Improving diagnosis of central nervous system tumours using genetic and epigenetic tools

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Glioma-containing tissue microarray stained against mutant IDH1.

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To my beloved wife

To my wonderful children

To my fantastic mother and father

To my extraordinary brother

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ABSTRACT

Brain tumor diagnostics has traditionally been based on histopathology stains. The introduction of immunohistochemistry stains resulted in improved ability to classify these often devastating tumors. The understanding of molecular markers in central nervous system tumors has improved substantially in recent decades. Demonstration of specific genetic changes such as mutations can have crucial impact on selection of therapy. Genetic changes also play an increasingly important role when it comes to classification of these tumors. Correct classification and grading are important to be able to give correct prognostic and predictive information and are of fundamental importance for efficient clinical patient handling. In this thesis, we use several molecular techniques to improve tumor diagnosis and tumor classification and investigate the utility of these methods to give a deeper understanding of these neoplastic processes.

In Paper I, we investigated the DNA methylation profiling method for molecular classification of diffuse lower grade gliomas. We showed that DNA methylation profiling not only gave correct diagnostic and prognostic information but also were able to give reliable molecular information enabling molecular classification of the tumors according to the World Health Organization classification system.

In Paper II, we assessed changes in DNA methylation pattern over time in diffuse *IDH*-mutant gliomas. We showed that tumors accumulated methylation alterations during progression, but that the overall methylation patterns most often were maintained upon recurrence.

In Paper III, we explored if a proposed immunohistochemistry-based investigation of phenotype predicted survival and tumor recurrence in a clinical cohort of diffuse low-grade gliomas that were reclassified according to the 2021 WHO criteria.

In Paper IV, we describe a 16-month-old patient with a tumor in the third ventricle with a relapse two years after diagnosis. The tumor was initially classified as a low-grade glioma but was after methylation profiling reclassified as an infant-type hemispheric glioma. To search for druggable targets and for further refinement of the molecular background both whole genome sequencing and whole transcriptome sequencing were performed. A novel *TPR::ROS1* fusion gene was detected activating the *MAPK*-, *PI3K*- and *JAK/STAT*- pathways.

In Paper V, we present a cystic pilocytic astrocytoma with *KIAA1549::BRAF* fusion in a 16-year-old patient. The tumor showed ganglion cell morphology and different vascularization in a nodular component. With extended molecular examination we were able to prove that the cells with ganglion cell morphology were of neoplastic origin.

In conclusion, we further demonstrate the importance of adding molecular investigation in the histopathological diagnostic work-up. We also present arguments for the importance of evaluating molecular findings in correlation with the histomorphology picture.

Keywords: Histopathology, DNA methylation profiling, DNA methylation-based classification, Diffuse lower grade-glioma, Genomic analysis, Molecular biomarkers, Pediatric glioma

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Sammanfattning på svenska

Hjärntumördiagnostik har traditionellt baserats på histopatologisk färgning. Introduktionen av immunohistokemisk färgning resulterade i förbättrad förmåga att klassificera dessa ofta livshotande tumörer. Förståelsen av molekylära förändringar i tumör i centrala nervsystemet har avsevärt förbättrats under de senaste decennierna. Att påvisa specifika genetiska förändringar såsom mutationer kan ha avgörande inverkan på valet av terapi. Genetiska förändringar spelar också en allt viktigare roll när det kommer till klassificering av dessa tumörer. Korrekt klassificering och gradering är viktigt för att kunna ge korrekt prognostisk och predikativ information och är av fundamental betydelse för en effektiv klinisk patienthantering. I denna avhandling undersöker vi förmågan hos flera molekylära tekniker att ge förbättrad tumördiagnostik och tumörklassificering samt fördjupad förståelse av dessa neoplastiska processer.

I delarbete I undersöker vi DNA-metyleringsbaserad profilering som metod för molekylär klassificering av diffusa låggradiga gliom. Vi visar att DNA-metyleringsbaserad profilering inte bara gav korrekt diagnostisk och prognostisk information men också kunde ge tillförlitlig molekylär information för klassificering av tumörerna enligt WHO:s klassificeringssystem.

I delarbete II studerade vi förändringar i DNA-metyleringsmönster över tid i diffusa *IDH*-muterade gliom. Vi visade att tumörer ackumulerade metyleringsförändringar under progression, men att metyleringsmönstret oftast var bevarat vid recidiv.

I delarbete III undersökte vi om en föreslagen immunohistokemisk undersökningsmetod av fenotyp kunde förutse överlevnad och tumörrecidiv i en klinisk kohort med diffusa låggradiga gliom reklassificerade enligt WHO:s kriterier från 2021.

I delarbete IV beskriver vi en 16 månader gammal patient med en tumör i 3:e ventrikeln som recidiverade 2 år efter diagnos. Tumören klassificerades initialt som ett låggradigt gliom men reklassificerades efter metyleringsarray-profilering som ett så kallat infant-type hemispheric glioma. För att leta efter behandlingsbara genetiska förändringar och för utökad förståelse av den molekylära bakgrunden utförde vi helgenomsekvensering och heltranskriptomsekvensering. En tidigare okänd *TPR::ROS1* fusionsgen påvisades vilken aktiverade *MAPK*-, *PI3K*- och *JAK/STAT*-vägarna.

I delarbete V presenterar vi ett fall av cystiskt pilocytärt astrocytom med *KIAA1549::BRAF*-fusion hos en 16 år gammal patient. Tumören uppvisade gangliocytär cellmorfologi och annorlunda vaskularisering i en nodulär komponent. Med utökad molekylär undersökning kunde vi visa att cellerna med gangliocytär morfologi var neoplastiska.

Sammanfattningsvis ger vi ytterligare belägg för vikten av att addera molekylära undersökningar i den histopatologiska utvärderingen av tumör från centrala nervsystemet. Vi presenterar också argument som stödjer uppfattningen att det är av stor vikt att utvärdera molekylära fynd i korrelation med den histomorfologiska bilden.

List of papers

This thesis is based on the following studies, referred to in the text by the Roman numerals.

I. Ferreyra Vega S, **Olsson Bontell T**, Corell A, Smits A, Jakola AS, Carén H. DNA methylation profiling for molecular classification of adult diffuse lower-grade gliomas. *Clin Epigenetics 2021:13(1):102*.

II. Ferreyra Vega S, **Olsson Bontell T**, Teresia K, Jakola AS, Carén H. Longitudinal DNA methylation analysis of adult-type *IDH*-mutant gliomas. *Acta Neuropathologica Communications 2023:11(1):23*.

III. Dénes A*, **Olsson Bontell T***, Barchéus H, Ferreya Vega S, Carén H, Lindskog C, Jakola AS, Smits A. The Clinical value of proneural, classical and mesenchymal protein signatures in WHO 2021 adult-type diffuse lower-grade gliomas. *Manuscript submitted*. *Shared first author.

IV. Deland L, Keane S, **Olsson Bontell T**, Fagman H, Sjögren H, Lind AE, Carén H, Tisell M, Nilsson JA, Ejeskär K, Sabel M, Abel F. Novel *TPR::ROS1* Fusion Gene Activates MAPK, PI3K and JAK/STAT Signaling in an Infant-type Pediatric Glioma. *Cancer Genomics Proteomics 2022:19(6):711-726*.

V. **Olsson Bontell T**, Danielsson A, Dahr N, Deland L, Tisell M, Sjögren H, Sabel M, Carén H, Abel F. Formation of ganglion cells in a nodular component of a cystic infratentorial pilocytic astrocytoma carrying *KIAA1549::BRAF* fusion. *Manuscript*.

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i. Bergström P, Agholme L, Nazir FH, Satir TM, Toombs J, Wellington H, Strandberg J, **Bontell TO**, Kvartsberg H, Holmström M, Boreström C, Simonsson S, Kunath T, Lindahl A, Blennow K, Hanse E, Portelius E, Wray S, Zetterberg H. Amyloid precursor protein expression and processing are differentially regulated during cortical neuron differentiation. *Sci Rep 2016:6:29200*.

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Contents

ABBREVATIONS

5mC	5-methylcytosine
cDNA	Complementary DNA
CIMP	CpG island methylator phenotype
cIMPACT	the Consortium to Inform Molecular and Practical Approaches to CNS Tumor Taxonomy
CNS	Central nervous system
CNA	Copy number alteration
CpG	cytosine-phosphate-guanine
ddNTPs	dideoxynucleotide triphosphates
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
dLGGs	Diffuse lower grade gliomas
dNTPs	deoxynucleotide triphosphates
FFPE	Formalin fixed paraffin embedded
FISH	Fluorescence in situ hybridization
G-CIMP	Glioma CpG island methylator phenotype
gDNA	Genomic DNA
IDH	Isocitrate dehydrogenase
IF	Immunofluorescence
IHC	Immunohistochemistry
MGMT	O-6-methylguanine-DNA methyltransferase
MNP	Molecular neuropathology
mRNA	Messenger ribonucleic acid
NGS	Next generation sequencing
ncRNA	noncoding ribonucleic acid
PA	Pilocytic astrocytoma
PCR	Polymerase chain reaction
pHGG	pediatric high-grade glioma
pLGG	pediatric low-grade glioma
RNA	Ribonucleic acid
RT	Reverse transcriptase
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
RT-PCR	Reverse transcription polymerase chain reaction
RT	Reverse transcriptase
qPCR	Quantitative PCR
SNP	Single nucleotide polymorphism
TMA	Tissue microarray
WB	Western blot
WGS	Whole genome sequencing
WHO	World Health Organization

INTRODUCTION

DNA

The DNA molecule

Deoxyribonucleic acid (DNA) is a macromolecule consisting of two polynucleotide chains coiled around each other forming a double helix carrying the genetic instructions for development, growth, functioning and reproduction [1]. DNA is found within the nuclei of almost all cells in the human body. The polynucleotide chains consist of rows of nucleotides. Each nucleotide is composed of one of four nucleobases, a sugar called deoxyribose and a phosphate group [2, 3]. The double helical structure of the DNA molecule was described by Watson and Crick in the 1950s [4, 5]. The structure was solved using data from X-ray diffraction images obtained by Rosalind Franklin [6, 7]. They also described the implications for genetic information transmission and how the molecule structure makes self-duplication possible [8].

Genes

One often used definition of a gene is a DNA-sequence coding for a protein, for isoforms of a protein or for non-coding ribonucleic acid (RNA) [9]. The connection between genes and proteins was already established in the 1940s [10]. In the early 1960s it was demonstrated that genes are first transcribed into messenger RNA (mRNA) [11-13]. This early concept of a gene can be summarized in the statement one gene – one mRNA – one polypeptide. This hypothesis was challenged when it was noticed that a single gene can produce more than one mRNA and that one gene can be involved in several transcription units. This can be achieved by multiple transcription initiation sites with alternative promoters so that the single gene can produce several transcripts [14]. Another way of producing different transcripts from one gene is the alternative splicing phenomenon [15, 16]. Genes are interrupted by introns, and this is called split genes. From these split genes one pre-mRNA molecule is produced. From this molecule, introns are removed during a maturation process called pre-mRNA splicing. By excluding individual exons from the mature mRNA and joining different exons in a nonconsecutive fashion, different mRNA molecules are created. There are two other ways that one gene can result in proteins with different amino acid sequences. One is so called mRNA editing, a process in which the structure of an RNA molecule is modified by post transcriptional processing [17]. The other one is gene sharing. Gene sharing describes the phenomenon that identically sequenced polypeptides coming from the same gene can have totally different functions in different cells because they are so differentially configured that they perform totally different functions [18]. The genome is transcribed from both DNA strands [19]. The amount of DNA in the genome paradoxically shows little correlation with an organisms size or complexity [20]. This is because a huge part of the genome does not contain protein-coding genes. Only approximately 2% of the genome encodes proteins. First the noncoding sequences were considered as junk but now it is viewed upon differently, and it is thought that as much as 70 to 90% of the genome is transcribed at some point [19, 21]. RNA molecules that are not translated into a protein are called noncoding RNA (ncRNA) [22]. Examples of ncRNA are ribosomal RNA (rRNA), transfer RNA (tRNA), small nuclear RNA (snRNA), long non-coding (lncRNA), microRNA (miRNA), small interfering RNA (siRNA), and extra cellular RNA (exRNA). They are all viewed upon as having important housekeeping- or regulatory functions.

Mutations

A mutation is a change in the DNA sequence of an organism. Mutations can be divided into germline or somatic mutations [23]. Germline mutations occur in eggs and sperms and can potentially be passed on to offspring while somatic mutations occur in body cells and are not passed on to the offspring. Mutations can be beneficial, neutral or contribute to disease [24-26]. There are many types of mutations. Some examples are missense, nonsense, insertion, deletion, duplication, frameshift, chromosomal rearrangements, polyploidy, loss of heterozygosity and many others [27]. Another way of dividing mutations is into loss-of-function and gain of function mutations. They can both contribute to cancer [28]. Loss-of-function mutations must affect both alleles and are in cancer often related to tumor suppressor genes. Gain-of-function mutations are in cancer often related to growth factors or growth factors related molecules.

Epigenetics

The term epigenetics refers to stable phenotypic changes that do not involve alterations in the DNA sequence [29]. The epigenetic machinery is one important factor making it possible to have a diversity of cell types with different gene expression, morphology, and function even though they have the same genetic code [30]. There are three main epigenetic mechanisms namely DNA methylation, histone modifications and non-coding RNA. DNA methylation is the most studied epigenetic mechanism and is described more in detail below. Histones are proteins that contribute to efficient packaging of DNA into chromosomes in the nucleus. Histones can be modified by for example acetylation, phosphorylation, ubiquitination, and methylation leading to activation or repression of gene transcription [31, 32]. Non-coding RNA are RNA molecules that are not translated into proteins but are able to regulate gene expression via chromatin modulation and binding [33, 34].

DNA methylation

DNA methylation was described already in the 1940s. It implies the addition of a methyl group (CH_3) to the carbon of the fifth position in a cytosine, most frequently occurring in a CpG site where a cytosine DNA base is followed by a guanine base (cytosine-phosphate-guanine) [35]. This reaction is catalyzed by a family of enzymes named DNA methyltransferases (DNMTs) which methylate the cytosine in the CpG site by transferring a methyl group from a donor molecule [36]. DNMTs are often called methylation writers. DNMT1 is a maintenance DNA methyltransferase which maintains already established patterns whereas DNMT3A and DNMT3B are called *de novo* DNA methyltransferase because they contribute with new patterns [37-39]. There are also other mechanisms such as methylation erasers counterbalancing the DNMTs by erasing the modifications introduced by the writers [40]. The methylation pattern created by the writers and the erasers are read by so-called readers.

A CpG island is a region with many CpG sites in proximity whereas regions close to such islands are called shores [41, 42]. Regions further away from the islands are called shelves and genome domains even further away are called open sea [43]. The frequency of CpG sites diminish with the distance from the CpG island. The human genome is considered to carry approximately 30 million CpG sites of which the majority, in human somatic cells, are methylated. When cytosine has been modified with the addition of a methyl group to its 5th carbon it is called 5-methylcytosine (5mC). In vertebrates this typically occurs at cytosine in CpG dinucleotides meaning where a cytosine nucleotide is followed by a guanine nucleotide. 70 to 80% of CpG cytosines are methylated in mammals [44]. As mentioned before CpG islands are areas with high frequency of CpG sites and they are very often associated with promoter regions [45]. In general, CpG dinucleotides occur in lower frequency in the genome than expected by random chance [46]. When multiple CpG sites are methylated in CpG islands in promoters there is effective silencing of the gene in question [41].

There are a number of ways to profile the DNA methylation pattern [47]. This can be done on a genome-wide scale in tissue, cells or even at single cell level. When combined with next-generation sequencing, DNA methylation at single base resolution is possible [48].

DNA sequencing, RNA sequencing and DNA methylation status analysis

There are many techniques to determine the order of the nucleotides in a part, parts of, or the whole genome or in RNA-molecules. The first sequencing methods were invented in the 1970s. Sequencing of RNA molecules was one of the earliest forms of nucleotide sequencing [49]. RNA sequencing has traditionally demanded the generation of a complementary DNA (cDNA) molecule but recently methods directly sequencing RNA molecules have been invented [50]. The first described methods for DNA sequencing invented in the 1970s involved DNA polymerase and location specific primers [51, 52]. This method was further developed and made faster by Frederick Sanger [53, 54]. Further technical development has made sequencing of full genomes possible and this to an increasingly reduced price [55-57]. One very important technical development was the introduction of the shotgun sequencing technique [58]. This paved the way for sequencing of the whole human genome [46, 59]. One way to speed up sequencing is to perform massive parallel sequencing, also called next generation sequencing (NGS) to distinguish them from earlier methods such as traditional Sanger sequencing [60]. There are multiple methods that can be used to perform NGS. With NGS it is possible to sequence an entire human genome within a day. One important sequencing method is pyrosequencing, first described in the early 1990s and then further developed [61-63]. This method relies on light detection of light released when DNA polymerase incorporate nucleotides. It is an important technique as it has higher sensitivity, is faster and more cost effective compared to Sanger sequencing [64]. One disadvantage with pyrosequencing is that the length of the sequence that can be sequenced is quite short. Another disadvantage is that the output data can be complex and hard to analyze.

Fusion genes are important contributors to cancer [65]. They can be diagnosed with fluorescence in situ hybridization (FISH) or reverse transcription polymerase chain reaction (RT-PCR) based methods. These techniques, however, typically only test for a single fusion gene at the time. It is now possible to sequence the whole transcriptome (RNAseq) and with this method look for many different fusion genes. This method has, because of the large size of the transcriptome, poor sensitivity. Targeted RNAseq is a similar method but here one only pinpoint RNA transcripts of interests. This technique enables sensitive detection of lowly expressed or rare transcripts [65]. A drawback with this technique is that one needs to decide which partner genes to look for in advance. One way to circumvent this obstacle is to use another technique called open-end RNA-sequencing [66]. When this method is applied a combination of a gene specific primer and a universal primer is used, making it possible to detect new fusion gene combinations.

There are several methods to determine the methylation status of DNA molecules [67]. The different methods have certain pros and cons. It is possible to perform whole genome methylation profiling, or one can choose to only look at certain areas of the genome. For the identification of differentially methylated regions there are a more limited number of methods available. One example of how this can be done is bisulfite sequencing where bisulfite treatment of DNA mediates deamination of cytosine into uracil. The converted residues will be read as thymine following PCR-amplification and Sanger sequencing. One then can compare results from Sanger sequencing from an untreated DNA sample with results from a sample that has been bisulfite treated. This enables detection of methylated cytosines. Bisulfite conversion reduces the genome complexity to three nucleotides. It is very important to ensure complete conversion of non-methylated cytosines. Whole genome bisulfite sequencing is possible and is like whole genome sequencing but with the addition of bisulfite conversion. The cost for this analysis is, however, high. Also, only a small fraction of the genome has the potential to be differentially methylated, which is why whole genome sequencing is rarely required. By only sequencing the 5-mC-

enriched fraction of the genome the analysis will be less expensive and with an increase in sequencing coverage. With array-based profiling approaches it is possible to analyze a large number of samples at an affordable cost. One such approach is the Illumina Infinium HumanMethylation450 BeadChip technology where more than 480,000 probes targeting 96% of CpG island regions and 99% of genes are included [68-70]. This technique has been further developed into Illumina Infinium HumanMethylation EPIC BeadChip array where more than 850,000 methylation sites across the human genome are interrogated and in an updated version more than 935,000 sites.

CNS TUMORS

WHO Classification system

Tumors in the central nervous system (CNS) are most often classified according to the World Health Organization (WHO) classification of tumors system presented in a series of books called the WHO Blue Books. The latest version is the sixth volume (fifth-edition) and it was published in 2021 [71, 72]. Before that there was a version published in 2016 and one in 2007 [73]. What was new in the 2016 version was the incorporation of well-established molecular findings into the classification system. Before that, genetic status served as supplementary information and was not included in the definition of neoplasms. In the fifth edition from 2021 the role of molecular findings in the classification has moved even further but is still based on histology and immunohistochemistry. Because of the rapid progression in understanding of molecular characteristics in human nervous system tumors a group of neuropathologists and neurooncologists formed a consortium to improve the diagnosis and classification of CNS tumors in advance of the publication of a new WHO classification [74, 75]. The consortium was called "the Consortium to Inform Molecular and Practical Approaches to CNS Tumor Taxonomy" (cIMPACT-NOW). Many of the recommendations from the consortium were included in the fifth edition.

Gliomas, glioneuronal tumours, and neuronal tumours

There are many groups of primary CNS tumors. The largest group is the "Gliomas, glioneuronal tumours, and neuronal tumours" [71, 72]. This group is further subdivided into many subgroups. The most important subgroup in adult patients is the adult-type diffuse gliomas containing isocitrate dehydrogenase (*IDH*) mutant astrocytomas, *IDH*-mutant and 1p/19q-codeleted oligodendrogliomas and *IDH*-wildtype glioblastomas. These tumors all grow in a diffusely infiltrative manner and are non-curable. According to the WHO classification system, most CNS tumors can be graded from grade 1 to grade 4 correlating with their clinical aggressiveness. Grade 1 tumors are slow-growing and in many cases curable. Grade 4 tumors are in general fast growing and considered malignant. Adult-type diffuse gliomas are according to WHO graded from grade 2 to grade 4. The most prevalent subgroup in children are the circumscribed astrocytic gliomas among others containing pilocytic astrocytomas (PA). PA are most often slow growing, in many cases curable and are grade 1 tumors according to WHO. Another quite common low-grade glioma is ganglioglioma, which is also a grade 1 tumor but traditionally has been considered as more likely to malignify than pilocytic astrocytomas. Gangliogliomas are sorted under the subgroup glioneuronal and neuronal tumours. In general, pediatric tumors have fewer mutations compared to tumors in adults. This is also true for CNS tumors. CNS tumors in children are often driven by fusion genes [76].

DNA methylation-based classification

In recent years it has become clear that groups of tumors in the central nervous system can be separated by comparison of their methylation pattern. It is even, in many cases, possible to identify specific tumor entities with this technique. This is called DNA methylation array profiling and has in recent years become an important tool for classification of CNS tumors [68-70]. The most widely used classifier is the classifier published by the German Cancer Research center together with Heidelberg University Hospital and other research institutes [77, 78]. This method takes advantage of the Illumina Infinium Human Methylation BeadChip technology [70]. The method has been recognized by the WHO as an important tool and has for some entities been included in the fifth edition of the WHO classification as a golden standard method. There are many pros and cons with this classifier. One advantage is that almost all entities in the WHO classification are included. One disadvantage with the method is that it requires high tumor cell content for reliable results. There are also other classifiers based on methylation data [79].

A universal feature of solid cancers is global hypomethylation which leads to chromosomal instability and increased frequency of mutations [80, 81]. This seems to be a very early event in tumorigenesis [82, 83]. There are other, more specific alterations that contribute to tumor formation, such as activation of oncogenes via hypomethylation and silencing of tumor suppressor genes via hypermethylation [84, 85]. More than twenty years ago it was discovered that in some colorectal cancers there was a genome-wide hypermethylation of CpG islands [86]. This was called *the CpG island methylator phenotype* (CIMP). Later it was shown that in a subset of adult type diffuse gliomas of lower grade, a similar hypermethylation pattern could be detected and this was called glioma CIMP (G-CIMP) [87]. A couple of years later it was shown that a mutation in *isocitrate dehydrogenase 1* is sufficient to establish this glioma hypermethylator phenotype [88].

Biomarkers in gliomas

There are many molecular findings that are considered as biomarkers in gliomas. Two examples are the above mentioned *IDH*-mutation and 1p/19q-codeletion. Another important biomarker is the O-6-methylguanine-DNA methyltransferase (*MGMT*) gene which codes for a DNA repair enzyme counteracting alkylating temozolomide treatment [89]. If the promoter is active and the enzyme is expressed, patients will not benefit from temozolomide treatment and will instead receive radiation treatment. Patients with an inactive promoter often receive temozolomide treatment in combination with radiotherapy [90-93]. The methylation status of the promoter determines if the gene is active or silenced. Analysis of MGMT-promoter methylation status is widely used in the clinic for glioblastoma, IDH-wildtype tumors. The clinical benefit of analyzing *MGMT*-promoter status in *IDH*-mutated astrocytomas are debated.

Another important biomarker is homozygous deletion of *CDKN2A/B*. According to the CNS WHO 2021 system, presence of homozygous deletion of *CDKN2A/B* in *IDH*-mutant astrocytomas will make a tumor a grade 4 tumor even if it lacks necrosis or microvascular proliferation [72, 94, 95].

AIMS

The overall aim of this thesis was to improve diagnosis, modeling and treatment of primary brain tumors. Accurate diagnosis of a tumor is necessary for appropriate clinical management and efficient treatment decisions. Research models based on human cells are probably necessary to reach a better understanding of brain tumors. Such models would also offer an opportunity to evaluate new treatment regimes.

The specific aims were:

Paper I: To investigate the value of DNA methylation profiling in adult patients with diffuse lower grade gliomas and evaluate the method as a tool for molecular stratification of adult diffuse lower grade gliomas.

Paper II: To investigate how DNA methylation changes in association with progression, with or without malignant transformation, of *IDH*-mutant gliomas over time.

Paper III: To investigate whether immunohistochemistry profiling with key markers is sufficient to subtype diffuse lower grade gliomas into clinically relevant phenotypes.

Paper IV: To describe a patient with an infant-type pediatric glioma with a novel *TPR::ROS1* fusion gene, elucidate the molecular events driving tumorigenesis and map the downstream pathways activated by the fusion to identify potential therapeutic targets.

Paper V: To describe a pilocytic astrocytoma containing an area with ganglion cell morphology and determine if these cells are neoplastic and part of the tumor.

METHODS AND METHODOLOGICAL CONSIDERATIONS

The main methods used in this thesis are described below. Detailed descriptions are found in the method sections of the attached papers.

Patients and samples (Paper I, II, III, IV, V)

The studies included in this thesis were approved by the Regional Ethics Committee in the Västra Götaland region in Sweden (Dnr. 1067-16 (Paper I, II, III) and Dnr. 239-13 (Paper IV and V)).

Archived formalin-fixed paraffin-embedded (FFPE) tumor tissues were received from the pathology department at the Sahlgrenska University Hospital. In paper I, II and III, FFPE tumor samples from adult patients with diffuse lower grade gliomas according to WHO 2007 were used. In Paper II, a group of primary tumors present in paper I and their matched tumor recurrences were analyzed. Paper III included the same samples used in paper I and to some extent also in Paper II. In Paper IV and V, FFPE and fresh-frozen tumor tissue and blood samples were used. Tumor tissue and blood was collected from patients undergoing CNS tumor resection at the Sahlgrenska University Hospital and used after signed informed consent from the patients' parents.

Tissue micro array – TMA (Paper I, II, III)

Tissue micro array (TMA) is constructed by combining core tissue biopsies from multiple blocks with paraffin embedded tumor tissue into a recipient paraffin block [96]. The core tissue biopsies often have a diameter between 0.6- and 1-mm. Multiple core tissue biopsies can be placed in the recipient paraffin block. Sections cut from the recipient block will then contain tissue from all donor blocks allowing parallel detection of proteins by immunohistochemistry, investigation of morphology with histochemistry stains, DNA with fluorescence in situ hybridization or RNA with RNA in situ hybridization. The biopsies are 3-4 mm in height making it possible to extract at least 200 sections from one donor block.

One advantage with the TMA-technique is that valuable tissue can be saved since hundreds of different tissue samples can be placed upon one microscope glass slide. Another advantage is that expensive substances and compounds can be saved. As an example, much less antibodies are of course needed to stain one TMA-section compared to hundreds of individual slides. Time is also spared both when it comes to performing experiments but also when evaluating them. It is also much easier to automatize the evaluation process of a TMA-section. A drawback of the technique is that only a small part of for example a tumor is investigated. A tumor can differ in multiple ways in different areas, and this is not represented in a small core biopsy. Making a TMA is also time consuming and needs special equipment. Choosing the right area is crucial and needs an experienced assessor. We used the TMA-technique in paper I, II and III to screen many tumors with immunohistochemistry against various epitopes.

Immunohistochemistry – IHC (Paper I, II, III, IV, V)

Immunohistochemistry is a widely used technique for identification and localization of antigens in tissue sections. It is based on antigen-antibody interaction where the antigen of interest is detected by an antibody [97, 98]. When the antigen-antibody binding has occurred it is visualized with a colored histochemical reaction that can be viewed and evaluated in a light microscope. It is possible to stain for

more than one antigen per sample. The stained slides can be digitalized and evaluated on a computer screen and the evaluation can in some cases be automatized.

Advantages with immunohistochemistry are that it is an old well-established technique that is relatively simple to perform, cheap and in many cases quite straightforward to evaluate. It can give information not only about the presence of a protein but also about its cellular localization. It is also very effective when working with tumor samples with low tumor cell content. One disadvantage is that for various reasons it can give both false positive and false negative results. We used immunohistochemistry in paper I, II, III, IV and V for multiple purposes.

Fluorescent in situ hybridization - FISH (Paper I, II, III, IV)

Fluorescent in situ hybridization (FISH) is a technique where DNA strands incorporated with fluorophore-coupled nucleotides called probes are hybridized to a complementary sequence in cells and then visualized with a fluorescence microscope [99]. The technique has high sensitivity and specificity and works at the single gene level. The probes come in many different flavors making the technique a valuable tool to answer many different research questions. In cancer research it is often used to look for losses, gains and translocations of genomic material.

Advantages with FISH is that it can work at the single cell level. It is an old technique and therefore also well established. The probes can be almost infinitely varied making the technique very flexible. There is a risk for both false positivity and false negativity. False positive results can be related to probes that bind in a non-specific manner. False negative results when it comes to the investigation of sections can be related to sectioning of the nuclei cutting away too much of the genomic material. We used FISH in paper I, II, III and IV for various purposes.

Mutation analysis with Sanger sequencing (Paper I, II, III, IV)

Sanger sequencing is a method of DNA sequencing invented by Frederick Sanger and colleagues in the 1970s [53, 54]. In Sanger sequencing, amplified DNA or cDNA is annealed to an oligonucleotide primer and then extended by a DNA polymerase that incorporates a mixture of four deoxynucleotide triphosphates (dNTPs) or four chain-terminating dideoxynucleotide triphosphates (ddNTPs) [100]. When ddNTPs are incorporated the elongation reaction stops and DNA fragments of various lengths are created. The ddNTPs are tagged with different fluorochromes. In this way distinguishable DNA fragments of various lengths are created. The samples can then be analyzed using capillary electrophoresis giving the DNA sequence, a process that can be automated.

Sanger sequencing is a well-established method. It is well suited for small-scale projects and for validation of deep sequencing results and it has a very low error rate with very high accuracy. It is a cheap method. The most important limitations are low quality sequences within the first base pairs, sometimes an inability to distinguish single base pair differences in very long segments and problems with DNA secondary structures. We used Sanger sequencing in paper I, II, III and IV.

Mutation analysis with targeted panel-based sequencing (Paper V)

In targeted panel-based sequencing, also called gene panel sequencing, some special areas of interest in the genome, often a group of genes, are simultaneously sequenced. The sequencing is usually performed with next generation sequencing technology. NGS is a collective name for a group of technologies where massive parallel sequencing of hundreds or thousands of fragments of DNA are performed in parallel. The NGS based gene panel tests can identify gene alterations that potentially can be targeted making

personalized treatment possible [101]. It can also give important diagnostic-, prognostic- and predictive information.

Gene panel sequencing allows for deeper sequencing than whole genome sequencing or whole exome sequencing enabling more sensitive detection. It is faster and more cost effective compared to whole genome sequencing and whole exome sequencing. Gene panel sequencing does rarely identify novel disease causative genes since it is limited to the genes included in the panel. Unlike whole genome sequencing it will not detect copy number variants or structural rearrangements. We used targeted panel-based sequencing in paper V.

Mutation analysis with whole genome sequencing (Paper IV, V)

Whole genome sequencing (WGS) refers to DNA sequencing of the entire genome so that both coding and non-coding regions are included [102]. WGS is based on short-read next generation sequencing where patients DNA is fragmented, and sequencing data generated for the entire genome. All or parts of the generated data can then be analyzed in multiple ways. When it comes to cancer research, paired tumor and blood lymphocytes are sequenced so that it is possible to differ between somatic and germline mutations.

Some advantages with WGS are that this is the most comprehensive genomic test present and that it can be used to test a wide range of DNA alterations such as single nucleotide variants, insertions, deletions, copy number variants and structural rearrangements. Since the whole genome is sequenced it can identify novel causes of genetic diseases. One disadvantage with WGS is that it generates a very large amount of data that can be hard to handle and analyze. It can also give information that is not wanted and not asked for regarding, for example, inherited diseases. Short read sequencing can have problems with some genome regions for example areas containing repetitive elements. Copy number variation detection and structural variant detection ability is not as good as in other techniques such as arrays. Limitations in read depth makes detection of mosaicism imperfect. We used WGS in paper IV and V.

Whole transcriptome sequencing (Paper IV)

The genes that are transcribed are known as the transcriptome. Some genes are expressed more than others, and this can vary over time. Transcriptome sequencing is the process of determining the genetic codes in the transcriptome and the relative proportions of these [103]. First all mRNA and ncRNA molecules are extracted, and corresponding cDNA created. This cDNA is then sequenced with NGS method.

One advantage with transcriptome sequencing is that it not only gives information about which genes are expressed but also about gene expression levels. This gives information about how active the certain gene is in a tissue or an individual at a certain time. It is also possible to apply the technique on individual cells. This information is in some ways closer to a phenotype compared to the information received from gene sequencing. RNA is more rapidly degraded than DNA and therefore more difficult to work with. Another disadvantage is that this technique does not give you any information about non-transcribed DNA. We used whole transcriptome sequencing in paper IV.

Targeted open-end RNA-sequencing (Paper IV)

Targeted RNA-sequencing is a method where specific transcripts are selected and sequenced [65]. It gives both quantitative and qualitative information about the transcript but with higher sensitivity than

whole transcriptome sequencing. Such targeted RNA-sequencing can be based on both enrichment and amplicon dependent approaches. The enrichment-based method can detect both known and new fusion partners also in FFPE material. When the open-end method is used, target-specific PCR with a combination of a gene-specific primer for a known fusion partner in combination with a universal primer for an unknown fusion partner is used [66].

This method targets RNA and therefore gives information about what is really transcribed from the genetic material and at what levels at a certain point of time. Compared to whole transcriptome sequencing, target sequencing, for good and bad, only gives information about a focused set of genes. This generates less information to analyze but on the other hand does not detect genomic alterations in other genes. Compared to FISH, immunohistochemistry, and RT-PCR this technique is more expensive and turnaround times higher. The open-end version of targeted RNA sequencing can detect gene partners in fusions even though the partner gene is not included in the panel. We used targeted open-end RNA-sequencing in paper IV.

Genome-wide DNA methylation analysis (Paper I, II, III, IV, V)

There are a number of DNA methylation profiling techniques [47]. They all have different advantages and disadvantages in aspects such as DNA input, resolution, genomic region coverage etcetera. This data can then be used to cluster the profile against formerly analyzed tumor tissue. There are multiple DNA methylation-based classifiers for central nervous system tumors with pros and cons [78, 79]. One advantage with the DNA methylation-based classification system from Capper et al. is the large number of included tumors and its similarities with the WHO classification system. At the same time as a proposed methylation class is received a copy number profile is also generated. This also gives very important information and can in many situations replace investigation with other techniques such as FISH. The analysis can also give information about important biomarkers. One disadvantage with the method is that it needs quite high tumor cell content. If it is performed on fresh frozen, non-mirrored tissue one cannot be sure that it is representative for the lesion in question. DNA methylation profiling demands advanced laboratory equipment, is expensive, takes a lot of time and is lab intensive. We used genome-wide DNA methylation profiling in paper I, II, III and V.

Chromosomal copy number analysis (Paper I)

Copy number alterations (CNAs) are alterations in the genome that result in abnormal number of copies of a specific segment of DNA. Such regions may or may not contain a gene. This can be caused by different forms of rearrangements such as inversions, deletions, translocations, and duplications. Some copy number variations are strongly linked to cancer and other diseases. Copy number alterations can also include larger parts of chromosomes, whole chromosomes, or many chromosomes [104, 105]. Information about gains and losses of parts of chromosomes or whole chromosomes can give important diagnostic, prognostic, and predictive information.

There are a variety of methods that can give information of chromosomal copy number variations. Such information can for example be given as a bonus when a genome-wide DNA methylation profile analysis is performed. A chromosomal copy number profile is also possible to extract after whole genome sequencing. Other examples of techniques that can be used are single nucleotide polymorphism microarray, microsatellite analysis and FISH. In paper one we analyzed chromosomal copy number variation with both FISH and genome-wide DNA methylation.

DNA and RNA extraction and cDNA synthesis (Paper I, II, IV, V)

To be able to sequence DNA or RNA they first must be isolated from the tissue or the cells in question. This process involves lysis of cells followed by the separation of the nucleic acid from other components such as proteins, cell membranes, lipids, and other nucleic acids [106]. The nucleic acid then needs to be purified and the concentration measured. Cell disruption can be performed by physical or chemical methods. A wide range of approaches and tools are used for both chemically and mechanically based methods depending on the properties of the sample that is going to be analyzed [106]. There is also a variety of devices used for the extraction part. Some examples are spin columns, magnetic beads, liquid handling robots and microfluidics [106]. The different methods have advantages and disadvantages when it comes to pricing, sensitivity, specificity, labor demand, risk for cross contamination, sample compatibility and demand on equipment and infrastructure.

We extracted DNA from FFPE-tissue for Sanger sequencing (paper I), for methylation analysis (paper I, II, IV, V) and for targeted panel-based sequencing (paper IV, V). DNA was extracted from fresh-frozen tissue and blood (paper IV, V) for whole genome sequencing. We extracted RNA from fresh-frozen tissue (paper IV) for whole transcriptome sequencing and RT-PCR. We extracted RNA from FFPE-tissue for targeted open-end RNA-sequencing (paper IV) and for targeted next generation sequencing (paper V).

Histological staining of FFPE tissue (Paper IV and V)

Histological staining is an old and commonly used technique where a tissue first is fixed to preserve the structure of the cells and its surroundings and then dehydrated, embedded, sectioned in thin slices, stained, and evaluated in some sort of microscope [107]. There are many different staining methods with different capacity to visualize different structures. Histologic evaluation can give important information about cell morphology, histologic pattern, tumor cell content, tumor cell purity, inflammation, degree of vascularization, presence of metals, lipids, and proteins in a sample etc. The most used histological stain in medical diagnostics is the hematoxylin and eosin staining method. The hematoxylin stains the cell nuclei blue, and eosin stains the cytoplasm and many extracellular components pink.

We used histological stains, to some extent, in all the five included studies. Many stains were used but most of all we used hematoxylin and eosin stain. In paper V we also used the elastin van Gieson stain, which is a method good at visualizing smooth muscle, connective tissue, and elastic fibers, and therefore effective in delineating for example blood vessels.

Polymerase chain reaction – PCR (Paper I, IV)

Polymerase chain reaction (PCR) is a fundamental and widely used method in life science. It is a laboratory technique where a specific segment of DNA is amplified, and millions of copies are made which can then be further studied [108]. It involves DNA fragments called primers selecting which part of the genome that should be amplified and a DNA polymerase that enzymatically assembles new DNA strands using the first DNA molecule as a template. The original DNA segment is then exponentially amplified. Most PCR methods are based on thermal cycling and use a heat stable DNA polymerase called *Taq* polymerase. There are a variety of variations of PCR. Allele-specific PCR is a method based on single-nucleotide variation used to detect mutations involving single base changes [109]. Here, successful PCR amplification is only achieved when a SNP-specific primer binds to the template. Quantitative PCR (qPCR) is a PCR method used to measure, not only the presence of a sequence in a sample, but also the quantity [110]. In this method a fluorescent dye such as Sybr Green or fluorophore containing DNA probes such as TaqMan, are used. This is often done in real-time. One variant of qPCR

is quantitative real time RT-PCR, which is a method to measure levels of RNA, most often mRNA. Multiplex PCR means that multiple different DNA sequences are amplified simultaneously in one reaction using multiple primer pairs [111].

We used PCR in paper I, II, III, IV and V.

Reverse transcriptase PCR (RT-PCR) is a method for amplifying DNA from RNA [112]. First a reverse transcriptase transcribes RNA into cDNA. This cDNA is then amplified by PCR. One application for RT-PCR is expression profiling. We used RT-PCR in paper IV.

Transfection (Paper IV)

Transfection is a process where nucleic acids are introduced into cells [113]. The genetic material can be DNA or RNA molecules. The procedure involves opening of the cell membrane allowing influx of the molecule in question. This can be done with chemical, physical, or viral based methods. Some examples of chemical methods involve the use of liposomes, nanoparticles and calcium phosphate. Examples of physical methods are electroporation, sonoporation, microinjection, gene gun and magnetofection. When viruses are used as carriers the most used are adenoviral vectors followed by lentiviral vectors. Transfection can be either stable or transient. Transiently transfected cells express the transfected DNA for a short period of time and do not pass it on to their daughter cells. Stable transfection on the other hand means that the transfected cell continues to express the transfected DNA and pass it on to their daughter cells.

We used transient transfection on cultured human embryonic kidney cells in paper IV as a method to elucidate the activated oncogenic pathways. We transfected the cells with a lipofection-based method (Lipofectamine 2000) because of its efficiency, simplicity, and its well proven ability to carry larger constructs.

Western blot - WB (Paper IV)

Western blot (WB) is a technique widely used in cell and molecular biology where specific proteins, in a mixture of proteins, can be separated and identified [114]. Most often a cell lysate is the sample that is investigated. The proteins are first separated by size with gel electrophoresis, moved to a membrane and then marked with primary and secondary antibodies for visualization. Unbound antibodies are washed away so that only antibodies bound to the protein of interest are left. A negative and a positive control to exclude nonspecific antibody binding and confirm correct antibody binding are included. The secondary antibody is visualized with different methods, most often chemiluminescent or fluorescent molecules [115]. By comparing the thickness of the visualized band with a standard, the amount of the protein in the sample can be indicated and semi-quantified. We used WB in paper IV to evaluate our transfection experiments.

Data and statistical analysis (Paper I, II, III, IV, V)

Statistical analysis in paper I was performed with IBM SPSS® Statistical software version 25. A Kaplan-Meir survival analysis and overall survival curves were compared with log-rank test.

In paper II the statistical software R version 4.1.2 with R studio was used to analyze the DNA methylation data. The proportion of neoplastic cells were estimated with the R package InfiniumPurify. Hierarchical clustering of the most deviating 1000 CpG sites were used to identify G-CIMP positive and negative tumors. The R package TCGAbiolinks was used to further discriminate the G-CIMP status into

high and low. R package MGMTSTP27 was exploited to predict the methylation status of the *MGMT* promotor. Differentially methylated CpG positions between primary and first recurrence were identified and also between groups of samples. Wilcox two-sided t-test was used for evaluation of statistical significance between different groups. We also used Pearson´s correlation to calculate the estimated overall survival probability. The Benjamin-Hoechberg method was used to adjust P-values for multiple comparison.

In paper III IBM SPSS® Statistical software version 28 was used for statistical analysis. The Kaplan-Meier method was used to estimate the post-operative survival and log-rank test for comparison between groups.

Since whole genome sequencing, whole transcriptome sequencing, and DNA methylation profiling was used in paper IV the work included a lot of data handling. For details, see the paper. Western blot data was normalized, and differences were determined with Ordinary one-way ANOVA test followed by the Dunnett's multiple comparison test. The statistical analyses were performed with GraphPad Prism version 9.1.1.

Whole genome sequencing and DNA methylation profiling was also used in paper V. For DNA methylation profiling raw methylation data (IDAT files) were generated from the methylation arrays and the IDAT files were uploaded into the publicly available DNA methylation-based classifier (MNP, version 11b4 and 12.5, https://www.molecularneuropathology.org/mnp). For prediction class scores in version 11b4 ≥ 0.9 was used as threshold for methylation class family and ≥ 0.5 for methylation class family member. For version 12.5 ≥ 0.9 was used as threshold for superfamily, family, class, and subclass.

RESULTS AND DISCUSSION

Paper I: DNA methylation profiling for molecular classification of adult diffuse lower-grade gliomas

For many years the classification of diffuse gliomas relied on histopathological criteria alone and was subject to high inter-observer variability [116-119]. Recent advances in genomic analysis have expanded the understanding of the molecular alterations characterizing diffuse lower-grade gliomas (dLGG) [120-122]. In the WHO 2016 classification incorporation of molecular biomarkers such as *IDH*-mutations and chromosomal alterations such as 1p/19q codeletion in combination with histological properties together lay the foundation for diagnosis and clinical prognostic stratification [123]. The influence of molecular alterations in the diagnosis of CNS tumors has since then grown even stronger [72, 74, 95, 124-128]. Genome-wide DNA methylation profiling has emerged as an important tool for characterization of CNS tumors and has been shown to be a robust and reproducible method for profiling tumors [129, 130].

In paper I we investigated the value of DNA methylation profiling in a cohort of adult patients with dLGG. We also evaluated the methylation-based classification for prognosticating outcome in patients with dLGG and compared it with the WHO 2016 grading system. We analyzed 166 cases with genome-wide DNA methylation analysis and performed classification using molecular neuropathology (MNP) version 11b4 [78]. We compared the robustness of methylation profiling in detecting diagnostic biomarkers compared to clinically used molecular routine techniques. The *IDH*-mutation status inferred from the DNA methylation array data showed 100% sensitivity and specificity when compared to Sanger sequencing. The DNA methylation profiling method also showed high sensitivity and specificity (100%) when analyzing 1p/19q-codeletion status. This was a better result than the clinically used techniques at the time of diagnosis achieved. Next, we investigated how well a DNA methylation-based classifier could assign a defined DNA methylation class to the dLGG samples [78]. Of the 166 profiled cases, 79% were assigned a defined DNA methylation class with a class prediction score $\geq$0.84. For 96% of those, the classifier assigned a specific methylation subclass with a prediction score $\geq$0.50 (n=126). The classifier was not able to predict a methylation class with a prediction score >0.30 (denoted as unclassified) in 6% of the cases. Methylation profiling reclassified and further subtyped some of the tumors. When it came to predicting overall survival, DNA methylation profiling provided similar prognostication compared to what was achieved after reclassification according to WHO 2016.

Limitations of this study were that we used a relatively small cohort and that the cases were classified according to WHO 2016 retrospectively. It would also be interesting to see how the results would have changed if a later classifier version (especially v12.5, not available at the time of the study) had been used.

In conclusion, we demonstrate that methylation-profiling is a valuable technique for providing diagnostic and prognostic information in patients with dLGG.

Paper II: Longitudinal DNA methylation analysis of adult-type *IDH*-mutant gliomas

IDH-mutant gliomas have a better prognosis than *IDH*-wildtyp. *IDH*-mutant gliomas are recognized by global hypermethylation at CpG islands. This is called G-CIMP (CpG island methylator phenotype). Individuals with tumors that lose the hypermethylation phenotype have a worse prognosis and faster disease progression compared to patients where the phenotype is preserved.

In this study, we examined how DNA methylation changes are related to tumor progression in *IDH*-mutated gliomas. We compared DNA methylation array data from primary tumor tissue with data related to relapse tumors. We evaluated methylation-based classification changes and assessed different methylation-based biomarkers and differential methylation status.

We showed that during progression of *IDH*-mutant gliomas, DNA methylation patterns are most often retained but alterations can occur. In some *IDH*-mutant astrocytomas, there was a shift in methylation subclass from low-grade to high-grade. In a few cases larger methylation differences between primary and relapse tumor resulted in tumors from the same individuals that did not group together in the clustering analysis. In some cases, there was also a subclass switch in the relapse tumor. In one patient the primary tumor was classified as an oligodendroglioma, but the relapse was classified as an oligosarcoma, probably reflecting malignant transformation [131, 132]. Oligosarcoma is not listed as an entity in the latest CNS WHO classification. This is an example where there is a discrepancy between the WHO classification system and the methylation profiling-based classification system from MolecularNeuropathology.org.

CDKN2A/B homozygous deletion is, according to CNS WHO 2021 system, a biomarker for grade 4 in *IDH*-mutant astrocytomas. This was also one of the most commonly occurring genetic alterations in our *IDH*-mutated astrocytoma relapse cohort. However, some *IDH*-mutated astrocytomas with morphology compatible with grade 4 and homozygous *CDKN2A/B* deletion was classified as lower-grade astrocytoma, *IDH*-mutant by the methylation array-based classifier tool. This might be an indication that *CDKN2A/B* deletion is an early event in the malignant transformation process.

One limitation of this study is that we use a relatively small cohort.

In conclusion our results show that DNA methylation patterns most often remain stable over time in *IDH*-mutant gliomas. Some tumors gained DNA methylation alterations during progression. Accumulation of site-specific methylation changes over time, associated with glioma subtype, were also detected.

Paper III: The clinical value of proneural, classical and mesenchymal protein signatures in WHO 2021 adult-type diffuse lower-grade gliomas

Adult-type diffuse gliomas are incurable primary tumors of the central nervous system characterized by infiltrative growth ultimately leading to the death of the patient. In the latest WHO classification from 2021, *IDH*-mutated tumors are separated from *IDH*-wildtype tumors [72]. *IDH*-wildtype tumors generally show a much more aggressive clinical behavior than *IDH*-mutated [95, 125, 133].

There are, however, differences in how tumors within a certain well-defined group behave in the clinical situation. Therefore, there is a need for additional classification systems that can anticipate if a tumor will behave in an aggressive manner and therefore needs more active treatment. One such system is the Verhaak classification system, which was described for GBM, based on mRNA-expression data [134]. In the clinical situation there is an advantage if such subclassification can be performed with immunohistochemistry. In 2014, Popova *et al* showed that immunohistochemical analysis with five key markers were sufficient to subgroup gliomas into so-called proneural, classical and mesenchymal phenotypes [135]. The clinical implications of this were not further studied.

In this study we evaluated this classification method in a clinical cohort including 183 patients (≥ 18 years of age) with morphological diffuse lower grade gliomas (dLGGs), re-classified according to CNS WHO 2021. In total 183 primary tumors and 49 recurrent tumors were examined. The tumors were re-classified using a combination of histology, immunohistochemistry, FISH, Sanger sequencing and DNA methylation profiling as previously described [136]. Tissue microarrays were constructed and

immunohistochemistry with the five markers performed. The stains were evaluated and annotated in a blinded fashion. The proportion of immune-positive cells for each core was graded on a scale from 0 to 3. Subtyping of the glioma samples into proneural, classic and mesenchymal was preformed according to an algorithm presented by Popova *et al*. We found a significant difference in survival between the different subtypes in the total cohort of morphological dLGGs, but not when we analyzed *IDH*-mutated and *IDH*-wildtype tumors separately. When analyzing tumor relapses, we found that most *IDH*-mutated tumors kept the proneuronal phenotype at recurrence, and *IDH*-wildtype tumors mainly kept or switched to the mesenchymal subtype at relapse.

Limitations of this study are that when working with human evaluation of immunohistochemistry stains there is a certain amount of subjectivity and inter-observer variability involved. Another factor when working with TMAs is the relatively small amount of tissue that is examined. The relatively small patient cohort is another weakness. In this study we focused on a small number of proteins and studied them at the protein level. A broader evaluation at the protein and mRNA level would perhaps give different results.

In conclusion, we demonstrate that the clinical usefulness and prognostic information given by this subtype procedure, in our hands, was not obvious when current relevant molecular subclasses were taken into consideration.

Paper IV: Novel *TPR::ROS1* Fusion Gene Activates MAPK, PI3K and JAK/STAT Signaling in an Infant-type Pediatric Glioma

Central nervous system tumors are the most common solid malignancies in children [137]. Of these central nervous system tumors gliomas is the largest group, accounting for 40 to 50% of the cases. Gliomas can be divided into pediatric high-grade and pediatric low-grade gliomas (pHGG and pLGG). For pediatric low-grade gliomas the prognosis is generally positive with a 10-year overall survival between 70-96%. However, the survivors often suffer from cognitive, neurological, psychological, social, and endocrine complications both related to the tumor and therapy [138]. In pediatric CNS tumors it is common to find chromosomal rearrangements causing fusion genes. The most common is the *KIAA1549::BRAF* fusion detected in the majority of pilocytic astrocytomas (70-80 %) and present in a third of all pediatric low-grade gliomas [139, 140]. There are also other common genetic alterations in pediatric low-grade gliomas such as *FGFR1/2* fusions, other *BRAF* fusions, *RAF1* fusions, and *MYB*/*MYBL1* fusions [141-147]. Most of them activates the Mitogen-Activated Protein Kinase (MAPK) pathway [148]. Some pLGG have fusion genes involving other receptor tyrosine kinases (RTK) such as *ROS1*, *MET*, *ALK* and *NTRK1/2/3* [72, 149, 150]. Recent research indicates that RTK-driven pediatric gliomas have poor outcome compared to the MAPK-driven [149]. Therapies targeting tumors driven by *ROS1*, *ALK* or *NTRK* fusions have been developed with several approved inhibitors such as larotrectinib, lorlatinibe and others [151]. Several ROS inhibitors are in clinical trials for adult patients with non-small cell lung carcinoma [152].

In paper IV we describe a patient with an infant-type pediatric glioma with a novel *TPR::ROS1* fusion gene and describe the molecular events driving tumorigenesis. We aimed to map the downstream pathways activated by this fusion to identify potential therapeutic targets. Fresh frozen tumor and FFPE tumor material was used from both the first surgery and one relapse. Histological stains, immunohistochemistry stains, mutation analysis and *BRAF* fusion analysis were performed. Paired whole genome sequencing on fresh tumor tissue from the first surgery and on normal blood lymphocytes and whole transcriptome sequencing was performed. Furthermore RT-PCR, Sanger sequencing, targeted open-end RNA-sequencing, DNA-methylation profiling and FISH were executed. Human embryonic kidney cells were cultured, transiently transfected with different constructs, and analyzed. Immunohistochemistry stains related to cell signaling pathways were performed.

Whole genome sequencing detected one somatic non-synonymous variant of unknown significance, seven structural variants and many copy number changes. Whole transcriptome sequencing detected two potential in-frame fusion transcripts that matched the structural variants that were identified in the whole genome sequencing analysis; *TPR(4)::ROS1(35)* and *ING5(7)::NFKBIE(2)*. *ING5* and *NFKBIE* are considered tumor suppressor genes and *ROS1* fusion genes oncogenic drivers, hence we focused on the *TPR::ROS1* fusion. The *TPR::ROS1* was abundantly expressed, and RT-PCR verified the junction between *TPR* and *ROS1*. FISH with break apart probes for *ROS1* confirmed the involvement of *ROS1* in a fusion. Targeted open-end RNA-sequencing on tissue from the relapse confirmed the *TPR::ROS1* fusion. Functional analysis using HEK293 cells involving transient transfection with TPR::ROS1 constructs revealed activation of MAPK-, PI3K- and JAK/STAT-pathways. This was confirmed with immunohistochemistry on both primary and relapse tumor tissue.

Limitations of this study are that we only describe and study one tumor case and that we have followed the patient for a limited time. The functional experiments were performed using HEK cells and not cells related to the human central nervous system. We also did not perform any experiments with targeted therapy against the fusion in our study.

In summary we described a novel *TPR::ROS1* fusion gene in a pediatric glioma classified as an infantile hemispheric glioma by methylation-based classification. We also demonstrated that the *TPR::ROS1* fusion activates the oncogenic MAPK, PI3K and JAK/STAT pathways.

Paper V: Formation of ganglion cells in a nodular component of a cystic infratentorial pilocytic astrocytoma carrying *KIAA1549::BRAF* fusion

Pilocytic astrocytomas are the most common tumor in the group *"Circumscribed astrocytic gliomas"* and are in fact the most common childhood brain tumor [72, 137]. In PA, alterations in genes related to the MAPK pathway are found in most cases [147, 148]. Gene fusions involving different combinations of *KIAA1549* and *BRAF* exons are the most frequent rearrangement in pilocytic astrocytomas and *KIAA1549::BRAF* fusion can be found in more than 60% of PA and is especially common in tumors found in the cerebellum [140, 153]. Another much less common fusion found in gliomas is the newly described *PTPRZ1::ETV1* fusion [154]. Gene fusions cannot only be used to strengthen a diagnosis but they, and the signaling pathways they are involved in, can be targeted by therapy. There has been a debate whether neoplastic ganglion cells can be formed in pilocytic astrocytomas.

Gangliogliomas and gangliocytoma are also low-grade primary brain tumors according to CNS WHO 2021 but are sorted in the group *"Glioneuronal and neuronal tumours"*. Gangliocytomas consists of clusters of mature neoplastic ganglion cells [155-160]. These ganglion cells often have dysplastic properties. Gangliogliomas are slow-growing, well-differentiated tumors containing a combination of neoplastic glial and ganglion cells. Both gangliocytomas and gangliogliomas are CNS WHO grade 1 tumors. These tumors can occur throughout the central nervous system even though a majority occur in the temporal lobes. Ganglioglioma can have anaplasia in the glial component including rich mitotic activity, elevated Ki-67 index, microvascular proliferation and necrosis at recurrence but also in material from the first presentation [155, 157, 161-166]. Many of the studies claiming presence of anaplastic ganglioglioma were made in an era where molecular analysis was lacking.

In this study we describe a case of a pilocytic astrocytoma in a 16-year-old patient with a nodular component exhibiting cells with ganglion cell morphology giving this part morphology to some extent imitating a ganglioglioma. We compared this component with areas with more traditional morphology in terms of histopathological stains and immunohistochemistry outcome. We used FISH analysis to demonstrate presence of *KIAA1549::BRAF* fusion in the tumor but also to prove that the cells with ganglion cell morphology truly were neoplastic. Applying DNA methylation profiling on the tumor area

with more traditional morphology with version 11b4 and 12.5 both gave high scores for posterior fossa/infratentorial pilocytic astrocytoma. In the copy number profile numerical changes were seen including focal duplication on chromosome 7, in conjugation with *KIAA1549* and *BRAF*, indicating presence of BRAF-fusion. DNA methylation profiling of the area containing ganglionic cells gave scores with match for "control tissue, reactive tumor microenvironment" with both version 11.b4 and 12.5.

Mutation analysis on the DNA and RNA level verified *KIAA1549::BRAF* fusion transcript in both components. A *PTPRZ1::ETV1* fusion transcript was also detected in the sample with classic morphology. This could not be verified when repeated with another extraction method, whole genome sequencing or FISH. In the analysis of tumor tissue not containing cells with ganglion cell morphology a *PTPRZ1::ETV1* fusion transcript was also detected. Whole genome sequencing also detected the KIAA1549::BRAF fusion.

Limitations of this study is that we only describe and study one tumor. This makes it impossible to say anything about the frequency of the described morphology pattern. There is also uncertainty how great the risk for malignant transformation in gangliogliomas is, which makes clinical implications hard to assess. We were not able to confirm the *PTPRZ1::ETV1* fusion. The patient was followed for a limited time span.

In summary we demonstrate a case with a nodular component with a mixture of astrocytic and ganglion cell-like morphology. We showed that these cells were truly neoplastic and not included nerve cells. This supports the view that ganglion cell morphology can exist in pilocytic astrocytomas. The distinctive morphology can be a phenomenon related to atypical local fluid circulation.

CONCLUDING REMARKS AND FUTURE PERSPECTIVE

In the future we foresee a continued rapid evolution in the field of neuropathology. Deeper understanding of the genetic changes causing CNS tumors will continue and the diagnosis that solely are based on histology and immunohistochemistry will gradually decrease. There will probably also be a further subdivision of tumor entities that today are looked upon as one entity. There will likely also be a continued harmonization between the CNS WHO classification system and the epigenetic methylation array-based system. The continuous need for an increase in sequencing capacity in the healthcare system will be a huge challenge both economically and when it comes to the requirement of human labor. It will be very important to evaluate the effectiveness of adding broad genetic testing both in economical and medical perspective [167-170].

In the future more focus will probably be put on the tumor proteome profile, i.e. all the proteins that is expressed by a tumor [171, 172]. Mass spectrometry-based proteomics has so far not been established as a clinically used tool as genome and transcriptome profiling methods have but the technical development in this field is progressing very fast and will probably in a near future be added to the now established DNA- and RNA-based methods [173-175]. Attempts to apply mass spectrometry-based protein profiling in the field of brain tumor biology has been made for some time [176, 177]. In recent years this field has rapidly progressed and has to some extent already been introduced in clinical work [178-181]. Profiling based upon proteomics will probably be a supplement, and not a replacement, to DNA and RNA-based methods.

Methylation array-based profiling will continue to develop on multiple levels. Faster and cheaper laboratory processes and equipment will probably make intraoperative diagnosis not only possible but perhaps standard of care [182-184]. Hopefully development of the method will make it less dependent on tissue with high tumor cell content. Since many primary CNS tumors are diffusely infiltrative this is an important factor. If a biopsy is taken from the periphery of a tumor, the tumor cell content can be very low. Hopefully there will also be technical progress involving development of chips with fewer and greater number of samples than the today used 8-well chip, making the analysis procedure more flexible.

Histology is an old hand cheap method that can be performed without advanced equipment and is therefore also feasible in developing countries. Immunohistochemistry is also a quite simple method that can be performed in many third world countries. Methods that demand sequencing of DNA or RNA or mapping of epigenetic patterns or profiling of the proteasome are on the contrary dependent on very expensive equipment. The methods are also very time-consuming, and interpretation of the results requires very competent staff. The new methods therefore might introduce a new injustice between patients living in countries with well-developed compared to patients in countries with less developed healthcare systems. On the other hand, the very rapid technological development in the field will probably reduce the cost for these methods and the equipment needed tremendously [185-187]. This can then open an opportunity for also making these advancements beneficial for the population in poorer countries [188, 189]. Internet and the possibility to reach cloud-based services can also be a way to catch up and instead equalize prerequisites between developed and developing countries.

Another area that I anticipate rapid and important development is in the field of liquid biopsies. This is today mostly performed on blood samples [190-192]. Research is also performed to evaluate the technique in cerebrospinal fluid [193-196]. To make a diagnosis by analyzing circulating tumor DNA without the need of tissue biopsy is a tempting approach. Circulating tumor DNA from the cerebrospinal fluid can potentially not only be used for characterization of a tumor but hopefully also to monitor tumors over time [197-199]. Hopefully, whole-genome methylation profiling can also be performed on

cell free DNA from the cerebrospinal fluid samples [200, 201]. Careful evaluation of these techniques when it comes to sensitivity and specificity will be very important. There is ongoing research on methods to improve the sensitivity of the technique so that diagnosis potentially also can be made on blood samples [202]. In a more distant future noninvasive techniques, such as radiomics and radiogenomics, where advanced imaging is combined with artificial intelligence, might be able to make a reliable diagnosis without the need of tissue or DNA [203].

There are already indications that artificial intelligence-based image analysis can detect cancer with high accuracy and efficiency and reduce interobserver variability in the field of prostate cancer [204]. It also seems to be possible to work with other tumors such as breast cancer [205]. Applying artificial intelligence-based image analysis on non-epithelial tumors such as gliomas will probably be a much more difficult task, but attempts are already made, and it is probably only a matter of time before such diagnostic platforms can be applied in the clinic [206, 207]. The best results will probably be achieved when a combination of histomorphology, radiology, genetic, epigenetic, and clinical data are put together in an AI based system.

An advancement that probably will be achieved more closer in time is improvements in the ability to perform large-scale sequencing, such as whole genome sequencing, using formalin-fixed paraffin-embedded tissue [208, 209]. This would be an important improvement in clinical practice as today large-scale sequencing to a large extent demands fresh frozen tissue [210]. This introduces logistic and practical problems. Fresh tissue must quickly be frozen or put in RNA-later to not be degraded. This hinders traditional histopathological diagnosis on that specific specimen. One also does not know if the analyzed tissue is representative of a tumor or if it only contains reactive, non-invaded, surrounding tissue. Now, there is often a dilemma on how much tissue one dares to prioritize for molecular diagnostics without jeopardizing a basic histopathology diagnosis.

The future will probably bring many new opportunities in ways that we are not able to foresee.

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