REVIEW OF LITERATURE

Chapter 2
REVIEW OF LITERATURE

2.1. Medulloblastoma: Brief history and Nomenclature

The term ‘medulloblastoma’ was introduced by Percival Bailey and Harvey Cushing in the June of 1925, during their course of a survey of 400 gliomas from the Peter Bent Brigham Hospital [27]. Twenty nine patients, primarily children, were reported with “a very cellular tumor of a peculiar kind” of which in 24 the tumor was in the cerebellar vermis, arising over the roof of the fourth ventricle. Initially considered a subtype of glioma, this soft, suckable midline cerebellar lesion was termed as ‘spongioblastoma cerebelli’. Globus and Strauss, however, had used the term ‘spongioblastoma multiforme’ to describe a series of cerebral tumors that showed considerable cellular differentiation, a feature absent in the cerebellar tumors. To avoid further confusion, the tumor was renamed as medulloblastoma to describe the group of undifferentiated tumors, with a distinct microscopic appearance, that set them apart from all the other tumors of the glioma series [27]. These tumors were so named, as they were thought to arise from a hypothetical, central nervous system (CNS) precursor cell, the ‘medulloblast’, with the capacity to differentiate along both glial and neuronal lines as against the spongioblast and apolar neuroblast [28-29].

2.2. Epidemiology

Medulloblastoma is the most common malignant brain tumor of childhood, accounting for 20 % of all pediatric brain tumors and 40 % of childhood posterior fossa tumors [2, 30]. The overall incidence of medulloblastoma is approximately 1.5 per million population in the USA per year and the incidence worldwide seems to approximate that in the United States [31]. Approximately 70 % of medulloblastoma cases occur in childhood (3-15 yr of age), with 10-15 % cases in infants (< 3 yr of age). Although medulloblastoma is known to
occur in adults, they account for <1% of all adult CNS tumors. There is a bi-modal
distribution in the age of incidence, with peaks at 3-4 years and 8-9 years of age.
Medulloblastomas have been shown to be predominant in males than in females, with a
gender ratio of about 1.5-2:1. Males > 3 yr of age have been reported to have the worst prognosis [6, 30, 32].

2.3. Classification of medulloblastomas

Although the new term ‘medulloblastoma’ provided uniformity of classification to pediatric posterior fossa tumors, it was controversial as the existence of the medulloblast had never been proven [28]. This led Rorke to include medulloblastomas in a group of histologically similar CNS tumors, called primitive neuroectodermal tumors (PNETs) and then subdivide them on the basis of location, and other histological or clinical features, such as evidence for cellular differentiation [33]. Gene-array data by Pomeroy et al, however, confirmed that medulloblastomas are molecularly distinct from other brain tumors including PNETs, atypical teratoid / rhabdoid tumors (AT/RTs) and malignant gliomas [34]. World Health Organization (WHO) classifies medulloblastoma as a grade IV embryonal tumor owing to its aggressive behavior and further recognizes five distinct histological variants: classic, desmoplastic / nodular (DN), medulloblastoma with extensive nodularity (MBEN), large cell and anaplastic medulloblastoma [4].

Classic medulloblastoma is by far the most common and is characterized by sheets of small uniform cells with a high nuclear-to-cytoplasmic ratio. The desmoplastic / nodular medulloblastomas in contrast combines nodules of differentiated neurocytic cells with a low growth fraction separated by reticulin-rich desmoplastic inter-nodular zones of moderately pleomorphic cells with a high growth fraction. MBEN are closely related to nodular / desmoplastic medulloblastomas, and contain particularly large nodules and
advanced neuronal differentiation. This variant presents most often in infants and has been associated with favorable prognosis. The large-cell medulloblastoma contains groups of cells with large pleomorphic nuclei, a prominent nucleoli and abundant cytoplasm, high mitotic and apoptotic rate. The anaplastic medulloblastoma is marked by nuclear pleomorphism, nuclear moulding, cell-cell wrapping, and high mitotic and apoptotic activity. Both large cell and anaplastic histology in medulloblastomas has been associated with poor prognosis. Because large-cell and anaplastic medulloblastomas share morpho-phenotypes and an aggressive biological behavior, they have been typically grouped as large-cell / anaplastic (LC/A) tumors in studies of medulloblastoma [8, 29].

2.4. Risk stratification and treatment

Risk stratification for the selection of treatment for medulloblastoma places patients into either average risk or high risk categories based on 3 clinical criteria (i) age at diagnosis, (ii) extent of resection (iii) Chang metastasis staging (Table 2.1). According to this classification, patients older than 3 years of age with non-metastatic disease and totally or near totally resected tumors (<1.5 cm² of postoperative residual tumor) are considered ‘average risk’ while all others are regarded as ‘high risk’ [5].

<table>
<thead>
<tr>
<th>Clinical Criteria</th>
<th>Average Risk</th>
<th>High Risk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at diagnosis</td>
<td>&gt;3 yr</td>
<td>&lt;3 yr</td>
</tr>
<tr>
<td>Post-operative residual Disease</td>
<td>&lt;1.5 cm²</td>
<td>≥1.5 cm²</td>
</tr>
<tr>
<td>Metastasis at diagnosis</td>
<td>Absent</td>
<td>Present</td>
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Table 2.1: Post-operative risk stratification of medulloblastoma patients.
Significant progress has been made over the past 80 years towards the treatment of what was once considered a fatal disease. Earlier, with surgery as the only treatment, the survival of medulloblastoma patients was dismal. This was primarily due to the propensity of medulloblastomas to metastasize and disseminate into the craniospinal axis with approximately 30% of children demonstrating cerebrospinal fluid (CSF) metastasis at diagnosis [8]. The first breakthrough in medulloblastoma treatment came with the introduction of craniospinal irradiation (CSI) in the 1950s [35]. This treatment strategy was proposed as a result of metastasis in the brain and spinal cord found on postmortem examination. By treating the whole craniospinal axis to a radiation dose of about 35 Gray (Gy), delivered in fractions of 1.6 Gy per day, and 50 Gy to the posterior fossa, almost two thirds of patients went on to become at least 3-year survivors [28]. Average risk medulloblastoma patients are currently treated with a low-dose craniospinal radiation (24 Gy) in combination with chemotherapy, following surgery [7, 36]. In contrast to other brain tumors in which the treatment is primarily surgery, medulloblastoma is sensitive to not only radiation but also chemotherapy. Current treatment therefore includes surgical resection followed by craniospinal radiation and chemotherapy [37].

Children < 3 yr age are considered a separate group, unfortunately also with a poor prognosis due to a likely combination of more aggressive disease and lower tolerance to treatment compared to older children. The devastating effects on the developing brain caused by the radiotherapy in young children have been well documented. As a result, surgery followed by chemotherapy alone is the most widely accepted treatment, with the focus to defer or prevent radiotherapy altogether [37-38]. Recently it was shown that young children (<5 yr at diagnosis) with desmoplastic / MBEN histology have a favorable outcome with 8 yr event free survival (EFS) rates of 55 % as compared to 27 % and 14 % in children with classic and large cell / anaplastic histology respectively [10].
2.5. **Challenges in medulloblastoma treatment**

Although the multi-modal treatment has improved the 5-yr survival rate, around one-third of the patients with medulloblastoma remain incurable. For those that survive, current treatments have significant morbidity. Surgery carries a high risk of the development of post-operative cerebellar mutism [39]. Radiation therapy leads to neurocognitive impairment, endocrine dysfunction, psychiatric and developmental deficits and in some cases secondary malignancies [9, 40]. Chemotherapy at the current doses used to treat medulloblastoma patients most often results in hearing loss, infertility and neuropathies [6]. These treatment sequelae become especially pronounced the younger a patient is at the time of treatment. The current parameters for risk-stratification are inadequate for accurately classifying patients to average risk and high risk, thus failing to treat high risk patients with a more aggressive therapy whilst over-treating the average risk patients causing unnecessary treatment sequelae [5]. Considerable efforts have been focused towards identification of molecular markers which could help in better risk-stratification and treatment, that would ultimately lead to improved patient outcome with reduced long-term sequelae.

2.6. **Molecular genetics of medulloblastoma**

Genetic alterations in common oncogenes / tumor suppressor genes, liked TP53 mutation, EGFR mutation / amplification, p16\(^{INK4A}\) deletion are relatively rare in medulloblastoma [11]. The most common genetic alteration reported in medulloblastoma is the isochromosome 17q (i17q), a rearrangement that is brought about by simultaneous loss of chromosome 17p and gain of 17q. i17q has been identified in around 40-50 % of tumors and has been associated with an unfavorable prognosis [8, 41].
Medulloblastoma was thought to be caused by deregulated nervous system development, due to its prevalence in children, in the region of the brain that develops post-natally and the presence of both neuronal and glial differentiation markers, suggesting neural stem cells as the cells of origin [42]. Molecular analysis of two familial cancer syndromes, Gorlin and Turcot syndromes provided valuable insights into the molecular pathogenesis of medulloblastoma. Germline mutations in \textit{PTCH1 (PATCHED1)} gene that encodes a protein which is a membrane-bound receptor in the Sonic Hedgehog (SHH) pathway, were identified in patients with Gorlin syndrome, wherein the affected individuals developed basal cell carcinoma and had an increased incidence of medulloblastoma [13]. In Turcot syndrome, affected individuals were found to possess germline mutations in Adenomatous Polyposis Coli (\textit{APC}), a tumor suppressor gene that negatively regulates β-catenin, which is the key effector of the WNT signaling pathway. These patients had a predisposition to develop colorectal cancers and brain tumors, including medulloblastoma [12]. Through these analyses the SHH and WNT signaling pathways that are critical for normal cerebellar development, were implicated in the development of medulloblastoma.

2.6.1. Sonic-Hedgehog signaling pathway

The Hedgehog signaling pathway plays an important role in embryonic development with involvement in stem-cell maintenance, tissue polarity, cell differentiation and proliferation. Originally defined in 1980 through genetic analysis in \textit{Drosophila melanogaster}, the hedgehog gene (\textit{Hh}) was named after the short and “spiked” phenotype of the cuticle of the \textit{Hh} mutant \textit{Drosophila} larvae [43]. Subsequently, three mammalian homologues of the Hh gene were identified: \textit{Desert Hedgehog (DHH)}, \textit{Indian Hedgehog (IHH)}, and \textit{Sonic Hedgehog (SHH)}, with SHH being the most broadly expressed mammalian Hh signaling
molecule [44]. The SHH pathway in addition to its many important roles, controls the normal development of the external granular layer (EGL) of developing murine cerebellum. During normal SHH pathway signaling, SHH, produced by Purkinje cells, binds to its receptor Patched 1 (PTCH1), a 12-pass transmembrane protein and thereby relieves the inhibition of the 7-pass transmembrane effector protein Smoothened (SMO) resulting in the downstream activation of the GLI family of transcription factors. These transcription factors activate target genes like *Cyclin D1 (CCND1), N-Myc (MYCN)* that induce the proliferation of cerebellar granule neural precursor (CGNPs) cells. In the absence of the ligand SHH, PTCH1 inhibits the activity of SMO and Suppressor of Fused (SUFU) inactivates the GLI transcription factors to prevent the SHH target genes from being transcribed (Figure 2.1) [45-46]. Mutations in the SHH pathway genes (*PTCH, SUFU, SMO*) have been identified in ~25% of sporadic medulloblastomas [14].

![Figure 2.1: The Sonic-Hedgehog signaling pathway [47].](image)

The mechanism by which the SHH pathway can drive tumorigenesis has been elucidated in several mouse models of medulloblastoma till date. One of the most widely
studied models of medulloblastoma is the *Ptc* mutant mouse. Mice heterozygous for the *Ptc* mutation (*Ptc*+/−) developed medulloblastomas at an incidence of 10-15 % and expressed high levels of *GLI1*, consistent with the activation of SHH pathway [48]. Another mouse model used, *Smo*, the activator component of the SHH receptor complex wherein, mice homozygous for activating mutation in *Smo* were shown to develop medulloblastomas at an incidence of 94 % by around 2-4 months of age [49]. These models have been instrumental in the discovery of several drugs, that have entered clinical trials, which inhibit proteins activated by the SHH pathway. However, efforts have been undermined largely because of the rapid emergence of resistance mutations [50].

### 2.6.2. WNT signaling pathway

The WNT signaling pathway has been implicated to play a role in a wide array of vital biological processes ranging from embryogenesis to stem cell pluripotency and cell fate decisions during development to cell behavior and in several diseases, especially cancer. Back in 1982, Roel Nusse and Harold Vamus identified a new proto-oncogene named *Int1* (integration 1), upon infecting mice with mouse mammary tumor virus (MMTV) in order to identify genes that could cause breast cancer. *Int1* was found to be conserved across species from humans to *Drosophila*. Subsequently, in 1987 it was found that the mammalian *Int1* homologue in *Drosophila* was actually *Wingless* (*Wg*), a segment polarity gene involved in embryonic development in *Drosophila*. Therefore, a new hybrid term ‘*Wnt*’ (for Wingless-related integration site) was coined to denote genes belonging to the *Int1/Wingless* family, with *Int1*, now called *Wnt1* [51]. Wnt ligands, a family of secreted cysteine-rich glycosylated proteins, signal by two pathways: canonical (Wnt-1, Wnt-3a and Wnt-8) and non-canonical (Wnt-4, Wnt-5a and Wnt-1) [52].
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The key component of the canonical WNT signaling pathway is β-catenin. In the absence of the Wnt ligands, cytoplasmic β-catenin is recruited into the destruction complex where it is N-terminal phosphorylated by Casein kinase-1 (CK-1) and Glycogen synthase kinase – 3 beta (GSK-3β). Upon phosphorylation, β-catenin is recognized by E3 ubiquitin ligase beta-transducin repeat containing protein (β-TrCP) which targets it for proteasomal degradation, ensuring that cytoplasmic levels of β-catenin remain low. The T-cell factor / lymphoid enhancer factors (TCF/LEFs) form a complex with Groucho and histone acetylases to repress WNT target genes. Activation of the canonical WNT signaling pathway is initiated by binding of the ligand Wnt to a receptor complex composed of a seven-pass transmembrane receptor Frizzled (FZD) and its co-receptor low density lipoprotein receptor-related protein 5 (LRP5) or LRP6 in the plasma membrane. This interaction can be inhibited by Secreted frizzled-related proteins (SFRPs), Dickkopf (DKK) family proteins and WNT-inhibitory factor 1 (WIF1) that act as negative regulators of this pathway. Next, Dishevelled (DSH) is recruited to the plasma membrane where it interacts with Frizzled to mediate the translocation of AXIN to the membrane and destabilization of the multiprotein destruction complex, APC/AXIN/GSK-3β. This inactivation enables the stabilization and further nuclear translocation of β-catenin to the nucleus where it forms a complex with TCF/LEF transcription factors. Binding of β-catenin to TCF/LEF alleviates the repressive activity of Groucho, activating WNT target genes such as MYC, CCND1, etc. (Figure 2.2) [53-54]. Mutations in the WNT pathway genes [CTNNB1 (β-catenin gene), APC, and AXIN] have been identified in approximately 10 % of sporadic medulloblastomas [14]. β-catenin nucleo-positivity has been reported to be a predictor of favorable outcome in medulloblastomas with mutations in CTNNB1 being present exclusively in these nucleo-positive tumors [56].
2.7. Expression profiling of medulloblastomas

High-throughput, integrative studies using gene expression profiling, array comparative genomic hybridization (aCGH) and single nucleotide polymorphism (SNP) have proved useful in identifying molecular subgroups of medulloblastomas. Thompson et al. performed expression profiling of 46 medulloblastomas using Affymetrix gene chip HG-U133A that contains 18,400 transcripts and variants [57]. This study identified five distinct subgroups of medulloblastomas enriched for specific genetic alterations. Although the study clearly identified tumors with WNT and SHH pathway activation profiles, these tumors did not segregate into distinct subgroups. The tumors with WNT and SHH activation profiles however, were strongly associated with CTNNB1 mutation, monosomy 6 and PTCH1, SUFU mutations respectively using immunohistochemistry (IHC), fluorescence in situ hybridization (FISH), and mutational screening data.
The first study classifying medulloblastoma into distinct molecular subgroups based on their gene expression profiles came from the study by Kool et al. on 62 medulloblastomas using a more updated Affymetrix chip (HG-U133 plus2.0 array) with twice as many probe sets than the one used by Thompson [58]. Five molecular subgroups (A-E) were identified wherein WNT (A) and SHH (B) pathway activated tumors formed two distinct subgroups. The three remaining subgroups (C/D/E) were more related to each other and characterized by elevated expression of neuronal differentiation genes, glutamate and gamma-aminobutyric acid receptors (C/D) and photoreceptor genes (D/E). Both WNT and SHH subgroup clusters were supported by close to 100% bootstrap support while the remaining clusters seemed more closely related and hence with a weaker support. Importantly, Kool et al. also re-analyzed the data by Thompson et al. and identified four subgroups of medulloblastomas viz., A (WNT), B (SHH), C and DE. This discrepancy was due to the inclusion of mismatched probes in the analysis by Thompson et al. that must have contributed to the false high intensity hybridization signals while Kool et al. used only one probe set having the highest signal intensity for the analysis. The study by Kool et al. also for the first time showed a strong association between patient age, gender, histology and molecular subgroups. Subgroups C/D/E were shown to be more associated with an increased incidence of metastatic disease.

In 2010, Northcott and colleagues reported a study on 103 samples integrating the gene expression data with DNA copy number alterations using high density SNP arrays, confirming results from previous studies [16]. This study was the first comprehensive study which correlated the molecular subgrouping with survival outcome in addition to other parameters like demographics, clinical presentation, gene expression profiles and genetic abnormalities. Unsupervised hierarchical clustering identified the same molecular subgroups as described by Kool et al. except that the two related subgroups C and D, seen
as two distinct subgroups in the study of Kool et al., were now seen as one subgroup, called Group D.

Cho and colleagues, in 2011, performed the largest gene expression and SNP array analysis on 194 medulloblastomas, and identified six subgroups (c1 to c6) [59]. WNT (c6) and SHH (c3) subgroups revealed characteristics similar to that reported by other groups. Importantly, their analysis further subdivided Northcott Group C (c1 and c5) and Group D (c2 and c4) indicating ‘subgroups within subgroup’. Notably, they showed that subgroup c1 characterized by high levels of MYC expression was associated with MYC amplifications as well as increased expression of photoreceptor associated transcripts and GABRA5, had poorer survival as compared to subgroup c5. Their study thus emphasized the importance of treatment stratification based on molecular subgroups with intensive treatment restricted to the c1 component of Group C.

Concurrent with the study by Northcott et al. in 2010, our group also identified 4 molecular subgroups (A-D) of medulloblastoma by performing gene expression profiling of 19 medulloblastomas using Affymetrix Gene 1.0 ST array. These four molecular subgroups closely matched those reported in Northcott et al. study. Strikingly, the WNT tumors appeared to be more common in our cohort as compared to that reported by other groups [15].

2.8. Current consensus in medulloblastoma subgrouping

Variations in the number and nature of the molecular subgroups reported by different groups resulted in a meeting of all investigators with the consensus agreement to view medulloblastoma as four core molecular subgroups: WNT, SHH, Group 3 (Group C) and Group 4 (Group D) [17]. The consensus meeting led to a meta-analysis of molecular and clinical data from 550 medulloblastomas from seven independent studies, including those
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described above [14]. The evidence from this analysis consistently pointed towards the understanding that medulloblastoma is a heterogeneous disease comprised of four core molecular subgroups with distinct histological features, molecular profiles, and clinical outcome, which have been detailed below [17, 60].

2.8.1. WNT subgroup

WNT subgroup tumors are characterized by activated WNT signaling pathway, mostly caused by mutations in CTNNB1. They have an excellent outcome with survival rates of > 90%. Genomically they are very stable, except that almost all cases have lost an entire copy of chromosome 6. WNT subgroups primarily comprise of older children and adults cases and have an equal male: female ratio. Nearly all WNT tumors are of classic histology.

2.8.2. SHH subgroup

SHH medulloblastomas are characterized by mutations in PTCH1, SMO, or SUFU, and/or amplifications of GLI1 or GLI2, which all lead to constitutive activation of the SHH signaling pathway. Genomically they are characterized by frequent loss of chromosome arm 9q, which harbors the PTCH1 gene. A subset of SHH tumors show MYCN amplification, which in this subgroup is associated with a poor outcome. Histologically these tumors can be classic, desmoplastic or LC/A however, desmoplastic histology is associated only with SHH tumors. SHH subgroup comprises of all age groups with a predominance of infants and adult. The gender ratio is equivalent to the WNT subgroup with almost equal distribution of male and female patients. Although there exist strong differences in prognosis depending on the histology, as described earlier, these patients in general have an intermediate outcome.
2.8.3. Group 3 and Group 4

Group 3 and Group 4 tumors are less well characterized, with no association with any signaling pathway. However, most of these tumors have the chromosome 17 aberrations that are frequently found in medulloblastomas. While Group 3 tumors have been shown to express retinal differentiation genes, Group 4 tumors express genes involved in neuronal differentiation. MYC overexpression and amplifications are predominantly seen in Group 3 tumors while Group 4 tumors have relatively low expression of both MYC and MYCN oncogene. Small subsets of Group 4 tumors show MYCN amplifications too. Both Group 3 and 4 were shown to have overexpression and also amplification of the oncogenes OTX2 and FOXG1B [16]. Group 3 comprises of primarily infants and children while Group 4 comprises of all age group patients with predominance of older children. Metastasis and LC/A histology, both markers of poor prognosis, are most common in Group 3 cases. Group 3 cases have been associated with the worst outcome as compared to all other subgroups, while Group 4 tumors have more of an intermediate outcome similar to the SHH subgroup. As compared to both the WNT and SHH subgroup, there is a gender imbalance seen in Group 3 and 4, with predominance of male patients. This probably explains the known poor prognosis of males with medulloblastomas as compared to females. Figure 2.3 summarizes the distinguishing characteristics of the four molecular subgroups of medulloblastoma.

2.9. Cell of origin in medulloblastoma

Originally hypothesized to arise from undifferentiated embryonal precursor cells in the ependymal lining of the fourth ventricle of the cerebellum [27], refinement in the understanding of medulloblastoma has led to the proposal that the different subgroups of medulloblastomas arise from different progenitor cells [29]. There has been intense
Figure 2.3: The four molecular subgroups of medulloblastoma. The figure shows the distinct demographics, clinical features, genetics and gene-expression signature characteristic of each subgroup and the reported studies on medulloblastoma molecular subgrouping [17].

research focused towards understanding the cell of origin in medulloblastoma.

Medulloblastomas showing constitutive activation of the SHH pathway have been shown to originate from granule cerebellar progenitors (GCPs) in the EGL in murine models [29, 61]. In their quest to identify the cell of origin of the WNT subgroup tumors, Gilbertson and colleagues targeted activating mutations of CTNNB1 to specific locations. While mutations in the cerebellum had little impact on the granule progenitor cell population, mutations in the dorsal brainstem caused abnormal accumulation of cells. Moreover, 15 % of mice in which Tp53 was concurrently deleted, developed medulloblastoma that recapitulated the anatomy and gene expression profiles of the human WNT subgroup medulloblastoma. The study suggested that WNT-subgroup
medulloblastomas arise outside the cerebellum from a distinct germinal zone of the hindbrain in the lower rhombic lip and embryonic dorsal brainstem that develops into structures within the brainstem [62-63]. The cellular origins for the two other subgroups are still unclear. A recent mouse model generated by transducing Trp53-null cerebellar progenitor cells with Myc closely mimicked the molecular features of the human MYC-subgroup medulloblastomas (Group 3) which were significantly different from the mouse models of the SHH and WNT subgroups [64]. In another mouse model of MYC-driven medulloblastomas it was suggested that cerebellar stem cells can give rise to MYC-driven medulloblastomas [65]. Further studies on mouse models based on newly identified genetic alterations could pave way for the development of new targets for therapy [18, 66-67].

2.10. Assays for molecular subgrouping of medulloblastomas

Since the establishment of molecular subgroups of medulloblastoma, efforts have been underway to develop assays for molecular classification that are essentially rapid, robust, and more importantly, applicable on formalin-fixed, paraffin-embedded tissues (FFPE), so that it can be useful in routine clinical practice.

Northcott et al. proposed four antibodies, DKK1, SFRP1, NPR3 and KCNA1 that could specifically identify the WNT, SHH, Group 3 and Group 4 subgroups, respectively [16]. The study demonstrated that 98 % of samples stained positive for one antibody, suggesting a high specificity. Survival analyses in a separate tissue microarray cohort classified by these antibodies confirmed that Group 3 tumors had the worst prognosis, regardless of M-stage. Ellison et al. also described an immunohistochemistry (IHC) based assay using the markers, GAB1, CTNNB1, filamin A, and YAP1, for identification of WNT, SHH, and non-WNT / non-SHH subgroups. Nuclear and cytoplasmic CTNNB1 staining was exclusive to the WNT tumors [68]. These tumors were immunoreactive to
filamin A and YAP1, but not GAB1. SHH tumors displayed cytoplasmic staining of CTNNB1, and exhibited positive immunostaining for filamin A, GAB1, and YAP1. Non-WNT / non-SHH tumors showed cytoplasmic CTNNB1 staining and were immunonegative for filamin A, GAB1, and YAP1.

However, transition of these IHC-based assays to the clinics has not been a success possibly due to lot-to-lot variability of antibodies, differences in tissue fixation and embedding protocols and technical and image interpretation variability. Schwalbe et al. suggested an assay for rapid diagnosis of medulloblastoma subgroups based on multiplex RT-PCR (GeXP assay) and a 13-gene mRNA signature [69]. The assay although rapid and cost-effective than microarray methodologies, was neither directly evaluated on samples belonging to published cohorts nor tested on FFPE tissues for its clinical utility. More importantly the 13-gene signature failed to differentiate between Group 3 and Group 4 tumors, which have been confirmed in multiple studies to be genetically and clinically distinct [16, 58-59]. More recently, a novel assay for medulloblastoma subgrouping analyzing 22 subgroup-specific genes using the nanoString technology was described [70]. The nanoString nCounter System is a non-enzymatic multiplexed assay that uses sequence-specific probes to digitally measure target abundance within a given sample. Using fresh frozen tumors, the authors reported 98 % accuracy in subgroup assignment and demonstrated that the assay could reliably predict the subgroups of 88 % of recent FFPE cases too.

2.11. Beyond protein-coding genes: Non-coding RNAs - MicroRNAs

The last 10 years has seen a surge in the field of microRNA research with miRNAs emerging as novel regulators of many aspects of cellular biology and development. MicroRNAs are a class of endogenous, small, non-coding, single-stranded RNA
molecules, ~ 22 nucleotides in length. They function by regulating target gene expression through imperfect base-pairing with the 3’-UTR of target mRNAs leading to translational repression or mRNA degradation. To date, many miRNAs identified are highly conserved across species including worms, flies, plants and humans, which implies that these miRNAs direct essential processes both during development and in the adult body. MiRNAs have been shown to regulate a wide array of cell functions ranging from cell proliferation, differentiation, apoptosis, fat metabolism, neuronal development etc. [19].

The first miRNA was discovered in 1993 by the joint efforts of Ambros and Ruvkun’s laboratories [71-72]. The heterochromic gene, lin-4, was identified in Caenorhabditis elegans through a genetic screen for defects in the temporal control of post-embryonic development. Most genes identified from the mutagenesis screens were protein-coding, but lin-4 was found to encode a 22-nucleotide non-coding RNA that contained sequences partially complementary to 7 conserved sites located in the 3′-UTR of the lin-14 mRNA. This complementarity was both necessary and sufficient to inhibit the translation of the lin-14 mRNA. Almost 7 years after the finding of the first miRNA, let-7, also required during C. elegans larval development, was identified in the year 2000 [73]. The finding that let-7 was conserved across species from flies to humans [74] and targets the RAS oncogene homolog in C. elegans [75], triggered a revolution in the research of miRNAs.

Subsequently, hundreds of miRNAs and their biological functions have been identified, and thus far (June 2013: Release 20), 24,521 hairpin sequences and 30,424 mature miRNAs, including more than 2000 mature human miRNAs, have been catalogued in the miRNA database, miRBase (http://microrna.sanger.ac.uk). MiRNAs account for 1-4 % of the currently known genes in the human genome, making them one of the largest classes of gene regulators. Majority of the human miRNAs are found within introns of
either protein-coding or noncoding mRNA transcripts. The remaining miRNAs are either located far from other protein-coding genes in the genome, within the exons of non-coding RNA genes, within the 3' UTRs of protein-coding genes, or they are clustered with other miRNA encoding genes [76].

2.11.1. MicroRNA biogenesis

The miRNA biogenesis is a multistep process comprising of three main events: cropping, nuclear export and dicing (Figure 2.4). The miRNA genes are initially transcribed by RNA polymerase II in the nucleus into primary capped and polyadenylated precursors called primary miRNAs (pri-miRNAs) which contain multiple stem loop / hairpin structures and are typically > 1000 bp long. The enzyme Drosha / DGCR8 then crops the pri-miRNA at the stem and releases a hairpin-structured 60-100 nucleotides long precursor-miRNA (pre-miRNA) with a ~2-nt 3' overhang. DGCR8 is a double-stranded RNA-binding protein that recognizes the proximal ~ 10 bp of the pri-miRNA hairpin stem, positioning the catalytic sites of the RNase III enzyme Drosha. The ~2-nt 3' overhangs of the pre-miRNA are further recognized by Ran-GTP and the export receptor Exportin 5 enabling its nuclear export. Subsequently, another RNase III enzyme, Dicer processes the pre-miRNA in the cytoplasm to release a ~ 22 nucleotide miRNA: miRNA* duplex. This miRNA duplex is then loaded into an RNA induced silencing complex (RISC), which includes the Argonaute proteins, and the mature single-stranded miRNA. The mature miRNA then binds to complementary sites in the mRNA target to negatively regulate gene expression. The mechanism of inhibition depends on the degree of miRNA-mRNA complementarity (imperfect or perfect) that results in inhibition of protein synthesis or mRNA degradation [20, 77].
2.11.2. MicroRNAs in cancer

A single miRNA is believed to target on an average more than 100 mRNAs. Moreover, 60% of human protein-coding genes have been predicted to contain miRNA-binding sites within their 3’-UTRs. Given their wide impact on gene expression, it is not surprising that they play a uniquely important role in disease phenotypes such as cancer [20].

The first report indicating a role of miRNAs in human cancers was in 2002. This study found miR-15 and miR-16 to be located in chromosome 13q14, a region frequently deleted in chronic lymphocytic leukemia (CLL). Subsequently, frequent deletion or down-regulation of these miRNAs was detected in greater than 60% of CLL cases [78]. Half of
the known miRNAs were reported to be located inside or close to fragile sites and in minimal regions of loss of heterozygosity, minimal regions of amplifications, and in common breakpoint regions associated with cancer [79]. Cimmino et al. reported that miR-15 and miR-16-1 function as tumor suppressors and their expression inversely correlates with anti-apoptotic BCL2 expression [80]. Let-7, downregulated in lung cancer, has been reported to target the proto-oncogene RAS [75]. MiR-34 downregulated in gastric cancer, lung cancer, ovarian cancer, colon cancer has been reported to induce apoptosis and target BCL2, NOTCH and HMG2 [20]. In contrast, there are few miRNAs that have been identified as oncogenes. MiR-155, one of the first oncogenic miRNAs described, is overexpressed in several malignancies like breast cancer, colon cancer, lung cancer, CLL, and acute myeloid leukemia (AML). MiR-21 was the first miRNA to be coined an oncomiR due to the rather universal overexpression of this miRNA in several cancers including AML, CLL, breast cancer, glioblastoma, and medulloblastoma, with PTEN and PDCD4 identified as its targets [81]. Like miR-21, the polycistronic miR-17 / 92 cluster, also known as oncomiR-1 has also been found to play an oncogenic role in several tumor types including medulloblastoma [82-83]. The tumor-suppressors PTEN and p21 and the anti-apoptotic protein BIM have been shown as its targets [81]. There are only a handful of studies till date that have reported miRNAs deregulated in medulloblastoma and the functional roles of a few have been demonstrated in medulloblastoma biology. These studies are discussed in detail in the Discussion chapter.

Transgenic mouse models experimentally demonstrated the causative roles of many of the miRNAs deregulated in cancer. Transgenic mice overexpressing miR-155 in early B-cells have been shown to exhibit pre-leukemic expansion of the pre-B-cell population ultimately resulting in full-blown B cell tumors [84]. Studies in miR-21 knockout mice have demonstrated reduced lung tumor burden following activation of a mutant K-
Ras\(^{G12D}\) allele [85]. In a separate transgenic line, mice conditionally expressing miR-21 developed a pre-B malignant lymphoid-like phenotype, thus demonstrating its oncogenic potential [86]. Retroviral overexpression of the miR-17/92 cluster has been shown to accelerate lymphoma formation in collaboration with \(MYC\) [87]. Transgenic mice overexpressing miR-17-92 cluster in B cells have been reported to develop lymphoproliferative disease and autoimmunity [88]. Loss of miR-17-92 using knockout mice have shown reduced tumorigenicity and increased cell death and \(PTEN\) has been reported as a target of miR-19 [89-90]. These data clearly suggested that deregulation of a single miRNA can lead to malignancy.

2.12. Application of differential miRNA expression as markers for diagnosis, prognosis and therapy

MicroRNA profiling has helped uncover deregulation of miRNA expression in several cancers to date including colorectal, leukemia, lung, and breast cancer [21]. MiRNA profiling compared to mRNA expression profiling has been shown to be a more accurate method of classifying tumor subtypes. MiRNA expression profile has successfully classified poorly differentiated tumors (of non-diagnostic histology), wherein the mRNA profile was highly inaccurate [22]. MiR-200 family has been reported to positively associate with the well-differentiated breast cancer phenotype (luminal) and is underexpressed in the malignant myoepithelioma of the breast [91]. The miRNA expression profile has been shown to be associated with tumor development, progression and response to therapy [22]. In various cancers, miR-21 has been reported as a predictor of poor outcome [92-93]. High miR-21 expression levels have been shown to be predictive of response to gemcitabine in pancreatic cancer patients [94]. In lung cancer, a large miRNA expression analyses reported that the levels of both miR-155 and let-7a-2 were
associated with poor survival [95]. Upregulation of miR-214 has been shown to cause increased resistance to cisplatin in ovarian cancer [96]. Liver cancer patients with low miR-26 expression have been reported to have a shorter overall survival but have been shown to respond better to interferon-α treatment than patients with high expression of miR-26 [97]. These reports and many more suggest the possible use of miRNAs as diagnostic, prognostic and predictive biomarkers. Recently, it has been reported that alterations in chromatin-modifying genes seems to be a more consistent phenomena that occurs across all subgroups of medulloblastoma, suggesting a prominent epigenetic deregulation (DNA methylation, histone modifications, miRNA expression) in medulloblastoma [18]. Epigenetic therapy is being looked upon as a promising therapeutic approach in medulloblastoma [98]. MiRNA-based anti-cancer therapies using miRNA mimics or antagonists are being exploited. Few miRNAs like miR-34 and let-7, which target a broad spectrum of solid tumors, have already entered pre-clinical development [99-100]. The ability of the miRNAs to concurrently target multiple-protein coding genes and function as master regulators of the genome with an apparent lack of adverse effect to the normal tissue have made miRNAs a promising new class of therapeutics.