Chapter 8

General discussion and future directions
GENERAL DISCUSSION

After acute leukemia, brain tumors constitute the most common form of childhood cancer. On average, 120 children are diagnosed with a brain tumor every year in the Netherlands. Although the implementation of dose-intensive cytotoxic chemotherapy and radiotherapy regimens has led to a large increase in survival in other areas in pediatric oncology, the overall survival rates for children with a high-grade brain tumor are still poor, especially in patients suffering from high-grade gliomas and anaplastic ependymomas. For some types, such as medulloblastoma, survival has improved over recent decades, but quality of life is often compromised by debilitating long-term sequelae in these patients. There is thus a great urgency to boost preclinical and clinical research in the field of childhood brain tumors, investigating new, tumor specific, targets for therapy, not only to improve the survival of these children, but also to reduce the deleterious late effects. During the writing of this thesis, phase I/II clinical trials (listed in Table 1) have explored therapies directed against several tumor-specific targets, mostly extrapolated from other cancers, to study their effect in (mainly relapsed) high-grade pediatric brain tumors and de novo DIPG. Thus far, with limited success.

This thesis has therefore explored new treatment targets for high-grade (pediatric) brain tumors by use of in silico genomics and proteomics, and literature studies, expanding even to other types of other cancer.

As AMPA-type glutamate receptors were described in the literature to have a tumor-promoting role in adult glioblastoma multiforme, with glioma cells being able to produce large amounts of the neurotransmitter glutamate, these receptors were investigated as targets for therapy (Chapter 2). In discrepancy with previously published data, we found these receptors to be down-regulated in adult glioblastoma compared to normal brain in silico and to be electrophysiologically non-functional in cultured glioma cells. Our observations partly explained why these cells are able to survive in their self-created glutamate-rich environment, but did not support a rationale for AMPA receptor inhibition, since no effect of these inhibitors on cultured glioma cells was observed. Recent research however, describes that subpopulations within these tumors, glioblastoma stem or tumor-initiating cells, might actually benefit from AMPA receptor targeting, since these specific cells do show up-regulation of these receptors. This emphasizes that potential drug target expression should also be investigated in subpopulations of tumor-initiating cells, which could be overlooked when focusing on ‘whole tumor’ target gene expression. Furthermore, the mechanism by which high-grade glioma cells release glutamate, via the glutamate-cysteine antiporter system xc, causing excitotoxic cell death of neurons, provides an interesting option for targeted therapy. Specific inhibitors against non-AMPA type (ionotropic and metabotropic) glutamate receptors are subject to investigation. Whether these will have significant anti-tumor effects remains to be seen, since we found
that these receptors were also not differentially expressed in high-grade gliomas compared to normal brain².

Identification of new targets in high-grade pediatric brain tumors depends on the availability of cell biological data from these tumors. Publicly available data on the genome, transcriptome and proteome are rich sources of information, allowing for the identification of potential genes or pathways for therapeutic targeting. These sources were used in this thesis for in silico analyses, comparing data from pediatric or adult high-grade brain tumors with normal brain tissues. PBXIP1 was discovered as a protein overexpressed in high-grade astrocytomas by use of The Human Protein Atlas, in which a large number of tissue micro-arrays of multiple cancers and normal tissues are banked (Chapter 5). Using this in silico proteomic approach, differential protein expression between glioma and normal brain can be investigated, allowing for the identification of new therapeutic targets with potential low toxicity to the normal brain. The disadvantage of this proteomic database is, that it currently does not include pediatric brain tumors. Interestingly, inhibition of PBXIP1 via RNA interference resulted in strong reduction of viability and motility of high-grade astrocytoma cells, which will be subject for further research within our group. Ultimately, new agents directed against this target may provide new therapies in high-grade astrocytomas.

Recently, new studies were published by other groups exploring the transcriptome of pediatric high-grade glioma⁵, DIPG⁶, ependymoma⁷⁸ and medulloblastoma⁹-¹². These data revealed that the classically ‘histology defined’ disease entities can now be redefined by molecular profiling, classify these diseases in distinct subgroups with distinct prognostic characteristics. For further research and diagnostics, histopathology will be redefined by new molecular techniques, allowing for better outcome prediction and definition of patient subgroups for treatment stratification.

In particular, for medulloblastoma, four prognostic subgroups have been defined based on gene expression: a subgroup with aberrant Sonic Hedgehog (SHH) pathway signaling, a subtype with aberrant signaling in the Wingless (WNT) pathway, Group 3 tumors (harbouring strong MYC amplification) and Group 4 tumors⁹-¹². Group 3 and 4 tumors are often metastasized and have a poor prognosis, whereas WNT-type tumors have a good outcome. Young children under the age of three years, presenting with medulloblastoma have an excellent prognosis when biology shows SHH pathway activation (with a desmoplastic or extensive nodularity histopathology). Based on these risk profiles, therapy needs to be adapted accordingly. As a result, in the very near future, WNT-type tumors will be treated with less intensive treatment regimens, hopefully resulting in excellent outcome, with less long-term side effects. Relapsed SHH-type tumors, often characterized by a mutation in the PTCH1 gene will be subject to targeted therapy with SHH inhibitors, in addition to standard cytotoxic therapies. For Group 3 and Group 4 tumors, current therapies are insufficient and therefore more intensive and/or targeted therapy approaches are needed for these tumors.
For childhood ependymoma, molecular classification studies are currently underway. Prognostic modeling of these tumors based on histopathological WHO grading has proved to be a difficult task, with inter-observer variation and irreproducibility over trials. Looking for better prognostic markers, recent molecular profiling has resulted in the definition of two distinct prognostic subgroups (A and B) of posterior fossa ependymomas. Also for infratentorial ependymomas, based on gene expression, two specific subgroups could be defined, whereas in supratentorial ependymomas, using array CGH, gain of 1q, and homozygous deletion of CDKN2A were found to be strong, negative, prognostic markers.

Expression profiling in high-grade glioma and DIPG have revealed focal high-level amplifications in PDGFRA and MET in a substantial number of tumors. In DIPG, based on its transcriptome, a mesenchymal / pro-angiogenic subgroup and an oligodendroglial subgroup were defined, with distinct prognostic differences.

Following up on expression profiling, more recently, exome and whole genome sequencing studies in pediatric high-grade brain tumors have identified interesting new mutations in genes that involve epigenetic regulators, such as MLL2 and KDM6A in medulloblastoma, and H3F3A and HIST1H3B in up to 80% of DIPG and non-brainstem high-grade glioma, which have prognostic significance. 450k DNA methylation analyses in medulloblastoma also indicate that these tumors are characterized by epigenetic deregulation of normal developmental processes. Likewise, the recently discovered histone mutations in pediatric high-grade glioma seem to influence the epigenome in these cancers to a large extent. Furthermore, also in childhood ependymoma, epigenomic arrays have revealed DNA methylation as an important oncogenic mechanism, for which therapies targeting the epigenetic machinery might be of benefit. Interestingly, in our study on SIRPα in medulloblastoma, we found that targeting of epigenetic modulator genes strongly affected viability of these cells. Because of their epigenetic deregulation, targeting the epigenetic machinery seems to be an interesting option in medulloblastoma, ependymoma and pediatric high-grade glioma, but fine-tuning these therapies is important, since they might interfere with normal developmental processes. Therefore, the biggest challenge may be to render this into a tumor-specific therapy, leaving normal cells unharmed.

Mutations or genomic aberrancies in high-grade pediatric brain tumor cells can also be ‘indirectly targeted’ through synthetic lethality, which can be induced in tumor cells when a certain signal transduction pathway is deregulated by a mutation of a particular gene. Inhibition of the salvage pathway, that the tumor cells now depend on, results only in lethality of the tumor cells and not of normal cells which still have the intact signal transduction pathway available. An example of this approach is the inhibition of the DNA repair gene PARP1. The targeting of genes which are synthetically lethal to a cancer-relevant mutation has the advantage of killing tumor cells and sparing normal cells, thereby avoiding treatment-related
toxicity. In this respect, defects in tumor cells’ double strand break repair or cell cycle regulation provide a rationale for PARP inhibition\textsuperscript{35}. Using \textit{in silico} analyses, \textit{PARP1 (Chapter 3)} and \textit{EGFR} and \textit{ERBB2 (Chapter 4)} overexpression compared to normal brain tissues was observed in adult and pediatric high-grade brain tumors and inhibition of these genes sensitized these tumors to radiation\textsuperscript{36,37}. PARP-inhibitors have entered the arena of new drugs in the treatment of pediatric brain tumors, both in combination with temozolomide and radiotherapy. Currently, in controlled Phase II and III studies, combining radiotherapy with inhibitory monoclonal antibodies directed against EGFR, encouraging response rates and longer survival time of high-grade glioma patients are being observed\textsuperscript{38}. Since high-grade gliomas in adults seem to be more EGFR-driven than their pediatric counterparts\textsuperscript{39}, the role of combining these agents in pediatric high-grade glioma and DIPG remains to be elucidated. In pediatric high-grade glioma however, interesting responses were observed combining EGFR inhibition with radiotherapy and vinorelbine, warranting further Phase III studies\textsuperscript{40}.

\textit{In vitro} radiosensitization studies have some drawbacks. Potential increased neurotoxicity upon treatment with radiosensitizers is difficult to assess. Furthermore, radiosensitization \textit{in vitro} is determined by classical dose-response curves, in which tumor cells are exposed to a radiosensitizer and increasing doses of radiation. In clinical practice, pediatric high-grade brain tumor patients are treated with fractionated radiotherapy schedules, with daily doses of 1.8 Gy, up to a cumulative dose of 54 Gy, depending on the tumor site and normal brain regions at risk. How these fractionated schedules used in the clinic relate to \textit{in vitro} single dose radiation tumor cell sensitivity assessment is unclear. Future \textit{in vitro} and \textit{in vivo} research into potential radiosensitizers in brain tumors should therefore also take into account these radiation schedules used in clinical practice. This might provide additional clues for optimal radiosensitization.

**FUTURE DIRECTIONS**

For an outstanding translational research program in pediatric neuro-oncology, collaboration between pediatric neurosurgeons, pediatric neuro-oncologists, neurologists, radiotherapists, neuropathologists, nuclear medicine physicians, radiologists, molecular (radio)biologists, bioinformaticians and statisticians is essential. Integrating clinical patient data, genomic profiles from patients’ normal and tumor material and by use of patient-derived cell lines and xenografts, knowledge on potential treatment targets will undeniably increase. With Connectivity Mapping, gene signatures from pediatric high-grade brain tumors are linked to corresponding drug sensitivity patterns from drug screens and data from genetic manipulations, such as RNA interference studies.
Besides their value as new prognostic tools in groups of patients, these novel molecular techniques also allow for patient-tailored, quick analyses of potential treatment targets in newly diagnosed and relapsed tumors, enabling personalized treatment with the best possible, individualized, targeted therapy. Using drug libraries and loss of function RNAi screens in patient-derived cell lines, mechanisms of resistance to targeted therapy can be investigated, and drug combinations can be explored that have synergistic, or even synthetic lethal effects, for use in these patients.

In the current era, ever-increasing amounts of genomic data of normal and tumor tissues are a challenge to the human mind. Unbiased analysis of these large and complex ‘Big Data’ ask for new technologies. Computer networks and grids will be used to process these large quantities of data within shorter timeframes. These systems are able to integrate information from a multitude of databases, such as published data (e.g. Pubmed), mRNA-, miRNA-, methylome-, proteome-datasets, RNAi screens and exome and whole genome sequencing from tumors and normal tissues. Unbiased data analysis, searching for patterns, will potentially provide answers in oncogenic mechanisms and the discovery of new targets for therapy.

New treatment target discovery at the same time raises the question whether drugs directed against these targets are actually able to reach their brain tumor targets, as the blood-brain barrier (BBB) prevents a lot of pharmaceuticals from entering the brain, by virtue of the ultrastructure of the BBB with tight junctions and expression of ATP-binding cassette (ABC) drug transporters. The BBB is usually, at least partly, disrupted in high-grade brain tumors, but one can question whether the extent of BBB disruption observed in these tumors is enough to provide ample availability of drugs which normally would not cross this barrier. Tumor cells use the intact BBB as a shelter from harm, suggesting that the inefficacy of cytotoxic therapies in the past is likely to be, at least partly, explained by the inability of these agents to reach the tumor cells. This could also imply that drugs, which have previously been unsuccessful in clinical trials, might be effective when given via a different route, or modified into e.g. nanoparticles. Research into methods of enhancing brain tumor uptake of new and ‘old’ agents give new opportunities for previously depreciated medication for the treatment of brain tumors. Future studies should take into account how to circumvent the BBB to accomplish therapeutically relevant concentrations of a drug. This could be reached by redesigning drugs to enhance BBB transport, e.g. optimizing size and increasing hydrophobic properties and modifying the charge of molecules. Nanotechnologies are currently being explored, making use of encapsulated drugs in polymers and liposomes, which enhances passage over the BBB.

Another possible solution to overcome the BBB is convection-enhanced delivery (CED). With CED, drugs are delivered into the brain tumor via microcatheters under a continuous pressure gradient. This technique yields high concentrations of drugs in and around the tumor, impossible to obtain via systemic delivery without causing toxicity. This method is suitable for
drugs which are selectively toxic to tumor cells and leave normal cells unharmed, and have excellent convection capacity. Translation of *in vitro* data on drug sensitivity of brain tumor cells and toxicity on normal astrocytes is of help in choosing the ideal candidate drug for delivery via CED. These candidate drugs, both new agents and more ‘conventional’ cytotoxic agents, can then be validated *in vivo*, allowing for swift translation into clinical studies.

In conclusion, the studies performed in this thesis have explored several targets for therapy, using literature and *in silico* data mining and validation of these potential targets in functional assays. For some targets, such as the AMPA-type glutamate receptors, more research is needed to establish whether they could serve as targets for therapy. Potential treatment target PBXIP1 will be subject to extended *in vitro* and *in vivo*, and, possibly, clinical studies. For other targets, such as PARP1 and the ErbB family, clinical trials with inhibitors against these targets, combined with radio- and/or chemotherapy in pediatric high-grade brain tumors are already underway. Interesting new avenues are to be explored in targeting the deregulated epigenome in medulloblastoma and pediatric high-grade glioma, as described in our study of SIRPα in medulloblastoma.

Research into pediatric high-grade brain tumors should be focused on novel drug target discovery, using large scale, bioinformatic analysis of ‘Big Data’, *in vitro* and *in vivo* models, and studies on the optimal delivery method of the (targeted) drugs. Ideally, Academia also develops new anti-cancer agents for small subgroups of patients, for whom the incentive for pharmaceutical companies to do so may be lacking. Hereto, accelerating translational research is of the highest importance. To this purpose, in the very near future, in the Netherlands, the Princess Máxima Center for Pediatric Oncology will be opened. In this center, all Dutch children with a malignancy will be diagnosed and treated according to standardized evidence-based protocols. With an in-house Research Institute with its own Neuro-oncology Research Facility, preclinical research programs will be highly interwoven with patient care and clinical studies, from bench to bedside and back again, ultimately improving survival and quality survival of children with a high-grade brain tumor.
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General discussion and future directions

REFERENCES

18. Puget, S. et al. Mesenchymal transition and PDGFRA amplification/mutation are key...


