A brain tumour is mainly considered intracranial tumour originated from abnormal and uncontrolled cell-division, normally either originated in neuron, glial cells (astrocytes, oligodendrocytes, ependymal cells, lymphatic tissue, blood vessels), in the cranial nerves (myelin-producing schwann cells), in the brain envelopes (meninges), skullly pituitary and pineal gland or spread from cancer primarily located in other organs (metastatic tumours). Primary brain tumours are commonly located in the posterior cranial fossa in children and in the anterior two-third of the cerebral hemi-sphere in adults. Brain tumours mainly include astrocytoma, piolocytic astrocytoma, dysembyoplastic neuroepithelial tumour, oligodendrogliomas, ependymoma, glioblastoma multiforme, mixed giomas, oligoastrocytomas, medulloblastoma, retinoblastomas, medulloblastoma, retinoblastoma, neuroblastoma, germinoma and teratoma.

In this thesis, we have studied two kinds of brain tumours, medulloblastoma and astrocytoma. Medulloblastoma is an extremly malignant and invasive PNET localizad at the cerebellum (Kleihues and Sobon, 2000). It corresponds histologically to WHO grade IV tumour and the most common malignant brain tumour of childhood, representing 20% of all childhood brain tumours.

The annual incidence of medulloblastoma has been estimated at 0.5/100,000 children younger than 15 year old.

The astrocyte, one form of glial cells which comprises much of the background substance of the brain and spinal cord, gives rise to large category of primary brain tumours, the diffusing and infiltrating astrocytomas. There are the most frequent intracranial neoplast that account for more than 60% of all primary brain tumour. These neoplasma can occur in all areas of the brain and spinal cord among children and adults. The incidence of astrocytomas is approximately 7/100,000 cases.

The World Health Organization (WHO) classification recognizes four grades of astrocytoma; Grade I- astrocytomas are growing, non-infilrative neoplasm, occurring mainly in children and young adults and include juvenile epilocytic astrocytomas and ganglioglioma.

Grade II- mainly well-differentiated fibrillary astrocytoma

Grade III- anaplastic astrocytoma is a more aggressive neoplasm.

Grade IV- Most malignant form of astrocytes named glioblastoma multiforme, it is the most common primary malignant brain tumour of adults.

Brain tumours are highly heterogenous in nature and depicting phenotypically and genotypically. Brain is itself a complex system and complete physiology is not very much clear and because of this reason, we are still unable to explore clear clues to brain tumour
development and its progression. Tumour heterogeneity varies in several ways. It changes with organ site and cell origin. Heterogeneity shows cellular differences within a single neoplasm. Phenotypic heterogeneity shows variation in antigen expression (Cikes, M and Klein, G 1972; Everson et al., 1974), membrane composition (Bosman and Winston, 1970; Pasternak et al.; 1971), response to chemotherapy (Valeriote and Putten; 1975), and metastatic proclivity (Suzuki et al., 1978, Weiss, 1980). In case of tumour heterogeneity among tumour subpopulations differences are extensive, viz cellular morphology; tumour histology; karyotype and other cytogenetic markers; growth rate; cell products; receptors; enzymes; immunological characteristics; metastatic ability and sensitivity to therapeutic agents.

Even phenotypic and genotypic heterogeneity is shown at cellular level in metastatic and primary tumours of the brain tumours. In fact, Shapiros’ Laboratory (Shapiro et al., 1981) used karyotype heterogeneity as a marker for subpopulation in fresh primary human gliomas and then this is used as a criteria for distinctive identification among subpopulation of tumours. Even other parameters are also involved including sensitivity to drug (Yung et al., 1982) and genetic stability (Shapiro and Shapiro, 1983).

Genetic instability is also one of the specific mechanisms for tumour and cause many errors in tumour cell DNA replication including point mutation, genomic rearrangement, chromosome losses, gene amplification etc. all of which are reflected in increased phenotypic variability. Recent investigation showed epigenetic mechanism as the chief source of variability in tumours. The most important hurdle of this tumour heterogeneity comes at the time of treatment of tumours because this heterogeneity causes failure of therapy.

In this study we have tried to get answer of some unsolved questions like; what are the genetic changes and pathways of oncogenesis and progression that account for the heterogeneity of brain tumours? Which genes, genetic changes and genetic pathways are important to the initiation, maintenance and progression of brain tumours?

For this study we have chosen sonic hedgehog signaling pathway in both brain tumours and study this pathway heterogeneity in medulloblastoma and astrocytoma. This pathway is well known in medulloblastoma development and other tumours including prostate cancer (Sanchez et al., 2005; Stecca et al., 2005), oral squamous cell carcinoma (Nishimaki et al., 2004), colorectal carcinoma (Zhu et al., 2004) however, in astrocytoma it is not well explored except for a few reports (Clement et al., 2007).

The present study is divided into three chapters.

In the first chapter we have transfected a medulloblastoma cell line Daoy and an astrocytoma cell line U87MG with GLI1 siRNA. After this transfection and sufficient knockdown (>60%) of
GLI1 in both cell lines, we have checked the other downstream target of GLI1 gene including PTCH1, Cyclin D2, Plakoglobin, PAX6, NKX2.2 and Bmi-1.

We have found downregulation of PTCH1 gene in both tumour cell lines, down regulation of Cyclin D2 in medulloblastoma cell line and Upregulation in Cyclin D2 in astrocytoma cell line U87MG, downregulation of Plakoglobin in medulloblastoma cell line and upregulation of plakoglobin in astrocytoma cell line, compared to negative transfected and untransfected cell lines. These genes are direct target of GLI1 transcription factor and our results support the PTCH1 regulation by GLI1 in both the tumours. However the regulation of Cyclin D2 and Plakoglobin varies from the previous report, where the scientists have mentioned the upregulation of Cyclin D2 and downregulation of Plakoglobin in GLI1 transfected epithelial cell (Yoon et al., 2002). These differences in regulation of Cyclin D2 and Plakoglobin in these tumours further support regional differential gene expression and regulation of sonic hedgehog as well in these tumours (Marc Fuccillo, 2007).

We also checked other putative target genes of GLI1 including PAX6, NKX2.2 and Bmi-1 in these tumours cell lines. We found upregulation of PAX6 in medulloblastoma cell line Daoy, downregulation in astrocytoma cell line U87MG, compared to negative siRNA and untransfected cell lines. PAX6 gene is considered as a tumour suppressor gene in glioblastoma (Zhou, YH et al., 2005) and its downregulation by GLI1 indicated the possibility of tumourogenesis in astrocytoma by Shh pathway. But the upregulation of PAX6 in medulloblastoma cell line showed a differential and multifaced regulation of Shh.

The NKX2.2 is a homeobox gene and is included among transcription factor II (TF.II). It is also putative immediate target gene of GLI1 factor and shows upregulation during early embryonic ventral patterning (March Fuccillo, 2006; Vokes SA, 2007; Takahashi, M 2002). After GLI1 knockdown in medulloblastoma cell line Daoy, PAX6 transcript expression was decreased compared to negative siRNA and untransfected cell lines. This decrease in the expression suggests that NKX2.2 is upregulated by GLI1 not only during early embryonic development but also in medulloblastoma progression. However, GLI1 knockdown astrocytoma did not show any changes in expression.

This Bmi-1 gene considered as an oncogene and assists the stem cell proliferative characteristic with sonic hedgehog signaling in medulloblastoma (Liu S et al., 2006). The regulation of Bmi-1 by GLI1 is not well defined. After GLI1 knockdown in medulloblastoma cell line Daoy, Bmi-1 expression was decreased, however its expression was increased in GLI1 knockdown astrocytoma cell line U87MG, compared to universal negative siRNA and
untransfected cell lines. Decreased expression of Bmi-1 after GLI1 knockdown in medulloblastoma revealed the possibility of upregulation of Bmi-1 by GLI1. Although, upregulation of Bmi-1 transcripts after GLI1 knockout in astrocytic cell line indicated that Bmi-1 could be negatively regulated by GLI1 in astrocytoma. Also few reports mention copy number alteration of polycomb gene Bmi-1 in glioma tumour (Hayry et al., 2008).

Remaining five cell lines of medulloblastoma including PFSK-1, Daoy, TE671c2, D283 and SK-PN-DW and seven cell lines of astrocytoma including A172, LN405, SW1783, T98G, SW1088 and GOS-3 showed similarity in expression pattern of the above said genes. We further checked the promoter methylation status of PTCH1 and Cyclin D2 in both medulloblastoma and astrocytoma cell lines and primary tumour samples.

The PTCH1 methylation had been reported in breast cancer cell line MCF-7 and primary samples, and this methylation were correlated with low expression of PTCH1 transcript (Idowolf et al., 2007). PTCH is also well known tumour suppressor gene and showed epigenetic regulation in many human malignancies (Berman DM et al., 2002, Calzada Wack et al., 2002, Toyota et al., 2001).

Medulloblastoma cell lines and medulloblastoma samples showed partial/hemi methylation in the promoter region of PTCH1 gene. This promoter hypermethylation was also correlated with low PTCH1 transcript expression in one cell line and 2/14 samples (p3 and p11) of medulloblastoma. Recently it was reported that proximal promoter of PTCH1 gene (variant exon 1B) showed methylation in some cases of medulloblastoma (Pritchard JI and Olson JM, 2008).

All of these studies support the possibility of methylation in PTCH1 promoter region and it could be one of the reasons for silencing of this gene in many cell tumours including medulloblastoma.

In glioblastoma, only one cell line and 3/44(7%) glioma samples showed partial/hemi methylation in the promoter region of PTCH1 gene. Moreover, their methylation showed correlation with low transcript expression in glioma cell lines and samples.

This PTCH1 methylation in few cell lines and samples of all these major brain tumours comprise medulloblastoma, and glioblastoma further support the previous investigation which had assumed two way of sonic hedgehog pathway activation i.e. one is ligand dependent and the other one ligand independent in which PTCH1 shows inactivation(Cohen et al., 2005).

Cyclin D2 is a member of the D-type Cyclins, implicated in cell-cycle regulation, differentiation and malignant transformation. Many cases of Cyclin D2 promoter methylation had been reported in pancreatic cancer (Matsubayashi, et al 2003), breast cancer (Evron, et al., 2001,
Evron et al., 2001, Lehmann et al., 2002, Sharma, et al., 2007) and Human epithelial Ovarian Cancer (Sakuma, et al., 2007).

Our studies establish that the promoter hypermethylation of Cyclin D2 gene plays a major role in brain tumours of medulloblastoma and glioblastoma tumours cell lines and primary tumour samples.

In cell lines A172, SW1783, T98G, CCF-STTG-1 and GOS-3, some showed low or no expression of Cyclin D2 and Cell lines U87MG, A172 and SW1783 showed low or no expression of PTCH1. The 5-aza-2'-deoxycytosine and TSA treatment showed increase in expression of these silent cell lines showing additional control at an epigenetic level (p=0.0014).

Promoter methylation of two CpG rich sites of Cyclin D2 in cell lines is that associated with low expression. In some conditions, these two sites showed hypermethylation and that was by 5-aza-2'-dC and TSA showing role of epigenetic control.

In second chapter, we have checked the expression and epigenetic regulation of other associated genes with sonic hedgehog including SMO, HHIP, SUFU, SFRP1 and GLI3 in medulloblastoma and astrocytoma cell lines and primary tumour samples.

This high expression of SMO itself is an indication of the oncogenic in nature of SMO and sonic hedgehog pathway activation. In medulloblastoma, sonic hedgehog pathway is well studied though this sonic hedgehog pathway activation is not very much explored in the astrocytomas. In this study, we have checked the role and activation of Shh signal activation in the astrocytoma. This high expression of SMO in astrocytoma cell lines could suggest the possibility of sonic hedgehog pathway activation in astrocytoma. However, few cell lines and samples showed promoter methylation in SMO which has not been discussed in thesis thesis.

HHIP acts as an antagonist of Shh pathway, the upregulation of Shh pathway leads to downregulation in the HHIP1 expression. In this study, low expression of HHIP transcript in medulloblastoma and astrocytoma cell lines was compared with normal brain tissue. It was highly significant (p<0.0001). This low expression of HHIP1 in these tumours cell lines indicate the possibility of sonic hedgehog pathway activation, because high expression of HHIP inhibits the Shh pathway in similar way as PTCH inhibition (McMahon et al 1999a and McMahon et. al. 1999b). It was also reported that HHIP showed epigenetic regulation in many cancers including Pancreatic cancer cell lines (Beachy et al., 2002) (McMahon et al., 1999). It also further establishes the possibility of epigenetic regulation of this HHIP gene in medulloblastoma, astrocytic cell lines and primary tumour samples.
SUFU acts as one of the tumour suppressor genes (TSGs) of sonic hedgehog pathway and blocks the nuclear accumulation of GLI1 and GLI2 (Barnfield et al., 2005) by facilitating phosphorylation in them. It was found to show overall low expression of SUFU in medulloblastoma and astrocytoma cell lines (p=0.0090). High SUFU expression showed negative effect on Shh signaling by inhibiting GLI1 and GLI2 transcriptional activation. Most of the tumours cell lines and samples showed low expression of SUFU. This low expression indicated that there could be possibility of inactivated SUFU in medulloblastoma and astrocytoma cell lines. This did not inhibit the transcriptional activator GLI1, consequently GLI1 is accumulated in the nucleus without any hindrance, and gets activated by other downstream target genes of this pathway.

SFRP1 protein is an antagonist of canonical pathway of WNT. This WNT pathway is also responsible for many cellular processes, organ development and malignant transformation (Bejsovec A, 2005; Brembeck et al., 2006; Kikuchi et al., 2006 and Lee et al., 2006). In another finding, it was reported that GLI1 also binds with the promoter region of SFRP1 and downregulates its expression (Katoh and Katoh, 2005).

This low expression indicates that SFRP1 is acting as a tumour suppressor gene and downregulates the activation of Shh signaling in medulloblastoma and astrocytoma. However, our results were contrary to previous reports, which explained the process of upregulation of SFRP1 in medulloblastoma development (Lee et al., 2003).

The Drosophila Shh pathway is mediated by downstream transcriptional molecule cubitus interruptus (Ci), having both activator and repression domain and their functions are according to the signal activation. The homologue of Vertebrate Ci has three GLI1, GLI2 and GLI3 transcriptional factors. Thus GLI1 and GLI2 are activators, and GLI3 has both activator and repressor domains.

It has been reported that GLI3 has two forms, one is full length (190kDa) and acts as activator(GLI3A) of Shh and another one is truncated and acts as a repressor(GLI3R) (89kDa) of Shh (Ping Dai, 1999). This activator showed high expression due to activation of Shh signaling and increased the expression of GLI1 by binding at 5'promoter region of GLI1.

In this study, it was determined that all six medulloblastoma cell lines showed high expression of GLI3 transcript as compared to normal adult brain tissue (p=0.0554). All eight astrocytoma cell lines also showed high fold expression of GLI3 transcripts compared to normal brain tissue. Astrocytic cell lines also showed full-length expression of GLI3 protein (190kDa). High expression of GLI3 in medulloblastoma and astrocytoma cell lines support the expression of full-length GLI3 transcript, which acts as an activator for sonic hedgehog pathway.
The HHIP is also regulated epigenetically and was reported to show promoter methylation in pancreatic neoplasm (Martin et al., 2005) and gastrointestinal cancer (Taniguchi et al., 2007). It was found that both medulloblastoma and astrocytoma tumour cell lines and primary samples showed methylation and their methylation correlated with low or no expression of HHIP. Moreover, HHIP acts as a negative regulator of Shh signaling and its methylation in these tumours, besides other tumours, suggested the possibility of two kinds of regulatory mechanisms controlling the expression of HHIP. Henceforth, HHIP is under two levels of regulation; one is epigenetic and another is under influence of Shh signaling activation.

The candidate SUFU is a newly identified tumour suppressor gene, which is predisposed in individual to develop medulloblastoma tumour (Taylor et al., 2002). Hence it is critical for embryonic development and tumour suppression.

It was found that only 2/44 glioma samples showed partial/hemi methylation in the promoter region of SUFU. This gene did not show any methylation in the 6 medulloblastoma, 8 astrocytoma cell lines, 14 medulloblastoma and remaining 42 glioma primary tumours samples. Moreover, there was no report, which has mentioned SUFU promoter methylation in any tumour so far. Henceforth, SUFU may not be under epigenetic control in these major brain tumours.

The SFRP1 encodes a Wnt/β-catenin signaling antagonist and showed frequent promoter methylation in many tumours. SFRP1 is also one of the target molecules of Shh signaling and is repressed by Shh activation (Ingram, et al., 2002).

In this study, we have determined the methylation of SFRP1 in medulloblastoma and glioblastoma cell lines and primary tumour samples. In medulloblastoma, one cell line TE671 and 5/14 (36%) samples showed partial/hemi methylation in the promoter region of SFRP1. However, this medulloblastoma methylation result is contrary to previous investigation, which showed no methylation in the promoter region of SFRP1 gene in medulloblastoma (Chang et al., 2005). Moreover, this hypermethylation results were in correspondence to no expression of SFRP1 transcripts in respective cell line and primary tumour samples.

In astrocytoma, 5/8 cell lines (A172, T98G, SW1088, CCF-STTG-1 and GOS-3) and 14/44 of glioma samples showed partial/hemi methylation in the promoter region of SFRP1 gene; however this methylation was supported by low or no expression of SFRP1 transcript. Moreover, it was reported that GLI1 binds to the promoter region of the SFRP1 gene and down-regulates its activity (Katoh, Y and Katoh, M, 2006). From this result, it is found that SFRP1 shows promoter hypermethylation in both brain tumours, medulloblastoma and astrocytoma, and this methylation is correlated with low expression of SFRP1 in these tumours.
Taken together, it has revealed two levels of controls, which regulates SFRP1 expression gene, one is regulated by sonic hedgehog signaling and the other is under epigenetic regulation.

In the third chapter, we have checked the effect of specific inhibitor of Shh signaling pathway cyclopamine in medulloblastoma cell line Daoy and astrocytoma cell line U87MG. The effect of cyclopamine was measured with MTT assay, cell cycle analysis and apoptosis analysis. In this study, cyclopamine showed inhibitory effects on sonic hedgehog signaling in these cell lines, which is manifested as toxic in nature, anti-proliferative effect and increased apoptosis in these two cell lines. These results further support the sonic hedgehog activation in glioblastoma besides medulloblastoma.

Our study confirmed the possible role of sonic hedgehog signaling in the development of astrocytoma tumour besides medulloblastoma. Sonic hedgehog shows differential expression and multi-facet action, not only in the early embryonic development but also in the progression of brain tumours.

Many sonic hedgehog signaling downstream targets genes showed two level of regulation: one at transcriptional level and another one at epigenetic level. And signaling pathway of sonic hedgehog shows heterogeneity between medulloblastoma and glioblastoma tumours even at molecular level.