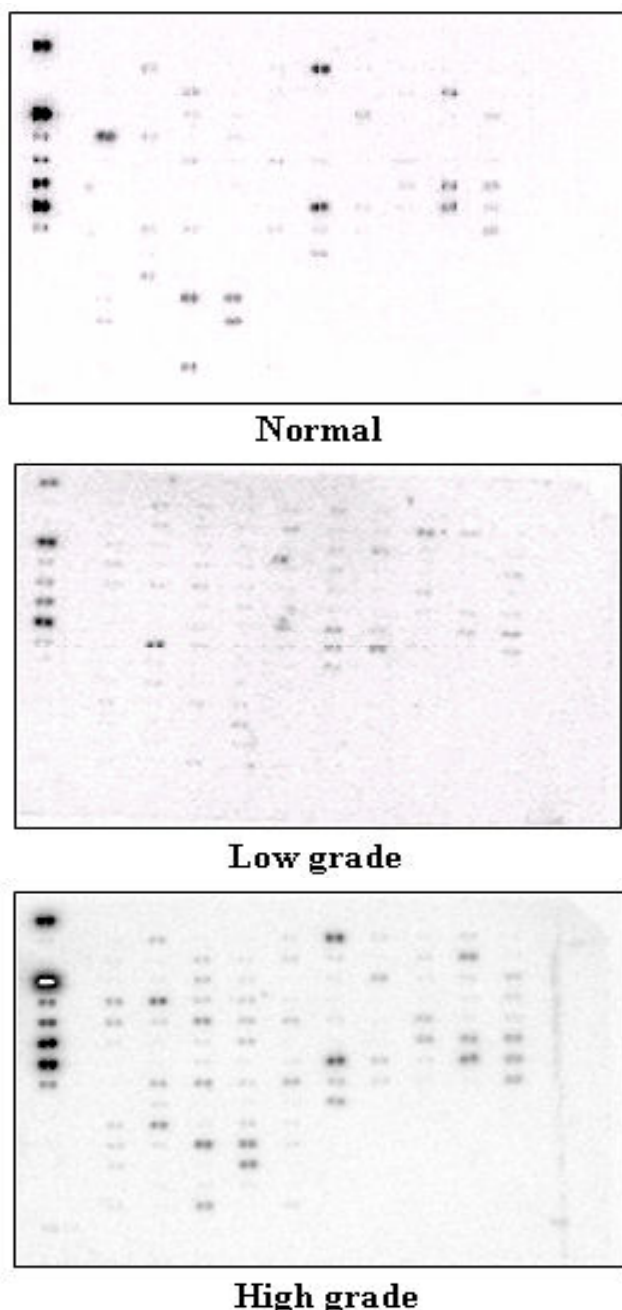
Figure 1
Macroarray profiles of cell cycle genes in different grades of Glial tumors

wash solution II (0.1 × SSC, 0.5% SDS). All the washings were carried out at 68°C for 30 min each. The membrane was finally rinsed in 2 × SSC, wrapped in a Saran wrap and exposed to a phosphor imager screen. The membrane was

exposed for 3–4 hrs and the image was documented in a Phosphor Imager (BIO-RAD).

Membrane stripping
The macroarray membrane was stripped and reused for a maximum of three times. The membrane was boiled in 0.5% SDS for 10 min. It was then cooled and rinsed in wash solution I. The membrane was wrapped in a Saran wrap and stored in -20°C.

Analysis
The resultant macroarray spots were normalized by a two step normalization process in order to control the background and have uniform signal intensity. Background normalization was done by checking the signal intensities of negative controls; normalization for uniform signal intensity was evaluated against known "housekeeping genes" in the expression array that have a known, stable binding efficiency. Expression uniformity among the housekeeping genes was observed in all the hybridization experiments. The qualitative scores of differential expression assigned to each transcript measurement were according to the following system: The fold increase (+) or decrease (-) in the range of (+/-) 0–0.5 were considered as No Change (NC), (+/-) 0.6–2.0 as Marginally Increased (MI) or Marginally Decreased (MD) and (+/-) 2.0 and above as Increased (I) or Decreased (D). Heterogeneity of expression within the grades was considered only when the expression differed by a minimal threshold of two fold.

Results
Transcriptional Assay of Cell cycle genes
Transcriptional assay of 114 cell cycle genes and 7 housekeeping genes were done using a macroarray system for astrocytoma grade II, anaplastic astrocytoma and normal Glial tissue (Fig 1). Differential expression pattern was observed in 39 (34%) gene transcripts in glioma when compared to normal tissue. Majority of these transcripts belong to intracellular kinase networks, cell cycle regulating kinases, transcription factors and transcription activators. 28 (24.5%) transcripts showed overexpression in low grade gliomas and seven (6%) transcript showed decreased expression (Appendix). However, in high grade gliomas 16 (14%) transcripts showed down regulation while 5 (4.3%) transcripts showed increased expression. Six of the overexpressed transcripts of low grade gliomas reversed to decreased expression in high grade (Fig 2).

In low-grade gliomas, upregulation of transcription factors and transcription activators were observed. Among the overexpressed intracellular kinase network transcripts, MAP Kinases form a major cluster of which, MAP Kinase 12 (15%) and MAP Kinase p38 beta (50%) contribute the most. Junction Plakoglobin, a cytoskeletal protein was

Figure 2
Upregulated and downregulated transcripts of expression in cell cycle genes

observed to have very high level of expression in low grade gliomas (91-fold). Other significantly upregulated transcripts in low grade gliomas include replication factor C3 (RFC3), jun B proto oncogene, GADD45, Chromatin Assembly factor1 p48 subunit, purine-rich element binding protein A and RBQ retinoblastoma-binding protein. Downregulated transcripts in low grades include CDK10, akt-proto oncogene and CDC37. In high grade gliomas, none of the functional clusters showed predominance of expression patterns. Significant upregulation was observed in CDKN1A, CDK5 and PCTAIRE protein kinase. Majority of the transcripts showed decreased expression in high grade, which include Cyclin D (7-fold) and CDK4 inhibitor 2D (4-fold). Heterogeneity was observed, but was within the minimal threshold and therefore, is not represented in the present data.

Discussion

In the field of neuro-oncology, new methods are desperately needed to complement current methods for disease detection, tumor grading, and determination of response to brain tumor therapy [11,12]. Conventional analysis of expression using Northern Blotting, RT-PCR methods [13] and gel based differential display methods are cumbersome, time consuming and very low throughput methods. In the present study, an attempt has been made to understand the involvement of different cell cycle genes in various grades of gliomas using a macroarray system. DNA microarray method provides a tool for massive parallel analysis of several genes using cDNAs, ESTs or oligo DNAs on a glass support; however, this method involves high system cost and data reliability is still a matter of concern. On the other hand, the reliability of data interpretation in filter based macroarray system used in the present study is very high; it is highly cost effective and uses a very well proven technology of spotting cDNA on nylon membranes. cDNA arrays can provide the powerful analysis of differing molecular profiles at various time points in the progression of the same tumor [14].

Presently, there are neither reliable prognostic indicators for brain tumors nor any circulating or *in situ* brain tumor markers that can be used to determine response to treatment. Earlier studies were based on individual grades of gliomas and represented wider spectrum of transcripts [15-18]. The mammalian cell cycle is exquisitely controlled by a 'machinery' composed of cyclin-dependent kinases and their binding partners, the cyclins. These kinases regulate transitions into DNA synthesis and mitosis; their inactivity contributes to cellular quiescence, differentiation and senescence [19]. Cell cycle transitions are, in turn, controlled by checkpoints that monitor ribonucleotide pools, oxygen tension, the extracellular environment, growth signaling programmes, the status of DNA replication, and the mitotic spindle apparatus.

Genes positively controlling cell cycle checkpoints can be targets for oncogenic activation in cancer, whereas, negative regulators, such as tumor suppressor genes, are targets for inactivation [10]. The observations made in the present study reveals that the majority of the alterations in the genes of cell cycle array belong to intracellular kinase networks, cell cycle regulating kinases, transcription factors and transcription activators. Based on the observation in the expression pattern in low grade and high grade gliomas, it can be suggested that the upregulation of cell cycle activators are seen as an early event in glioma. Once these cells become malignant, it is not the cell cycle activators alone that are involved in tumorigenesis that provide a bad prognosis. In an earlier study the authors reported that the correlation of c-Myc with cellular proliferation is dependent on tissue type and differentiation status [20,21]. Therefore, understanding the molecular details of cell cycle regulation and checkpoint abnormalities in cancer could offer an insight into potential therapeutic strategies.

Mitogenic signals provided by upregulation of cyclins and cyclin dependent kinases induce stress, which results in the crosstalk of intracellular kinases. In the present study, the transcripts of MAP Kinases, which belong to the intracellular kinase network, are overexpressed in low-grade gliomas. This overexpression in low grade gliomas may lead to initiation of proliferation through MAP Kinase pathway, which seems to be an early event in gliomas.

Proliferation through the MAP Kinase pathway is regulated by Tyrosine kinases [22]. Plakoglobin (Pg) is the only cytoplasmic protein component common to both junctional complexes mediating cell-cell adhesion, adherens junctions and desmosomes. It was found to be overexpressed in low grade gliomas by 91-fold. The regulatory functions of plakoglobin are still not well characterized. However, it could be suggested that overexpression of MAP Kinases and Plakoglobin is mediated by tyrosine kinases in the pathogenesis of gliomas.

Functional genomics approach in brain tumor research will help us to identify transcriptional aberrations that are causative or are caused by brain neoplasia, which in turn will facilitate the understanding of functionally relevant pathways involved in brain tumor pathogenesis and provide for relevant prognostic, diagnostic, and/or therapeutic tumor markers. Based on this limited experience, we conclude that DNA macroarray analysis is a powerful tool for studying the pathogenesis of gliomas.

Summary and Conclusion

Macroarray analysis of 114 cell cycle genes in different grades of glioma indicated differential expression pattern in 34% of the gene transcripts, when compared to the

normal tissue. Majority of the transcripts belong to the intracellular kinase networks, cell cycle regulating kinases, transcription factors and transcription activators. Based on the observation in the expression pattern in low grade and high grade gliomas, it can be suggested that the upregulation of cell cycle activators are seen as an early event in glioma; however, in malignancy it is not the cell cycle activators alone, which are involved in tumorigenesis.

Authors' contributions

AA and MB carried out the transcriptional profiling; RNB did the biopsy and provided the sample while VVR provided the pathological diagnosis. All the authors contributed equally towards interpretation.

Additional material

Additional File 1

Differential display of cell cycle genes in response to normal glial tissue
Click here for file
[http://www.biomedcentral.com/content/supplementary/1477-3163-3-11-S1.doc]

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