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Medulloblastoma biology in the post-genomic era

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Abstract

Medulloblastomas, the most common malignant pediatric brain tumors, are comprised of four molecularly distinct subtypes. However, treatment has yet to exploit these molecular vulnerabilities. Three recent studies sequenced a total of 310 primary tumors and identified that two of the four medulloblastoma subtypes are concomitantly associated with subtype-specific mutations as previously characterized. In contrast, the overwhelming majority of mutations occurred only once in the entire cohort and just 12 genes were recurrently mutated with statistical significance. Perturbations in epigenetic regulation are emerging as a unifying theme in cancer and similarly recurring mutations in epigenetic mechanisms were distributed across all subtypes in medulloblastoma. Designing targeted therapies to such a molecularly diverse disease in the post-genomic era presents new challenges. This will require novel methods to link these nonrecurrent mutations into pathways, and preclinical models that faithfully recapitulate patient driver events. Presently, medulloblastoma reinforces epigenetic mechanisms as a tantalizing therapeutic target in cancers.

Keywords

cerebellum; chromatin; epigenetics; epigenomics; medulloblastoma; Swi/Snf; systems biology

Medulloblastomas are the most common malignant pediatric brain tumors. Treatment is currently limited to surgical resection, radiation and cytotoxic chemotherapy, which frequently devastates cognition in survivors. Recent analysis of transcription profiles has shown that medulloblastomas are comprised of four molecularly distinct subtypes that each have distinct clinical outcomes, histology, age groups and gender biases [1–5]. A consensus has been reached to label these subtypes as Wnt and SHH for the subtypes with a defined mechanism of tumorigenesis, and Group 3 and Group 4 for those without [6,7]. Each subtype is so distinct it has been argued that they can be considered different diseases requiring independent treatment protocols. Designing targeted therapies predicates understanding the oncogenic events behind each subtype.

The four distinct subtypes of medulloblastoma are concomitantly associated both with subtype-specific mutations and copy number events. In three recently published papers,

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researchers from St. Jude Children's Research Hospital (TN, USA), the German Cancer Research Center (Heidelberg, Germany) and our group sequenced a total of 310 primary tumors from patients and compared them with their matched normal blood. This included 76 whole genomes, 113 whole exomes and 121 partial exomes based on candidate genes from discovery cohorts [8–10]. Altogether, 2102 mutated genes were identified. Medulloblastomas were found to have a low overall mutation rate of 0.35 nonsilent mutations per megabase, consistent with other pediatric cancers [8,9,11].

These data uncover four major findings: the overwhelming majority of mutations occurred only once in the entire cohort; just 12 genes were recurrently mutated with statistical significance out of the 2102 genes mutated at least once in the cohort; mutations or chromosomal copy number changes predicted to activate a specific signaling mechanism were confined to a specific subtype; and recurring mutations predicted to modulate epigenetic mechanisms were distributed across multiple subtypes. Since most mutations occur only once, it is difficult to resolve which mutations drive oncogenesis.

Subtype-specific mutations that dysregulate molecular signaling pathways are most clearly linked to the Wnt and SHH subtypes. The Wnt subtype is the best understood subtype and is nearly universally driven (over 90%) by a stabilizing mutation in *CTNNB1* (β-catenin) that activates Wnt target genes. Patients with this subtype have an excellent prognosis. This subtype has a relatively stable genome, with monosomy chromosome 6 as the only consistent copy number variant. Additionally, 50% of Wnt tumors also have mutations in the RNA helicase *DDX3X*. Previously linked to other cancers and viral infections, DDX3X has roles in cell migration, cell cycle progression and chromosome separation [12–14]. In both *in vivo* and *in vitro* functional assays, *DDX3X* mutations contribute to oncogenesis by increasing cell proliferation, and potentiate Wnt signaling when they occur in combination with *CTNNB1* mutations [9,10]. SHH-subtype patients have an intermediate prognosis. These tumors have mutations in *PTCH1*, *SMO, Gli2* or *SUFU* that deregulate and promote constitutive activation of the SHH pathway. The most frequent copy number alteration in the SHH subtype is loss of chromosome 9q at the site of the *PTCH1* locus. This subtype has greater chromosomal instability than the Wnt subtype, but it does not exhibit a high degree of copy number variation overall.

By contrast, Group 3 has a high rate of copy number alterations, paucity of significant mutations and the worst patient outcome. Group 3 is classified by expression of *MYC*, photoreceptor and *GABA* signatures. Sequencing and single nucleotide polymorphism profiling verified that a subset of Group 3 has increased or high copy number amplification of *MYC*, which would account for high levels of *MYC* expression. The photoreceptor signature may be caused by mutations of *OTX2* in some patients. OTX2 is involved in optic placode specification and hindbrain patterning during cerebellar development [15,16]. BMP signaling is similarly instructive in the cerebellar primordium and indeed an inhibitor to BMP signaling, *CTDNEP1*, is mutated in Group 3 tumors [17]. This subtype has frequent gains of chromosome 17q with loss of 17p resulting in isochromosome 17q (i17q). Since *CTDNEP1* is at 17p13.1, it is nominated as a probable tumor suppressor for certain subtypes of medulloblastoma.

Group 4 also has a high degree of chromosomal copy number variation with nearly universal occurrence of i17q, and with frequent gains in chromosome 7 and 4, and loss of 8, 10 and 11. Group 4 is characterized by a neuronal and glutamatergic molecular signature and amplification of *MYCN*. This subtype has a striking 3:1 male:female predominance. Epigenetic regulators of histones are the only recurrent event in this subtype with mutations to histone demethylase *KDM6A*, and histone methyltransferases *MLL2* and *MLL3*.

Epigenetic regulators as a general class are the shared peak in the genomic landscape of medulloblastoma, as they are mutated in every subtype.

Epigenetic mechanisms are universally altered

'Epigenetic', which comes from Greek roots to mean 'above the genes,' describes modifications to how DNA is packaged into chromatin and ultimately transcribed. The major epigenetic regulators are DNA methylation, histone modifications, Swi/Snf and Polycomb chromatin remodelers, and noncoding RNAs [18]. Epigenetic modifications are inheritable, but not irreversible, and are therefore ideal master regulators for maintaining or restricting cell fate potential during normal development [19]. As passengers during mitosis, epigenetic marks accumulate during cell fate commitment and ultimately differentiation.

DNA packaging further regulates transcription through interplay of numerous different types of histone modifications. DNA is packaged into chromatin by wrapping around histones, which have four subunits. The hallmarks of active euchromatin are acetylation or phosphorylation of histones while silenced heterochromatin has methylation and sumoylation of histones. Both active and inactive states share methylation and ubiquitination histone modifications, but also residue location that confers the accessibility to transcription. While previously thought to be a simple biphasic active versus silent state, we now know there to be a poised state that has both activating and silencing histone marks present at the same time and place [20]. Ultimately it is the relative level of each that confers transcriptional regulation. Principally, it is the combination of modifications to H3K27, H3K4, H3K9 and H3K20 that classify promoters as active, repressed or poised [21,22]. In embryonic stem cells for example, pluripotency is maintained by activating acetylation marks to H3K9 near propotency genes while lineage-specific genes are deactivated and marked with H3K27me3 by Polycomb group proteins [23]. During differentiation, these deactivating H3K27me3 marks are erased, for example, by the demethylase KDM6A [24,25]. An interplay between histone methyltransferases, histone deacetylases (HDACs) and histone acetyltransferases (HATs) regulate these modifications on the over 100 modifiable residues. Altogether, transcription regulation emerges as a context-dependent system that exemplifies how combinatorial control drives specialization of the multitudes of cell types in the body. Global regulators of cell fate such as these are prime candidates to be exploited during cancer development.

Mutations in epigenetic mechanisms emerge from these large cohort sequencing initiatives as a unifying theme across all subtypes and are prime candidates to be driver events for oncogenesis in embryonal CNS cancers such as medulloblastoma that retain characteristics of embryonic progenitors. Medulloblastoma maintains many undifferentiated progenitor-like features, such as a large nucleus to cytoplasm ratio, which gives its characteristic 'small blue cell' histology, and expression of early neural markers such as *Sox4*, *Sox11* and *CIC* [26]. Mutations in at least one epigenetic mechanism are found in every subtype. Perturbed epigenetic mechanisms in medulloblastoma include histone modifiers, chromatin remodeling complexes and RNA helicases. Histone modifiers encompass enzymes that add (*MLL2/3*; SHH, Groups 3 and 4) or remove methyl (*KDM6A*; Groups 3 and 4) or acetyl groups (*CREBBP*, *HDAC2;* Wnt and Group 4) to specific residues, and their associated corepressor complexes (*BCOR, NCOR2, GPS2, LDB1*; SHH, Groups 3 and 4) that regulate them.

The fact that these regulators are so frequently mutated in medulloblastoma across subtypes should not be a surprise as deregulation of DNA methylation and histone modifiers has been well documented in medulloblastoma and other cancers [27,28]. It is likely that these mutations can substitute for each other to maintain cell fate potency of embryonal tumors.

For example, H3K27 is trimethylated by Polycomb group proteins, such as the subunit EZH2, to inactivate lineage-specific genes, while KDM6A preferentially demethylates H3K27me3 during differentiation [24,25]. Group 3 and 4 medulloblastoma have perturbed this interplay by either overexpressing *EZH2* or deactivating *KDM6A* [10]. The outcome of either overexpression of *EZH2* by gains of chromosome 7q, or loss-of-function *KDM6A* mutations is the same – to maintain repressive H3K27me3 [10]. This limits differentiation and maintains a stem-cell-like progenitor status. As these events are mutually exclusive in patients, this suggests that the outcome of the methylation marks is a shared mechanism of action between either gains in *EZH2* or mutations of *KDM6A* and that perhaps other genomic events could also phenocopy them. Taken together, this suggests a probable link between a gain or loss of mechanisms to restrict fate choices, by either copy number or mutation events.

Mutations in epigenetic regulators are emerging as the next major chapter in the biography of cancer, not just medulloblastoma [29] . Many large-cohort whole-exome sequencing projects in cancers from all germ layers are similarly identifying perturbations in epigenetic mechanisms, as reviewed in [30]. Novel mechanisms have been identified; for example, in addition to the occurrence of *DDX3X* mutations, mutations in other RNA helicases are found in 15% of tumors suggesting that RNA helicases more generally may have a driver role in medulloblastoma and possibly other cancers. More recently, pediatric rhabdoid tumors were found to have the lowest mutation rate of any cancer to date and with the only recurrent event to ablate function of the Swi/Snf ATPase *SMARCB1* either through mutation or loss of the gene loci [31]. Melanoma sequencing involving 121 tumors identified mutations in the Swi/Snf subunits *ARID*, *SMARCA4* and polycomb *EZH2* [32–34]. Pediatric glioblastoma sequencing identified a histone 3.3 chromatin remodeling pathway to be mutated in nearly half of all patients [35,36]. Furthermore, they demonstrated that tumors with the mutation corresponded with increased methylation at H3K36 [35]. These sequencing data nominate epigenetic mechanisms as a consistent theme in cancer.

Current preclinical limitations

With a lack of targeted therapies, treatment of medulloblastoma is still limited to surgical resection, radiation and broad-spectrum cytotoxic chemotherapy drugs such as vincristine. If the investigators behind these sequencing initiatives were hoping to identify a consistent druggable target for a significant percentage of patients, they have been sorely disappointed. The main consistency is how heterogeneous these tumors are from patient to patient, the Wnt subtype being the exception. All these data have added layers of complexity to unraveling and treating medulloblastoma.

Ploidy variations

Catastrophic genomic shattering and rearrangement, or ‘chromothripsis,’ is another challenge in medulloblastoma, both in terms of understanding its biology and for clinical drug targets. Genomic stability is widely variable among the subtypes, with Groups 3 and 4 exhibiting much higher rates of chromosome copy number variation, which correspond with fewer somatic mutations. This suggests that the copy number events themselves are the drivers for these subtypes. The sequencing paper spearheaded by the International Cancer Genome Consortium PedBrain Tumor Project further identified that tetraploidy is common in Group 3, maybe in as many as 54% of tumors, and also in Group 4 with 40% reported [8]. The low allelic fraction of mutations in the tetraploid tumors suggests these whole-genome duplication events preceded accumulation of mutations. Furthermore, tumors with *TP53* mutations in the SHH subtype were found to have increased rates of chromothripsis. Following genomic shattering, novel gene fusions can be made that combine function or regulation of multiple disparate and previously unrelated proteins [37]. A predilection for

chromosomal instability will make these tumors more challenging to treat as chemotherapy agents could potentially increase this instability. However, this does propose an opening for therapeutic intervention if mitotic checkpoints can be targeted to ameliorate the effects of chromosomal instability.

Animal models genocopy few patients

Recently, animal models have been developed for the Wnt, SHH and Myc signature subtypes, which are simultaneously major leaps forward to understanding the underlying biology of the disease and of mixed preclinical utility. Animal models are instrumental for preclinical development of targeted drug therapies. However, for the animals to accurately phenocopy how patients will respond to novel drugs, they may need to accurately represent the patient populations.

The Wnt animal models are best positioned to be an accurate representation of the Wnt patient population. As almost every patient in the Wnt subtype has a stabilizing mutation in *CTNNB1*, the targeted drug therapies designed to target this pathway are likely to be of some benefit to the vast majority of patients in this subtype. Indeed, a rodent model develops medulloblastoma in the posterior fossa via expression of stabilized *CTNNB1* from a lineage-specific promoter in radial glial cells of the hindbrain in combination with *TP53* knockout [38]. This model has been instrumental in identifying the lower rhombic lip progenitors as the cell of origin for this subtype. However, *TP53* mutations occur infrequently in the Wnt subtype, which may decrease the significance of the model for preclinical drug screening.

Two animal models for the MYC-driven subset of Group 3 have recently been reported in the same issue of *Cancer Cell*. Transplanted granule neuron precursors either knocked-out for *TP53*, or overexpressing dominant-negative *TP53*, in combination with overexpressing *MYC* form tumors in the recipient rodents that resemble patient tumors [39,40]. Furthermore, these tumors appear to be of a more primitive lineage than the cells they were derived from and behave similarly to induced pluripotent stem cells [39]. That the induced pluripotent stem cell genes synergize to be sufficient drivers of neural fate in lower vertebrates could account for the similarity in presence of a stem cell state and an embryonal CNS tumor [41]. The MYC signature is undoubtedly a driving event for poor outcome in medulloblastoma with those patients having just a 20% 5-year survival rate [3]. However, no Group 3 patients have mutations in *TP53* so it is unclear the extent to which patients would recapitulate the response to targeted drugs designed in these rodents.

SHH animal models have identified that these tumors originate from granule neural progenitors within the external granule layer. These rodents are generated by activating the SHH pathway from perturbing its receptors *PTCH1*$^{+/-}$ or *ND2-SMOA1* [42–44]. Inhibitors of the SHH receptor SMO are undergoing clinical trials for medulloblastoma and other cancers, such as basal cell carcinoma, which can similarly be caused by mutations in *PTCH1* as in the case of Gorlin's syndrome. These drugs are based on cyclopamines, such as GDC-0449, and target the SHH receptors [45]. How useful these drugs will be for the patients of the SHH subtype is debatable as only approximately 20% patients have mutations in *PTCH1* or *SMO* receptors. If SHH signaling is activated downstream of the cell surface receptors, such as via mutations in *SUFU* or *Gli2* transcription factors, the SMO inhibitors are insufficient to antagonize the signaling cascade [46].

Rapid resistance to targeted therapies

A key challenge for treating cancers with targeted therapies has been how quickly the tumors become resistant to the drug even after initial remission. The SMO inhibitor

GDC-0449 is no exception. A patient with metastatic medulloblastoma had an initial response to the inhibitor; however, remission was short-lived and the tumors returned to their original size within 3 months of initial treatment [47]. An acquired point mutation in *SMO* was found to be the mechanism for resistance in the patient [48]. This type of resistance is more striking in melanoma. Half of melanomas are driven by a stabilizing mutation in *BRAF* at V600E [32], which constitutively activates the RAS–MAPK–MEK pathway. Targeted therapies to inactivate the pathway, such as vemurafenib (also known as PLX4032) or imantinib, cause cell death in tumors expressing these mutations resulting in remission in approximately 80% of patients [49]. However, all patients ultimately relapse with survival extended by mere months [50]. The tumors with the V600E *BRAF* mutation acquire novel mutations to quickly become resistant to the inhibitor and return in a more aggressive form [51].

Drug development for epigenetic regulators

It is becoming clear that a single targeted therapy is insufficient both in a given patient and even for a subtype. Genome-wide sequencing results are giving us cause to re-evaluate the vision for developing subtype-specific therapies that target hyperactive signaling pathways causative of the subtypes. As the epigenetic machinery is so frequently perturbed across all subtypes and indeed other cancers, targeting these mechanisms may be a more cogent approach.

Dysregulation of epigenetic mechanisms offers a potential window of vulnerability for targeted therapies in cancer, and there is already a growing body of evidence for drugs that target epigenetic machinery [52]. Two epigenetic drugs have already been approved by the US FDA targeting DNA methylation and HDACs, and many more are under development [30]. HDAC inhibitor LBH589 (panobinostat) has recently been shown to have broad efficacy across a large screen on the Cancer Cell Line Encyclopedia against 479 cell lines from 39 tumor types [53]. The HDAC and DNA methylation inhibitors function by altering transcription of genes either in a generalized manner, such as for HDACs, or in a context-dependent manner, as for histone methyltransferases [54]. Given their broad range of efficacy and mechanisms, these inhibitors are attractive candidates for combinatorial drug therapy.

Systems biology approach

While these major sequencing initiatives have confirmed the penetrance of many well-documented oncogenes, such as *TP53* and *MYCN*, only a minority of patients in the cohort share one of the recurrent mutations. Clearly, recurrent mutations are only one piece of the cancer puzzle. How can a consistent picture of cancer be drawn from so much heterogeneity? It has been proposed that a clearer picture will emerge with larger data sets. While this may be informative, waiting for more data is impractical. Alternatively, these data can be analyzed using a systems biology approach to uncover connections, and data from each level of gene regulation needs to be merged.

Exome sequencing's promise to explain cancer development originated with Knudsen's multiple hit hypothesis [55]. Knudsen's model posits that cancer forms by slowly accruing mutations in proto-oncogenes and tumor suppressors, *MYCN* and *TP53,* respectively, being the classic examples. However, gain or loss of protein function can be accomplished by multiple different mechanisms, not just mutations. For example, in rhabdoid tumors *SMARCB1* is either mutated or the loci is lost in every patient [31]. An oncogeneic event can occur at any level of the entire process from copy number changes, chromatin regulation, transcription regulation and mRNA processing by miRNA or splice forms, to protein degradation [56,57]. Proper cell fate choice, proliferation, differentiation and commitment

require an unbroken sequence of events from gene regulation to expression and protein regulation, which feeds back to gene regulation. Perturbations in any one of these long sequence of events can be seen in cancer development.

Novel driver events have recently been reported in medulloblastoma by merging copy number events and mutations. To investigate the extent to which copy number events drive tumorigenesis, the Medulloblastoma Advanced Genomics International Consortium (MAGIC) studied over 1200 tumors by single nucleotide polymorphism array [58]. They identified 62 significant driver events across all samples and 110 that were subtype-specific. In the SHH subtype, for example, amplified genes included *GLI2*, *MYCl1*, *YAP1* and *MDM4*. Group 4 has mutations in *KDM6A* and MAGIC similarly identified deletions of its loci, which suggests its role as a tumor suppressor.

As most of the mutations in the medulloblastoma cohorts were singletons, or so called 'one-offs,' there is a necessity for novel methods to interweave these into networks to identify shared pathways. This has been used in glioblastoma to identify major network modules by leveraging existing protein–protein interaction databases [59]. However, these networks do not take into account other types of interactions, such as gene regulation.

Taking into account these other types of interactions is a significant undertaking as most complete data sets do not exist for a given system, and the methods to merge mRNA expression, mutations, methylation status and miRNA expression have yet to mature. There is a current trend to propose to collect data for the complete picture of a tumor or cell line. St. Jude's Pediatric Cancer Genome Project has been refocused to prioritize linking expression data with exome sequencing [60]. Once these data sets have been acquired, the next challenge for the field will be to develop methods to link the data types computationally. There have been some early success at linking gene expression profiles using gene set enrichment analysis [61], protein–protein interaction networks and signaling pathways [58]. Additionally, these types of network analysis are being used to identify common nodes shared among many tumors [62].

In summary, its predilection to afflict young children is the underpinning of medulloblastoma's complexity. Since these tumors develop in brains that are themselves still developing, the disease can take many different forms as there are many vulnerable windows of opportunity for the disease to develop. This also makes the tumors complicated to treat as the treatment stunts, often permanently, cognitive development leaving many of these children without the ability to care for themselves if they survive to adulthood. Creating targeted therapies for medulloblastoma requires identifying its weaknesses and characterizing its underlying biology. A key assumption about cancers in general is that profiling their molecular characteristics can excavate their origins. Like a ghostly imprint of their former cells, a growing tumor retains evidence of its cell of origin and its developmental milestones. Following a cancer's first conception, the tumors themselves may continue to evolve and gain more genetic alterations, which allow for resistance. Overall, medulloblastoma is a parsimonious cancer as it exhibits a low mutation rate compared with other cancers [8,9,11]. A lower mutation rate suggests that the recurrent mutations are more likely to be driver, rather than passenger, events. This makes medulloblastoma an ideal model system to study driver events. Consequently, what is learned from the oncogenic drivers of medulloblastoma can be leveraged to other cancers and all evidence nominates epigenetic mechanisms as a key pan-cancer theme.

Future perspective

Today we have arguably more data on the molecular and genomic landscape of medulloblastoma than many other cancers, but this has yet to translate to a significant leap

forward in the clinic. In this post-genomic era of medulloblastoma research, translating the mutation, copy number and molecular signature of these tumors into druggable targets will require novel methods to stitch together these diverse events into unifying mechanisms. As a field, our ability to generate large amounts of data has outstripped our ability to fully analyze it. The next decade of cancer research will be dependent on our ability to interpret and model heterogeneous driver events to identify targetable hot spots in the majority of patients. Furthermore, rapid evolution of drug resistance in the clinic is the major hurdle of targeted therapies. Moving forward, new drugs or combinations of drugs are needed to thwart resistance. Drugs to target epigenetic machinery are a logical next step not only in medulloblastoma but also across many cancers. The post-genomics era of cancer research is transitioning into an epigenomics era as more and more cancers are found to share hits in their epigenetic machinery.

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Executive summary

- Medulloblastoma sequencing has identified mutations in subtype-specific signaling pathways but otherwise few recurrent mutations.
- Epigenetic mechanisms are universally altered in each subtype and in many other cancers.
- Current preclinical limitations to targeted therapies:
  - Ploidy variations and unstable genome make a single drug for medulloblastoma unlikely.
  - Animal models genocopy few patients.
  - Many cancers demonstrate a rapid resistance and relapse to target therapies, such as SMO and BRAF.
- Drugs to epigenetic regulators may have a broad efficacy in medulloblastoma and other cancers.
- A systems biology approach is needed to link nonrecurring mutations.