Chapter 1

Introduction and outline of thesis

1.1 Introductory résumé
1.2 Genetics and pharmacogenomics of diffuse glioma
   Pharmacology & Therapeutics (2013)
1.3 Outline of thesis captured in four questions
1.4 Abbreviations
CHAPTER 1.1 INTRODUCTORY RÉSUMÉ

Cancer is a disease caused by alterations in DNA and disturbance of associated processes. This thesis concentrates on cancer of the brain, and in particular on low-grade glial neoplasms. Brain tumors of this type originate from the supporting tissue of the central nervous system. The thesis commences with a review of the implementation of molecular markers in oncology in general, and continues to define glioma subtypes, address developments in molecular analysis and present the state of the art molecular applications in diffuse glioma in 2013 (chapter 1.2). The glial tumor ‘canvas’ lead us to draw up four questions listed in chapter 1.3 and tackled in chapters 2 to 5 of this thesis ‘molecular characterization of low-grade glial neoplasms’. The discussion in chapter 6 seeks for the answers to these questions integrated with more recent insights published by other groups, which is followed by a future perspective.
CHAPTER 1.2 GENETICS AND PHARMACOGENOMICS OF DIFFUSE GLIOMA

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ABSTRACT
Rapidly evolving techniques for analysis of the genome provide new opportunities for cancer therapy. For diffuse glioma this has resulted in molecular markers with potential for personalized therapy. Some drugs that utilize pharmacogenomics are currently being tested in clinical trials. In melanoma, lung-, breast-, gastric- and colorectal carcinoma several molecular markers are already being clinically implemented for diagnosis and treatment. These insights can serve as a background for the promise and limitations that pharmacogenomics has for diffuse glioma. Better molecular characterization of diffuse glioma, including analysis of the molecular underpinnings of drug efficacy in clinical trials, is urgently needed. We foresee exciting developments in the upcoming years with clinical benefit for the patients.
INTRODUCTION
The role of the genetic code, of a patient or a tumor, has been appreciated for a long time as an important factor in efficacy of pharmaceutical agents. Although the terms pharmacogenetics and –genomics are used interchangeably in literature (1), we refer to pharmacogenetics as the constitutional chromosomal variations and mutations that can influence metabolism of drugs. Pythagoras already noted in 510 B.C. that ingestion of fava beans was lethal to only some individuals; if the fava beans would have been eaten for medical purposes, we would categorize this congenital trait here as pharmacogenetics. Pharmacogenomics is the discipline that comprises all therapies which exploit information of somatic, molecular alterations of the cancer genome. Pharmacogenomics gained enormous interest in the past decades and is the focus of this review. Specific molecular alterations can serve as markers for survival, called prognostic marker or for response to treatment (predictive). In personalized therapy a tumor is tested for the presence or absence of such a marker which enables the clinician to decide on the right timing of treatment and, when indicated, to prescribe a drug that targets this specific alteration. Drugs are often designed to target pathways in which these alterations act. Not all pathways are well-defined and ‘accidental’ discoveries may also result in the identification of a relation between a molecular alteration and increased sensitivity to a specific agent, while the biological interaction is not (yet) explained. In other scenarios, a drug is known to target molecular features, but the specific marker needed to select patients that are sensitive to this treatment has not been identified. We still consider these drugs to have pharmacogenomic features, but true personalized therapy is performed when prescribing drugs on the basis of genomic information of the tumor of an individual patient (companion diagnostics).

In recent years, several drugs with pharmacogenomic features have been designed, most of which belong to the family of kinase inhibitors. More than 500 kinases have been identified; among other functions, these cellular proteins mediate transduction of signals that influence proliferation, migration and survival of (cancer) cells (2). Although the success rate of agents that target these kinases varies, they are promising compounds as the knowledge on pathways involved in tumorigenesis rapidly increases. At present, the possible therapeutic effects of drugs targeting these kinases have already changed experimental approaches considerably. There is a diversity in markers for tumor (sub) types; in addition to classic genomic alterations, markers can be of epigenetic or non-coding RNA origin.
PHARMACOGENOMICS: BOOSTED BY THE NEW GENERATION OF LABORATORY TECHNIQUES

When genomic applications in clinical care are discussed, a short description on the technical aspects is required to comprehend the rapidly growing potential as well as the limitations of the genetic code as a diagnostic tool. In the 1950s, after the fundamental discovery that normal human cells contain 46 chromosomes (3), chromosomal alterations were detected using karyotyping. Karyotyping enables identification of gross chromosomal abnormalities in diseases such as cancer and congenital disorders. A limitation of this early cytogenetic technique was the need for chromosomes in metaphase (dividing cells) and the inability to detect small aberrations. It took 20 more years before substantial improvement was achieved through the introduction of fluorescence in situ hybridization (FISH) (4). FISH uses chromosome region-specific probes with a fluorescent signal. These probes hybridize to the complementary DNA-sequence in the sample, and then the presence and number of DNA molecules in the specific region in the tumor can be analyzed. However, with this technique only one or few chromosomal locations could be studied in one experiment. In the 1990s comparative genomic hybridization (CGH) was developed which offered a much higher spatial resolution compared to karyotyping for the analysis of chromosomal aberrations on a genome-wide basis. With CGH, signal of fluorescently labeled patient-tumor DNA (e.g. red) is compared to fluorescently labeled normal human DNA (e.g. green). The ratio of these signals was translated to copy number changes for each location yielding a resolution of 5-10 Mb (5;6). The technique was soon replaced by array CGH (7) to yield ever increasing resolution (8). Array CGH was still limited to assessment of numerical copy number changes alone, omitting detection of balanced translocations and (point) mutations (9).

Since 1977, mutations in genes of interest have been analyzed by Sanger sequencing. Often, these techniques were used in complementary fashion; array CGH to detect chromosomal aberrations, while genes within regions of interest were analyzed one-by-one by sequencing analysis. Next-generation sequencing (NGS), or massively parallel sequencing (MPS) is the new kid on the block in genome research. This technique allows high resolution analysis of the entire genome of multiple samples in one experiment whilst yielding chromosomal translocations, copy number measurements and point mutations. MPS has shown to work robust in many laboratories on DNA isolated from routinely collected clinical material (usually formalin fixed paraffin embedded, (FFPE)) (9;10). A major challenge of this technique is to cope with the enormous volume and complexity of data it provides. The constant and dramatic drop in costs for this technique (11) combined with the development of application to FFPE samples are now making MPS accessible for implementation in the routine diagnostic setting (Figure 1).
MOLECULAR MARKERS THAT INFLUENCE CLINICAL DECISION-MAKING IN MEDICAL ONCOLOGY

Cancer researchers and scientists at pharmaceutical companies are currently interested in the development of companion diagnostics: the detection of markers for clinical or therapeutic decision-making. Ideally, this enables unequivocal stratification of patients and selection of the proper medication and dosage. The presence of several markers already influences clinical decision-making in medical oncology, e.g. ERBB2(17q21-22) amplification in breast cancer, EGFR(7p11) mutations in non-small cell lung carcinoma (NSCLC), KRAS(12p12) mutations in colorectal carcinoma (CRC), c-KIT in gastrointestinal tumors (GIST), and BRAF mutation in melanoma (12). Here, we highlight the corresponding agents, because their discoveries are prime examples of personalized therapies with pharmacogenomics features.

With respect to breast cancer, the ERBB2 gene encoding HER2 (human epidermal growth factor Receptor 2) is amplified in 20% to 30% of all breast tumors. The monoclonal antibody trastuzumab inhibits proliferation of HER2 positive cancer cells by selectively blocking the receptor and is in use for early and metastatic breast cancer. Trastuzumab was approved by FDA in 1998 and is since 2010 also in use for gastric cancers showing HER2 overexpression (13-15). It took seven more years before gefitinib, a ´small molecule´ EGFR tyrosine kinase inhibitor (EGFR-TKI) (16) was introduced for treatment of advanced NSCLC with EGFR-mutation. In CRC, KRAS mutation is highly predictive for response to antibodies such as panitumumab or cetuximab. Only patients with EGFR-mutated tumors not bearing KRAS mutation seem to benefit from treatment with these agents (17). Another example of pharmacogenomics in personalized cancer therapy is the small molecule kinase inhibitor imatinib which is effective in chronic myeloid leukemia.

Fig. 1. Example of a chromosomal copy number profile of a low grade oligodendroglioma with complete 1p/19q co-deletion, and with additional (partial) losses of chromosome 4, 9p, 13q and 20p. This profile is created using DNA isolated from formalin fixed paraffin embedded tumor tissue, using whole-genome-shallow-MPS (Illumina Genome Analyzer IIx; Illumina Inc), 0.2 fold coverage; the y-axis represents normalized log 2 read counts (number of sequence tags) per 50 kb bins. On the x-axis, bin values ordered by genomic position.
(CML) patients with a BCR-ABL translocation (18). Due to high homology between
binding sites, c-Kit mutations in GIST can also be targeted by imatininb. Recurrence-free
survival in GIST-patients improved significantly after the introduction of imatinib (19;20).
More recently vemurafenib was introduced for treatment of metastatic melanoma. This
BRAF inhibitor targets the pathway affected by BRAF V600E mutation which results
in transient remission and prolonged progression-free survival in these patients (21).
These promising developments set the stage for application of pharmacogenomics in
other cancer types.

THE NEED FOR MOLECULAR MARKERS OF DIFFUSE GLIOMA

Diffuse glioma are the most frequent primary tumors of the central nervous system.
Diffuse glioma are thought to originate from (progenitor) glial cells and are, in contrast
to other glioma variants, characterized by diffuse infiltrative growth. Current WHO
classification of diffuse glioma relies on the microscopic analysis of tumor material.
Three malignancy grades (WHO grade II, III, IV) and three subtypes (oligodendroglioma,
astrocytoma, oligoastrocytoma) are defined (22)(Table 1). WHO grade II glioma are
denominated as low grade glioma (LGG), while grade III and IV tumors are referred
to as high grade glioma (HGG). The most malignant glioma, WHO grade IV glioma or
glioblastoma (GBM), is also the most frequently occurring. A distinction can be made
between primary and secondary GBMs; the first manifest themselves from the beginning
as high-grade tumors, whereas secondary GBMs derive via malignant progression from
lower grade diffuse glioma. Primary GBMs have a molecular profile that distinguishes
them from other glioma subtypes (23). WHO grade II and III astrocytomas and
oligoastrocytomas often also progress to a WHO grade IV glioma, then called secondary
GBM (22). Despite this well-documented distinction between subtypes and grades,
diffuse glioma are considered to belong to a spectrum of tumors with overlapping
histopathology and inter-observer variability among neuropathologists can be high.
Still, a higher WHO malignancy grade is strongly related to shorter survival, while within
the group of WHO II and III glioma, tumors with oligodendrogial features tend to have a
better prognosis than their astrocytic counterparts. However, patients with similar WHO
classified glioma might still show a wide variation in survival times; for instance, survival
of patients with a WHO grade II glioma varies from a few months to two decades.
Thus, the current histopathology based WHO classification does not always allow for
unequivocal clinical decision-making.
Table 1: Schematic representation of the different classes of diffuse gliomas as defined by histopathological assessment of subtype (oligodendroglioma, oligoastrocytoma, astrocytoma) and malignancy grade. LGG=Low Grade Glioma, HGG= High Grade Glioma.

Current optimal treatment for glioma patients consists of resective surgery, and postoperative irradiation and/or chemotherapy in most cases. Up till now, choice and timing of therapy rely on the histopathological (WHO) diagnosis combined with the overall clinical course and radiologic features in an individual patient. The clinician does not want to postpone necessary interventions, while premature treatment might lead to serious cognitive deficits (irradiation) or serious complications due to chemotherapy in the short and long-term (24). An even more serious problem is that glioma will not be cured by either of these therapies; almost all patients eventually die of their tumor. This implicates that a better distinction of diffuse glioma subtypes is urgently needed for a more optimal guidance of the current treatment options, and for development of new treatment strategies. In the light of these facts, it is important that molecular markers are identified and evaluated for their potential use in diagnostics with prognostic and/or predictive value. While prognostic information may aid in timing of treatment, predictive markers may help in making a choice of particular therapeutic regimens and are even more desirable. Genome techniques are likely to provide such molecular markers. Using data from The Cancer Genome Atlas (TCGA), a gene expression-based molecular classification was proposed, stratifying GBM into four distinct molecular subtypes with prognostic value (25;26). Although it is not clear if such an RNA based classification should be introduced into clinical practice, it does provide an example of the possibilities molecular markers may have for diffuse glioma.

PROMISING MOLECULAR MARKERS OF DIFFUSE GLIOMA

Diffuse glioma belong to a spectrum of morphologies from pure oligodendroglial to pure astrocytic features. With some overlap, molecular markers within this spectrum fit features of one of these histological subtypes. Mixed (i.e. oligoastrocytic) diffuse glioma generally resemble one or another. Here, we provide a concise summary of promising markers (Figure 2) and discuss their (future) role in companion diagnostics for diffuse glioma patients.
a. Copy number aberrations

Copy number aberrations (CNAs) occur when cell division errors, causing a surplus or shortage of a part of a chromosome. Deletions in tumors may unmask genes involved in tumor suppression, whereas gains may point to oncogenes involved in tumor initiation or progression. As it is almost impossible to mention all CNAs throughout the genome and differences between diffuse glioma subtypes, we highlight only the most frequent or prognostic CNAs, at chromosomes 1, 7, 9, 10, 13, 19 and 22 in table 2. Focal CNAs of the EGFR pathway and the co-deletion of 1p/19q are discussed separately below. In general, progression from low to high grade glioma can be recognized in consecutive chromosomal profiles (of repeated surgeries of one glioma patient) as additional CNAs are observed (27). Still, there is no direct relation between number of CNAs and malignancy grade. Additional CNAs can represent ‘passenger’ alterations and do not contain driver genes per se.

<table>
<thead>
<tr>
<th>CNA</th>
<th>Comments</th>
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<tbody>
<tr>
<td>Partial deletion 1p</td>
<td>Related to short survival in astrocytic tumors (42)</td>
</tr>
<tr>
<td>Gain 7q</td>
<td>Most commonly altered chromosome arm in astrocytic tumors</td>
</tr>
<tr>
<td></td>
<td>In primary GBMs often accompanied by amplification of EGFR (44)</td>
</tr>
<tr>
<td></td>
<td>Also present in subset of LGGs, but then without amplification of EGFR (45)</td>
</tr>
<tr>
<td></td>
<td>In oligodendrogial tumors related to short survival, usually without concurrent 1p deletion (46)</td>
</tr>
<tr>
<td>Deletion 9p</td>
<td>Common in anaplastic gliomas and GBMs (47)</td>
</tr>
<tr>
<td></td>
<td>Impaired functioning of TSGs on 9p21 such as CDKN2A, CDKN2B and p14ARF (44;48)</td>
</tr>
<tr>
<td>Deletion 10/ deletion 10p / Deletion 10q</td>
<td>Common in primary GBMs, often accompanied by PTEN loss</td>
</tr>
<tr>
<td></td>
<td>Related to shorter survival in LGGs, anaplastic gliomas and primary GBMs Houillier 2010 Neuro Oncol (44;49)</td>
</tr>
<tr>
<td></td>
<td>Involvement of 10q25-qter associated with progression (23)</td>
</tr>
<tr>
<td>Deletion 13q</td>
<td>Typically includes the locus of RB1 (50)</td>
</tr>
<tr>
<td>Deletion 19q</td>
<td>Associated with tumor progression to secondary GBM (in tumors without concurrent 1p loss) (50)</td>
</tr>
<tr>
<td>Deletion 22q</td>
<td>Common in astrocytic gliomas, including secondary GBMs (82%), less common in primary GBMs (6%); 22q12.3-13.2 and 22q13.31 often involved, including TSG RRP22 (51).</td>
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Table 2: Frequent chromosomal copy number aberrations(CNAs) detected in diffuse glioma.

GBM=glioblastoma, LGG= Low Grade Glioma, HGG= High Grade Glioma, TSG= Tumor Suppressor Gene, LOH= Loss Of Heterozygosity, qter= terminal end of q arm.

Epidermal growth factor receptor (EGFR) is involved in the EGFR/PTEN/Akt/mTor pathway. Focal CNAs, such as deletion of PTEN (10q23) and amplification and over-expression of EGFR result in glioma cell proliferation, migration and inhibition of apoptosis (23). The deletion that results in EGFRvIII (variant 3), is the most commonly mutated EGFR
amplicon in GBMs. EGFRvIII expression is associated with increased mitotic activity and tissue invasiveness. Alterations in EGFR (60%) or PTEN (40%) are typical for primary GBMs, and rare in WHO grade II and III glioma and secondary GBMs. In fact, EGFR status can be helpful for the pathologist to distinguish anaplastic astrocytomas from primary GBMs in histological borderline cases. The prognostic impact of EGFR-pathway alterations and of EGFRvIII expression within the group of primary GBMs are not clear (28-30).

Co-deletion of 1p/19q is a hallmark of the typical oligodendroglioma, i.e. diffuse glioma showing the histological ‘halo’/fried egg -phenotype (round nuclei and clear perinuclear halo). The mechanism of this co-deletion is unbalanced translocation of t(1;19)(q10;p10)(31) and is generally accepted as a positive prognostic indicator in LGG. Furthermore, 1p/19q co-deleted WHO III oligodendroglioma are also more sensitive to chemotherapy and irradiation (32-35). This information can help clarify whether a histologically hybrid oligoastrocytoma will behave like an oligodendroglioma, which is prognostically more favorable, or as an astrocytoma. 1p/19q co-deletion occurs in 50-80% of oligodendroglioma, in 30-50% of oligoastrocytomas, and in only 10% of astrocytomas. Several studies have been performed to find driver genes that are involved in this co-deletion (36;37). Recently, mutations in the genes FUBP1 (1p31.1) and CIC (19q13.2) were identified. The frequency of mutations in CIC in 1p/19q co-deleted glioma is highest in oligodendroglioma and is, though at a lower frequency, also present in oligoastrocytic and astrocytic tumors bearing deletion of these two chromosome arms. Mutations of FUBP1, involved in regulation of the oncogene c-Myc, occur less frequent (38). The prognostic value of these genes has not been consolidated yet due to small study cohorts (39;40). Silencing of the pH regulatory protein NHE-1, has also been related to the distinctive and prognostically favorable biology of oligodendroglioma. The resulting neutralization of acidosis possibly prevents tumor growth (41). Of note, in some glioma only parts of these chromosome arms are deleted, which relates to a worse prognosis compared to LGGs not bearing CNAs at 1p/19q (42). Also, co-occurrence of deletions of 1p/19q and 10q is related to a worse prognosis compared to those diffuse glioma not bearing any of these three CNAs (43). It may very well be that in these tumors there is no true ‘whole-arm’ 1p/19q co-deletion, but again only partial deletions. Ideally, a molecular diagnostic tool should be able to verify a complete versus a partial deletion of both arms.

b. **Pivotal genes and epigenetic features**

This paragraph lists promising and well-established markers, which have high potential to serve purposes such as diagnosis, establishment of prognosis and prediction of therapy response (Figure 2). Similar to CNAs and genetic mutations, alterations in
epigenetic regulation can lead to tumor development and progression. In addition, various types of interactions between genetic and epigenetic alterations have been revealed (52). DNA methylation and posttranslational histone modification are the two most common epigenetic phenomena affecting DNA replication, transcription and repair. Methylation is the process of adding a methyl group to cytosine to create 5-methylcytosine. Altered promoter methylation results in changed gene expression. In tumorigenesis and tumor progression, a change in promoter methylation can affect activity of oncogenes and tumor suppressor genes. This area of interest provides new insights in gliomagenesis, meanwhile it adds to the ever-increasing complexity of our understanding of tumorigenesis. Also, future classification of diffuse glioma could incorporate epigenetic features (53). For example, diffuse glioma bearing an 1p/19q co-deletion have a distinct methylation profile (54).

RB1 is a possible survival marker in diffuse glioma; survival was significantly shorter in two studies of anaplastic astrocytomas and GBM-patients with a disrupted RB1 pathway (55;56). RB1 protein (13q14-13q32) is involved in the tumor-related RB1/p15INK4b/p16INK4a pathway. In several integrative genome analyses of diffuse glioma this pathway was consistently related to regulation of gliomagenesis (57-60). The function as a tumor suppressor gene is lost by promoter methylation (61) and disruption of the pathway results in uncontrolled cell growth. When taking into account all members of the pathway, primary and secondary GBMs are equally affected. The few LGGs that show alterations in the RB1 pathway generally lack 1p/19q co-deletion, IDH1/2 mutations and TP53 mutations (so-called triple-negative) and are reported to carry an unfavorable prognosis (62;63).

TP53 is one of the most frequently mutated tumor suppressor genes in various cancer types. We refer to three excellent reviews for further reading on TP53 mutation and its function in diffuse glioma (48;64;65). Methylation of the promoter of TP53 results in reduced TP53 expression in all three subtypes, i.e. astrocytomas, oligodendroglioma, and oligoastrocytomas. In a study of 109 LGGs TP53 promoter methylation was detected in 60% of samples, almost equally distributed over the three main subtypes (66). This suggests that, although TP53 mutations are primarily present in astrocytomas, TP53 function can be affected in other subtypes through epigenetic changes. When taking into account the other genes which act in the TP53/MDM2/p14ARF pathway and epigenetic deregulation, nearly 100% of secondary GBMs show alterations in one of these ‘teamplayers’ (67;68). Primary GBMs show a much lower rate of TP53 mutation (28%) and this is thought to represent general genomic instability (23). TP53 mutated low grade diffuse glioma are related to a worse prognosis (69). Currently, IDH1 (2q32) and IDH2 (15q21) mutations are hot topics in glioma research and diagnosis. IDH1 wild-type functions in the citric acid cycle, IDH1 mutation affects angiogenesis, glucose metabolism and developmental apoptosis. Altered IDH1 is an early marker
of gliomagenesis and mainly detected in grade II and grade III glioma and secondary glioblastomas (70;71). IDH2 mutations are less common than IDH1 mutations and most often occur in low-grade oligodendroglial tumors (72). Moreover, altered IDH1 status affects the epigenetic landscape, and may result in the glioma CpG-island methylator phenotype (G-CIMP) (73). This phenotype is characterized by a genome-wide change in methylation causing chromosomal breakages and copy number aberrations. G-CIMP is frequent in astrocytomas, oligodendroglioma and oligoastrocytomas as well as in secondary GBMs, but rare in primary GBMs (54). The phenotype is already present in LGGs and stays stable throughout progression (52). This molecular subtype strengthens general opinion that IDH1 mutation correlates with a lower degree of molecular malignancy and favorable prognostic significance (27;74). IDH1 serves as an example for the possible interaction between genetics and epigenetics. This relation may be present for other markers as well and could lead to discovery of mechanisms that play an important role in the genetics, and possibly pharmacogenomics, of diffuse glioma. Finally, approximately 80% of all primary glioblastomas show global hypomethylation (Figure 2). This alteration promotes proliferation and the degree of hypomethylation is correlated to the rate of genomic instability and prognosis. For example; the oncogene MAGEA1 (Xq26) is reactivated only in those GBMs with <50% methylation compared to normal brain tissue. MAGEA1 plays an important role in tumorigenesis and treatment efficacy; activation results in inhibition of p53 function and diminishes response to chemotherapy (75). In contrast to the G-CIMP group, patient survival is shorter in hypomethylated glioma (76).

In 2005, O6-methylguanine-DNA methyltransferase (MGMT) was introduced as a very promising epigenetic marker in diffuse glioma (77). The discovery resulted in a large number of studies dedicated to this marker, with variable success. In its natural habitat, MGMT (10q26) acts as a repair enzyme and protects cells from mutagenic effects. Hypermethylation of the promoter of MGMT has a silencing effect and enhances efficacy of alkylating agents such as temozolomide (TMZ) as well as increased sensitivity to irradiation (although to a lesser extent) (64;65;78). Positive treatment effects have been reported in both low- and high-grade diffuse glioma where MGMT was silenced by hypermethylation (77;79-81). The exact mutational effect of radiotherapy is unclear, but one could hypothesize that damaging effects are sustained when the DNA repair system is impaired. However, survival advantages can also be explained by other prognostically favorable genetic alterations that often co-exist with MGMT promoter methylation, like 1p/19q co-deletions and IDH1 mutations (82). Compared to 1p/19q co-deletion, less ‘strong’ evidence has been collected for the value of MGMT in the clinical setting, despite the relatively long time since discovery and the large numbers of studies dedicated to
this topic. However, recent insights are promising; in 2012 results of a German phase III trial emphasized the importance of using MGMT as a predictive biomarker. In this study, MGMT methylation was related to longer overall survival in elderly patients treated with TMZ, compared to those patients with non-methylated tumors and compared to the use of irradiation in patients with methylated tumors (79).

Another epigenetic mechanism is histone modification. Histones associate with DNA to form nucleosomes. These nucleosomes represent repeating units in chromatin. Post-translational histone modification affects chromatin structure, and results in altered binding of effector molecules and changes in gene transcription. Genes affecting histone post-translational modifications are gaining interest in literature. Exciting is a recent paper that points out the first cancer-related recurrent mutation in a histone itself; the mutated histone H3F3A exerts its effect across the genome and results in alternative

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**Fig. 2. Schematic representation of genomic alterations in diffuse gliomas.** Early events in LGGs and additional markers detected in the course of molecular progression to HGGs are depicted separate from alterations found in primary GBMs. * Especially detected in classic oligodendroglial tumors. ** Especially detected in astrocytic tumors. *** MGMT methylation has positive predictive value in gliomas treated with chemotherapy or irradiation. LGG=LowGrade Glioma, HGG=High Grade Glioma, CNAs=chromosomal Copy Number Aberrations. Other abbreviations are explained in text.
chromatin remodeling in primary GBMs of children and young adults (83). The types of histone modification are variable and a few mutations in genes encoding proteins involved in this type of epigenetic regulation, such as histone deacetylases (HDAC) in diffuse glioma, have been identified in diffuse glioma. During progression to a higher malignancy grade, expression levels of various HDACs change (52). The prognostic value of these alterations has not been fully elucidated yet, but down-regulation of the RRP22-gene via hypermethylation and histone modification has been related to a worse prognosis in GBM patients (51).

c. miRNAs
The final set of molecular markers that is reviewed here is the class of non-coding RNAs, of which microRNAs (miRNAs) have so far received most attention (84). These small, ~22-nucleotide-long, single stranded, RNA molecules regulate translation and degradation of target messenger RNAs and are thought to control approximately 30% of human genes. Over 1,000 miRNAs have been identified in the genome and most are located in tumor-associated regions. Apart from that, many more non-coding RNAs have been identified (84). The importance of miRNAs was exemplified by a publication in 2005, where the authors state that cancer cells show overall lower miRNA expression compared to normal tissue. Moreover, they emphasize that poorly differentiated tumors of histological uncertain origin can be classified by miRNA expression patterns (85). miRNAs have the ability to promote tumorigenesis; the loss of tumor suppressive miRNAs enhances the expression of target oncogenes, whereas increased expression of oncogenic miRNAs (oncomiRs) can repress tumor suppressor genes (86). In glioma, several miRNAs are active. Here, we will list a few of them. The first miRNA to be identified as a potential oncomiR in glioma is miR-21 (87-89). miR-21 was found to be highly expressed in GBM and its inhibition lead to reduced migration and invasion by targeting inhibitors of metalloproteinases (88-90). The tumor suppressor miR-101 was recently discovered to be downregulated in GBM. miR-101 is responsible for the translational repression of the Polycomb group (PcG) protein EZH2, implicated in bone morphogenetic protein signaling, which is important in the differentiation capacity of glioblastoma cells (91). Increased expression of EZH2 has been shown to correlate with glioma grade and recurrence (92;93). Also miR-10b has been proposed as a global regulator of glioma cell proliferation and glioma cell death, by repressing the cell cycle-related genes BCL2L11/Bim, TFAP2C/AP-2γ, CDKN1A/p21, and CDKN2A/p16 (94). The level of miR-128 is reduced in glioma cells and related to up-regulation of the oncogene Bmi-1. This can promote tumorigenesis via an increase in histone methylation and upregulation of Akt phosphorylation and p21 levels (95). miR-17 and miR-184 are associated with progression of low-grade astrocytic tumors to secondary GBM (96).
In addition to oncomiRs and tumor suppressive miRNAs, angiomiRs are also involved in glioma. AngiomiRs were shown to influence the angiogenic capacity of the glioma endothelial cells \textit{in vitro} and \textit{in vivo}, such capacity was first described for miR-296 in glioma-associated endothelial cells (97). Interestingly, it was reported that miR-101 is also down-regulated in primary endothelial cells exposed to vascular endothelial growth factor (VEGF) or glioblastoma cells, and in blood vessel endothelial cells isolated from glioblastoma patient samples (93). Thus, miR-101 can also be assigned to the class of angiomiRs.

In the context of pharmacogenomics, several \textit{in vitro} studies reported on the role of miRNAs in the effect of TMZ in glioma cells. For instance miR-21 was reported to sensitize U251 cells to TMZ (98), but miR-21 was also described to block TMZ-induced apoptosis in U87 glioma cells (99). In addition, miR-195, miR-455-3p and miR-10a are reported to be implicated in acquired TMZ resistance (100). Another miRNA, miR-181d was described to predict TMZ response and to downregulate MGMT (101;102), and miR-125b-2 caused resistance to TMZ in glioblastoma stem cells through the mitochondrial pathway of apoptosis (103). Meanwhile, clinical utility of miRNAs in glioma pharmacogenomics remains to be shown.

\section*{PHARMACOGENOMICS: GENERAL CONSIDERATIONS}

Current opinion is that it is impossible to cure all cancers, but changing the lethal ones to a chronic condition would mean a huge leap forward. Unfortunately, the road to effective personalized therapy is hindered with various obstacles. Using companion diagnostics to select the right patients is essential in this era with rapidly increasing opportunities for more personalized therapy (9). Essential features of such a diagnostic tool are accuracy (including reproducibility and robustness) and speed. Massively parallel sequencing (MPS) may play an important role in this process (104). Another challenge is to select a biologically active compound that is similar to an endogenous ligand of the target (105). Oncogenes and tumor suppressor genes that are difficult to target directly could possibly be affected through upstream regulator or downstream effector molecules. Discussion on the perfect strategy is still ongoing; upstream molecules call for a combination of several drugs to overcome resistance mechanisms, while downstream targets are often not tumor-specific (because not mutated) which explains why these drugs also affect normal cells. The search for new cancer therapies has recently been accelerated by two pharmacogenomic studies by Barretina and Garnett. In these studies large panels of human cell lines (of common tumor types, including GBMs) profiled at DNA, RNA and chromosomal levels were tested for sensitivity and resistance to both clinically available drugs and promising drugs that are in the pipeline. This collection has resulted in the identification of genetic, lineage, and
Drug resistance is an important notion that also applies to pharmacogenomics and has several aspects. First, there can be intrinsic resistance due to congenital differences in drug metabolism. This phenomenon actually is genetic and a concern for all cancer therapies. Second, there is the possibility of acquired resistance, where initially responsive tumor cells present with alternative pathways or additional mutations that promote tumor growth, possibly induced by the harsh environment chemotherapy creates. A third important aspect is intra-tumoral heterogeneity: the presence of multiple, genetically different clones of tumor cells or even stem cells within one tumor which would explain partial response to treatment. Acquired tumor resistance and intra-tumoral heterogeneity are overlapping challenges in tumor treatment. An important pitfall of the use of cell lines in preclinical research is that this setting does not (fully) account for intratumoral heterogeneity. Also, environmental selection forces may induce temporal or spatial changes in characteristics of a gene. Consequently, there is no clear-cut distinction between driver and passenger genes. This adds to the complexity in the design and use of targeted drugs. Multi-drug combinations might limit resistance to a certain extent, but also multiply costs and risks of side-effects. GBMs might very well be the most difficult subtype of diffuse glioma to treat, due to its molecular intra- and inter-tumor heterogeneity. Moreover, phase I and II trials are usually designed for recurrent high-grade glioma where additional alterations caused by previous treatment of TMZ and irradiation, might hamper extrapolation of the results to newly diagnosed tumors. Theoretically, molecular targeting could be very efficient, but response rates in large cohorts are often lower than expected. Trials use large cohorts and are therefore expensive, resulting in high costs of targeted drugs after FDA approval. Currently, most patients are not yet molecularly stratified before enrollment. This ‘trial and error’ approach is only acceptable as long as the marker that predicts sensitivity to a pharmacogenomically acting drug is unknown. Awareness is growing that responsive subgroups in trials should not be overlooked, as drugs could be effective in small subpopulations carrying specific genomic alterations. New agents should be tested in patients with tumors that are likely to respond; i.e. tumors positive for the targeted marker(s). This is essential to bring pharmacogenomics to the personalized therapy-level. Elucidation of the molecular subpopulations defined by functional processes or genomic alterations will improve companion diagnostics. Furthermore, the need for large cohorts for testing efficacy of targeted therapy will be less, thereby reducing costs.
PHARMACOGENOMICS OF DIFFUSE GLIOMA: CURRENT STATE OF THE ART

Although major advances in glioma research have been made, treatment benefits lag behind. Here, agents currently tested in clinical trials for glioma patients are discussed. So far, finding new treatments for HGG and especially GBMs has received most attention. This is explained by a higher prevalence compared to LGG and a more urgent clinical need. Additionally, their short survival has the ‘relative advantage’ that treatment benefits can be evaluated in a short time span.

There are a few ongoing glioma-trials that exploit companion diagnostics. Two markers that bear predictive features for diffuse glioma are used in clinical practice; 1p/19q co-deletion and MGMT status. 1p/19q co-deleted oligodendroglioma are more sensitive to combined procarbazin, lomustin and vincristin (PCV) chemotherapy and irradiation (32;111). The value of this marker is currently further evaluated in two ongoing trials; one focusing on co-deleted tumors (CODEL) and the other on non-co-deleted tumors (CATNON) (112;113). The long-term additive effect of PCV, compared to solely irradiation in treatment of anaplastic diffuse glioma was recently reinforced by results of an EORTC trial that showed positive outcome, especially for 1p/19q co-deleted tumors (114).

Despite the lack of a biological explanation of the 1p/19q co-deletion, evidence for the predictive value is currently much stronger than for MGMT (32-35). The biological mechanism underlying MGMT methylation, however, is more evident than for 1p/19q, since DNA repair is impaired when the MGMT promoter is altered. Irradiation and chemotherapy, particularly alkylating agents such as TMZ, both disrupt the genomic code (115) and these effects are sustained if MGMT does not function properly. Currently, trials are conducted to compare efficacy of TMZ to other agents in HGG with and without hypermethylated MGMT. One of these agents is paclitaxel poliglumex, a member of the taxane family (116). Another promising marker is EGFRVIII. Recurrent GBMs that are EGFRVIII-positive, can possibly be targeted by subcutaneous injections of the vaccine rindopepimut. First results of this phase II trial are expected in 2014 (117).

Other studies fit in pharmacogenomics but do not use companion diagnostics for the presence of a certain molecular marker in advance. Similar to the positive predictive effects of MGMT hypermethylation, drug-based inhibition of DNA repair can enhance toxicity in cancer cells. This concept is called ‘synthetic lethality’ and is based on the assumption that co-occurrence of two non-lethal mutations can result in a lethal situation for cancer cells (118). PARP inhibitors are leading in this field (119); olaparib, which is effective in breast tumors, is tested in diffuse glioma patients (120;121). Many of the markers in GBMs cluster in the p53, RB1 and/or the PI3K/Akt pathway which act downstream of receptor tyrosine kinases (23;70). There is a focus on therapies with an inhibiting effect on tyrosine kinases (TKI) or receptors of kinases (RTKi), because of the key role TKIs play in cancer pathways (122). Such therapies may disrupt signaling...
pathways on which the tumor depends (oncogene addiction) (123). Some agents
directly target VEGF or EGFR in diffuse glioma, such as axitinib and erlotinib (124;125),
which are approved for treatment of SCLC. Others target downstream components,
for example mTOR inhibitors like temsirolimus and everolimus (126;127). The efficacy of
antibodies for VEGF in tumor treatment is evaluated in bevacizumab- and ramucirumab-
trials (128;129).

Another example of pharmacogenomics-driven therapy is enhancing efficacy by
targeting epigenetic features. In contrast to genetic alterations, ‘epimutations’ are
theoretically reversible by drugs. DNA methyltransferases (DNMT) and histone
decaytase inhibitors (HDACi) may have the potential to exert this effect. Only HDACi
are currently tested in diffuse glioma. These drugs act on core histones and non-
histone proteins (130). Thus, their effect is two-fold; first they open chromatin structures
enabling better access for DNA damaging agents, and subsequently they re
verse epigenetic silencing and re activates the cell cycle protein p21 which in turn enhances
cell cycle arrest and apoptosis. The effect of the addition of HDACi such as vorinostat
and valproic acid to regular treatment regimens is currently evaluated in several trials
(131-133). The downside of an epigenetic approach in therapeutics is the current lack
of specificity; unintentional silencing in normal cells and activation of oncogenes are
major concerns for the design of these drugs.

PHARMACOGENOMICS OF DIFFUSE GLIOMA: THE FUTURE

Pathologists first determine the histology in hematoxylin-and-eosin stained sections
and when indicated, request additional immunohistochemical staining or molecular
tests. It is foreseen that when pharmacogenomics gains more clinical significance and
eventually guide clinical decisions, detailed information of the genome sequence data
from MPS will arrive on the pathologists’ desk together with the histological sections.
Molecular markers of interest can be selected by opening a particular virtual hatch door;
a DNA marker which is ready for use in diagnostics. This combination of histology with
the complete genomic picture saves time for the pathologist, and more importantly will
improve diagnostics due to the more comprehensive information on each tumor.

Eventually, molecular markers may serve several purposes both for individual patients
and cancer research in general; they will (1) allow for improved tumor tissue characterization
and development of non-invasive diagnostics that withhold high-burden surgeries, (2)
 improve prognostication and support adequate timing of additional treatment, and (3)
identify the most optimal targets for treatment of the tumor in an individual patient.

The vast majority of biomarkers are currently identified in tumor tissues. Biomarker
discovery can be conducted in numerous types of functional screens (134). However,
the search for biomarkers using non-, or minimally, invasive diagnostics tools (1) is
emerging (allowing for easier and frequent access to biomarkers in e.g. body fluids). These platforms include the detection of circulating tumor cells (135), cell-free nucleic acids (136) or tumor-derived microvesicles (exosomes) (137). Microvesicles are small protein and RNA containing membranous vesicles secreted by a number of cell types (138), and can be isolated from multiple bodily fluids including malignant effusions (139), urine (140) and peripheral blood (141;142). Also glioblastoma cells release microvesicles that can be detected in a non-invasive manner (141;142). Microvesicles isolated from the blood of glioma patients were used to determine the EGFRvIII status of the corresponding tumor (142). Recently, it was shown that blood platelets (thrombocytes) also contain glioma-derived (mutant) RNAs, and mutant EGFRvIII was detected in blood platelets of EGFRvIII-positive glioma (143). Continued research in such non-invasive diagnostics is warranted in order to establish the robustness of such novel biomarker platforms. However, molecular characterization in tumor tissue currently still is the gold standard.

A gene-expression based classifier with prognostic value has already been created for GBMs (25). However, the assessment of prognosis and the timing of postoperative treatment of glioma can still be difficult, in particular for LGGs. 1p/19q co-deletion has prognostic value in oligodendroglioma, but additional markers are required to improve robustness and enable further classification of astrocytomas. Future prognostic classifiers will probably use information on a combination of chromosomal copy number aberrations, mutations in genes, and epigenetic changes, as such a combination provides more insight into the underlying biology of a particular tumor than a single dimension of DNA information. A new diagnostic tool with prognostic value for LGGs, that exploits these three dimensions of DNA, is under construction in our laboratory. We prefer to use DNA over RNA, since DNA tests are better feasible in our experience and can be applied more widely (9).

In previous paragraphs we have already illustrated how identification of pathways has resulted in matching therapies for some tumors (3), unfortunately, progress in glioma treatment is lagging behind. In the nearby future, we expect that tyrosine kinases and antibodies which target specific and well-defined molecular alterations will play an important role in treatment of patients with diffuse glioma. While listing the most promising agents can be compared to gazing into a crystal ball, it may well be that a combination of drugs that e.g. target EGFRvIII and an mTor inhibitor with an epigenetically acting agent has the highest potential. Although beyond the scope of this review, integration with immunotherapy may also prove to be of benefit for glioma patients, an example of which is the use of the EGFRvIII vaccine (144). Surely in the beginning, strategies like these will be supported by conventional treatment such as irradiation and classical chemotherapies. Eventually, however these may be replaced by new treatment options.
In conclusion, we anticipate that future therapeutics for patients with a diffuse glioma will consist of a combination of agents that act on the basis of identification of specific genomic alterations. Introduction of pharmacogenomics in diagnostics will enable the clinician to practice ‘playing chess’ and think a few steps ahead rather than ‘whack-a-mole’. Where in case of tumor recurrence, any time the tumor succeeds in escaping attacks due to tumoral heterogeneity and by developing resistance mechanisms (108). Several ingredients of a successful introduction of pharmacogenomics are already there: the rapidly developing techniques (such as MPS) help designing tools and finding markers (such as EGFRvIII), and an increase in collaborations that allows integrative genomic analysis with validation cohorts and web-based data-networks enabling widely accessible data (e.g. TCGA and AACR-FDA-NCI Collaborative (26;145)). These ingredients can be used to design and optimize personalized strategies for the treatment of diffuse glioma. Design of glioma-specific companion diagnostics will be of great help to the clinician. In the past ten years a considerable part of the genetics involved in gliomagenesis has been unraveled and we foresee high potential for pharmacogenomic applications in diffuse glioma patients. Hopefully, these new insights will soon lead to the development and clinical use of drugs that substantially improve the prognosis for patients with diffuse glioma.
CHAPTER 1.3: OUTLINE OF THESIS CAPTURED IN FOUR QUESTIONS

This thesis addresses four questions that find their origin in the review preceding this chapter (146). Glial neoplasms encompass a wide spectrum of histological morphologies. Molecular analysis may contribute to subtyping glial neoplasms and improve clinical relevance. Particularly the high variability in survival of patients with diffuse low-grade glioma (LGG) requires markers allowing stratification for expected prognosis. Better perception of a patient’s lifetime expectation is not only beneficial for clinical decision making but also reduces the uncertainty for patients. Markers with predictive value could identify tumors sensitive or resistant to a particular therapy. Meanwhile, genetic variability within a tumor, i.e. spatial heterogeneity, is a separate issue, which complicates the interpretation of molecular characterization and therefore should be studied simultaneously.

1. Are there copy number aberrations (CNAs), other than 1p/19q co-deletion, with prognostic value for patients with LGG? (chapters 2 and 3)

The future of patients diagnosed with LGG is very uncertain, because of the wide variability in overall survival. Clinical and histopathological parameters are insufficient to provide for an accurate prognosis, emphasizing a need for additional markers. It has been shown that complete co-deletion of chromosomal arms 1p and 19q is a marker strongly indicative of a favorable prognosis (147). However, it does not suffice for classification of all LGG; even after stratification by 1p/19q co-deletion, a wide distribution in duration of survival is observed in both the group with as well as in the group without this aberration. IDH mutation is strongly associated to favorable survival, but IDH wild type LGG are rarely encountered. Prognostically favorable or unfavorable CNAs could be of additional value to define prognosis and thereby adjust timing and choice of postoperative treatment. For example, irradiation may eventually cause cognitive deficits and should preferably not be applied at an early stage in those patients who are anticipated to survive for a long time (148).

2. What can we learn from spatial and temporal analysis of CNAs in LGG and could these analyses provide insight into evolution to a higher malignancy grade? (chapter 3)

‘Spatial heterogeneity’ in molecular alterations in tumor cells, is a consequence of a variety of subclones within the neoplasm. It is conceivably an important cause of lack of response to chemotherapies; one subclone of tumor cells may be sensitive to a therapeutic agent, whereas other subclones continue to grow or even progress to a higher malignancy grade (149). Detailed molecular analysis of spatially distinct areas in a tumor could reveal the characteristics of these subclones. Combining this information
with analysis of recurrent tumors may expose molecular alterations involved in
tumor progression, i.e. ‘temporal evolution’. Therefore, knowledge of the distribution
of clinically relevant CNAs in time and space in LGG could provide insight into their
notoriously unpredictable behavior.

3. Are DNA repair deficits involved in temozolomide (TMZ)-associated
hypermutation of LGG? (chapter 4)
Various regimens of chemotherapy for patients with LGG are currently under
investigation in clinical trials. We evaluated the effect of TMZ, since it is an effective
part of the standard of care regimen in high-grade glioma and is also frequently
administered to patients with LGG at time of progression. TMZ induces a specific
type of mutations in DNA in order to stimulate apoptosis of tumor DNA. In a subset
of TMZ-treated low- and high-grade glioma, however, an exceptionally high number
of these TMZ-associated mutations has been detected in cells that obviously did not
succumb after treatment (150;151). This observation is referred to as ‘hypermutation’
and, in high- grade glioma, often corresponds with mutation of a gene involved in
DNA repair (150;152;153). Comprehensive study of DNA repair related genes may
clarify this mechanism and eventually enable selection of patients with LGG that are
prone to hypermutation. So far, the implications of TMZ-associated hypermutation in
recurrent tumors of LGG are unknown. If future studies provide evidence for a clinical
disadvantage of hypermutation, TMZ should be avoided in this subgroup of LGG.

4. Can shallow whole genome sequencing for CNAs in various glioneuronal
tumors (GNT) help to establish a less observer-dependent classification?
(chapter 5)
The advent of shallow whole genome sequencing enabled a new approach to CNA-
analysis in archival material. Using the Dutch pathology registry (PALGA), databases and
basements of several medical centers in the Netherlands were searched to assemble a
representative cohort of LGG, including detailed clinical information such as survival.
After optimizing this technique with LGG samples (154) we applied it to GNT, another
group of glial neoplasms. GNT encompass various subgroups, such as ganglioglioma
and dysembryoplastic neuroepithelial tumors (155;156). Inter-observer variability
among pathologists can be explained by the variable histology in these tumors
and the lack of unequivocal criteria for diagnosing neoplasms that do not show the
prototype histology. Also, some GNT are difficult to distinguish from LGG because they
show a similar diffuse growth pattern. An incorrect histological diagnosis will have
consequences for the treatment strategies and associated side-effects for both groups
of patients. A study of CNAs in GNT may provide insight into their molecular variability
and thereby aid in development of a new classification that overcomes these issues.
CHAPTER 1.4 ABBREVIATIONS

- aCGH: array comparative genomic hybridization
- CNAs: copy number aberrations
- DNT: dysembryoplastic neuroepithelial tumor(s)
- FFPE: formalin-fixed paraffin-embedded
- GBM: glioblastoma
- GG: ganglioglioma
- GNT: glioneuronal tumor(s)
- LGG: diffuse low-grade glioma
- MGMT: O-6-Methylguanine-DNA Methyltransferase
- MMR: mismatch repair
- TCGA: The Cancer Genome Atlas
- TMZ: temozolomide
- WGS: whole genome sequencing