Chapter – II: Results
Expression of other sonic hedgehog genes including Smoothened (SMO), HHIP, SUFU, GLI3 and SFRP1 in six medulloblastoma and eight astrocytoma cell lines by RT-PCR and Real Time RT-PCR

R. 20 (SMO)
Medulloblastoma cell lines

RT-PCR: All six cell lines (TE671, PFSK-1, Daoy, TE671c2, D283 and SK-PN-DW) showed high expression of SMO transcript as compared to normal brain tissue (Figure 28A).

Real Time RT-PCR: Similarly, all of the six cell lines of medulloblastoma showed high fold expression of SMO transcript compared to normal brain tissue and this differences was highly significant (p=0.0009) (Figure 28B).

Astrocytoma cell lines

RT-PCR: In seven cell lines of astrocytoma SMO transcript expression was high and one cell line U87MG did not show the expression of SMO transcript compared to normal brain tissue (Figure 28C).

Real Time RT-PCR: Similarly, except one cell line (U87MG) all remaining seven cell lines showed high fold expression of SMO transcript, compared to normal brain tissue. (Figure 28D).

R.21 HHIP
Medulloblastoma cell line

Real Time RT-PCR: All 6 cell lines showed low expression of HHIP transcript as compared to normal brain tissue and it was highly significant (p<0.0001) (Figure 29A).

Astrocytoma

Real Time RT-PCR: All 8 glioblastoma cell lines showed low expression of HHIP transcript as compared to normal brain tissue and the difference was highly significant (p<0.0001) (Figure 29B).
Fig. 28 A-B. RT-PCR and Real Time RT-PCR comparative expression of Smoothened (SMO) transcript in six medulloblastoma cell lines (M1-TE671, M2-PFSK-1, M3-Daoy, M4-TE671c2, M5-D283 and M6-SK-PN-DW) Fig.A. All six medulloblastoma cell lines showed high expression of SMO transcript compared to normal brain tissue. L and B stand for lung and brain as normal control. Transferin Receptor (TFR) was taken for internal control of RNA expression in RT-PCR. Fig. B. All six medulloblastoma cell lines showed high fold expression of SMO compared to normal brain tissue (p=0.009). All data from Real Time RT-PCR were normalized by GAPDH.
R.22 SUFU

Medulloblastoma

Real Time RT-PCR: In this 2/6 cell lines TE671 and TE671c2, we showed 2-4 high-fold expression and remaining 4/6 cell lines showed either equal (SK-PN-DW) or low (PFSK-1, Daoy and D283) expression of SUFU transcript as compared to normal brain tissue (Figure 30A).

Astrocytoma

Real Time RT-PCR: All 8 cell lines showed low expression of SUFU transcript as compared to normal brain. It was highly significant (p=0.0009) (Figure 30B).

R.23 SFRP1

Medulloblastoma

Real Time RT-PCR: cell line SK-PN-DW showed high-fold expression, Daoy cell line showed equal expression, three cell lines PFSK-1, TE671c2 and D283 showed low expression and one cell line TE671 showed no expression of SFRP1 compared to normal brain tissue. It was not significant (Figure 31A).

Astrocytoma

Real Time RT-PCR: One cell line(SW1783) showed high-fold, 5 cell lines showed low (U87MG, A172, T98G, CCF-STTG-1 and GOS-3) and remaining 2 cell lines did not show expression of SFRP1 transcript compared to normal brain tissue.(Figure 31B).

R.24 GLI3

Medulloblastoma

RT-PCR: All 6 cell lines showed high expression of GLI3 transcript as compared to normal brain tissue (Figure 32A).

Real Time RT-PCR: Similarly, all six cell lines also showed high-fold expression of GLI3 as compared to normal brain tissue.(Figure 32B).

Astrocytoma

RT-PCR: All eight cell lines showed high fold expression of GLI3 transcript as compared to normal brain tissue (Figure 32C).
Fig. 28C-D. RT-PCR and Real Time RT-PCR comparative expression of SMO transcript in 8 astrocytic cell lines: (G1-U87MG, G2-A172, G3-LN405, G4-SW1783, G5-T98G, G6-SW1088G7-CCF-STTG-1 and G8-GOS-3) Fig. 28C. Most of the cell lines except U87MG showed high expression of SMO as compared to normal brain tissue. L and B stand for lung and brain, taken as a normal control. Transferin receptor (TFR) was taken for internal control of RNA expression in RT-PCR. Fig. 28D. Most of the cell lines except U87MG, showed high fold expression of SMO compared to normal brain tissue \((p=0.0586)\). All data from Real Time RT-PCR were normalized by GAPDH.
Real Time RT-PCR: In astrocytic cell lines, all eight cell lines showed high fold expression of GLI3 compared to normal control brain tissue (Figure 32D) significant (p=0.0247).

R.25 Protein Expression of GLI3:

Western blot: Most of the cell lines except A172 cell line showed protein expression of GLI3 (Figure 33A). Most of the astrocytic samples also showed GLI3 protein expression (Figure 33B).

R.26 Expression of other sonic hedgehog genes including SMO, HHIP, SUFU, GLI3 and SFRP1 in fourteen medulloblastoma and twenty seven glioma primary tumour samples.

Most of the medulloblastoma samples and glioma samples showed high expression of SMO transcript compared to normal brain tissue (p=0.53) (p=0.013). (Figure 34A-B)

All medulloblastoma and glioma primary tumours samples showed either no expression or low expression of HHIP transcript. Highly significant (p<0.0001) (Figure 34C-D).

SUFU expression was not checked in medulloblastoma samples because of insufficient RNA quantity. In astrocytic tumours, 25/27 of the samples showed low expression, only 2/27 samples showed high-fold expression as compared to normal brain tissue. It was not significant (Figure 35A).

In medulloblastoma 2/11 samples showed high-fold of expression, 5/11 samples showed low expression and remaining 4/11 samples showed no expression of SFRP1 transcript compared to normal brain tissue (Figure 35C).

In astrocytic tumour samples, 2/27 primary tumours showed high-fold expression and remaining 25/27 primary samples showed low expression of SFRP1 transcript as compared to normal brain tissue. (Figure 35D).

GLI3 transcript expression was not checked in medulloblastoma samples because of insufficient RNA quantity.

In astrocytic tumour, 6/27 samples showed high-fold expression of GLI3 transcript and remaining 21/27 samples showed more or less equal expression compared normal brain tissue (Figure 35B).
Fig. 29 A-B: Real Time RT-PCR comparative expression of HHIP transcript in six medulloblastoma and eight astrocytoma cell lines. Fig. A-B. All 6 cell lines of medulloblastoma and 8 astrocytoma cell lines showed low expression of HHIP compared to normal brain tissue (p<0.0001). All data were normalized by GAPDH.
Promoter hypermethylation analysis:

Methylation Results:
Epigenetic regulation plays a very significant role in the progression of many cancers, because this phenomenon silences many tumour suppressor genes (TSGs). In this section we have checked the epigenetic regulation of these putative tumour suppressor genes (TSGs): HHIP, SUFU and SFRP1.

R27 HHIP1 Promoter Methylation:
In this case, methylation analysis was done by MSP-Meth method. For this we used single pair primers designed with no CpG in its sequences. These primers amplify both unmethylated and methylated sequences. In this case in vitro methylated DNA (IMD) melting peak (green color) was considered as positive control for methylated DNA and normal blood (red color peak) as a positive control for unmethylated DNA region and both controls have given different melting temperature, 81° and 77.5° respectively. Cell lines and samples were given melting peak (blue color) temperature which varies with the CpG methylation in the promoter region.

Medulloblastoma: In this case, two cell lines TE671 and TE671c2 showed methylated peak and methylated melting temperature(80.5° degree) and remaining four cell lines PFSK-1, Daoy, D283 and SK-PN-DW showed hemi/partial methylated peak and hemi/partial melting temperature range(79.5°-80° degree) (Figure 36A-B) (Table 16).

In medulloblastoma samples, only 2/14 samples showed hemi/partial methylation peak and melting temperature (78.5° degree) (Figure 36C-D) (Table 16).

Astrocytoma: In this case, two cell lines (CCF-STTG-1 and GOS-3), showed complete promoter methylation and their melting temperature was at 81.0° degree, four cell lines (U87MG, A172, LN405 and T98G) showed hemi/partial promoter methylation and their melting temperature was at 79.5° degree. One cell line (SW1088) did not show promoter methylation which gave melting temperature was at 77.5° degree (Figure 37A-D).

In glioma, more than 75% (32/44) of the primary tumour samples showed methylation in the promoter region of HHIP (15/44 samples showed complete promoter methylation; 17/44 samples showed hemi/partial methylation) and remaining 25% of the samples did not show methylation in the promoter region (Figure 37E-H) (Table 17).
Real Time RT-PCR

Fig. 30 A-B. Real Time RT-PCR expression of SUFU in six medulloblastoma and eight astrocytoma cell lines. Fig. A. Most of the medulloblastoma cell lines except (TE671 and TE671c2) showed low expression of SUFU transcript as compared to normal brain tissue. Fig. B. All 8 astrocytoma cell lines showed low expression of SUFU transcript as compared to normal brain tissue (p=0.009). All data were normalized by GAPDH.
R.28 SUFU Promoter Methylation:
In this case, we also used MSP-Meth method for SUFU promoter methylation analysis. Positive control for complete methylated region was \textit{In vitro} (IMD) showed green peak being given melting temperature was at 83° and positive control for unmethylated DNA region showed red peak, being given melting temperature was at 80° degree.

\textbf{Medulloblastoma:} In this case all of the 6 cell lines and 14 samples showed unmethylated melting peak (blue color) being given melting temperature at 80° degree (Figure 38A-B)(Table.16)

\textbf{Glioblastoma:} In this case all 8 cell lines showed unmethylated melting peak (blue color) and their melting temperature was at 80° degree (Figure 38A-B) (Table 17). In glioma 2/44 primary tumour samples showed hemi/partial methylated peak (blue color) being given melting temperature in the range of 81°-83° degree.

R.29 SFRP1 Promoter Methylation:
This methylation study was also done with MSP-Meth method. The IMD was taken as a positive control for the methylated promoter region, which gave melting temperature of 82° degree. Normal blood DNA was taken as positive control for unmethylated promoter region which gave melting temperature of 77.5° degree.

\textbf{Medulloblastoma:} only TE671 cell line showed hemi/partial methylation in the promoter region of the SFRP1 promoter region, the remaining cell lines did not show any methylation. In medulloblastoma samples, 5/14 samples showed partial/hemi methylated in the promoter region of SFRP1 gene. The melting temperature of methylated cell line and samples is 78.5 degree (Figure 39A-C) (Table 16).

\textbf{Glioblastoma:} In glioblastoma, 5/8 cell lines (A172, T98G, SW1088, CCF-STTG-1 and GOS-3) showed partial/hemi methylation in the promoter region of SFRP1 gene. In glioma, 14/44 samples showed hemi/partial methylation in the promoter region of SFRP1 gene. It showed melting peak temperature at 78.5 degree (Figure 39A-C) (Table 17).
Fig. 31 A-B. Real Time RT-PCR comparative expression of SFRP1 transcript in six medulloblastoma and eight astrocytoma cell lines. Fig. A. Most of the cell lines except (SK-PN-DW) showed low/no expression of SFRP1 transcript as compared to normal brain tissue (p=0.0831). Fig. B. Except one cell line (SW1783) all remaining cell lines showed either low no expression of SFRP1 transcript compared to normal brain tissue. All Data were normalized by GAPDH.
Fig. 32 A-B RT-PCR and Real Time RT-PCR comparative expression of GLI3 transcript in six medulloblastoma cell lines (M1-TE671, M2-PFSK-1, M3-Daoy, M4-TE671c2, M5-Daoy, and M6-SK-PN-DW). Fig. A. All six cell lines of medulloblastoma showed high expression of GLI3 as compared to normal brain tissue. L, B and NTC stands for lung, brain and no-template control respectively. Transferin receptor (TFR) was taken for internal control of RNA expression in RT-PCR. Fig. B. All six medulloblastoma cell lines showed high fold expression of GLI3 compared to normal adult brain tissue. All Real Time RT-PCR data were normalized by GAPDH.
Astrocytoma cell lines

Fig. 32 C-D

C  RT-PCR

Mr  B  G1  G2  G3  G4  G5  G6  G7  G8  NTC

324 bp

Fig. 32 D. RT-PCR and Real Time RT-PCR of GLI3 transcript in eight astrocytoma cell lines (G1-U87MG, G2-A172, G3-LN405, G4-SW1783, G5-T98G, G6-SW1088, G7-CCF-STTG-1, G8-GOS-3. Fig. C. All 8 astrocytoma cell lines showed high expression of GLI3 compared to normal brain tissue. L, B, and NTC stands for lung, brain and no-template control respectively. Transferin receptor (TFR) was taken for internal control of RNA expression in RT-PCR. Fig. D. All eight cell lines of astrocytoma showed high fold of GLI3 transcript expression as compared to normal brain (p=0.0247). All data from Real Time RT-PCR were normalized by GAPDH.
Western blotting:

Fig. 33 A. Astrocytoma cell lines

Fig. 33 B. Glioma samples

Fig. 33 A-B. Protein expression of Gli3 showed by astocyteoma cell lines and glioma samples. Fig. A. Western blotting showed Gli3 protein expression in all eight astrocytoma cell lines (G1-G8). Among 8 cell lines 2 glioma cell lines (G2-A172, G3-LN405) did not show very distinct band of Gli3 protein. Fig. B. In glioma samples (1-4 and 6), all samples showed good expression of Gli3 protein. C+ was HeLa cell lysate of GLI3 protein taken for normal control. GAPDH protein was taken as loading control.
Real Time RT-PCR of medulloblastoma and glioma samples

Fig. 34A-D

A. SMO

B. SMO

\[ p = 0.03 \]

C. HHIP

D. HHIP

\[ p < 0.01 \]

Fig. 34 A-D. Real Time RT-PCR comparative expression of SMO, and HHIP genes in 14 medulloblastoma and 27 glioma samples. **Fig. A-B.** Most of the primary tumour samples of medulloblastoma and astrocytoma showed high fold expression SMO compared to normal brain tissue. **Fig. C.** Most of the medulloblastoma samples showed either low or no expression of HHIP transcript compared to normal brain tissue \((p < 0.001)\). **Fig. D.** All glioma samples showed low expression of HHIP transcript compared to normal brain tissue \((p < 0.001)\).
Real Time RT-PCR expression of SUFU, GLI3 and SFRP1 in medulloblastoma and glioma samples

Fig. 35 A-D

A. SUFU

B. GLI3

C. SFRP1

D. SFRP1

Fig. 35 A-D. Real Time RT-PCR comparative expression of SUFU, GLI3 and SFRP1 in 27 glioma samples and 11 medulloblastoma samples. Fig. 35A. Most of the glioma primary tumour samples showed low expression of SUFU transcript as compared to normal brain tissue. Fig. 35B. Among glioma primary tumors (27) 26% of the samples showed high and remaining 76% of the samples showed more or less equal expression of GLI3 transcript compared to normal brain tissue. Fig. 35C. Among medulloblastoma, 79% (10/14) samples showed low expression and remaining 21% (4/14) samples showed high fold expression of SFRP1 compared to normal brain tissue. Fig. 35D. Among glioma, 24/27 of the samples showed low expression of SFRP1 compared to normal brain tissue. All data were normalized by GAPDH.
Fig. 36 A-B. HHIP Promoter hypermethylation study by MCA-Meth in medulloblastoma cell lines. This case used single pair primers designed with no CpG in its sequences and melting peak temperature varies with the percentage of methylation in the given sequences. Normal blood used as a positive control for unmethylated DNA region (red peak, 78° temp) using bisulphite primers in medulloblastoma cell lines (TE671 and Daoy) (Fig. A-B), in vitro methylated DNA (IMD) used as positive control for methylated DNA region (green color peak, 80.5° tem) (Fig. A-B) Fig. A-B. Medulloblastoma cell lines TE671 and Daoy showed complete promoter methylation and partial promoter methylation peaks respectively (blue color peak, 80.5° and 80° temps) No-template did not give any amplification by this primer (black color).
HHIP Promoter methylation study by MCA-Meth

Medulloblastoma samples

Fig. 36 C-D

C. p7

D. p29

Fig. 36 C-D. HHIP Promoter methylation study by MCA-Meth in medulloblastoma samples (p7 and p29). Fig. C-D. Unmethylated region in p7 sample (blue color peak, 78° temp) (Fig. C) and hemi/partial methylated region in p29 sample (blue color peak, 80° temp) (Fig. D). No-template control did not give any amplification by this primers (black color).
HHIP Promoter Methylation study by MCA-Meth

Astrocytoma cell lines

Fig. 37A-B

**A. U87MG**

**B. SW1088**

Fig. 37 A-B. HHIP Promoter hypermethylation study by MCA-Meth in astrocytoma cell lines (U87MG and SW1088). Normal blood used as positive control for unmethylated DNA region (red peak, 78° temp) (Fig. A and B), in vitro methylated DNA (IMD) used as positive control for methylated DNA region (green color peak, 81° temp) (Fig. A and B), partial methylated DNA region showed in U87MG cell line (blue color peak, 80.° temp) (Fig. A) and unmethylated DNA region showed in SW1088 cell line (blue color peak, 78° temp) (Fig. B). No template control did not give any amplification by this primers (black color).
Fig. 37 C-D. HHIP Promoter hypermethylation study by MCA-Meth in astrocytoma cell lines (CCF-STTG-1 and GOS-3). Normal blood used as positive control for unmethylated DNA region (red peak, 78°C temp) (Fig. C and D), in vitro methylated DNA (IMD) used as positive control for methylated region (green color peak, 81°C temp) (Fig. C and D), methylated region showed by CCF-STTG-1 cell line (blue color peak, 80.5°C temp) (Fig. C) and methylated region showed by GOS-3 cell line (blue color peak, 81°C temp) (Fig. D). No-template control did not give any amplification by this primers (black color).
HHIP Promoter hypermethylation study by MCA-Meth

Glioma samples

Fig. 37E-F

Fig. 37 E-F. HHIP Promoter hypermethylation study by MCA-Meth in glioma tumour samples no. (5 and 15). Normal blood used as positive control for unmethylated DNA region (red peak, 78° temp) (Fig. E and F), in vitro methylated DNA (IMD) used as positive control for methylated DNA region (green color peak, 81° temp) (Fig. E and F), unmethylated promoter region in samples no. 5 (blue color peak, 78° temp) (Fig. E) and hemimethylated methylated region in sample no. 15 (blue color peaks, 78° and 80.5° temp) (Fig. F). No-template control did not give any amplification by this primers (black color).
Fig. 37 G-H. HHIP Promoter hypermethylation study by MCA-Meth in glioma samples (24 and 31). Normal blood used as positive control for unmethylated promoter region (red peak, 78° temp) (Fig. G and H), in vitro methylated DNA (IMD) used as positive control for methylated promoter region (green color peak, 81° temp) (Fig. G and H), partial methylated promoter region showed by samples no. 24 (blue color peak, 80° temp) (Fig. G) and methylated promoter region showed by samples no. 31 (blue color peak, 81° temp) (Fig. H). No-template control did not not give any amplification by this primers (black color).
Fig. 38A-B. SUFU Promoter methylation study by MSP-Meth. Fig. 38 A-B. In this case used single pair primers designed with no CpG in its sequence. These primers amplify both unmethylated and methylated sequences and melting peak temperature varies with the percentage of methylation in the given sequences. Blood used as positive control for unmethylated DNA region (red peak, 80° temp) (Fig. A and B), in vitro methylated DNA (IMD) used as positive control for methylated DNA region (green color peak, 83° temp) (Fig. A and B), unmethylated DNA region in cell line/sample (blue color peak, 80° temp) (Fig. A) and hemimethylated/partially methylated DNA region in cell line/sample (blue color peaks, 80° and 84° temp) (Fig. B). No template control did not give any amplification by this primers (black color).
SFRP1 Promoter hypermethylation study by MSP-Meth

Glioma samples

Fig. 39A-B

A. 22

B. 23

Fig. 39 A-B. SFRP1 Promoter hypermethylation study by MSP-Meth. Normal blood used as positive control for unmethylated promoter region (red peak, 78° temp) (Fig. A and B), in vitro methylated (IMD) used as positive control for methylated promoter region (green color peak, 81° temp) (Fig. A and B), hemimethylated/partial methylated region in samples GT-22 and GT-23 blue color peak, 80° temp) (Fig. A and B). No-template control did not give any amplification by this primers (black color).
SFRP1 Promoter hypermethylation study by MSP-Meth

Glioma samples

Fig. 39C

GT-33

**Fig. 39C.** SFRP1 Promoter hypermethylation study by MSP-Meth Method. Normal blood used as positive control for unmethylated promoter region (red peak, 78° temp) (Fig.C), *in vitro* Methylated DNA (IMD) used as positive control for methylated promoter region (green color peak, 81° temp) (Fig. C), unmethylated region showed by sample no. (33) (blue color peak, 78° temp) (Fig. C) and no-template control did not give any amplification by this primers.
Chapter – II: Discussion
Expression of SMO, HHIP, SUFU, GLI3 and SFRP1 in medulloblastoma and astrocytoma cell lines.

D.19 Smoothened (SMO)
It is a seven transmembrane receptor of sonic hedgehog pathway and this gene is located at 7q32.3 locus of the chromosome. It is responsible for the downstream activation of Shh pathway and is considered as an oncogene of this pathway. Upregulation of Smoothened gene indicates the sonic hedgehog signal activation and leads to cancer development (Xie et al., 1998). Through this study, the expression of this gene was determined in medulloblastoma cell lines and it was found that all 6 medulloblastoma cell lines TE671, PFSK-1, Daoy, TE671c2, D283 and SK-PN-DW showed high expression of SMO transcript while this high expression level was significant (p=0.0009) compared to normal brain tissue (Figure 28A and Figure 28B). Even some cell lines (TE671 and TE671c2) reached up to >80 fold high expression of SMO transcript compared to normal brain tissue.

In astrocytic cell lines, most of the cell lines except one cell line U87MG showed high fold SMO transcript expression. In LN405 cell line, the expression reached >80 fold high compared to normal brain tissue (p=0.0586) (Figure 28C and Figure 28D).

It was determined that most of the cell lines of medulloblastoma and astrocytoma showed high level expression of SMO mRNA except one cell line of astrocytoma U87MG compared to normal brain tissue. These comparative expressions between cells lines and normal brain tissue were highly statistically significant. This high expression of SMO itself is the indication of an oncogenic activity of SMO and sonic hedgehog pathway activation. In medulloblastoma, sonic hedgehog pathway is well studied but this sonic hedgehog pathway activation is not very much explored in these astrocytomas, in this study, we 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. In case of astrocytoma, one cell line U87MG, showed very low expression of SMO and this low expression was due to epigenetic regulation discussed in other chapter.

D.20 HHIP1 (Hedgehog interacting protein 1)
It acts as an antagonist of Shh pathway, the upregulation of Shh pathway leads to downregulation in the HHIP1 expression. In this study, overall low expression of HHIP
transcript in medulloblastoma and astrocytoma cell lines was compared with normal brain tissue. It was highly significant (p<0.0001) (Figure 29A-29B). The 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 (Chuang and McMahon 1999a; Rowitch et al., 1999b). It was also reported that HHIP showed epigenetic regulation in many cancers including Pancreatic cancer cell lines (Martin et al., 2005). It also further establishes the possibility of epigenetic regulation of this HHIP gene in medulloblastoma, astrocytic cell lines and primary tumour samples.

D.21 SUFU (Suppressor of Fused)
This gene 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 felicitating phosphorylation in them. It is located at 10q24 locus of the chromosome and shows loss of hetrozygosity (LOH) in many tumours including prostate cancer, breast cancer, medulloblastoma and lung cancer (Latini et al., 2001; Leube et al., 2002). Here in this study, it was found to show overall low expression of SUFU in medulloblastoma (Figure 30A) and astrocytoma cell lines (p=0.0090) (Figure 30B). High SUFU expression showed negative effect on Shh signaling by inhibiting GLI1 and GLI2 transcriptional activation. Most of the tumours cell lines and samples had low expression of SUFU. This low expression indicated there could be possibility of inactivated SUFU in medulloblastoma and astrocytoma cell lines. This did not inhibit the transcriptional activator GLI1 and consequently GLI1 gets accumulated in the nucleus without any hindrance and gets activated other downstream target genes of this pathway.

D.22 SFRP1 (Secreted frizzled-related protein1)
This protein is an antagonist of canonical pathway of WNT. It is considered as a TSG and shows frequent loss of hetrozygosity (LOH) in the region 8p12-p11.1 of human hepatocellular carcinoma (HHC) (Finch et al., 1997).

This WNT pathway is also responsible for many cellular processes, organ development and malignant transformation (Uren et al., 2006). The SFRP1 gene showed low expression in breast cancer (Ugolini et al., 2001), ovarian cancer (Takada et al., 2004) and many more.
Recent cancers studies had revealed the interrelation in SHH and WNT pathway, with the nexus of phosphorylation status of GSK-3(Serine-threonine kinase) (Kalderon et al., 2002).

In another finding, it was reported that GLI1 also binds with the promoter region of SFRP1 and down regulates its expression (Katoh and Katoh, 2005). Thus, the study of transcript expression of SFRP1 was checked in major brain tumours comprising medulloblastoma and astrocytoma cell lines.

In medulloblastoma, most of the cell line except SK-PN-DW showed high expression of SFRP1 transcript compared to normal brain tissue (p=0.0831) (Figure 31A).

In astrocytic cell lines, most of the cell lines except SW1783 showed either no expression or very low expression compared to normal brain tissue (Figure 31B). The results obtained show that most of the cell lines of medulloblastoma and astrocytoma show low expression of SFRP1 transcript as compared to normal brain. This low expression indicates that SFRP1 is acting as a tumour suppressor gene and down regulates the activation of Shh signaling in medulloblastoma and astrocytoma. However, our results are contrary to previous reports which had explained the process of upregulation of SFRP1 in medulloblastoma development (Lee et al., 2003).

D.23 GLI3
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 domain.

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 (Dai, et al., 1999). This activator showed high expression due to activation of Shh signaling and increases the expression of GLI1 by binding at 5'promoter region of GLI1. In the absence of Shh signaling, the processing of GLI3 into shortened form acts as a transcriptional repressor in cultured cells and limb explants culture (Wang et al., 2000).

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) (Figure 32A-B). All
eight astrocytoma cell lines also showed high fold expression of GLI3 transcripts compared to normal brain tissue (Figure 32C-D). Astrocytic cell lines also showed full length expression of GLI3 protein (190kD) (Figure 33A).

High expression of GLI3 in medulloblastoma and glioblastoma cell lines support the expression of full length GLI3 transcript which acts as an activator for sonic hedgehog pathway.

D.24 Expression of SMO, HHIP, SUFU, SFRP1 and GLI3 by medulloblastoma and glioma primary tumours:

Similar to medulloblastoma and astrocytoma cell lines, tumour samples of medulloblastoma (Figure 34A) \( p=0.0053 \) and glioma (Figure 34B) \( p=0.013 \) showed many fold expression compared to normal brain tissue. This high expression further extends the possibility of sonic hedgehog activation in astrocytoma including medulloblastoma.

Most of the medulloblastoma (Figure 34C) \( p<0.001 \) and glioma (Figure 34D) \( p<0.001 \) primary tumours showed either low or no expression of HHIP compared to normal brain tissue. This low expression correspondence to cell lines expression of HHIP in these respective tumours. Low expression of HHIP showed the activation of Shh signaling in these tumours.

Most of the glioma samples showed low expression of SUFU compared to normal brain tissue (Figure 35A) and this low expression is akin to astrocytoma cell lines. Low expression of SUFU indicates non-functional inhibition on GLI gene which further activates sonic hedgehog signaling in glioma tumours. Due to insufficient RNA quantity of medulloblastoma samples, SUFU expression was not check.

SFRP1 transcript expression (except 1-2 samples) was either very low or nil in most of the samples of medulloblastoma (Figure 35C) and glioma primary tumour samples (Figure 35D) compared to normal brain tissue. Low expression itself indicates suppressed inhibitory effect in sonic hedgehog pathway; this result was very much similar to cell lines of these tumours.

In GLI3, 7/27(26%) of the glioma samples showed high-fold and remaining 20/27 samples showed equal expression compared to normal brain tissue (Figure 35B). This high expression revealed the expression of full length GLI3 activator and it is assisting sonic hedgehog activation. Most of the glioma samples also showed GLI3 full length protein (190Kd) expression (Figure 33B).
Promoter Methylation Analysis

In this study, some genes of the pathway including HHIPP, SUFU and SFRP1 were selected and their promoter methylation status was determined besides its correlation with expression in medulloblastoma cell lines and tumour samples.

The HHIP, SUFU and SFRP1 are considered as tumour suppressor genes (TSGs), however recently HHIP and SFRP1 have been reported for promoter hypermethylation in some tumours.

D.25 HHIP Promoter Methylation in medulloblastoma and astrocytoma cell lines and primary tumour samples:

The Human hedgehog interacting protein (HHIP) is a negative regulator of hedgehog signaling. It encodes a glycoprotein which binds to all 3 human hedgehog proteins with a similar affinity to the membrane receptor protein PTCH1 (Kayed, et al., 2004, Chuang et al., 2003). HHIP expression is under influence of ectopic expression of Shh and is lost in mutant Shh expression (Chung et al., 1999). This indicate that HHIP is transcriptional target of Shh and acts as key molecules for negative regulatory feedback. It 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).

In this study, HHIP promoter hypermethylation has been checked in major brain tumours comprising medulloblastoma and astrocytoma cell lines and primary tumour samples. It was found that both medulloblastoma and astrocytoma tumours 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 one is influenced by Shh signaling activation.

D.26 SUFU Promoter Methylation in medulloblastoma and astrocytoma:

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 is indispensable during development, one allelic mutation of SUFU +/- p53/- in mice develop tumours including medulloblastoma and rhabdomyosarcoma which show aberrant Shh signaling (Lee et al., 2007).
In the present study, we checked the promoter hypermethylation of SUFU gene in medulloblastoma and astrocytoma cell lines and samples. 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.

D.27 SFRP1 Promoter Methylation in medulloblastoma and astrocytoma:
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). This SFRP1 showed promoter methylation in hepatocellular carcinoma (Shih et al., 2006; Huang et al., 2007). It was also reported that this gene did not show methylation of the promoter region in medulloblastoma, however, it was show found promoter methylation supratentorial primitive neuronectodermal tumours (SPNETs) (Chang et al., 2005). The gene SFRP1 also showed methylation in the colorectal cancer (Suzuki et al., 2002).

In this study, we 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 glioblastoma, 5/8 cell lines (A172, T98G, SW1088, CCF-STTG-1 and GOS-3) and 14/44 of glioma samples (Figure 39A-C) 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 [316](Katoh and Katoh 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.