Recent Advancement of the Molecular Diagnosis in Pediatric Brain Tumor

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Recent discoveries of brain tumor-related genes and fast advances in genomic testing technologies have led to the era of molecular diagnosis of brain tumor. Molecular profiling of brain tumor became the significant step in the diagnosis, the prediction of prognosis and the treatment of brain tumor. Because traditional molecular testing methods have limitations in time and cost for multiple gene tests, next-generation sequencing technologies are rapidly introduced into clinical practice. Targeted sequencing panels using these technologies have been developed for brain tumors. In this article, focused on pediatric brain tumor, key discoveries of brain tumor-related genes are reviewed and cancer panels used in the molecular profiling of brain tumor are discussed.

Key Words : Brain neoplasms · Pediatrics · Molecular diagnostics · High-throughput nucleotide sequencing · Next generation sequencing.

INTRODUCTION

Since the end of the Human Genome Project, a number of genomic studies of human disease such as The Cancer Genome Atlas (TCGA) and International Cancer Genome Consortium have been activated25). Such large scale genome studies were enabled by the development of massively parallel sequencing technology, also known as next-generation sequencing (NGS), which can rapidly generate high-throughput data with low per-base cost5,40).

In addition, targeted anti-cancer therapy has been highlighted with the discovery of cancer driver genes32,67). Such clinical needs, in turn, prompted the development of sequencing technologies and cancer genome studies. TCGA, the large scale cancer genome study consortium, started its three-year pilot project in 2006, especially about glioblastoma and now completed the characterization of 33 cancer types including 10 rare cancers71).

The acceleration of genomic studies in the field of brain tumor led to the discovery of key genes in brain tumor development, such as isocitrate dehydrogenase (IDH), H3F3A, and alpha thalassemia/mental retardation syndrome X-linked (ATRX)52,64). In addition, these genes have been found to be deeply involved in the diagnosis and prognosis of brain tumor. Brain tumor is the most common type of solid cancer in chil-
dren. Over the past decade, molecular research on brain tumors has made unprecedented progress in pediatric brain tumors. Unique genomic and epigenomic alterations are continuously discovered according to the patients’ age, tumor grade, and histologic differences of brain tumors in large-scale global collaborative studies. In addition, the therapeutic paradigm is changing as targeted therapies are developed to correct the genetic abnormalities, prompting a new sub-classification of brain tumors. In 2016, the new World Health Organization (WHO) classification of central nervous system (CNS) tumors incorporated the genetic abnormalities into the classification and diagnosis of the tumor. Therefore, some tumors must undergo molecular testing, which is essential for accurate diagnosis. Sometimes multiple tests are required for each patient, so multiplex panel tests using NGS method have begun to be used in clinical settings to meet the reasonable turnaround time and the price.

In this article, the important genetic abnormalities involved in brain tumors, especially in pediatric brain tumors, are reviewed and the existing brain tumor panels are analyzed to suggest the optimal design of the pediatric brain tumor panel.

**GENETIC ABNORMALITIES IN PEDIATRIC GLIOMAS**

Gliomas are the most common CNS tumors in children and adolescents. Children’s gliomas are mostly low-grade, classified as grade 1 or grade 2 according to the WHO classification of CNS tumors and appear to be slowly growing lesions. Low grade glioma (LGG) in children is fundamentally different from those of adult which are characterized by IDH mutation and have generally good prognosis. Gliomas are currently not fully cured, despite efforts to utilize all currently available treatment. Therefore, the purpose of the treatment of LGGs, which is pursued by neurosurgeons, pediatric oncologists and radiation therapists, is to improve the quality of life of patients and prevent long-term sequelae.

Among gliomas, the newly included tumors in the WHO classification revised in 2016 is the diffuse midline glioma, H3 K27M-mutant, a broad-spectrum central glioma within the astrocytic tumor category. RELA fusion-positive ependymoma was classified as a new subtype of supratentorial ependymoma.

**Low grade gliomas including other astrocytic tumors**

In the newly revised WHO classification in 2016, pilocytic astrocytoma (PA), pleomorphic xanthoastrocytoma (PXA), subependymal giant cell astrocytomas are belonging to the “other astrocytic tumor”. About 50% of optic pathway PA and about 4% of cerebellar PA occurs in families with mutations in the neurofibromatosis type 1 (NF1) gene and the rest occurs sporadically. In these gliomas, the most common somatic point mutation is BRAF V600E mutation causing BRAF activation, which is also observed in 33% of ganglioglioma, 70% of PXA, and approximately 15% of pediatric LGG. The 70% of PA showed one copy gain of BRAF gene, by the fusion between BRAF gene and KIAA1549 gene located on chromosome 7q34. As a result of overactivation of MEK and ERK genes in the down-stream of BRAF signaling pathway, gliomagenesis is known to occur. BRAF gene duplication is known to occur in more than 80% of PA of posterior fossa and 22% of the pilomyxoid astrocytoma. Other BRAF fusion partners (FAM131B, SRGAP3, MACFI, RNF130, CLCN6, MRKN1, and GNAI1) result in equally strong BRAF activation through the loss of the N-terminal of autoregulatory domain. However, the effect of specific BRAF abnormalities on the prognosis is unclear. In one study of 164 childhood PAs, BRAF-KIAA1549 fusion was associated with a good prognosis while other studies do not show any association with prognosis.

The mutations of other genes, including FGFR1, MYB, MYBL1, and ATRX, have been identified through whole exome sequencing (WES) of these gliomas.

**Diffuse midline glioma, H3 K27M-mutant (WHO grade IV)**

Gliomas with histone H3 K27M mutation, formerly called infiltrating brainstem or pontine glioma have been named as “diffuse midline glioma, H3 K27M-mutation” in the revised WHO classification. These tumors are classified as astrocytic and oligodendroglial tumor category and are classified as WHO grade IV glioma of the pediatric population, which occur in the midline of CNS, such as thalamus, pons and spinal cord. It has been shown that high grade gliomas in children have genetic abnormalities and gene expressions different from adults and their prognosis is different. In 2014, Histone gene mutation was found to be a driver mutation through WES. This tumor usually differentiates into astrocytes.
and is morphologically similar to WHO grade IV human astrocytoma. In addition to H3 K27M mutation, the mutation of TP53 (50%), PPM1D (15%), ACVR1 (20%), PDGFRA (10%), and SMARCA4/B (<5% of cases) can be present.\(^{44,68}\). It is known that tumors with this mutation are worse than those without the K27M-mutation among the same grade glioma in the same region. Two-year survival rate is generally less than 10% when treated with current therapies.\(^{64,76}\).

Ependymomas

It is known that the genetic aberration of ependymoma varies depending on the tumor site of the CNS and the biology of the tumor follows genetic characteristics.\(^{50,77}\). Although the morphology of the lesion may be identical, it can be divided into three groups according to the location of the tumor because the gene abnormality is different.\(^{50}\). About 70% of cases of supratentorial ependymomas are characterized by RELA-C11orf95 fusion and 30% by YAP1 gene fusion.\(^{43,77}\). It is known that the LAMA2 overexpression group show worse prognosis than NELL2 overexpression group among the cases of posterior fossa ependymomas.\(^{46}\). In the spinal cord, familial ependymomas are known to associate with NF1 gene mutation, while the other spinal cord ependymomas do not show any specific mutation but it is known that they show three types of copy number variation.\(^{50}\).

Ependymoma, RELA fusion-positive

This C11orf95-RELA fusion ependymoma is a new genetic subtype of the supratentorial ependymoma which has been newly included in the 2016 WHO classification. The C11orf95-RELA gene fusion, which is one of the genetic features of the ependymomas above the tent, is associated with the activation of NF-κB pathway.\(^{31,51}\). The partner gene can be a gene other than C11orf95. The grade of this tumor follows a pathologic grade that is evaluated according to existing morphologic features, but the prognosis is worse in the case of RELA fusion than YAP fusion.\(^{43,51}\).

NEURONAL AND MIXED NEURONAL-GLIAL TUMORS

Thirteen tumors belong to mixed neuronal-glial tumors, including dysembryoplastic neuroepithelial tumor, ganglion cell tumor, papillary glioneuronal tumor, rosette-forming glioneuronal tumor, central neurocytoma, extraventricular neurocytoma, cerebellar liponeurocytoma and paraganglioma. Diffuse leptomeningeal glioneuronal tumor was newly included in the revised 2016 WHO classification.

Gangliocytoma and ganglioglioma

It is known that approximately 20–43% of neuroepithelial neoplasms and ganglioglioma show BRAF V600E mutation and the frequency of this mutation increases with the degree of malignancy.\(^{31,51}\). BRAF V600E mutation is related to the use of a targeted therapeutic agent for this mutation, such as vemurafenib. The BRAF V600E mutation also has been reported to be associated with the worse prognosis of ganglioglioma.\(^{39,81}\).

Diffuse leptomeningeal glioneuronal tumor (International Classification of Diseases for Oncology [ICDO] code and WHO grade not yet assigned)

There are few reported cases of diffuse leptomeningeal glioneuronal tumors (DLGNT). WHO grade has not yet been established in this tumor.\(^{20}\).

A recent report showed the presence of BRAF duplication in about 44% and BRAF V600E mutation in about 11%.\(^{15}\). Other aberrations in MAPK/ERK pathways including RAF1, FGFR1, NF1, and MYB or MYBL1 were also reported.\(^{46}\). Low grade nature of DLGNT was reported, but cases having nuclear atypia, high Ki-67 index and glomerular vascular proliferation may show bad prognosis.\(^{31}\).

CNS EMBRYONAL TUMORS

The most significant change in the revised 2016 WHO classification is the CNS embryonal tumor, previously referred to as the CNS primitive neuroectodermal tumor (CNS PNET). The reason for the change in the name of the tumor is to prevent confusion with the extracranial PNETs, such as Ewing sarcoma. Among CNS embryonal tumors, medulloblastoma (MB) is classified according to the combination of tumor genetics and morphological subtype.\(^{28}\). Embryonal tumor with multilayered rosettes (ETMR; chromosome 19 microRNA cluster [C19MC] altered, and not otherwise specified [NOS]) is newly added to the 2016 WHO classification, which is characterized by gene amplification at the site of the microRNA clusters on chromo-
some 19<sup>38,69</sup>. However, if morphologically CNS PNET, described above, is not genetically clear, it is classified as "CNS embryonal tumor, NOS". ETMR can strongly express Lin28 or show the amplification of C19MC locus on 19q13.42 chromosome region or both<sup>49,50</sup>.

**Medulloblastomas**

MB is the most common embryonal tumor of the CNS and is common in children. Most occur in the cerebellum but exceptionally, WNT activated tumors can occur in the dorsal brainstem<sup>23</sup>. In accordance with the known genetic abnormalities and morphologic features, it is classified by two classification systems, i.e., genetically determined and morphologically determined classification<sup>30</sup>.

Genetic MB classification is composed of WNT-activated, Sonic Hedgehog (SHH)-activated, group 3 and group 4. CTNNBI mutation and monosomy 6 are the characteristics of WNT subtype<sup>49</sup>. The germline or somatic mutation of the TP53 gene is observed in SHH-activated MB, but not observed in the WNT-activated MB or group 3 and group 4 MBs<sup>69</sup>. The SHH MB with TP53 gene mutation is known to have a poor prognosis<sup>82</sup>.

In contrast, group 3 and group 4 MBs are immunohistochemically and molecular-genetically overlapped and they show copy number variations in variable chromosomal loci<sup>23</sup>. In many hospitals, these two groups are classified as non-WNT/non-SHH groups because group 3 and group 4 cannot be easily categorized by conventional laboratory tests. Tumors with either MYC or N-MYC gene amplification or anaplastic/large cell type have poor prognosis<sup>82</sup>. Generally, group 3 has the worst prognosis<sup>19,53</sup>. If there are cerebrospinal metastases, the prognosis is poor and the recurrence is common<sup>23,30</sup>. MB with extensive nodularity usually has a good prognosis<sup>9</sup>. If genetic testing is not possible or the results are ambiguous, it can be diagnosed as MB, NOS.

In order to genetically classify MBs, originally it is necessary to examine the mutation of several genes and chromosome copy number variation. Currently, immunohistochemistry can be used to classify MB<sup>23</sup>. However, it is not easy to interpret the immunoreactivity and match with genetic subgroup.

Recently, according to Cavalli et al.<sup>69</sup>, the genetic groups of MB can be further subdivided. That is, it is subdivided into WNT<sub>α</sub> to β, SHH<sub>α</sub> to δ, group 3α to γ, and group 4α to γ, which better reflect the prognosis and clinical characteristics<sup>9</sup>.

**Embryonal tumor with multilayered rosettes, C19MC altered, and NOS, WHO grade IV**

This tumor is one of the malignant CNS tumors. It is composed of multiple layers of rosettes and broad neuropil, and also has genetic characteristics of C19MC in chromosomal band 19q13.42 amplification or fusion with Tweety family member 1 (TTYH1) gene<sup>35,47</sup>. This can be confirmed by fluorescence in situ hybridization (FISH). These tumors are very rare and can occur in the cerebrum, brain stem, and cerebellum.

If the tumor is morphologically compatible but has not undergone genetic tests or has no abnormality of this gene by the molecular test, it is diagnosed as an "embryonal tumor with multilayered rosettes, NOS". This tumor is a very rapidly growing WHO grade IV tumor with a poor prognosis, and the average survival time with the current treatment is 12 months (reported up to 24–36 months), and the relationship between the gene alteration and the prognosis should be studied<sup>87</sup>.

**CNS embryonal tumor, NOS**

Most of the tumors diagnosed as "CNS-PNET" in the past are now diagnosed as "CNS embryonal tumor, NOS". These tumors are very rare CNS neuroepithelial tumor with poor differentiation, and the specific morphological features or classifiable genetic abnormalities of these tumors have not yet been revealed<sup>26,38</sup>. Most of these tumors express Lin28 immunohistochemically<sup>49,70</sup>. The prognosis of this tumor is very poor (WHO grade IV), and worse than that of the MB<sup>9</sup>.

**Atypical teratoid/rhabdoid tumor (AT/RT)**<sup>23</sup><br>

AT/RT are characterized by the mutation of SMARCB1 (95%) or SMARCA4 (about 2%) genes<sup>23</sup>. When the mutation of those genes is not examined or this mutation is not found in spite of compatible histology, it is diagnosed as "CNS embryonal tumor with rhabdoid features".

A rare case of a malignant neoplasm characterized by atypical morphology and rhabdoid features with acquisition of the secondary SMARCB1 mutations in PXA and GG was reported<sup>65</sup>.

**GERM CELL TUMORS**

**Germinoma**<br>

CNS Germ cell tumor (GCT) has been known to have a common chromosomal abnormality 12p redundancy, i(12p), which
was found in studies of malignant testicular tumors. The most common cytogenetic abnormality in extravaginal germinomas is the 12p overlap. However, specific genes on i(12p) associated with the development of GCT are not known.

Cytogenetic abnormalities as driver mutations in childhood GCT include the loss of 1p and 6q, the changes in sex chromosomes, and 12p abnormalities with some gains. The most common chromosomal imbalance is an increase in the X chromosome, as well as an increase in 1p, 8p, and 12q, and a loss in 13q and 18q. The most frequent gene abnormality in CNS GCTs is XXY, similar to Klinefelter syndrome which tends to develop GCTs in the intracranium.

In the intracranial pure germinomas, mutations of KIT/RAS gene were frequently detected, and mutations in the KIT gene exons 11, 13, and 17 as well as KIT amplification were found in 23–25% of intracranial GCTs. It is thought to contribute to the development of GCTs. MYC or MYCN amplification can be observed in a small number of GCTs.

Non-germinomatous germ cell tumor

In the yolk sac tumor (YST), chromosome lp36 gain, 6q loss and chromosome 1 and chromosome 20 abnormalities have been reported. In addition, i(12p), which is characteristic of other malignant GCTs of testis and ovary, can be detected. Embryonal carcinoma and choriocarcinoma show similar cytogenetic abnormalities, i(12p).

Immature teratomas are usually diploid, whereas YST can be diploid, tetraploid or aneuploid. Chromosomal abnormalities include gain of chromosomes X, 1, 3, 8, 12, and 14, i(12p) and loss of X and Y. There may be loss of 1q and rearrangement of 3q and 6q.

Current status of targeted next-generation sequencing in brain tumors

There has been a great progress in the understanding of molecular characteristics of brain tumors by genome-wide study, such as WES and whole genome sequencing (WGS). However, targeted NGS panel composed of limited number of genes is required for the routine clinical practice of brain tumor diagnosis and treatment. For the clinical NGS test of brain tumors, we should consider the spectrum of sequencing panel (pan-cancer panel or organ-specific panel), target enrichment method (hybrid capture or amplicon sequencing), type of tissues (fresh frozen [FF] or formalin-fixed paraffin-embedded [FFPE] tissues) and gene contents. The summary of targeted NGS panels used for brain tumors in recent publications is shown in Table 1. There are three types of panel: pan-cancer, brain tumor-specific and glioma-specific panel.

Pan-cancer panels and organ-specific panels have pros and cons. Pan-cancer panels are usually composed of more than 300 genes of major oncogenes, tumor suppressor genes and druggable genes frequently altered in various type of cancers. Because of large target region, pan-cancer panels show better performance in copy number alterations (CNA). However, pan-cancer panels usually require more time and cost. Moreover, genes solely mutated in certain type of cancer with rare frequency, such as HIST1H3B or HIST1H3C are not covered by pan-cancer panels. Organ-specific panels consist of lower number of genes than pan-cancer panel, so we can reduce the time and cost for the NGS test. In addition, organ-specific panel covers cancer type-specific genes with rare mutation rate. However, organ-specific panel have limitations in the clinical trial enroll and CNA analysis.

Because the target region of NGS panel is smaller than 1% of human genome, we should enrich the region of interest in the genome. There are two types of target enrichment method, which are hybrid capture and amplicon method. Hybrid capture method takes longer hands-on time and turn-around time, and usually requires more than 200 ng of genomic DNA. Amplicon sequencing method enriches target sequences by PCR amplification. Hybrid capture method is suitable for NGS panels with more than 50 genes as well as comprehensive genomic analysis including single nucleotide variation (SNV), indel, CNA and structural variation. However, hybrid capture method takes longer hands-on time and turn-around time, and usually requires more than 200 ng of genomic DNA. Amplicon sequencing method has easier workflow, short turn-around time and requires small amount of genomic DNA (more than 20 ng). However, amplicon sequencing is usually used for NGS panels composed of less than 50 genes, and has a limit to CNA analysis.

FFPE tissue is widely used in the histological diagnosis due to the preservation of morphology, fast tissue preparation, and low cost for storage. However, FFPE tissue has several issues in molecular testing, such as the fragmentation of DNA, crosslinking, and cytidine deamination. For those reasons, DNA extract-
ed from FF is preferred in NGS test. However, several commercially available DNA extraction kit for FFPE yield high quality of DNA from FFPE tissues. So, both of FF and FFPE tissues can be used in clinical NGS test. When the test was performed with FFPE tissues, C to T transition with low variant allele frequency could be false-positive call caused by cytidine deamination. Enzymatic removal of deaminated cytosine by UDP glucuronyl transferase can reduce that error.

Selection of gene contents for brain tumor panel is based on the classification of brain tumors and corresponding oncogenic pathways. For the diagnosis and classification of gliomas, IDH1, IDH2, ATRX, TP53, CIC, FUBP1, BRAF genes and telomerase reverse transcriptase (TERT) promoter are usually included. For the detection of 1p/19q co-deletion in oligodendroglioma, additional genomic regions which exist in 1p or 19q can be included in the panel. To diagnose diffuse midline glioma, H3F3A, HIST1H3B, and HIST1H3C should be tested. For the classification of MB, APC, CTNNB1, TP53, PTCH1, SMO, SUFU, KDM6A, MYC, MYCN genes and TERT promoter can be used. For the diagnosis of AT/RT, SMARCB1 (INI1) and SMARCA4 (BRG1) can be included. For the therapeutic intent, druggable or potentially druggable target such as HER2, ALK, MET, ROS1, KIT, PDGFRA, FGFR1, FGFR3, BRAF can be included in NGS panel.

For now, targeted NGS panel for brain tumors has several limitations. First, reliable detection of copy number alteration is limited due to uneven target coverage, the absence of matched normal data, or the lack of coverage uniformity. Second, nucleotide sequences with high GC content such as TERT promoter usually show lower depth of coverage. Third, exact classification of MB and ependymoma based on NGS panel test is limited because of the classification of those tumors are mainly based on transcriptome and methylome analysis.

**CONCLUSION**

The molecular abnormalities of pediatric brain tumors and current status of targeted brain cancer panels were reviewed. Due to the completion of the human genome project and the development of gene abnormality testing techniques, the revolution of genomic tests for human diseases are actively under-
way. Even if morphologically tumors are belonging to the same group, it is found that the prognosis and response to the treatment depend on the gene abnormality. Therefore, pathologic diagnosis has been transformed into the integrated diagnosis reflecting the gene abnormality in the revised 2016 WHO classification of CNS tumors. Brain cancer panel using targeted sequencing will be helpful to such integrated diagnosis and target therapies to cure diseases.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

INFORMED CONSENT

This type of study does not require informed consent.

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