Genetic and expression variations of cell cycle pathway genes in brain tumor patients

Anum Zehra Naqvi¹, Ishrat Mahjabeen¹, Saima Ameen¹, Malik Waqar Ahmad¹, Asad Ullah Khan¹, Zertashia Akram¹, Mahmood Akhtar Kayani¹*.

¹Cancer Genetics and Epigenetics Lab, Department of Biosciences COMSATS University, Islamabad.

Anum Zehra Naqvi and Ishrat Mahjabeen have contributed equally to this work.

Mahmood Akhtar Kayani
*corresponding author: mkayani@comsats.edu.pk+92-321-5357981
Cancer Genetics & Epigenetics Research Group, Department of Biosciences, COMSATS Institute of Information Technology, Park Road Chak shahzad Islamabad, Pakistan

Running Title: Cell cycle pathway genes and brain tumor
Abstract

This study was designed to determine the association between the genetic polymorphisms/expression variations of RB1 and CCND1 genes and brain tumor risk. For this purpose, 250 blood samples of brain tumor patients along with 250 controls (cohort I) and 96 brain tumor tissues (cohort II) with adjacent control section were collected. Mutation analysis of RB1 (rs137853294, rs121913300) and CCND1 (rs614367, rs498136) genes was performed using ARMS-PCR followed by sequencing and expression analysis was performed using real time PCR and immunohistochemistry. The results showed homozygous mutant genotype of RB1 gene polymorphism, rs121913300 (p=0.003) and CCND1 gene polymorphism rs614367 (p=0.01) were associated significantly with brain tumor risk. Moreover, significant downregulation of RB1 (p = 0.005) and upregulation of CCND1 (p=0.0001) gene was observed in brain tumor sections vs controls. Spearman correlation showed significant negative correlation between RB1 vs proliferation marker, Ki-67 (r= -0.291*, p<0.05) in brain tumors. Expression levels of selected genes was also assessed at protein level using IHC and signification downregulation of RB1 (p=0.0001) and upregulation of CCND1 (p=0.0001) was observed in brain tumor compared to control sections. In conclusion, it is suggested that polymorphisms/expression variations of RB1 and CCND1 genes may be associated with increased risk of brain tumor.

Keywords: RB1, CCND1, Polymorphism, Expression analysis, Brain tumor.
Introduction

Brain cancer develops in brain or in spinal cord and is categorized into different types on the basis of their location and origin such as glioma, meningioma, pituitary adenomas, medulloblastoma and schwannoma [1]. Among these types, glioma also referred as tumors of glial cells, are the most common type of primary brain tumors [2]. Gliomas are further classified into various categories as per their morphological appearance which includes astrocytomas, oligodendrogliomas, ependymomas and glioblastomas. According to WHO’s (World Health Organization) 2007 classification, gliomas are classified into four categories namely grade one, two, three and four which are decided on the basis of severity [3, 4]. Regardless of advancements in therapeutical procedures like radiotherapy, chemotherapy and surgical removal, the prognosis and survival of patients (especially glioblastoma) is still lacking [5, 6]. Therefore, it is significant to illuminate the exact molecular cause of glioma/brain tumor, which may possibly support in the diagnosis and prognosis of brain tumor patients. Radiation exposure to head and inherited syndromes (which are rare) are among the established risk factors of brain tumorigenesis [7]. The exact molecular mechanism of these risk factors to increase the risk of tumour development in brain is still unknown. Nevertheless, it has been observed that radiation disturbs the G1/S and S phase of the cell cycle pathway and ultimately resulted in increased proliferation and tumorigenesis.

G1/S transition phase defines the fate of the cell based on the proliferation or apoptotic signals. Genes which are involved in the transition of G1/S phase transition are cyclin dependent kinases, cyclins and retinoblastoma 1 gene [8, 9,10]. RBL gene confines the cell in gap phase in absence of proliferation signals. Activated pRB protein binds with E2F transcription factor and halt transcription mechanism. RB1/E2F complex suppresses the transcription in quiescent cells [11]. Any polymorphism or expression variation in RB1 gene may disrupt the transition of cell cycle phases. RB1 gene is found to be mutated in many cancers including brain tumor [12]. Selected polymorphisms of RB1 gene rs137853294 and rs121913300 are exonic SNPs. These SNPs result in aberrant mRNA structure and protein of RB1 gene in breast cancer patients [13].

Another important cell cycle pathway gene is CCND1, which make a complex with CDK4 and initiates the transition of gap phase in to synthesis phase. CCND/CDK complex inactivates pRb protein through phosphorylation resulting E2F transcription factor starts progression of cell from
G-phase to S-phase [14]. Any mutation in proto-oncogene CCND1 may lead to carcinogenesis through abnormal cell proliferation of cell. Its expression also varies most of the cancers including brain tumor [15]. CCND1 polymorphisms rs614367 and rs498136 are intergenic SNPs which affects the regulation and expression of CCND1 gene in breast cancer and malignant melanoma respectively [16,17].

A number of studies have been published for expression analysis of RB1 and CCND1 in different cancers including brain cancer. Till however, no study has been reported to screen out the hot spot polymorphisms of RB1 and CCND1 genes along with expression variations of respective genes in brain tumor and different subtypes of brain tumor. Present study was designed to find out whether the polymorphisms or expression variation in the RB1 and CCND1 genes can modify the risk for brain tumor, and if the effects of these polymorphisms differ in different pathological parameters of brain tumor patients.

Materials and Methods

Specimen Collection

Ethical board of both COMSATS Institute of Information Technology and collaborating hospital approved the proposal of present study. Two study cohorts of brain tumors were patients enrolled in present study. Study cohort 1 consisted of 250 blood samples of brain tumor patients and 250 age and sex matched controls. Selection criteria for patients included in cohort 1 comprised confirmed histological diagnosis of brain tumor, no preoperative therapy and availability of complete follow-up data. However, no restrictions related to histological subtypes of primary brain tumors were applied. Different subtypes such as anaplastic astrocytoma, anaplastic oligodendroglialoma, anaplastic meninoma, meniingioma, diffuse astrocyeoma, choroid glioma, oligodendroglialoma, ependymoma, meningiomas, atypical meningioma, GBM, diffuse medine glioma, meningiomas and pituitary adenomas were included in the present study. Inclusion criterion for controls included absence of prior history of cancer or precancerous lesions. Patients and controls suffering from any other familial disease (diabetes, blood pressure and cardiovascular impairment) were excluded from this study. Cohort 1 was used for screening of
hotspot polymorphisms of \textit{RB1} and \textit{CCND1} genes in brain tumor patients. Study cohort II consisted of 96 brain tumor tissues along with adjacent un-involved healthy area used as controls. Samples of tumor core, the invasive edge of tumor and microscopically healthy mucosa (control) were obtained from each surgical section and stored in RNA at-80°C. Presence of tumor cells in the collected tissues was rectified by examination of frozen sections following hematoxylin and eosin stain (HE stain) by a consultant pathologist. Whereas samples of control were obtained from macroscopically confirmed (by a pathologist) uninvolved healthy area more than 2cm away from the tumor. Cohort II was used for expression analysis of \textit{RB1} and \textit{CCND1} gene using the quantitative real-time PCR technique. Both brain tumor cohorts were collected after taking the consent from patients from Department of Neurosurgery, Pakistan Institute of Medical and Health Sciences (PIMS) Hospital in during 2015-2017. After obtaining informed consent, all individuals were personally interviewed using the specifically designed questionnaire. Information on age, gender, ethnic group and detailed exposure data on smoking was recorded.

\textbf{RNA&DNA Extraction}

Blood samples of cohort 1 were collected in ETDA vacutainers. DNA was extracted from blood samples through phenol chloroform method \cite{18}. DNA samples were stored in TE buffer at 4 \textdegree C for further mutational analysis. In case of cohort II tumor samples were collected in 15 ml eppendorf tube containing RNA later solution. RNA was extracted from fresh tissue samples of both tumor and control through TRIzol method \cite{19}. Extracted RNA was stored in DEPC at -20 \textdegree C for expression analysis of selected genes. Extracted RNA and DNA were visualized and confirmed by performing 1% agarose gel electrophoresis.

\textbf{Polymorphism Screening and Sequencing}

Polymorphisms for \textit{RB1} and \textit{CCND1} gene were selected from dbSNP database. The SNP selection criteria were based on minor allele frequency (MAF) > 0.05. The selected SNPs included rs137853294, rs121913300 for \textit{RB1}, rs614367 and rs498136 for \textit{CCND1}.

Genotyping was performed by allele-specific polymerase chain reaction (ARMS-PCR). Primers for PCR amplification were designed using WASP (web based allele specific primer designing tool) and purchased from Macrogen (korea). Primers specific for each polymorphism are given
in Table 1. PCR reaction was performed in a reaction volume of 10µl containing 50-100 ng genomic DNA, 100 µM of each primers and Solis BioDyne master mix. The thermal cycling protocol used was: 94°C for 5mins and 35 cycles at 94°C for 30sec, optimized annealing temperature for 45sec, 72°C for 1min and final extension for 10min. The PCR products were visualized on a 2% agarose gel electrophoresis (100V, 300A for 45mins). Presence or absence of expected product size bands, specific for wild or mutant primers, were evaluated using UV trans illuminator (Gel Doc BioRad, USA). β-Actin was used as internal control in each reaction. PCR products with homozygous wild, homozygous mutant and heterozygous mutant genotypes were further confirmed by DNA sequence analysis.

**Expression Analysis**

Expression variation of RB1, CCND1 and Ki-67 genes was analyzed by performing quantitative real-time PCR technique. cDNA was synthesized and confirmed by amplification of β-actin which was taken as endogenous control. Primers were designed for β-actin, RB1, CCND1 and Ki-67 genes by IDT software for real time PCR to estimate the relative expression of these genes in control and tumor samples.

Each qPCR was performed in a 20 µl reaction mixture containing ~1 µl of product from RT reaction, 10 µl of 2× Sybr Green, 1 µl of each primer and 7 µl RNase free water. qPCR was performed using Real-Time PCR system (Applied Biosystems Step one plus) under standard conditions. The relative mRNA levels of RB1, CCND1, Ki-67 and β actin were computed using the 2−ΔΔCt analysis method [19]. Melt curve analysis of selected genes was also performed to detect the heterogeneity in brain tumor cohort and specificity or qPCR [20].

**Immunohistochemistry**

Immunohistochemical analysis (IHC) was performed using the DAB kit (Sigma). Paraffin embedded sections of brain tumor sections and adjacent uninvolved control sections were used for this study after deparaffinization and rehydration steps using xylene and ethanol respectively, as described previously [21]. Sections were incubated for one hour with mouse anti-RB1 (Novus Biologicals, at a dilution of 1: 1,000), mouse anti-CCND1 (Novus Biologicals, at a dilution of 1: 1,000) and mouse anti-Ki-67 (Novus Biologicals, at a dilution of 1: 1,000). Species specific secondary antibody (Novus Biologicals) was also used for each selected gene. Additionally,
negative controls were prepared using the same procedure except that the primary antibodies were replaced with PBS. Negative controls were used for assessment of specificity of this current assay [21].

The relative intensities of the completed immunohistochemical reactions were also evaluated using light microscopy by three independent trained observers, unaware of the clinical data. Tumour cells were counted randomly in ten high power fields for measurement of immunoreactivity. The following formula was used for the evaluation of immunoreactivity:

Immunoreactive score = intensity score x proportion score

Structure prediction

Centroid secondary structure of mRNA of wild type RB1 transcript and its selected exonic SNPs were designed by dot block model using Veinna RNA Web Services software. Furthermore, protein structure of normal pRB and mutated pRB were predicted using SWISS-MODEL software.

Statistical Analysis

Statistical analysis was performed using MedCalc statistical software and GraphPad Prism (5.03) software. Comparison between the expected and actual genotypes was done using Hardy-Weinberg equilibrium test. Chi-square tests was applied to estimate the difference in genotypes and allele frequencies between patients and control individuals. Odds ratio (ORs) and 95% confidence intervals (CIs) were assessed through logistic regression. This predictive analysis was performed with respect to demographic features, smoking status, family history, tumor types and grading. SNPs analysis and their multiplicative interactions were also calculated by three genetic models (additive, dominant and recessive) and logistic regression model respectively. P-value < 0.05 represents the statistically significant difference between patients and controls. Genotyped data of SNPs were used to design their haplotypes using Haploview 4.2 software. Linkage disequilibrium (LD) between all selected SNPs was determined through expectation maximization (EM) algorithm.

For cohort II, Chi-square tests and one-way analysis of variance was used to assess the association of RB1 and CCND1 gene expression with clinical and histopathological parameters.
Spearman correlation coefficient was used to assess correlations among the gene expression and clinical and histopathological parameters.
Results

Retrospective Cohort Study

In study cohort I, 250 brain tumor patients and 250 control individuals were examined for four selected SNPs including rs137853294, rs121913300 (RB1), rs614367 and rs498136 (CCND1). In study cohort II, 96 brain tumor tissues and 96 healthy tissues were analyzed for the expression variation of RB1 and CCND1 genes. Demographic features of the study cohort are given in Table 2.

Genotype and Allele Frequencies of RB1 and CCND1 SNPs

Genotype and allele frequencies of RB1 polymorphisms are mentioned in Table 3. RB1 polymorphism rs137853294 showed significantly higher frequency of heterozygous mutant genotype (OR, 1.49; 95% CI, 1.02-2.16; p = 0.04) in patients as compared to controls. Mutant allele (T) frequency of rs137853294 was significantly higher (OR, 1.50; 95% CI, 1.11-2.01; p = 0.007) in tumor patients as compared to control individuals. In case of rs121913300 RB1 polymorphism, ~20.48-fold increased risk of brain tumor was associated with homozygous mutant genotype (OR, 20.48; 95% CI, 2.72-154.21; p = 0.003) in tumor patients. Moreover, significantly higher frequency of heterozygous mutant genotype (OR, 1.68; 95% CI, 1.05-2.66; p = 0.03) and mutant T allele (OR, 1.56; 95% CI, 1.22-2.01; p = 0.0005) was observed in sampled tumor patients as compared to controls.

CCND1 polymorphism’s genotype and allele frequencies are given in Table 3. Genotype frequency of CCND1 polymorphism rs614367 showed ~4.88-fold increased risk of brain tumor with homozygous mutant genotype (OR, 4.88; 95% CI, 1.39-17.21; p = 0.01) in sampled tumor patients. Furthermore, higher frequency of heterozygous mutant genotype (OR, 1.48; 95% CI, 1.04-2.12, p = 0.03) and mutant T allele (OR, 1.65; 95% CI, 1.23-2.22; p = 0.0007) was observed in tumor patients as compared to control individuals. In case of rs498136 polymorphism of CCND1, ~8.23-fold increased risk of brain tumor was observed associated with homozygous mutant genotype (OR, 8.23; 95% CI, 1.02-66.31; p = 0.047) in brain tumor patients compared to controls.
Logistic Regression Models Analysis

Three logistic regression models (dominant, recessive, additives) were used for further analyses of genotype frequencies of all SNPs as shown in Table 4. For RB1 SNP rs137853294, dominant (OR, 1.62; 95% CI, 1.13-2.33; p = 0.008) and additive genetic model (OR, 1.50; 95% CI, 1.11-2.01; p = 0.007) showed significant association with increased risk of brain tumor. Similarly, rs121913300 SNP showed the significant association of brain tumor risk with all three genetic models i.e. dominant (p < 0.0001), recessive (p = 0.003), and additives (p = 0.0005). Moreover, CCND1 polymorphism rs614367 showed significant association of all three genetic models i.e. dominant (p = 0.002), recessive (p = 0.01) and additive (p = 0.0007) with increased risk of brain tumor. Rs498136 polymorphism also showed significant association of recessive genetic model (OR, 8.23; 95% CI, 1.02-66.31; p = 0.047) with increased risk of brain tumor.

Association of RB1 and CCND1 SNPs with Different Parameters

Genotypes of all four selected SNPs of cell cycle genes were associated with demographic features, risk factors and histopathological subtypes by applying logistic regression model as shown in Table 5. Results showed that rs137853294 polymorphism (OR, 2.272; 95% CI, 0.088-0.084; p = 0.024) and rs121913300 polymorphism of RB1 (OR, 0.264; 95% CI, 0.082-0.0851; p = 0.026) showed positive association with tumor grades. For CCND1 gene, rs614367 and rs498136 also showed positive association with tumor grades (OR, 4.197; 95% CI, 0.054-0.771; p = 0.014) and OR, 2.137; 95% CI, 0.258-5.007; p = 0.03), as showed in Table 5.

Haplotype Analysis of RB1 and CCND1 SNPs

Linkage disequilibrium was estimated between all selected SNPs of RB1 and CCND1 genes. Haplotypes of these SNPs were also examined to determine their association with tumor risk. Thirteen common haplotypes were generated from these selected SNP, among brain tumor patients and controls as shown in Table 6. Haplotypes which significantly increased the risk of brain tumor included CCTG (p = 0.002), CCTT (p = 3.57e-006), CTCG (p = 5.41e-010), TCCT (p = 0.008), TCTG (p = 0.006), TCTT (p = 0.0006), TTTT (p = 0.01) and CTCT (p = 2.16e-006). Whereas, haplotypes which significantly reduced the risk of brain tumor included CCCG (p = 6.21e-012), CCCT (p = 1.09e-009), CTTG (p = 0.0005) and TTTG (p = 0.003).
Moreover, linkage disequilibrium plot showed strong LD between exonic \textit{RB1} SNP (rs121913300) and intergenic \textit{CCND1} SNP (rs614367) in tumor patients. Highly strong LD was also observed between \textit{CCND1} intergenic SNPs (rs614367 and rs498136) in brain tumor patients compared to controls as illustrated in Figure 1. Logistic regression analysis for SNP-SNP interactions among all four SNPs of \textit{RB1} and \textit{CCND1} genes showed positive significant correlation between s137853294 vs rs498136 (OR, 3.372; 95% CI, 0.520–21.872; \(p = 0.02\)) and rs614367 vsrs498136 (OR, 3.083; 95% CI, 0.559–17.00; \(p = 0.029\)) with increased risk of brain tumor as shown in Table 7.

\textbf{mRNA Secondary Structure and Protein Structure Prediction of \textit{RB1} SNPs}

Sequence of wild \textit{RB1} gene and its two exonic SNPs were used to predict their secondary structure of mRNA using ViennaRNA Web Service. Prediction showed that there is no visual change in the structure of wild \textit{RB1} mRNA and first missense mutated mRNA. While second mutated mRNA changed and shortened its structure as demonstrated in Figure 5. According to centroid secondary structure, minimum free energy (MFE) of wild mRNA and mutated mRNA were -826.91 kcal/mol and -828.11 kcal/mol respectively. Moreover, truncated mRNA had -765.41 kcal/mol minimum free energy. Protein structure of wild \textit{RB1} gene, SNP1 mutated \textit{RB1} and SNP2 truncated \textit{RB1} gene were predictive using SWISS-MODEL as illustrated in Figure 2.

\textbf{Expression analysis of \textit{RB1} and \textit{CCND1} gene at mRNA}

\textit{RB1} and \textit{CCND1} mRNA levels were observed in study cohort II including 96 brain tumor tissues and adjacent uninvolved healthy section used as control. \textit{RB1} expression has observed significantly lower in brain tumour (\(p= 0.005\)) than in normal tissue samples. Statistical significant decrease in \textit{RB1} mRNA level was observed in brain tumors with anatomical site of glioma (\(p= 0.008\)) compared to other anatomical sites. Significant decreased mRNA level of \textit{RB1} gene was also observed in grade IV (\(p=0.004\)) brain tumor as compared to other grades (Figure 3). In case of second selected gene \textit{CCND1}, significantly higher mRNA level was observed in brain tumors section compared to adjacent control section (\(p=0.0003\)). Further analysis showed significantly increased \textit{CCND1} mRNA level in grade IV (\(p=0.03\)) brain tumor patients compared to other grades (Figure 4).
Expression level of proliferation marker *Ki-67* was also observed to find out whether the change in expression level of cell cycle gene affected the proliferation process in brain tumorigenesis. Significant higher level of *Ki-67* was observed in brain tumor (*p* = 0.0001) compared to adjacent control section and this up-regulation was more pronounced in grade IV brain tumor tissue (*p* = 0.001) compared to other grades (Figure 5).

**Expression analysis of *RB1* and *CCND1* gene at protein level**

Expression level of *RB1*, *CCND1* and *Ki-67* was also observed in 96 brain tumor section and adjacent control section using IHC. Expression of *RB1* was observed lower in brain tumors compared to adjacent control sections as shown in Figure 6B and C. Measurement of immunoreactive intensity showed that weak immunoreactivity of *RB1* was observed significantly higher (*p* = 0.001) in brain tumor sections as compared to moderate and strong immunoreactive intensity as shown in Figure 6D. Expression analysis of *CCND1* at protein level showed the increased expression of *CCND1* in brain tumor section compared to control section as shown in Figure 6E and F. Measurement of immunoreactive intensity also showed that strong immunoreactive intensity of *CCND1* was observed significantly higher (*p* = 0.001) in brain tumor sections as compared to weak and moderate immunoreactive intensity as shown in Figure 6G.

Expression level of *Ki-67* was also observed at protein level using the IHC and expression level of *Ki-67* was observed higher in brain tumors section compared to adjacent control section as shown in Figure 6H and I. Measurement of immunoreactive intensity also showed that strong immunoreactive intensity of *Ki-67* was observed significantly higher (*p* = 0.001) in brain tumor sections as compared to weak and moderate immunoreactive intensity as shown in Figure 6J.

**Correlation between *RB1*, *CCND1* and *Ki-67* expression and clinicopathological characteristics**

Correlations were tested between *RB1*, *CCND1* and *Ki-67*, clinical and pathological features of brain tumor patients group II. The *RB1* mRNA level was negatively correlated with grades (*r* = -0.252**, p**< 0.01), however positive correlation was observed for *CCND1* mRNA level and grades
(r= 0.222*, p<0.05) of brain tumor patients. In case of gene to gene correlation, a significant negative correlation was observed between CCND1 vs RB1 (r= -0.337**, p< 0.001) and RB1 vs Ki-67 (r= -0.291*, p<0.05) in brain tumor patients as shown in Table 8.

Correlations was also tested at protein level between selected genes and clinicopathological parameters of gastric cancer patients as shown in Table 8. A significant negative correlation was observed between CCND1 vs RB1 (r= -0.54***, p< 0.0001) and RB1 vs Ki-67 (r= -0.29*, p< 0.05) in brain tumor patients. However, a significant positive correlation was observed between CCND1 vs Ki-67 (r= 0.43**, p< 0.001) in brain tumor patients as shown in Table 8.

**ROC Curve analysis**

To assess the diagnostic value of both selected genes ROC curve analysis was performed. After the generation of ROC curve, area under the curve (AUC) and 95% confidence interval (CI) was also calculated. The area under the curve for RB1 gene was 63.5 (95% CI: 0.562-0.703; p<0.001) and for CCND1 gene was 64.0 (95% CI: 0.567 to 0.709; p<0.0006) as shown in Figure 7.

**Discussion**

This study was designed to explore the correlation between the genetic polymorphism of RB1 and CCND1 genes to brain tumor risk in Pakistani population. This study encompasses the genetic analysis of RB1 exonic and CCND1 intergenic polymorphisms in brain tumor patients to find their association with brain carcinogenesis. This has followed by structure prediction of exonic variants performed to assess the effect of these polymorphism on transcription level. Expression levels of RB1 and CCND1 were also analyzed in brain tumor patients. Furthermore, expression levels of cell cycle pathway genes were also correlated with proliferation marker, Ki-67 to illuminate correlation between cell cycle pathway gene variation and proliferation process in brain tumorigenesis.

In present study we used two study cohort, cohort 1 consisted of 250 blood samples of brain tumor patients and cohort 2 consisted of 96 brain tumors. Both cohort of brain cancer contained higher number of glioma patients compared to other type of brain tumors such as meningioma and pituitary adenomas. Previous studies have also reported the increased incidence of glioma (77–81% of all primary malignant tumors of CNS) in the southeast, northwest, and Midwest of
USA [22], Asian population and Hispanic population [23] compared to meningioma and pituitary adenomas. Reasons bind these incidence differences can be variations in geographic regions, environmental factors, diet, occupational and personal exposures and lifestyle [22, 24]. In addition, ethnic/race variations are likely to contribute to observed differences [25, 26].

Retinoblastoma 1 gene has been found mutated or deregulated in many human cancers [27-35] and one subtype of brain tumor, glioma [36]. Present study examined the two exonic SNPs of RB1 gene (rs137853294 and rs121913300) in brain tumor patients. Exonic or coding region SNPs mainly influence the transcription of the gene by altering the structure and function of transcribed protein [37]. Among SNPs, rs137853294 showed the significant association in heterozygous mutant genotype to brain tumor risk in current study. This SNP alters the protein structure of RB1 by replacing amino acid Arginine to Tryptophan. As a result of this missense mutation, altered pRB protein is formed which disrupts the cell cycle checkpoints and contributes to tumorigenesis [13]. Furthermore, second exonic variant, rs121913300 was also observed significantly associated with brain tumor risk, indicated by genotypic frequency of both homozygous and heterozygous mutants in current study. This SNP stops the transcription of RB1 gene by inducing the stop codon. Resulting truncated pRB protein is formed which causes the abnormality in cell cycle control [13]. These findings of both SNPs agree with many earlier studies which state that genetic mutations in RB1 gene vary its expression and can contribute in retinoblastoma [13, 27].

Cyclin D1 gene, second important molecule of cell cycle pathway has also been found mutated in almost every human cancer by altering the normal cell cycle pathway. CCND1 gene polymorphisms have been found in several cancers [38-47] including gliomas also [48]. However, till now no study has been published with respect to brain tumor and different subtypes of brain tumor such as glioma, meningioma and pituitary adenoma. In this study, two intergenic SNPs (rs614367 and rs498136) of CCND1 were analyzed which can potentially influence the regulation and expression of CCND1 gene. Any polymorphism in intergenic regions may disrupt the transcription of CCND1 gene by altering its expression and may increase the susceptibility of diseases [49].

SNP rs613467 is present in the regulatory region of CCND1 which regulates the transcription of CCND1 gene. Alterations in that region can cause the abnormality in interpretation of regulating
signals resultantly producing transcription abnormalities [50]. rs614367, was found significantly associated with increased risk of brain tumor in both homozygous and heterozygous mutant genotypes. Similar trend of rs614367 association has earlier been found in breast cancer patients in earlier studies [51, 52]. SNP rs498136 is located in 5' promotor region of CCND1 at transcription binding site and resultantly alters the binding site of transcription factors for CCND1 gene and aberrant regulation of the gene [53]. Second selected SNP of CCND1 polymorphism rs498136, was also showed significant association in brain tumor patients in homozygous mutant genotype in present study. Findings of this study are in line with the previous study suggested the strong association of rs498136 with malignant melanoma [16].

To test the hypothesis that SNPs in coding region/intragenic region result in variations at transcription level and change expression level of respective genes. Expression levels of RB1 and CCND1 gene was also measured in cohort 2 of brain tumor patients and controls using qPCR and immunohistochemistry. Significant down-regulation of RB1 expression was observed in brain tumor patients when compared with adjacent controls, after qPCR and IHC. This downregulation was observed more pronounced in grade IV tumors compared to lower grade tumors. Expression pattern variations between the controls and patients may be due to genetic heterogeneity in RB1 gene by different environmental factors and genetic and epigenetic changes [54, 55].

Expression level of RB1 gene was also correlated with different type of brain tumors and significant downregulation of RB1 gene was observed in glioma compared to meningioma and pituitary adenomas. Deregulation of RB1 gene has been reported in different type of brain tumor such as glioma [56] and meningioma [57]. It has also been reported in previous study that downregulation of RB1 gene in glioma was associated with increased cell proliferation and decrease survival in more than one third of patients [58]. One of basic reason of downregulated expression of RB1 gene in glioma compared to meningioma and pituitary adenoma, can be that number of gliomas are significantly higher in our study cohort compared to other type of brain tumors. Other reasons of this downregulation are genetic variations [30, 59], RB1 promoter hyper methylation [31, 60-63], variations in microRNA binding affinity [57] and this downregulated/loss of expression ultimately resulted in increased invasion of tumor cells and more aggressive type of brain tumor in CNS carcinogenesis process [62].
In case of second selected molecule, *CCND1* gene, significant upregulation was observed in brain tumor patients compared to controls using qPCR and immunohistochemistry. Additionally, this up-regulation was observed higher in aggressive brain tumors in present study. *CCND1* overexpressed and gene amplification is reported in many cancers including the meningioma and pituitary adenomas [65-68]. Mechanisms which are involved in over expression of *CCND1* gene by upstream factors, remain unknown. Genetic variations in exonic region [42] and intragenic/UTR region resulted in abnormal binding of transcription factors. [67] has found the relation between abnormal binding of octamer-binding transcription factor 4 (OCT4) due to polymorphism in UTR region of *CCND1* with the octamer motif (ATTTTGCGAT) and promoted proliferation of cell cycle [66] and tumorigenesis. Additionally, expression levels of *RB1* and *CCND1* gene was also correlated with expression level of proliferation marker, *Ki*-67. Significant correlation was observed which is according to earlier findings that genetic aberrations and expression deregulation of cell cycle pathway genes results in uncontrolled cell cycle process and enhanced proliferation.

In conclusion, our study demonstrates a significant association between cell cycle pathway genes, polymorphisms and brain tumors in Pakistani population. Our study also confirms that the deregulated cell cycle pathway gene is linked with increased brain tumorigenesis, at least in Pakistani population. As the matter of fact, tumorigenesis and deregulated cell cycle genes are the major challenging items in neoplastic initiation and progression. Additional studies such as, a large-scale studies adjusting for a wide range of factors, [7, 10] functional alteration studies as single cell level will be recommended to validate these findings and to fully explore the molecular mechanisms that contribute to the deregulation of cell cycle pathway genes in brain tumorigenesis.

**Author contributions statements**

All of the authors read and approved the final version of manuscript. AZN, SA, MWA and AK collected and isolated the DNA/RNA samples. AZN, MWA and ZA performed the genotyping and drafted the manuscript. SA, AK and IM performed the relative expression analysis studies and drafted manuscript. IM performed the genotyping/expression studies, statistical analyses of the data and draft of the manuscript. MAK supervised the project, helped to analyze the
statistical data and provided critical revisions. All of the authors discussed the results and commented on the manuscript.

**Conflict of Interest**

The authors declare that they have no competing interests.

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| Primer Name | Primer Sequence | Product Size |
|-------------|-----------------|--------------|

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**Table 1:** Primer sequence of *RB1* and *CCND1* gene
### Table 2: Demographic parameters of brain tumor cases and controls.

| Variables          | Cases (N=250) | Controls (N=250) | Tumor Tissues (N=96) |
|--------------------|---------------|------------------|----------------------|
| **Age (Years)**    |               |                  |                      |
| Median (Range)     | 42 (8-88)     | 47 (9-88)        | 45 (21-71)           |
| **Age**            |               |                  |                      |
| ≤ 30               | 62 (24.8%)    | 49 (19.6%)       | 12 (12.5%)           |

**Primers specific for Real Time PCR**

| Primers | Sequence (Type) | Length (bp) |
|---------|----------------|-------------|
| CCND1 F | CCTCGGTGTCCTACTTCAAATG (Sense) | 100bp |
| CCND1 R | CACTTCTGTTCTCAGAC (AntiSense) | |
| RB1 F   | GCAGATATGCTTCCACCAG (Sense) | 108bp |
| RB1 R   | TAGATGTTCCTCCAGGAAT (AntiSense) | |

**Primers specific for ARMS-PCR**

**RB1 first SNP**

| Primers | Sequence (Type) | Length (bp) |
|---------|----------------|-------------|
| RB1 rs137853294 FI (T allele) | CAGTGTATCGGCTAGCTATCGCT | 188bp |
| RB1 rs137853294 RI (C allele) | AAGCGTTCAAAAGTGATTTAGACG | 257bp |
| RB1 rs137853294 FO (5'-3') | TGCAATTTCTTCTCATGTCATCCTGTGTT | 394bp |
| RB1 rs137853294 RO (5'-3') | GTAGGGGAGGAGAAGGTAAGTCG | |

**RB1 second SNP**

| Primers | Sequence (Type) | Length (bp) |
|---------|----------------|-------------|
| RB1 rs121913300 FI (T allele) | AAGGTGGAATACGCTTTTACAT | 150bp |
| RB1 rs121913300 RI (C allele) | AGATAAAATTCTTCTTGATGC | 214bp |
| RB1 rs121913300 FO (5'-3') | CAATTTCTTCTCATCCTTTT | 322bp |
| RB1 rs121913300 RO (5'-3') | CTAAAGCAATATCAATCAATAT | |

**CCND1 first SNP**

| Primers | Sequence (Type) | Length (bp) |
|---------|----------------|-------------|
| CCND1 rs614367 FI (T allele) | TTGGCTTCTCTGCAACGCT | 214bp |
| CCND1 rs614367 RI (C allele) | GGGGCCTAAAGAGATGTAATTG | 293bp |
| CCND1 rs614367 FO (5'-3') | TTACATAGAAGGAGGTGAAGCA | 465bp |
| CCND1 rs614367 RO (5'-3') | CGTCTCTGAGGTTGAGCAGCAT | |

**CCND1 second SNP**

| Primers | Sequence (Type) | Length (bp) |
|---------|----------------|-------------|
| CCND1 rs498136 FI (T allele): | CATTATTGTGCTTCTCAACGCT | 153bp |
| CCND1 rs498136 RI (G allele) | AAACCTCTGTTTACTGGTTGTC | 103bp |
| CCND1 rs498136 FO (5'-3') | CTGCCAGAGGACTTTAGAAAGTG | 208bp |
| CCND1 rs498136 RO (5'-3') | TAGACATGGAAAATATTCCAGCA | |
| Age      | Cases   | Controls | Total |
|----------|---------|----------|-------|
| > 30     | 188 (75.2%) | 201 (80.4%) | 84 (87.5%) |

**Gender**

| Gender | Cases   | Controls | Total |
|--------|---------|----------|-------|
| Males  | 165 (66%) | 172 (68.8%) | 59 (61.5%) |
| Females | 85 (34%) | 78 (31.2%) | 37 (38.5%) |

**Smoking**

| Smoking | Cases   | Controls | Total |
|---------|---------|----------|-------|
| Smokers | 83 (33.2%) | 120 (48%) | 44 (46%) |
| Non-Smokers | 167 (66.8%) | 130 (52%) | 52 (54%) |

**Family History**

| Family History | Cases   | Controls | Total |
|----------------|---------|----------|-------|
| Positive       | 26 (10.4%) | - | 13 (14%) |
| Negative       | 224 (89.6%) | - | 83 (86%) |

**Brain Tumor Types**

| Glioma                      | Cases   | Controls | Total |
|-----------------------------|---------|----------|-------|
| Diffuse astrocytoma (II)    | 170 (68%) | - | 68 (70.8%) |
| Anaplastic astrocytoma (III)| 35      | 19       | 12    |
| Choroid glioma (II)         | 64      | 05       | 04    |
| Oligodendroglioma (II)      | 06      | 03       | 03    |
| Anaplastic oligodendroglioma(III) | 04 | 03 | 03 |
| GBM (IV)                    | 27      | 11       | 07    |
| Diffuse medine glioma (IV)  | 06      | 04       | 04    |
| Ependoma (II)               | 03      | 07       | 07    |

| Meningioma                 | Cases   | Controls | Total |
|-----------------------------|---------|----------|-------|
| Meningioma (I)              | 64 (25.6%) | - | 21 (21.9%) |
| Atypical meningioma (II)    | 21      | 08       | 04    |
| Anaplastic meningioma (III) | 30      | 13       | 04    |

| Pituitary Adenoma           | Cases   | Controls | Total |
|-----------------------------|---------|----------|-------|
| Grade II                    | 16 (6.4%) | - | 07 (7.3%) |
| Grade III                   | 09      | 04       | 03    |

| Tumor Grade                | Cases   | Controls | Total |
|-----------------------------|---------|----------|-------|
| Grade I                     | 21      | 09       |       |
| Grade II                    | 108     | 43       |       |
| Grade III                   | 88      | 29       |       |
| Grade IV                    | 33      | 15       |       |

**Ionizing Radiation**

| Ionizing Radiation | Cases   | Controls | Total |
|--------------------|---------|----------|-------|
| Exposed            | 24 (9.6%) | - | 16 (16.7%) |
| Unexposed          | 226 (90.4%) | - | 80 (83.3%) |
Table 3: Genotypes and allele frequency of selected SNPs of *RB1* and *CCND1* in brain tumor patients

| Genotype/Allele | Cases n (%) | Controls n (%) | OR (95% CI) | P-value |
|-----------------|-------------|----------------|-------------|---------|
| *RB1* Gene      |             |                |             |         |
| rs137853294     |             |                |             |         |
| CC              | 135 (54%)   | 164 (65.6%)    | 1           | 1       |
| CT              | 95 (38%)    | 73 (29.2%)     | 1.49 (1.02-2.16) | 0.04*   |
| TT              | 20 (8%)     | 13 (5.2%)      | 1.60 (0.77-3.26) | 0.21    |
|                | C allele frequency | T allele frequency | OR (95% CI) | p-value |
|----------------|-------------------|-------------------|-------------|---------|
| rs121913300 CC | 17 (6.8%)         | 54 (21.6%)        | 1           | 1       |
| rs121913300 CT | 214 (85.6%)       | 195 (78%)         | 1.68 (1.05-2.66) | 0.03*  |
| rs121913300 TT | 19 (7.6%)         | 1 (0.4%)          | 20.48 (2.72-154.21) | 0.003** |
| rs614367 CC    | 119 (47.6%)       | 154 (61.6%)       | 1           | 1       |
| rs614367 CT    | 117 (46.8%)       | 93 (37.2%)        | 1.48 (1.04-2.12) | 0.03*  |
| rs614367 TT    | 14 (5.6%)         | 3 (1.2%)          | 4.88 (1.39-17.21) | 0.01*  |
| rs498136 GG    | 85 (34%)          | 104 (41.6%)       | 1           | 1       |
| rs498136 GT    | 157 (62.8%)       | 145 (58%)         | 1.22 (0.85-1.75) | 0.27   |
| rs498136 TT    | 8 (3.2%)          | 1 (0.4%)          | 8.23 (1.02-66.31) | 0.047* |
| rs614367 C allele frequency | 355 (71%) | 401 (80.2%) | 1 | 1 |
| rs614367 T allele frequency | 145 (29%) | 99 (19.8%) | 1.65 (1.23-2.22) | 0.0007*** |
| rs498136 G allele frequency | 327 (65.4%) | 353 (70.6%) | 1 | 1 |
| rs498136 T allele frequency | 173 (34.6%) | 147 (29.4%) | 1.27 (0.97-1.66) | 0.078 |

**Abbreviations:** n, number of samples; OR, ODDS ratio; CI, confidence interval; level of significance p-value <0.05*, <0.01**, <0.001***.
Table 4: Association of SNPs of RB1 and CCND1 gene with brain tumor risk based on genetic models.

| Genotype/Allele | Model  | OR (95% CI)       | P-value   |
|-----------------|--------|-------------------|-----------|
| **RB1 Gene**    |        |                   |           |
| rs137853294     |        |                   |           |
| CC vs CT + TT   | Dominant | 1.62 (1.13-2.33)  | 0.008**   |
| TT vs CC + CT   | Recessive | 1.58 (0.77-3.26)  | 0.21      |
| T vs C          | Additive | 1.50 (1.11-2.01)  | 0.007**   |
| rs121913300     |        |                   |           |
| CC vs CT + TT   | Dominant | 3.78 (2.12-6.73)  | < 0.0001**** |
| TT vs CC + CT   | Recessive | 20.48 (2.72-154.21) | 0.003** |
| T vs C          | Additive | 1.56 (1.22-2.01)  | 0.0005*** |
| **CCND1 Gene**  |        |                   |           |
| rs614367        |        |                   |           |
| CC vs CT + TT   | Dominant | 1.77 (1.24-2.52)  | 0.002**   |
| TT vs CC + CT   | Recessive | 4.88 (1.39-17.21) | 0.01*     |
| T vs C          | Additive | 1.65 (1.23-2.22)  | 0.0007*** |
| rs498136        |        |                   |           |
| GG vs GT + TT   | Dominant | 1.38 (0.96-1.99)  | 0.08      |
| TT vs GG +GT    | Recessive | 8.23 (1.02-66.31) | 0.047*    |
| T vs G          | Additive | 1.27 (0.97-1.66)  | 0.08      |

Abbreviations: OR, ODDS ratio; CI, confidence interval; level of significance p-value <0.05*; <0.01**; <0.001***.
Table 5: Association of selected SNPs of *RB1* and *CCND1* genes with different parameters.

| SNPs vs Parameters | B   | Std. Error | Wald | Sig.  | OR     | 95% CI       |
|--------------------|-----|------------|------|-------|--------|--------------|
| rs137853294 vs     |     |            |      |       |        |              |
| Gender             | -0.636 | 0.533    | 1.428 | 0.232 | 0.529 | 0.186-1.503 |
| Age                | 0.399  | 0.558     | 0.512 | 0.474 | 1.491 | 0.499-4.451 |
| Smoking            | 0.33  | 0.614     | 0.289 | 0.591 | 0.719 | 0.216-2.394 |
| Family History     | -0.877 | 0.783  | 1.255 | 0.263 | 0.416 | 0.090-1.929 |
| IR                 | -0.07  | 1.281     | 0.003 | 0.956 | 0.932 | 0.076-11.490 |
| Grade              | 3.3   | 0.577     | 5.077 | 0.024 | 2.272 | 0.088-0.844 |
| Types              | -0.536 | 0.603    | 0.790 | 0.374 | 0.585 | 0.180-1.907 |
| rs121913300 vs     |     |            |      |       |        |              |
| Gender             | -0.127 | 0.524    | 0.059 | 0.809 | 0.881 | 0.316-2.459 |
| Age                | 0.332  | 0.617     | 0.289 | 0.591 | 1.393 | 0.416-4.668 |
| Smoking            | 1.958  | 0.518     | 3.418 | 0.064 | 1.384 | 0.139-1.059 |
| Family History     | 0.247  | 0.509     | 0.235 | 0.628 | 1.28  | 0.472-3.470 |
| IR                 | 0.061  | 0.823     | 0.005 | 0.941 | 1.062 | 0.212-5.327 |
| Grade              | 4.333  | 0.598     | 4.976 | 0.026 | 3.264 | 0.082-0.851 |
| Types              | -0.405 | 0.596     | 0.462 | 0.497 | 0.667 | 0.208-2.144 |
| rs614367 vs        |     |            |      |       |        |              |
| Gender             | -0.017 | 0.616    | 0.001 | 0.978 | 0.983 | 0.294-3.285 |
| Age                | 0.168  | 0.659     | 0.065 | 0.798 | 1.183 | 0.325-4.304 |
| Smoking            | 1.589  | 0.678     | 5.496 | 0.019 | 0.204 | 0.054-0.771 |
| Family History     | 0.572  | 1.139     | 0.253 | 0.615 | 1.772 | 0.190-16.512 |
| IR                 | 0.334  | 1.159     | 0.083 | 0.773 | 1.397 | 0.144-13.537 |
| Grade              | 5.625  | 0.659     | 6.084 | 0.014 | 4.197 | 0.054-0.716 |
| Types              | -0.249 | 0.685     | 0.133 | 0.716 | 0.779 | 0.204-2.983 |
| rs498136 vs        |     |            |      |       |        |              |
| Gender             | 0.56  | 0.864     | 0.42  | 0.517 | 1.75  | 0.322-9.517 |
| Age                | -1.052 | 0.777    | 1.834 | 0.176 | 0.349 | 0.076-1.601 |
| Smoking            | -0.586 | 0.759    | 0.597 | 0.44  | 0.556 | 0.126-2.462 |
| Family History     | 0.124  | 1.162     | 0.011 | 0.915 | 1.132 | 0.116-11.049 |
| IR                 | -0.822 | 1.201     | 0.468 | 0.494 | 0.44  | 0.042-4.625 |
| Grade              | 2.128  | 0.756     | 0.029 | 0.035 | 2.137 | 0.258-5.007 |
| Types              | 0.788  | 1.117     | 0.498 | 0.480 | 2.200 | 0.247-19.633 |

OR, ODDS ratio; CI, confidence interval; level of significance p-value <0.05*; <0.01**; <0.001***.
Table 6: Haplotypes of cell cycle genes (RB1 and CCND1) in brain tumor patients.

| RB1 Haplotypes (SNPs) | CCND1 Haplotypes (SNPs) | Frequency | x² | P-value | OR | 95% CI |
|------------------------|--------------------------|-----------|----|---------|----|--------|
| rs137853294 rs121913300 | rs614367 rs498136 | Cases    | Controls |
| C C                  | C G*                      | 0.107 0.279 | 47.336 | 6.21e-012 | 0.31 | 0.219-0.437 |
| C C                  | C T*                      | 0.091 0.234 | 37.222 | 1.09e-009 | 0.329 | 0.228-0.476 |
| C C                  | C G*                      | 0.054 0.018 | 9.456 | 0.002113 | 3.127 | 1.458-6.707 |
| C C                  | T T*                      | 0.074 0.014 | 21.51 | 3.57e-006 | 5.717 | 2.506-13.040 |
| C T                  | C G*                      | 0.295 0.134 | 38.583 | 5.41e-010 | 2.711 | 1.966-3.739 |
| C C                  | T G*                      | 0.06 0.124 | 12.078 | 0.000513 | 0.453 | 0.288-0.715 |
| C C                  | C G*                      | 0.035 0.018 | 2.709 | 0.099806 | 1.962 | 0.867-4.440 |
| T C                  | C T*                      | 0.058 0.025 | 6.845 | 0.008905 | 2.403 | 1.223-4.721 |
| T C                  | C G*                      | 0.021 0.003 | 7.507 | 0.006161 | 8.424 | 1.361-52.138 |
| T C                  | T T*                      | 0.056 0.016 | 11.582 | 0.00067 | 3.642 | 1.648-8.044 |
| T T                  | C G*                      | 0.081 0.109 | 2.213 | 0.136876 | 0.724 | 0.473-1.110 |
| T T                  | T G*                      | 0.001 0.022 | 8.746 | 0.003113 | 0.053 | 0.007-0.376 |
| T T                  | T T*                      | 0.017 0.001 | 6.109 | 0.013468 | 11.843 | 1.815-77.292 |

Abbreviations: SNP, single nucleotide polymorphism; x², chi-square; OR, ODDS ratio; CI, confidence interval; p-value < 0.05 was statistically significant.
Table 7: SNP-SNP interactions of all selected SNPs with brain tumor risk based on logistic regression model.

| SNP-SNP Interactions | B    | Std. Error | Wald | Sig. | OR   | 95% CI       |
|----------------------|------|------------|------|------|------|--------------|
| rs137853294 vs       | 0.852| 0.756      | 1.269| 0.26 | 2.343| 0.532-10.315 |
| rs121913300          |      |            |      |      |      |              |
| rs137853294 vs       | 1.509| 1.153      | 1.715| 0.19 | 0.221| 0.023-2.116  |
| rs614367             |      |            |      |      |      |              |
| rs137853294 vs       | 3.329| 0.954      | 1.624| 0.0203| 3.372| 0.520-21.872 |
| rs498136             |      |            |      |      |      |              |
| rs121913300 vs       | 1.064| 0.738      | 2.076| 0.15 | 2.897| 0.682-12.314 |
| rs614367             |      |            |      |      |      |              |
| rs121913300 vs       | 0.078| 1.14       | 0.005| 0.945| 1.081| 0.116-10.106 |
| rs498136             |      |            |      |      |      |              |
| rs614367 vs          | 3.126| 0.871      | 1.67 | 0.0296| 3.083| 0.559-17.006 |
| rs498136             |      |            |      |      |      |              |

Abbreviations: SNP, single nucleotide polymorphism; OR, ODDS ratio; CI, confidence interval; p-value < 0.05 was statistically significant.
Table 8: Spearman correlations among clinical features and cell cycle pathway genes expression of brain tumors †.

| mRNA | Gender | Type | Survival | Family | Grade | I.R | Area | RB1 | CCND1 | Ki-67 |
|------|--------|------|----------|--------|-------|-----|------|------|-------|-------|
| Age  | 0.039  | -0.037 | 0.074    | 0.111  | 0.06  | -0.247*** | -0.22 | -0.33 | 0.126  | 0.124  |
| Gender | 0.02  | 0.416  | 0.067    | -0.04  | 0.016 | 0.195* | 0.009 | 0.104 | 0.33** |
| Type  | 0.056  | -0.058 | 0.146    | -0.027 | -0.016 | -0.067 | -0.132 | 0.07  |
| Survival | -0.057 | 0.01   | 0.108    | 0.049  | -0.150 | -0.05  | -0.171 |
| Family | -0.182* | -0.081 | 0.005    | 0.137  | -0.016 | -0.169 |
| Grade | 0.045  | -0.117 | -0.252*  | 0.222* | 0.103 |
| I.R  | 0.051  | -0.098 | 0.072    | 0.091  |
| Area  | 0.045  | 0.047  | -0.264*  |
| RB1   | -0.337** | -0.291* |
| CCND1 | 0.135  |

| Protein level | Gender | Type | Survival | Family | Grade | I.R | Area | RB1 | CCND1 | Ki-67 |
|---------------|--------|------|----------|--------|-------|-----|------|------|-------|-------|
| Age           | 0.039  | -0.037 | 0.074    | 0.111  | 0.06  | -0.247*** | -0.22 | -0.29 | 0.12   | 0.07   |
| Gender        | 0.02   | 0.416  | 0.067    | -0.04  | 0.016 | 0.195* | 0.32  | 0.09  | 0.05   |
| Type          | 0.056  | -0.058 | 0.146    | -0.027 | -0.016 | -0.12  | -0.11  | 0.13   |
| Survival      | -0.057 | 0.01   | 0.108    | 0.049  | -0.08  | -0.07  | 0.08   |
| Family        | -0.182* | -0.081 | 0.005    | 0.06   | 0.02  | 0.14   |
| Grade         | 0.045  | -0.117 | -0.394** | 0.19   | 0.54*** |
| I.R           | 0.051  | -0.16  | 0.06     | 0.16   |
| Area          | 0.08   | 0.12   | 0.21*    |
| RB1           | -0.54*** | -0.29* |
| CCND1         | 0.43** |

† Spearman correlation coefficients. The expression levels of RB1, CCND1 and Ki-67 for the patient cohort were based on the relative mRNA level and protein level. The p values were computed using one-way ANOVA and \( \chi^2 \) test. * p<0.05, ** p<0.01, *** p<0.001.
Figure legends

**Figure 1:** Linkage disequilibrium plot for *RB1* and *CCND1* polymorphism. (A) Control Sample; (B) Tumor patient samples; Site1 for rs137853294; Site2 for rs121913300; Site3 for rs614367; Site4; rs498136; Darker region shows higher $r^2$-value.

**Figure 2.** Protein and mRNA secondary structure prediction of *RB1* exonic SNPs. (a) Superimposed structure of normal and mutated *RB1* proteins (blue, wild; white, SNP1 mutated); purple, SNP2 truncated); (b) mRNA secondary structure of normal *RB1* gene; (c) mRNA secondary structure of SNP1 rs137853294 polymorphism; (d) mRNA secondary structure of SNP2 rs121913300 polymorphism.

**Figure 3:** mRNA expression of (A) *RB1* in brain tumor samples and normal control samples, (B) *RB1* in brain tumor samples with smoking status (C) *RB1* in brain tumor samples with IR. (D) *RB1* in tumor samples with family history, (E) *RB1* in different types of brain tumor (F) *RB1* in brain tumor samples with different grades. Among these grades, grade I included meningiomas (9), grade II included diffuse astrocytoma (07), choroid glioma (12), oligodandroglioma (05), ependoma (07), atypical meninigioma (08) and pituitary adenoma (04). Grade III included anaplastic astrocytoma (19), anaplastic oligodandroglioma (3), anaplastic meningioma (04) and pititutary adenoma (03). Grade IV included GBM (11) and diffuse medine glioma (04). (G) melt curve analysis of *RB1* gene.

**Figure 4:** mRNA expression of (A) *CCND1* in brain tumor samples and normal control samples, (B) *CCND1* in brain tumor samples with smoking status (C) *CCND1* in brain tumor samples with IR. (D) *CCND1* in tumor samples with family history, (E) *CCND1* in different types of brain tumor (F) *CCND1* in brain tumor samples with different grades. Among these grades, grade I included meningiomas (9), grade II included diffuse astrocytoma (07), choroid glioma (12), oligodandroglioma (05), ependoma (07), atypical meninigioma (08) and pituitary adenoma (04). Grade III included anaplastic astrocytoma (19), anaplastic oligodandroglioma (3), anaplastic meningioma (04) and pititutary adenoma (03). Grade IV included GBM (11) and diffuse medine glioma (04). (G) melt curve analysis of *CCND1* gene.

**Figure 5:** mRNA expression of (A) *Ki-67* in brain tumor samples and normal control samples, (B) *Ki-67* in brain tumor samples with smoking status (C) *Ki-67* in brain tumor samples with IR.
(D) Ki-67 in tumor samples with family history, (E) Ki-67 in different types of brain tumor (F) Ki-67 in brain tumor samples with different grades. Among these grades, grade I included meningiomas (9), grade II included diffuse astrocytoma (07), choroid glioma (12), oligodandroglioma (05), ependoma (07), atypical meningioma (08) and pituitary adenoma (04). Grade III included anaplastic astrocytoma (19), anaplastic oligodandroglioma (3), anaplastic meningioma (04) and pititutary adenoma (03). Grade IV included GBM (11) and diffuse medine glioma (04). (G) melt curve analysis of Ki-67 gene.

Figure 6: protein expression of RB1 gene (A) negative control (B) adjacent control section (C) brain tumor. Immunoreactive intesity of RB1 gene in brain tumor sections (D). protein expression of CCND1 gene (E) negative control (F) adjacent control section (G) brain tumor. Immunoreactive intesity of CCND1 gene in brain tumor sections (H). protein expression of Ki-67 gene (I) negative control (J) adjacent control section (K) brain tumor. Immunoreactive intesity of Ki-67 gene in brain tumor sections (L).

**Figure 7:** ROC curve analysis of RB1 gene (A) and CCND1 gene (B) in brain tumor patients.
