ISBN: 90-9020870-4

Medulloblastomas and ependymal tumours Impact of translational research on treatment perspectives H. J. Gilhuis

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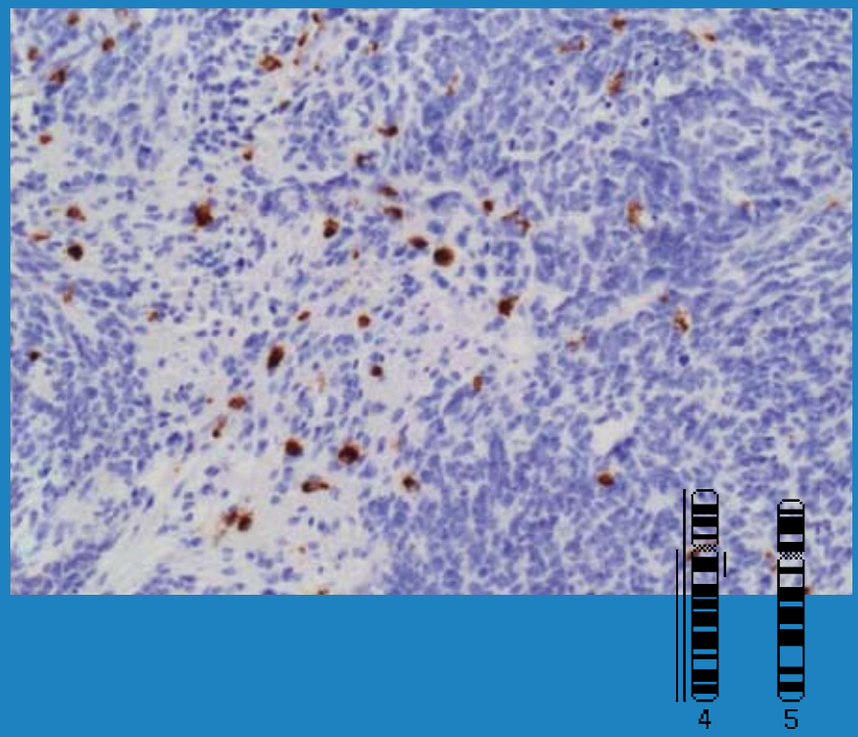

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Cover design by H.J. Gilhuis, based on figures 1 (pages 26 and 67)

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een wetenschappelijke proeve
op het gebied van de Medische Wetenschappen

proefschrift

ter verkrijging van de graad van doctor
aan de Radboud Universiteit Nijmegen,
op gezag van de Rector Magnificus,prof. dr. C.W.P.M. Blom
volgens besluit van het College van Decanen
in het openbaar te verdedigen op dinsdag 3 oktober 2006
des namiddags om 3.30 uur precies

door

Herman Jacobus Gilhuis
geboren op 21 april 1967
te Koog aan de Zaan

‘Zoals een omvangrijke, ongeordende bibliotheek niet zoveel nut afwerpt als een middelgrote die wel geordend is, zo is ook een grote hoeveelheid kennis die niet door ons eigen denken is verwerkt, veel minder waard dan een kleine hoeveelheid die wij zelf hebben doordacht. Want pas door wat men weet naar alle kanten te combineren, door het vergelijken van iedere waarheid, maken wij onze kennis volledig eigen en krijgen wij die in onze macht. Wij kunnen alleen doordenken wat wij weten, dus we moeten wel iets leren, maar we weten ook alleen wat we hebben doordacht’.

Arthur Schopenhauer (uit Parerga en paraliponema)

Promotors	Prof. dr. G.W.A.M. Padberg
Co-promotors	Dr. P. Wesseling
	Dr. A.C. Kappelle
Manuscript commissie	Prof. dr. A.J. van der Kogel
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This thesis was sponsored by Biogen Idec, Boehringer Ingelheim, Janssen-Cilag, Menarini Farma Nederland, Novartis Pharma, Sanofi-Aventis, Schering-Plough, TEVA Pharma, UCB Pharma.

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List of abbreviations

Tumours

AE	Anaplastic ependymoma
LgE	Low-grade ependymoma
ETs	Ependymal tumours
MB	Medulloblastoma
MpE	Myxopapillary ependymoma
SubE	Subependymoma

Techniques

CGH	Comparative genomic hybridisation
ISH	In situ hybridisation

Various

CSF	Cerebrospinal fluid
ChT	Chemotherapy
CNS	Central nervous system
MRI	Magnetic resonance imaging
RT	Radiotherapy
WHO	World Health Organisation

Chapter 1

Introduction and outline of thesis

GENERAL INTRODUCTION

While compared to other cancers primary brain tumours are relatively rare in adults, they constitute the most common paediatric solid tumours in children with an incidence of 1.7-4,1/100,000 [1,2]. Primary brain tumours are the leading cause of cancer related death in children under 16 years of age [1,2]. The significant improvements in survival as seen in children with haematological cancers such as leukaemia have not been achieved for most of the malignant central nervous system (CNS) tumours. The pathologic spectrum, distribution, and behaviour of paediatric CNS tumours differ considerably from the adult group of CNS tumours. The treatment of CNS tumours in children provides tremendous challenges because of the need to preserve the mental and physical development of the child. Apart from the astroglial tumours, these paediatric tumours are medulloblastomas (MBs) and ependymal tumours (ETs), which are both neuroepithelial tumours [3].

The majority of MBs occur in individuals younger than 16 years, with a mean age of 9 years, accounting for 15-30% of all intracranial tumours in this group [4]. The incidence in children is 5.5-7.0 per million [5,6]. The male to female ratio is 1.6:1 [4]. MBs were initially described by Bailey and Cushing in 1925 as tumours consisting of small, undifferentiated, round cells arising in the wall of the fourth ventricle and projecting into the centre of the cerebellum [7]. Similar tumours were described in adults in other sites of the brain and later classified as primitive neuroectodermal tumours (PNETs). MBs might be referred to as ‘posterior fossa PNETs’ [8]. The World Health Organisation (WHO) definition of MB is ‘a malignant, invasive embryonal tumour of the cerebellum with preferential manifestation in children, predominantly neuronal differentiation, and an inherent tendency to metastasise via cerebrospinal fluid (CSF) pathways’ [8].

ETs account for 6 to 12% of primary intracranial tumours in children and 1-3% of primary intracranial tumours in adults. Thirty percent of children affected are younger than 3 years old [9]. The incidence of ETs in children and adolescents is estimated to be 1.9 per million and are equally distributed over the two sexes [10]. ETs were first described in the 19th century by Virchow and in 1924 classified by Bailey and Cushing as glial tumours [11]. The WHO definition is ‘a tumour predominantly composed of neoplastic ependymal cells, with preferential manifestation in children and young adults’ [12].

MEDULLOBLASTOMAS

Clinical symptoms, diagnosis and treatment

Clinical symptoms usually occur when medulloblastomas (MBs) grow into or compress the fourth ventricle, with subsequent obstruction of the CSF flow, resulting in increased intracranial pressure. This obstruction, rather than the tumour itself, causes headache, lethargy, papilloedema, nausea and vomiting. Ataxia, nystagmus and 6th cranial nerve palsy develop as a result of direct infiltration of brainstem by MB, subarachnoid spread of tumour to the sixth nerve, or a diffuse increase in intracranial pressure. If the tumour invades the meninges at the level of the foramen magnum, or if there is cerebellar tonsil herniation from increased intracranial pressure, nuchal rigidity and a headtilt may result. Very young children with open sutures may have non-specific signs such as anorexia, failure to thrive and increased head size [13]. Symptoms caused by extraneural metastasis are seen in 7% of cases. Most of these metastases (88%) are found in bones and cause local pain [14,15].

The current procedure of radiological imaging is magnetic resonance imaging (MRI) using both T1-weighted images with and without contrast, and T2-weighted images. MBs demonstrate a well-demarcated hypointense signal on T1-weighted images with contrast enhancement and a hyperintense signal on T2-weighted images [16]. Leptomeningeal involvement can be diagnosed with a high degree of certainty on MRI. CSF cytology may be useful when the MRI is equivocal for the presence of leptomeningeal metastasis. Spinal MRI is relevant for staging of disease postoperatively according to criteria developed by Chang et al. (Table 1) [17].

Most MBs originate in the wall of the fourth ventricle. Although the cell of origin of these tumours is controversial, it is probable that MB takes its origin from germinative neuroepithelial cells in the roof of the 4th ventricle [3]. They appear macroscopically as soft grey-pink masses, often with local necrosis. Four MB subtypes are described in the WHO 2000 classification: Large cell/ anaplastic MBs, classic MBs, desmoplastic MBs, and MBs with extensive nodularity and advanced neuronal differentiation. Large cell/ anaplastic MBs are characterised by large, round and pleomorphic nuclei with prominent nucleoli and an abundant cytoplasm [8]. Classic MBs are composed of small densely packed cells with round-to-oval highly hyperchromatic nuclei surrounded by scanty cytoplasm [8]. The histological hallmark of desmoplastic MBs is the presence of nodules or pale islands, which are comprised of clusters of cells with round, regular nuclei and abundant, fibrillar cytoplasm [18]. MBs with extensive nodularity and advanced neuronal differentiation show intranodular nuclear uniformity and cell streaming in a fibrillary background.

Stage	Description
T stage	
T1	Tumour less than 3 cm in diameter and limited to the midline position in the vermis, the roof of the fourth ventricle and less frequently cerebellar hemispheres
T2	Tumour more than 3 cm in diameter, further invading one adjacent structure or partially filling the fourth ventricle
T3a	Tumour invading two adjacent structures or completely filling the fourth ventricle with extension into the aqueduct of Sylvius, foramen of Magendie or foramen of Luschka, thus producing marked internal hydrocephalus
T3b	Tumour arising from the floor of the fourth ventricle or brain stem and filling the fourth ventricle
T4	Tumour further spreading through the aqueduct of Sylvius to involve the third ventricle or midbrain or tumour extending to the upper cervical cord
M stage	
M0	No evidence of gross subarachnoid or haematogenous metastasis
M1	Microscopic tumour cells found in cerebrospinal fluid
M2	Gross nodule seedings demonstrated in the cerebellar, cerebral subarachnoid space or in the third or lateral ventricles
M3	Gross nodule seedings in the spinal subarachnoid space
M4	Extraneuroaxial metastasis

Table 1 *Chang staging system for posterior fossa medulloblastoma [17].*

No formal grading system of MBs has evolved and treatments are currently not stratified by histological grade [18,19]. Large cell/ anaplastic MBs are associated with aggressive behaviour, in casu CSF dissemination and extracranial metastases [20]. MBs with extensive nodularity and advanced neuronal differentiation have a better prognosis than the other histopathological variants. In a study of 330 MBs from the US Pediatric Oncology Group, increasing grade of anaplasia and extent of anaplasia were associated with aggressive clinical behaviour. Neither degree of nodularity nor desmoplasia were associated with longer survival [18].

The most common cytogenetic abnormality described in MBs is a deletion of 17p, alone or in combination with an isochromosome 17q, found in approximately 30% of tumours. Chromosome 17p possibly harbours a tumour suppressor gene [21]. The

clinical relevance of loss of 17p is unclear. Some retrospective reviews in the literature correlated loss of 17p with a poor outcome, whereas others did not find this association [21-24]. Loss of heterozygosity (LOH) studies trying to link loss of heterozygosity on 17p and adverse prognosis gave contradictionary findings [21,23,24].
Comparative genomic hybridisation (CGH) studies failed to identify specific genetic abnormalities or patterns associated with prognosis. An array of non-random genomic losses is found [25]. Repeated chromosomal imbalances most frequently involve chromosome 17 (loss of 17p and gain of 17q) and losses of chromosomes 10q, 11 and 8. In a study investigating 77 cases of MBs, all four patients with MYCC amplification died within months of diagnosis, compared with 50% of patients whose tumour did not show MYCC amplification [26]. However, in another study an association between MYCC and MYCN expression and prognosis was absent [27].

Combining clinical factors with tumour ERBB2 (epidermal growth factor receptor) expression (assessed by Western Blot) might allow a more accurate prediction. A recent study showed that all 26 children with ERBB2-negative disease were alive 5 years after diagnosis, against only 54% of children with ERBB2-positive tumours ($p < 0.0001$) [27]. Tumour DNA ploidy has been variably associated with outcome, and is not incorporated into prospective staging systems [28].

Therapeutic approaches are based on separation of patients into ‘average-risk’ and ‘high-risk’ categories. ‘Average-risk’ patients are those who are 3 years of age or older, with a total or near total resection and no evidence of tumour spread beyond the primary tumour site. ‘High-risk’ patients are younger than 3 years of age, with more than 1.5 cm^2 residual tumour after surgery and/ or spread of tumour outside the primary site [29]. The extent of the disease at the time of diagnosis is the most significant prognostic factor. In children with localised disease, the amount of residual disease present after surgery is the most important prognostic factor. The probable explanation for the poorer survival of children less than 3 years of age is that they receive a lower therapeutic radiation therapy (RT) dose rather than that their tumours possess different biologic characteristics [29].
In all patients, surgery is the initial step in treatment of MBs. The aim is gross total resection, which is attainable in approximately 75% of patients. A retrospective study demonstrated that gross total surgical resection results in an improved overall progression-free survival rate in patients with non-disseminated tumours [30]. After surgery, 10-20% of children suffer from ‘cerebellar-mutism syndrome’, a poorly understood combination of mutism, ataxia, personality change and cranial nerve deficits, following surgery. This syndrome is not related to the extent of resection. The cerebellum is probably involved in the mental initiation preceding any intentional bucco-phonatory movements. Median duration in a group of 8 affected children was 4 weeks. Recovery was near complete for all children [31].

For patients older than 3 years, surgery is always followed by craniospinal RT with a boost to the primary tumour site [29]. For 'average-risk' patients, the standard dose of craniospinal irradiation is 35-36 Gy (in fractions of 1,5-1,8 Gy, 5 times per week) plus an additional 18-20 Gy boost in 18 fractions to the posterior fossa [32-36]. In children aged 1.5-2.9 years, a reduced-dose RT of 45 Gy to the primary tumour and 23.4 Gy to the craniospinal axis is given [29]. Craniospinal irradiation reduces dissemination and improves outcome: the progression-free survival rate at 5 years in patients receiving local RT only is about 10%, whereas it is 50% in those children receiving craniospinal axis irradiation with local boost. The best survival rates have been reported for patients who received RT immediately following surgery [32-36].

Recent trials suggest that all patients should be treated with adjuvant chemotherapy (ChT) [29,32,35]. The addition of ChT improves event-free survival at 3 and 5 years [35]. Packer et al. treated 65 children with 'average-risk' MBs between 3 and 10 years of age, with reduced-dose craniospinal RT and adjuvant vincristine, lomustine, and cisplatin ChT. Progression-free survival was 86% +/- 4% at 3 years and 79% +/- 7% at 5 years. Sites of relapse for the 14 patients who developed progressive disease included the local tumour site alone in two patients, local tumour site and disseminated disease in nine, and non-primary sites alone in three. The overall survival rates compared favourably to those obtained in studies using full-dose RT alone or full dose RT plus ChT, suggesting that reduced-dose craniospinal RT and adjuvant ChT is a feasible approach for 'average-risk' children [34]. For this group of children, the combination of vincristine, cisplatin, and lomustine is considered the best choice of ChT [34]. A potential advantage of ChT is reduction of the total radiation dose without impairment of local control and curation rate, thereby reducing the long-term effects of RT on neuropsychologic and endocrine development.

For patients with 'high-risk' disease, recent clinical trials have focussed on intensifying ChT, including high-dose chemotherapy with autologous peripheral stem cell support [37]. In children younger than 3 years, the use of ChT has been investigated in an attempt to spare the developing brain from late adverse neurocognitive and neuroendocriene late sequelae due to RT. Craniospinal RT will result in significant intellectual deterioration in these children, with reductions in IQ of up to 40 points [32,38]. However, without RT, survival rates are disappointing. In a study of 35 children younger than 3 years who were treated with conventional ChT and without RT, 20 (57%) relapsed at a median time of 6.3 months [39].

In case of relapse after RT, the outcome is very poor. The vast majority dies within 18 months of relapse [21]. No standard therapy is available. In a study using high-dose thiotepa and etoposide with autologous bone marrow rescue, a partial response was observed in 2 of the 6 (33%) patients 28 days following the autologous bone marrow rescue [40].

Due to significant advances in surgical techniques, RT, and the introduction of ChT regimens, survival rates for children with MBs have improved in recent years [29,35].

Nowadays, a recurrence-free 5-year survival can be achieved in 60-65% of the patients [3,29,32,35]. Long-term survivors face life long sequelae from combined treatment, impairing their quality of life [32]. As RT and ChT impair the hypothalamic functions, endocrinopathy is common, with growth hormone deficiency in 75-85% and thyroid abnormalities in 35-69% of cases, resulting in the need for life long substitution [41-42]. The adverse effects of RT on neuropsychological status increase with treatment dose (35 versus 25 Gy, given in standard fractions of 1,8 Gy) [43]. Age at time of radiation predicts the decline in IQ following RT [32]. This is probably due to damage to the developing cerebral white matter by a mechanism not fully understood. Children treated with RT have reduced volumes of cerebral white matter on MRI as compared to non-RT treated age-matched controls [44].

EPENDYMAL TUMOURS

Clinical symptoms, diagnosis and treatment

Ependymal tumours (ETs) can be found anywhere in the CNS. About one-third of ETs are located in the spinal canal [45]. About two-thirds of the intracranial ETs in children are located infratentorially and one-third supratentorially, while in adults it is inverse [9]. Two-thirds of the intracranial ETs occur in children, 96% of spinal ETs are seen in adults, with a peak incidence between 20-40 years of age [46].

Intracranial ependymal tumours

Clinical manifestations of ETs are site-dependent. The most common initial symptoms of posterior fossa tumours (nausea, vomiting and headache) are caused by increased intracranial pressure due to obstruction of CSF flow. Involvement of posterior fossa structures can cause ataxia, dizziness and paresis. Patients with supratentorial ETs may have focal neurological deficits, seizures, or features of increased intracranial pressure [47]. Clinical signs include papilloedema, meningeal signs, ataxia, cranial nerve palsy, and in children below the age of two years an increased head size or a bulging fontanel [9,47].

Radiological investigation is usually performed by MRI scanning, using both T1-weighted images with and without contrast, and T2-weighted images. ETs demonstrate a hypointense signal on T1-weighted images. On T2-weighted images, they are hyperintense and generally well demarcated from surrounding brain. Inhomogeneous contrast enhancement occurs in most ETs [48]. CSF cytology and postoperative craniospinal MRI are needed for the stratification of patients into prognostic risk groups. Age of less than 3 years and posterior fossa localisation are associated with poor prognosis [49].

There are four ET histopatholoical subtypes. Low-grade ependymoma (lgE), the most common type of intracranial ET has two main characteristic histological features; anuclear perivascular collars of radiating cell processes (perivascular pseudorosettes) and true rosettes of tumour cells with a central lumen. LgEs are considered low grade (WHO grade II) [12]. Anaplastic or malignant ependymoma (aE) (WHO grade III) show higher cellularity, marked mitotic activity, and nuclear atypia with or without florid microvascular proliferation. Large areas of necrosis and high apoptotic rate are common findings [12]. Myxopapillary ependymomas (mpEs), (WHO grade I), are usually located in the conus-cauda region, and are characterised by cuboidal to elongated tumour cells radially arranged in a papillary manner around vascularised stromal cores [12]. Subependymomas (subE) are also considered low grade, and are classified as WHO grade I. They are characterised by clusters of isomorphic nuclei embedded in a dense fibrillary matrix of glial cell processes with frequent occurrence of microcysts. Mitosis are very rare or absent [12].

Molecular genetic findings of intracranial ETs are so far not incorporated into risk classifications. Most ETs show cytogenetic alterations. Abnormalities of chromosome 22, mainly monosomy, are found in 30% of the tumours [50]. A large CGH study in paediatric ETs revealed that patients with a CGH pattern showing few, mainly partial imbalances, had a worse prognosis compared to the group showing 13 or more chromosome imbalances with a non-random pattern of whole chromosome gains and losses [51]. In this study, genetic imbalances and surgical resection predicted clinical outcome, whereas age, histopathology and other clinical parameters did not. The most common genetic abnormalities found in these studies were loss of chromosome 22, gain of chromosome 1 and loss of chromosomes 6q and 17 [51-54]. Loss of chromosome 22, probably harbouring a tumour suppressor gene, is so far not related to outcome [52-54]. No underlying sequence of genetic events has been identified in the malignant progression from low to high-grade ETs so far.

Treatment of ETs starts with surgery aiming at radical resection. Radical resection is the most important factor for progression-free and overall survival [55-57]. The tumour residu is best estimated with post-operative MRI. Gross total resection is reported in 50-73% of cases [55,56].
Post-operative RT has long been used for ETs, although randomised prospective studies are not available [49,55,56,57]. By consensus, a tumour dose of 54-55 Gy for lgEs and 60 Gy for aEs is given [57]. Craniospinal irradiation is applied to patients with leptomeningeal seeding [58]. To avoid neurocognitive and neuroendocriene complications, patients younger than 3 years old are usually treated postoperatively with a multi-agent ChT regimen instead of RT. The role of ChT is controversial. Robertson et al. concluded on the basis of a prospective trial (a regimen of 2 drugs versus 8 drugs) involving 32 children between the ages of 2 and 17 years, that

adjuvant ChT had no impact on progression-free survival [55]. Another study of 48 children younger than 3 years of age treated with delayed RT and ChT suggested that ChT allows a delay in RT, and improve survival [59]. So far, ChT has no role in the adjuvant setting with the exception of children younger than 3 years [56,59].
Residual disease after surgery and CSF dissemination are the two most important clinical parameters affecting outcome [60]. In a study of 80 children, (aged 4 months to 15.8 years, 38 with a complete tumour resection), relapses occurred from 3-72 months after diagnosis (median 25 months). Survival at 5-years for patients who had complete resection of the tumour is 75% vs. 41% for those with incomplete resection. For those who received RT, the 5-years survival was reported 65% vs. 23% for those who did not receive RT [61]. The value of MRI in follow-up has been established although it is not clear how frequent these should be performed. Patients with a surgical removal of an early, asymptomatic recurrence have a better prognosis as compared to those with a symptomatic recurrence [62,63]. The prognostic significance of histological malignancy grade in ETs is unclear. Some retrospective studies show that aEs carry a worse prognosis, but this was not confirmed in other studies [55,59,64].
Most recurrences are at the site of the primary tumour (59-78%) and complete resection is a key factor in long-term survival [61,62]. The role of RT and ChT in relapsing ETs is not well defined yet.

Spinal ependymomas
About one-third of ETs are localised in the spinal cord. Sixty percent of all spinal cord tumours in children and adults are ETs [45].

The interval between the onset of symptoms and diagnosis ranges from days to years. Pain is the most common initial symptom and is seen in about two-thirds of patients. Neurological signs are found in 70% of patients and include hyperreflexia, motor and sensory dysfunction, and spasticity. Sensory disturbances are the second most common symptom and are seen in 60% of cases. Fifty percent of patients have limb weakness, and one-third have bladder/bowel dysfunction [60].

MRI is the imaging modality of choice and usually reveals a sharply circumscribed lesion. T1-weighted images may demonstrate cysts associated with the tumour. Contrast enhancement allows for the differentiation between tumour and surrounding oedema [65].

The vast majority of spinal cord ETs are lgEs (WHO grade II) or mpEs (WHO grade I). LgEs are found throughout the spinal canal and can be situated both intra- and extramedullary. MpEs are usually located extramedullary in the conus-cauda region [46,66,67].

A wide range of genomic alterations as detected by comparative genomic hybridisation is described in spinal lgEs and mpEs. Spinal lgEs relatively often show gain of chromosomes 2, 7, and 12 and loss of 14, and MpEs gain of chromosomes 9 and 18 [52,68]. Allelic loss on 22q is frequently found in spinal lgEs [69]. Although mpEs are designated as a WHO grade I tumour and spinal lgEs as a WHO grade II, mpEs have on average more genomic alterations (as detected by CGH) and a higher percentage of aneuploidy than spinal lgEs [70].

Local control is the main factor influencing disease-free survival and can be obtained by radical surgery [71,72]. Complete resection, however, is only possible in 57-76% of the cases because of the risks of surgery-related neurological deficits [73,74]. RT after incomplete resection increases local control. The dose used is 45-50 Gy (in doses of 1,8-2 Gy) and the target volume consists only of the tumour bed with a suitable margin for clinical target volume and planning volume, as this tumour is not likely to relapse at distant sites [75,76]. In general, spinal lgEs and mpEs have a good prognosis. Late recurrences and distant metastases are rare [77,78]. Long-term neurological outcome is related to pre-operative conditions without clear differences between partially and totally resected tumours, and without impact of exact location. Seeding is seen in mpEs and reported more often these days, probably due to the use of MRI. It is interesting to report that these seeded tumours do not cause clinical symptoms in most of the patients, probably due to the small size and the low proliferation rate [75].

AIM OF THIS THESIS

MBs and ETs are among the most common primary CNS childhood tumours. Although new multimodality treatment regimens have substantially improved survival in this group, there is still a mortality rate of one-third for MB patients and 40% for intracranial ETs patients [26,63]. Further progress in the therapeutic approach requires translational research; the application of basic scientific discoveries into clinical findings and, simultaneously, the generation of scientific questions based on clinical observations. Combined molecular and histopathological analysis of tumours together with clinical staging will identify groups of patients with specific treatment requirements, avoiding overtreatment and improving mortality rate and event-free survival. Fortunately the progress in molecular biology and genetics has given us a much deeper insight into tumour induction and progression. Genomic, proteomic, and angiogenic studies have become important aspects of cancer research.

The studies presented in this thesis focus on identifying new molecular, morphological, and clinical features related to prognosis in MBs and ETs. For this purpose several

techniques were used. CGH was performed on MBs and ETs, and in an experimental glioblastoma xenograft study (chapters 2, 3, 4, and 6). To further substantiate the relation between genetic alterations and histopathological grading in ETs, DNA ploidy assessment was performed (chapter 4). We studied the expression pattern of insulin-like growth factors (IGF)-I and -II mRNA, which may play an important role in tumour growth and development in MBs (chapter 5). Genetic alterations were correlated with prognostic factors and outcome. As angiogenesis, the outgrowth of new bloodvessels from the pre-existent vasculature, is a critical process for the local growth of tumours, we performed studies attempting to link the pattern of vascularisation with clinico-biological behaviour. As glioblastomas are very well vascularised, an experimental study was done on the relationship between genetic alterations (as detected by CGH), growth rate, and vascularisation in glioblastoma xenografts (chapter 6). A new technique was used for the three-dimensional visualisation of the vascularisation pattern of MBs and ETs. The vascularisation pattern of the histopathological subtypes of MBs and ETs were analysed and compared with those of normal cerebral white and grey matter (chapter 7). Finally, a retrospective study on the use of RT for partially resected spinal ETs is presented in chapter 8.

SUMMARY OF THE CYTOGENETIC TECHNIQUES USED

Comparative genomic hybridisation (CGH)
A molecular cytogenetic technique that screens chromosomal imbalances in tumours. CGH detects amplifications and deletions in a single experiment without the need for specific DNA probes or previous knowledge of chromosomal aberrations present. It provides an overview of chromosomal imbalances by comparing the hybridisation efficiency of tumour and reference DNA to normal metaphase chromosomes. Test and reference (tumour) DNA is differentially labelled (e.g. reference in red and test in green) with fluorochromes (direct labelling), or haptens (indirect labelling) which are later visualised by fluorescence. Both DNAs are mixed in a 1:1 ratio, and are in situ hybridised onto normal human metaphase chromosomes. During this co-hybridisation, tumour and reference DNA compete for the same targets on the metaphase chromosomes. The relative amount of tumour or reference DNA bound to a given chromosomal region is dependent on the relative abundance of these sequences in both DNA samples. Gains will show in green and losses will show in red, whereas regions that are normally present will show in yellow. The presence and localisation of these imbalances can be detected and quantitated by analysing the ratio test-fluorescence versus reference-fluorescence (green/red) along the chromosome. Fixed thresholds of 1.2 for gain and 0.8 for loss were used [79]. The sensitivity depends on the percentage of cells containing the aberration, and the quality of the CGH experiment. The lowest amount of DNA copy number change reported is 10-12 Mb [80].

Fluorescence in situ hybridisation (FISH)
Intracellular labelling of single-stranded DNA or RNA with an immunofluorescent particle attached to a pairing DNA or RNA strand, in morphologically preserved biological structures such as chromosomes, cells and tissues.

DNA ploidy assessment
The measurement of DNA content. Ploidy refers to the number of copies of the chromosome in a cell. A normal cell has two copies of each chromosome (diploid). A haploid cell has one copy, a tetraploid cell has four copies, etc. DNA ploidy assessment can reveal genetic instability in a (sub)population of cells.

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Chapter 2

Comparative genomic hybridisation of medulloblastomas and clinical relevance: eleven new cases and a review of the literature

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Reprinted from: Clin Neurol Neurosurg 2000;102:203-9
with permission from Elsevier Science B.V.
Grant sponsor: NIH (CA590905 to RBJ) and the Dutch Cancer Society (HJG, RHB)

ABSTRACT

Medulloblastomas are highly malignant primitive neuroectodermal tumours of the cerebellum that display a wide variety of histopathological patterns. However, these patterns do not provide an accurate prediction of clinical-biological behaviour and no satisfactory morphological grading system has ever been presented. Genetic alterations may provide additional diagnostic information and allow clinically relevant subgrouping of primitive neuroectodermal tumours. We examined ten medulloblastomas and one medulloblastoma cell line. One amplification site on chromosome 8q24 was detected in the cell line corresponding to the known amplification of the MYCC gene in this cell line. The gain of 2p21-24 in two tumours was shown to represent amplification of the MYCN gene by Southern blot hybridisation and fluorescence in situ hybridisation. The data show that the isochromosome 17 can be recognised using comparative genomic hybridisation (CGH) by the typical combination of loss of 17p combined with gain of 17q. No specific pattern of genetic alterations could be linked to the clinical behaviour of the tumours. We have compared our results with previous CGH studies on medulloblastomas.

INTRODUCTION

Medulloblastomas (MBs) are highly malignant primitive neuroectodermal tumours that probably arise from primitive neuroectodermal cells, constituting the external granule cell layer of the developing cerebellum. It is the most common paediatric brain tumour with an incidence of 5 cases per 1 million children each year [1]. The survival rate at 5 years is approximately 50% with a long-term survival rate of 30%, largely as a result of local re-growth and metastasis [2]. In addition, the survivors suffer from the adverse side effects of the treatment including mental retardation, stunted growth and leukoencephalopathy [3,4]. Designing new treatments aimed at achieving longer survival periods and fewer side effects will require individualised treatments based on prognostic factors. At present, only four such factors are available: age, extent of surgical resection, local spread to the brain stem, and the presence of metastases [5]. In contrast to gliomas, devising a grading system for MBs, which links pathological features to prognosis, has not been successful [6]. Genetic analysis of MBs could provide additional prognostic information because the biological behaviour of a tumour is ultimately determined by its genetic changes. For example amplifications of oncogenes MYCN, EGFR (Epidermal Growth Factor Receptor Gene) and MYCC appear to predict rapid progression of MBs [7-11]. In addition, the study of genetic alterations helps to understand the oncogenesis of MBs. Comparative genomic hybridisation (CGH) is the ideal technique for studying genetic changes in tumours, as it can detect amplifications and deletions in tumours without the need for specific

probes or previous knowledge of chromosomal aberrations present [6,12,13]. CGH provide an overview of all chromosomal imbalances by comparing the hybridisation efficiency of tumour and reference DNA to normal metaphase chromosomes [12]. The regions showing amplification or deletion by CGH can be further investigated using other genetic techniques such as fluorescence in situ hybridisation (FISH) and Southern blot assays. In this study, we used CGH followed by FISH and Southern blotting to identify genetic alterations in MBs and a MB cell line in order to detect genetic changes. We compared our results with other CGH studies on MBs to define the clinical significance of the detected chromosomal abnormalities.

MATERIALS AND METHODS

Patients and Tumours

The tumours for this study were obtained from the frozen brain tumour resource of Mayo Clinic. Ten fresh frozen tumours were selected from patients (3 female, 7 male) ranging in age from 7 months to 33 years. The clinicopathological findings are summarised in Table 1. In all cases, the diagnosis of MB was confirmed by a neuropathologist (B.W.S.) using haematoxylin/eosin (H&E) stains. Five of the 10 MBs had desmoplastic features. A MB cell line (cell line 341) was obtained from the MB of a 3.5-year-old male during craniotomy [14].

Case	Sex	Age at operation (years)	Outcome	Histology (follow-up period)	Metastasis
1314	F	0.6	A, 7 yr	Desmoplastic Mb	none
968	F	33	A, 6 yr	Desmoplastic Mb	none
616	M	13	D, 3 yr	Desmoplastic Mb	local recurrence and CSF
620	M	6	D, 5 yr	Classic Mb	local recurrence and frontal bone
309	M	8	D, 2 yr	Desmoplastic Mb	bone
622	M	12	A, 7 yr	Classic Mb	CSF
166	M	7	A, 9 yr	Classic Mb	None
167	M	10	A, 10 yr	Classic Mb	None
10	M	3	A, 7 yr	Desmoplastic Mb	None
24	F	5	A, 7 yr	Classic Mb	None
cell line 341	M	3	D, 0.8 yr	Classic Mb	bone and abdomen

Table 1 *Clinical Data on the Patients. F: female; M: male; A: alive; D: dead; yr: year; classic MB: classic medulloblastoma; desmoplastic Mb: desmoplastic medulloblastoma; CSF: cerebral spinal fluid.*

DNA samples and probe labelling

Reference genomic DNA from peripheral blood leukocytes of a healthy male donor was obtained and DNA was isolated from the tumour according to a standard protocol [15]. The probe was indirectly labelled by nick translation using the Vysis nick translation kit (Vysis, Downers Grove, IL). The tumour DNA was labelled with Biotin 16-dUTP (Boehringer Mannheim, Indianapolis, IN) and the normal DNA was labelled with Digoxigenin 11-dUTP (Boehringer Mannheim).

CGH

Metaphase chromosomes were cultured from Phytohaemagglutinin-stimulated blood lymphocytes of a normal, healthy male donor according to standard cytogenetic procedures. Fixed metaphase suspensions were dropped onto slides in a controlled environment chamber (Thermatron Industries, Holland, MI). CGH was carried out as described elsewhere [16]. Briefly, slides with metaphase spreads were incubated with RNase A (Boehringer Mannheim;100mg/mL in 2 x SSC) for 1 hour (hr) at 37°C, digested with pepsin (Sigma, St. Louis, MO, 0.1 mg/mL in 0.01 M HCl) and post-fixed with formaldehyde (Fisher Scientific, Pittsburgh, PA; 1% in PBS with 50 mM MgCl2). The slides were denatured in 70% formamide/2 x SSC for 2 minutes (min) at 80°C. The probe mixture consisted of 223 ng of labelled tumour and normal DNA together with a 100-fold excess of Cot-1 DNA (Vysis) in 50% formamide, 2 x SSC, and 10% dextran sulfate. The probe solution was denatured for 5 min at 80°C, quenched on ice, and allowed to preanneal at 37°C for 1 hr. The probe was then applied to the denatured slide and hybridisation was carried out under a sealed coverslip for 72 hr in a moist container. Post hybridisation washes consisted of 3 x 5 min in 50% formamide/ 2 x SSC at 37°C and 3 x 5 min in 0.1 x SSC at 60°C and once in 4 x SSC at room temperature. The slides were preincubated with 0.5% dry milk powder in 4 x SSC for 30 min at 37°C in a moist container. Avidin-fluorescein isothiocyanate (FITC, Boehringer Mannheim) was used to detect the tumour DNA and anti-digoxigenin rhodamine (Boehringer Mannheim) was used to detect the normal DNA (both diluted 100-fold in 0.5% dry milk powder in 4 x SSC). The slides were incubated for 60 min at 37°C in a moist container. Next, the slides were washed 3 x 5 min in 4 x SSC/0.05% Tween. Chromosome preparations were dehydrated and counterstained with 4'-6-diamidino-2-phenylindoledihydrochloride (DAPI) for identification.

Image analysis

Images were captured with a Zeiss Axiophot fluorescence microscope (Carl Zeiss Inc., Thornwood, New York) equipped with a 100 Neo-Fluar objective, a computer controlled filter wheel, and a cooled CCD camera (Photometrics, Tucson, AZ) using Smartcapture IPLab Extensions software (Vysis). Image analysis was carried out on a PowerMac computer using Quips Imaging Software (Vysis). The fluorescence ratio (FR) of the FITC-to-rhodamine signals were averaged from 8 to 12 chromosomes

of each type. The average FR for the CGH experiments was 1.0 with a standard deviation of 0.05. Based on our previous experiments and the published requirements for quantitative analysis of CGH, FR thresholds of 0.80 and 1.20 were chosen to distinguish numerical and regional loss and gain, respectively [16-18].

Controls

All experiments were repeated once. Male control DNA was used in all experiments. Control procedures included CGH experiments using normal versus normal DNA and normal male versus normal female. CGH was also performed on DNA isolated from a tumour cell line (MPE 600) provided in the Vysis CGH Nick Translation kit (Vysis). This DNA was used as a positive control in each CGH experiment. All of the known abnormalities present in the Vysis tumour cell line were readily detected in each CGH experiment.

Southern blot analysis

Because the CGH experiment suggested that the oncogene MYCN may be amplified in some MBs, we assessed MYCN gene dosage by Southern blot on the tumour DNA. First the digested tumour DNA was transferred to a nylon membrane according to standard procedures. Hybridisation was carried out as described elsewhere [15].

After wetting the filter in 0.2 x SSC with 0.5% SDS, it was pre-hybridissed at 42°C in 50% formamide containing 6 x SSC, 5x Denhardt's, 1% SDS, and 100 µg/mL denatured herring sperm DNA. The filter was then hybridised overnight at 42°C in a solution of the same composition, but containing 25 ng of plasmid pNB-1 (American Type Culture Collection, ATCC, Rockville, MD) labelled with 32P-dCTP to a specific activity of 5 x 108 cpm per µg by the random primer technique [19].

Following hybridisation, the filter was rinsed 2 x 5 min in 1xSSC with 0.1% SDS at room temperature, 1 x 5 min in 0.2 x SSC with 0.1% SDS at room temperature, 2 x 30 min in 0.2 x SSC with 0.1% SDS at 60°C, and 2 x 5 min in 0.2 x SSC at room temperature before exposing it to X-ray film.

In order to control for MYCN signal variation associated with sample loading, the membrane was stripped of plasmid pNB-1 in 0.4 M NaOH and then rehybridised with the control probe KM.19. Autoradiograms associated with each hybridisation were scanned and resulting images were analysed for relative MYCN signal response using the NIH Image 1.52 software program.

Chromosome studies

Chromosome studies were performed on fresh material from 3 tumours and on the cell line using previously described methods [10].

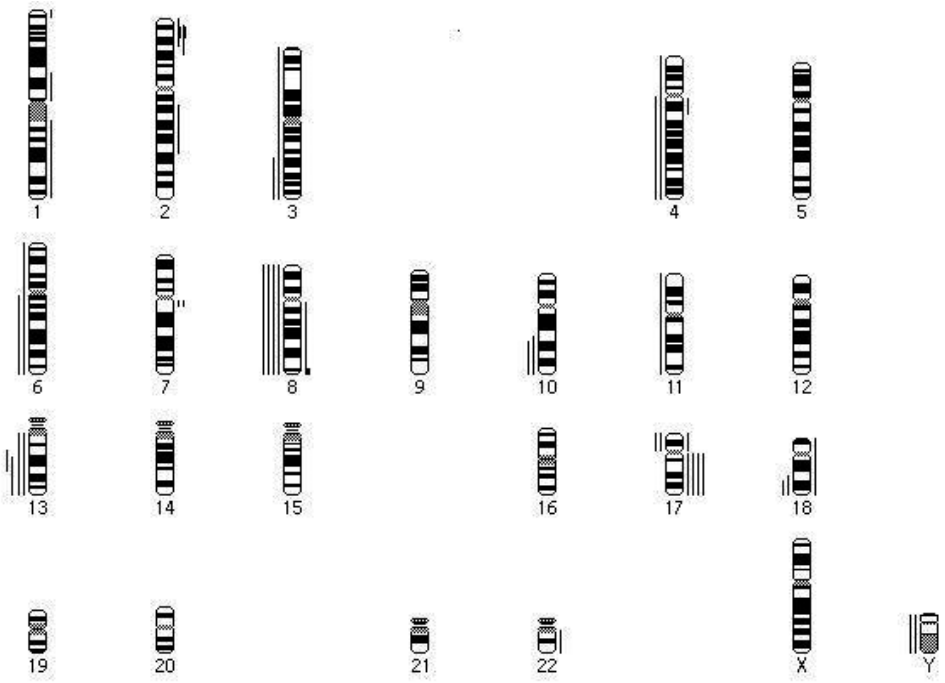

Figure 1 *Summary of genetic imbalances detected by CGH in 10 MBs and one MB cell line. Lines on the left of each chromosome indicate losses, whereas lines on the right represent gains. Amplification sites are indicated by squares.*

Fluorescence in situ hybridisation (FISH)

FISH was performed on paraffin-embedded tissue sections (5μm), which were mounted on glass slides. The slides were first deparaffinised (3 x 10 minutes in xylene), and dehydrated (2 x 5 min in 100% ethanol and 2 x 5 min in 95% ethanol). The slides were then treated with 0.2N HCl for 20 min at room temperature, washed in deionised water for 3 min and then in a phosphate buffered saline solution for 3 min. Next, the slides were placed in plastic coplin jars filled with 10mM citric acid (Sigma, pH 6.0) and microwaved on high-power for 10 min. The slides were removed from the jars, cooled to room temperature, and placed in 2 x SSC for 5 min. This was followed by tissue digestion in which 1 mL pepsin (Sigma, 4mg/mL) was placed on each slide and incubated at 37°C in a moist container for 15 min. The slides were washed in deionised water for 3 min and then placed in 2 x SSC for 5 min. Next, the slides were put into a coplin jar filled with 0.1M triethanolamine/acetic anhydride for 3 min at room temperature and then washed in 2 x SSC for 3 min. The hybridisation procedure has been described previously [6]. A biotin labelled alpha satellite chromosome 2 probe (Oncor, Gaithersburg, MD) and a SpectrumOrange labelled locus specific probe

for MYCN (Vysis) were the FISH probes used. Avidin-FITC was used for detection of the biotin probe. The signals were evaluated using a Zeiss Axioplan fluorescent microscope equipped for FITC, TRITC, and DAPI fluorescence.

RESULTS

CGH was performed using DNA from 10 primary fresh frozen tumours and 1 cell line. Figure 1 and Table 2 summarise the alterations detected by CGH for both the tumours and the cell line. The most frequently observed alteration involved chromosome 17. Gain of 17q was detected in 4 of the 10 primary tumours and in the cell line. In two cases (cell line 341 and case 622) gain of 17q was accompanied by a loss of 17p (Figure 2). The second most common aberration was the loss of chromosome 8, which was detected in 4 tumours. Also, 7q11.2 was gained in 2 tumours. Deletions of 13q were detected in 2 tumours; 1 of the whole arm and 1 of bands 13q21-qter. Deletions of 10q, 18q, and Y were each detected in 2 tumours. Gain of 1p and amplification of 2p21-24 (MYCN region) were each detected in 2 tumours.

CGH results were compared with routine cytogenetic data, MYCN dosage analysis by Southern blot, and FISH studies. The results of these experiments are summarised in Table 2.

The Karyotypes were available for cases 1314, 968, and 622, and the cell line. For case 1314, the deletion detected on 10q was nonclonal, yet the CGH result showed loss in that region of chromosome 10 q-arm. The complex karyotype of case 968 shows several numerical and structural alterations, including deletion of 17p. However, CGH only detected loss of 14q and 18q. No abnormalities were found in the karyotype of case 622 although several gains and losses were detected by CGH. In addition, the amplification of 8q, loss of 17p, and gain of 17q detected by CGH in cell line 341 also matched the alterations detected in the karyotype.

The results of the Southern blot analyses showed a 5- and 1.9-fold amplification of MYCN in both tumours (616 and 166) with amplification of 2p21-24 (MYCN region) by CGH. FR thresholds were 1.5 and 2.0, respectively. Similarly, FISH analysis showed MYCN amplification for both cases.

No single abnormality could be linked to clinical-biological behaviour. A trend relating the number of genetic alterations to the survival rate was observed: both cases 616 and 309 had short survival rates combined with a large number of genetic changes. Tumours with desmoplastic histology (1314, 968, 616, 309, 10) were not associated with specific genetic alterations.

Case	CGH Analysis		Karyotype	MYCN dosage**	MYCN amplification***
	Loss	Gain			
1314	10q23-10qter	None	46,XX,del(10)(q22q24)*	0.88	ND
968	14q, 18q21-qter	None	80-90,XXXX,-1,-9,-12,-14,16, del(17)(p11.2?p13),18,+mar	1.00	ND
616	10q22-qter, Y	1p11-22, 2p21-25, 2q14.131, 4q12, 21, 17p	ND	5.04	Yes
0620	None	7q11.2, 17q	ND	0.61	No
309	3q24-qter, 4q, 6q, 8, 13q1422, 18q22-qter	1p36.2-3, 7q11.22, 17q	ND	0.75	No
622	3, 8, 17p	17q	46,XY	1.00	No
166	8, 13q21-qter	2p16-24, 17q	ND	1.84	Yes
167	Y	None	ND	0.80	ND
10	4, 8, 11,	22	ND	1.04	ND
24	6	None	ND	1.54	ND
Cell line 341	17p	1q, 8q(q24 amplification), 17q, 18	45,XY,7,add(8)(q24.1), add(16)(q13),i(17)(q10), der(20)t(1;20)(q12;q13.3)	ND	ND

Table 2 *Genetic analysis of medulloblastomas.* The karyotype of case 1314 was nonclonal, ** MYCN dosage determined by Southern-blot hybridisation relative to standard, *** MYCN amplification determined by FISH (see text for explanation), ND: not done.*

DISCUSSION

In this study, CGH was used to obtain an overview of genomic alterations in 10 MBs and 1 MB cell line. Nonrandom losses (2 or more cases) were found on chromosome regions 10q23-qter, 13q14-q22, 17p, and 18q22-qter and nonrandom gains (2 or more cases) were detected for regions 1p, 2p21-24, 7q11.2, and 17q. Nonrandom whole chromosome losses (2 or more cases) were detected for chromosomes 8 and Y.

Gain of the distal chromosome 2 p-arm was detected in 2 tumours (616 and 166). This region is of great interest in MBs because it contains the MYCN gene, which has been detected in about 7% of MBs and may be associated with a poor prognosis [8,9,20]. The MYCN amplification was increased 2-5-fold in both tumours. The cell line 341 showed amplification on the distal end of the chromosome 8 q-arm. This corresponds to the 20-fold amplification of MYCC previously established by Southern hybridisation (results not shown) [14]. Thus the gains of 2p and 8q in MBs as detected by CGH represent amplifications of MYCN and MYCC, respectively. These alterations constitute important steps in the oncogenesis of MBs, but their clinical relevance is as yet unclear [10,11].

Aberrations of chromosome 17 are frequently detected in MBs [20-32]. We found gains of 17q in combination with a loss of 17p in two cases (622, cell line 341, Figure 2). The combination of 17q gain and 17p loss most likely represents the presence of

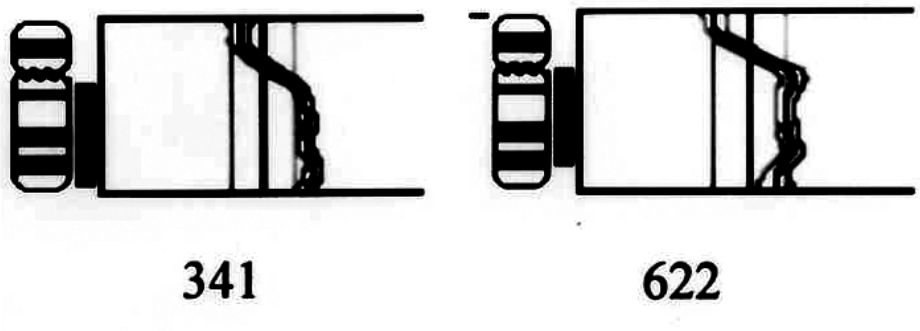

Figure 2 *CGH results for chromosome 17 indicating the presence of an iso chromosome 17(i[17q]). Average profiles are shown for cases 341 and 622 which both had a gain of 17q and a loss of 17p. The mode of the intensity ratios (middle line) and the thresholds for overrepresentation (right line) and underrepresentation (left line) are presented. FR thresholds of 0.80 and 1.20 were used to distinguish numerical and regional chromosomal loss and gain, respectively.*

isochromosome 17 (i[17q]) which is found in 30% of MBs [21,22,25]. This interpretation of the CGH pattern is supported by the presence of i[17q] in the karyotype of the cell line.

Several other anomalies were observed in this set of MBs by CGH. Tumour 616 had amplification on 1p11-22, which has also been described in gliomas [33]. Two tumours showed a deletion of 10q22-10qter and potentially including the recently described 'deleted in malignant brain tumour' (DMBT) and 'phosphatase and tensim homoloque delete on chromosome 10' (PTEN) genes [34]. The deletion of 18q21-qter potentially includes the 'deleted in colon carcinoma' (DCC) gene [35]. Two tumours showed a deletion of 13q with a minimally deleted region of 13q14-22 which contains the retinoblastoma 1 (RB1), 'disrupted in B cell malignancy 1' (DBM1), and breast cancer 2 (BRCA2) genes [36].

So far, 150 primary MBs have been analysed by CGH [20,28-33]. An array of nonrandom genomic losses was found in most samples. Patterns of chromosomal gains and losses unique to MBs were not observed. Chromosomal imbalances most frequently involved chromosome 17 (loss of 17 p and gain of 17q) and losses of 10q, 11 and 8. High-level amplifications were found on 2p24 and 8q24, suggesting involvement of MYCN and MYCC respectively [20,32] in concordance with our results. Three studies (62 patients) and one case report compared the clinical outcome of primary MBs with CGH results in the search for genetic alterations with potential prognostic value [27,29,31,32]. Nishizaki et al. suggested an association between the number of chromosomal changes and the unfavourable prognosis on the basis of only six cases [30]. The two larger studies (n = 56) and our results (n = 11) failed to identify specific genetic abnormalities or patterns associated with prognosis [27,31], although Nicholson et al. suggested an association of loss of chromosome 22 and a poor prognosis [31]. Much attention has been focused on aberrations of chromosome 17, the most typical genomic alteration in MBs. Although an association between loss of heterozygosity (LOH) on 17p and adverse prognosis has been reported [7], these findings could not be reproduced in the CGH series comparing CGH with clinical outcome [27,29,31].

Prognosis has previously been linked to histological variant, with desmoplastic variants tending to have a more favourable outcome than classic MBs [37, 38]. We found no difference in survival between patients with desmoplastic and non-desmoplastic tumours. Nicholson et al. also failed to find a significant difference in prognosis between 14 patients with desmoplastic MBs and 17 patients with non-desmoplastic MBs [31]. However, our number of patients is too small to make any conclusions about this matter.

Although CGH is a valid and reproducible technique for the detection of sub-chromosomal gains and losses in solid tumours [13,16], the results are not completely concordant with cytogenetic analysis. Both our group and others [13,16] have observed this. The reasons for the discordant results are likely due to weaknesses of both procedures. Cytogenetic analysis only evaluates a limited number of clones present within each tumour. Routine cytogenetic analysis does not provide a complete overview of all genetic aberrations. Culturing solid tumours is difficult, and the cultures may be overgrown by normal cells. CGH only detects copy number changes involved. Translocations and inversions cannot be detected by CGH as they represent balanced alterations not involving loss or gain of DNA. Multiploidy cannot be detected by CGH, because equal amounts of tumour and control DNA are used in CGH experiments.

In conclusion, we were able to confirm that the gains of 2p and 8q represent amplifications of MYCN and MYCC respectively. CGH studies have failed to identify a single genetic aberration or pattern of alterations relating to biological behaviour.

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Chapter 3

Correlation between localisation, age, and chromosomal imbalances in ependymal tumours as detected by CGH

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ABSTRACT

Ependymal tumours (ETs) are gliomas that arise from the ependymal lining of the cerebral ventricles and from the remnants of the central canal of the spinal cord. Both clinical and genetic studies suggest that distinct genetic subtypes of ETs exist, the subtypes being correlated with patient age and/or tumour site. In the present study the tumour genome of 20 ETs (15 adult and 5 paediatric cases) was screened for chromosomal imbalances by comparative genomic hybridisation (CGH). The most frequently detected imbalances were -22q (75%), -10q (65%), -21 (50%), -16p (50%), -1p (45%), +4q (45%), -10p (45%), -2q (40%), -6 (40%), -19 (40%), -2p (35%) -3p (35%), and -16q (35%). Comparison of the chromosomal imbalances detected in ETs to those previously reported in oligodendroglial and astrocytic tumours revealed that in this respect ETs show similarities to these other gliomas. By combining these results with those of a recent study of Zheng et al. and Hirose et al., it was found that, although ETs from different localisations and from adult and paediatric patients show overlap at the CGH level, some chromosomal imbalances occur predominantly in a certain category. In adult patients, spinal ETs relatively often showed +2, +7, +12, and -14q; infratentorial ETs -22; and supratentorial ETs -9. In addition, in posterior fossa ETs -6 and +9 were much more frequent in adults than in children. It is concluded that the genetic background of ETs is complex and partly determined by tumour site, histopathological subtype, and age of the patient.

INTRODUCTION

Ependymal tumours (ETs) are gliomas that are considered to arise from glial cells which normally cover the wall of the cerebral ventricles and of the central canal in the spinal cord. Although ETs are relatively common in childhood, they may occur at all ages [1,2]. According to the World Health Organisation (WHO) classification, ETs are subdivided into four categories: myxopapillary ependymoma (mpE, WHO grade I), subependymoma (subE, WHO grade I), low-grade ependymoma (lgE, WHO grade II), and anaplastic ependymoma (aE, WHO grade III). In addition, the most malignant glial tumour, i.e. glioblastoma multiforme (WHO grade IV), sometimes shows focal ependymal differentiation (GBM-E). Numerous studies have yielded conflicting results regarding the prognostic significance of histological parameters for patients with lgE or aE (for review see [3]). Clinical studies revealed that postoperative survival depends on age of onset, tumour site, and extent of tumour resection [1]. Furthermore, it is well established that tumour localisation and age of the patient are interrelated [1].

These clinical data suggest that distinct tumour entities exist among ETs, which may be age- or site-related. Indeed, age-related genetic pathways are suggested by loss

of heterozygosity (LOH) analysis, reporting LOH 22q to be a common finding in adult ETs that is only rarely present in a paediatric population [4]. Site-dependent genetic pathways are suggested by the observation that neurofibromatosis type-2 (NF-2) patients especially develop spinal ETs, and that sporadic spinal ETs are characterised by a high incidence of loss of 22q and NF-2 mutations [1,5].

In the present study we screened 20 glial tumours with ependymal differentiation for chromosomal imbalances throughout the entire tumour genome using comparative genomic hybridisation (CGH). We also analysed whether ETs show similarities to astrocytic and oligodendroglial tumours at the chromosomal level. Finally, the correlation between chromosomal aberrations, patient age, tumour localisation, and histopathological grading was analysed by combining our results with those recently reported by Zheng et al. in a group of 28 ETs [6], and with those of Hirose et al. in a group of 44 ETs [7].

MATERIALS AND METHODS

Surgical specimens of 15 adult and 5 paediatric tumours selected for the present study were obtained from patients treated at the University Medical Centre Nijmegen and the St. Elizabeth Hospital Tilburg, The Netherlands. During surgery, part of the tumour was snap-frozen in liquid nitrogen and stored at -80°C.

Hematoxylin and eosin-stained (H&E) paraffin sections of each tumour were classified according to the WHO 2000 classification [1] as mpE (n=2), lgE (n=12), or aE (n=4). For grading an ET as aE, the tumour had to show at least three of the following four histopathological features: high cellularity, marked nuclear polymorphism, brisk mitotic activity (10 or more per mm^2), and florid microvascular proliferation. In addition, two cases of glioblastoma multiforme with local ependymal differentiation in the form of perivascular pseudorosette formation were included in this study (GBM-Es). Apart from these ependymal features (that are illustrated in Figure 1), these two GBM-Es histopathologically showed astrocytic differentiation, necrosis, and all four features for high-grade malignancy as mentioned above.

CGH was performed essentially as previously described [8], with minor modifications. Tumour and normal DNA were differentially labelled using biotin-dUTP or digoxigenin-dUTP (Roche Molecular Biochemicals, The Netherlands) in nicktranslation and the probe was prepared as described [8]. The probe and metaphase spreads were denaturated simultaneously using the OmniSlide Thermal Cycler (5 minutes 37°C, 3 minutes 72°C followed by approximately 60 hours at 37°C) (Thermo-Hybaid, Middlesex, UK). Detection and analysis was performed as described [8] using

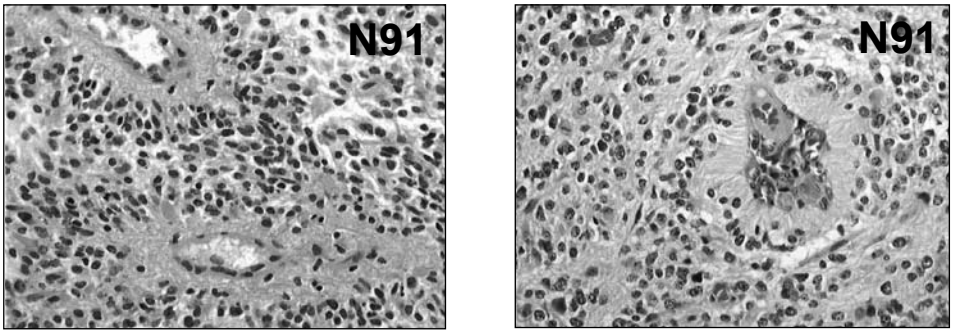

Figure 1 *Ependymal features in glioblastomas. Illustration of perivascular pseudorosette formation that was only focally present in two high-grade malignant glial tumours (N91, N213), included in the present study as glioblastomas with focal ependymal differentiation (GBM-Es).*

streptavidin-FITC and sheep-anti-digoxigenin-TRITC (Roche Molecular Biochemicals, The Netherlands) and the QUIPS CGH software (Applied Imaging, UK). The standard thresholds for gains (1.2) and losses (0.8) were used, aberrations of 1.4 and 0.6 were called clear copy number changes and aberrations (1.6 were called high copy number gains). The quality of our CGH experiments was monitored by including both a negative and positive control experiment [8].

Combining the CGH results of Zheng et al. (28 ETs) [6] and Hirose et al. (44 ETs) [7], with those in our group of 20 ETs, the correlation between chromosomal imbalances, patient age, tumour site, and histopathological grading was analysed. An overview was created of the frequency of gains and losses on each chromosome arm for different tumour localisations in adult and paediatric ETs.

RESULTS

Clinical data and histopathological diagnosis of the 20 analysed tumours are shown in Table 1. Histopathological analysis identified 2 mpEs, 12 lgEs, 4 aEs, and 2 GBM-Es (Table 1). In the group of adult tumours (n=15), 8 occurred in the spinal canal (53%) whereas the other 7 were intracranial tumours (47%) (3 in the posterior fossa and 4 supratentorial). In contrast, all paediatric tumours (n=5) were intracranially located (3 posterior fossa and 2 supratentorial). The CGH results are summarised in Table 1 and depicted in Figure 2. The mean number of aberrations per tumor was 11 (range 2 to 25). Frequently detected chromosomal imbalances (present in 7 tumours or more) were -22 (15/20), -10q (13/20), -16p (10/20), -21 (10/20), -1p (9/20), -10p (9/20), +4q

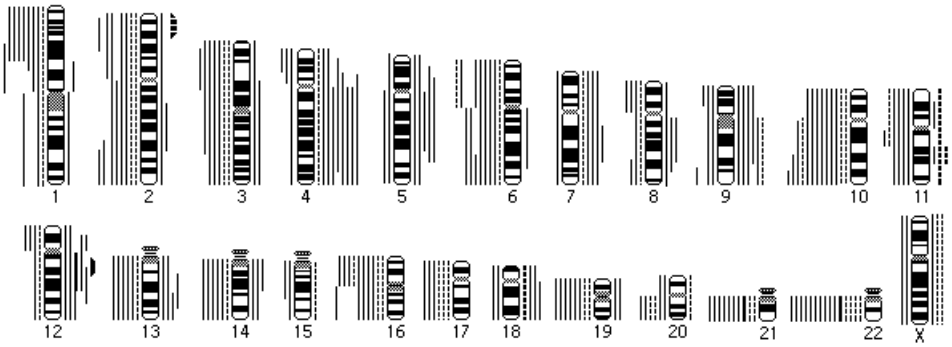

Figure 2 *Summary of all chromosomal imbalances as detected by CGH in adult and paediatric ETs. Lines on the left and right of the chromosome indicate losses and gains of these chromosomal regions respectively. A thin line indicates aberrations crossing the 0.8 or 1.2 threshold, a heavy line indicates a clear copy number change (crossing the ratios 0.6 and 1.4), and an additional spot on the bar indicates a high copy gain. Aberrations in adult ETs are indicated by a continuous line, aberrations in paediatric ETs by a discontinuous line.*

(9/20), -2q (8/20), -6p (8/20), -6q (8/20), -19 (8/20), -2p(7/20) -3p (7/20), and -16q (7/20). The common regions of overlap of these regions are -10q25-qter, -1p31-pter, +4q11-32, -2q33-qter, -6q23-qter, 2p16-24, and -3p14-24.

In Figure 3, an overview is shown of the frequency of gains and losses involving the individual chromosome arms for spinal, posterior fossa, and supratentorial ETs in adults, and for paediatric posterior fossa and supratentorial ETs. In this figure we combined our CGH results with those previously reported by Zheng et al. [6] and Hirose et al [7]. The mpEs and GBM-Es were not included in this overview.

DISCUSSION

Chromosomal imbalances detected by CGH in ETs

A loss of 22 was the most frequently detected aberration in our group of ETs (75%). Other chromosomal imbalances frequently detected were: -10q (65%), -21 (50%), -16p (50%), -1p (45%), +4q (45%), -10p (45%), -2q (40%), -6 (40%), -19 (40%), -2p (35%) -3p (35%), and -16q (35%). In general, our results are in concordance with those of Zheng et al. [6], whereas the frequencies reported by Hirose et al. [7] are

No	Sex	Age (years)	WHO Grade	Site	CGH losses	CGH gains	No. ab.
Adult tumours							
N270	M	31	mpE	spinal	1p31-pter, 1q, 2p16-24, 2q33-qter, 3p14-24, 6q23-qter, 6p, 10, 11, 14q, 16p, 17, 19, 22	4p14-qter, 5p14-q31, 9pter-q31, 12p12-q21, 18	19
N273	F	33	mpE	spinal	1p31-pter, 4p15-pter, 8p, 10, 12p, 13q, 14q, 16p, 19, 22	4p14-qter, 5p13-q31, 7q21-31	13
N222	M	57	lgE	spinal	16, 22	12	3
N221	M	49	lgE	spinal	21c, 22 c	7, 12	4
N226	F	57	lgE	spinal	1, 2, 3, 6, 8, 10, 11, 13q, 14q, 16, 19, 20, 21, 22	4, 5, 7, 9, 18	19
N229	M	39	lgE	spinal	2q, 6q, 13q, 22	19	5
N231	F	45	lgE	spinal	1p, 4p, 5, 6p, 9, 10, 19, 20q, 21, 22	2, 3, 4q, 8q11-23, 13q, 14q	16
N268	M	57	lgE	spinal	1p31-pter, 9q33-qter, 10q25-qter, 11q12-13, 15q, 16, 17, 19, 20q, 22, X	1p11-22, 2q21-32, 3p14-qter, 4p15-q32, 5q11-23, 6q11-24, 8q11-22, 11p11-14, 11q14-22, 12p11-12, 12q14-23, 13q14-31, 14q11-21, 18q	25
N225	F	32	lgE	post.fossa	2, 3, 6, 10, 16, 17, 21, 22	4, 9, 13q, 14q	12
N269	M	25	lgE	post.fossa	1p22-pter, 2p, 2q32-qter, 6, 8p, 8q23-qter, 10, 16q, 21, 22, X	4q, 9	13
N271	F	59	lgE	post.fossa	1p31-pter, 10q23-qter, 14q, 16p, 19, 20, 21, 22	4q	9
N267	F	25	lgE	suprat.	1p11-31, 2, 3, 4, 6, 7, 9, 10, 11, 12, 13q, 18, 21	1q, 5, 8, 19, X	18
N76	M	22	aE	suprat.	3pter-q24, 5q, 10q, 11, 13q, 18, 21	9q	8
N213	F	34	GBM-E	suprat.	21	12q, 12q11-21c, 13q22-qter	4
N91	M	60	GBM-E	suprat.	6q, 9p21-pter, 12p, 14q, 15q11-22	7, 12q13-15*	7

No	Sex	Age (years)	WHO Grade	Site	CGH losses	CGH gains	No. ab.
Paediatric tumours							
N224	M	2	aE	post.fossa	17, 19, 20q, 22	X	5
N227	M	2	aE	post.fossa	10q, 16p, 22	X	4
N266	F	12	lgE	post.fossa	1, 2, 3, 6, 8, 10, 12, 13q, 14q, 16, 17, 21, 22	7, 9q, 11c, 15q, 18c, 20	19
N228	F	17	lgE	suprat.	2, 3, 6p, 10, 11pter-q13, 16, 17, 19, 20q, 21, 22	4	12
N87	M	6	aE	suprat.	-	2p22-pter*, 11q14-22 c	2

Table 1.*Chromosomal imbalances detected by CGH in ependymal tumours and corresponding clinical characteristics. Sex: M= male; F= female. Age = age at surgery. WHO grade: mpE = myxopapillary ependymoma (WHO grade I); lgE = low-grade ependymoma (WHO grade II); aE = anaplastic ependymoma (WHO grade III); GBM-E = glioblastoma multiforme showing focal ependymal differentiation (WHO grade IV). Site: tumour localisation; spinal = spinal canal, post.fossa = posterior fossa, suprat. = supratentorial; No. ab = number of chromosomal aberrations; c = clear copy number change, ratio more than 1.4 or less than 0.6; * = high copy gain, ratio >1.6.*

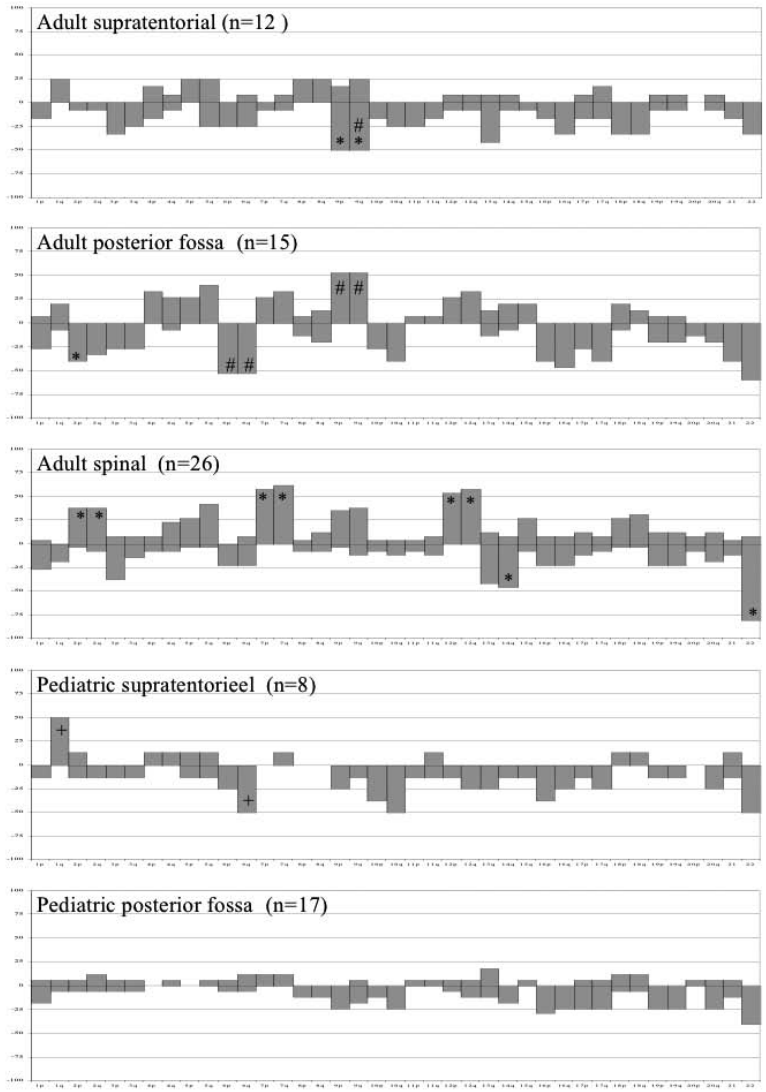

Figure 3. *An overview of the frequency of chromosomal imbalances in ependymal tumours at different sites, based on combination of the results of the present study and those of Zheng et al [6] and Hirose et al. [7]. A positive percentage represents the frequency of a gain, whereas a negative percentage represents the frequency of a loss. The chromosome arms are indicated at the bottom of the figure. Maximum differences between different tumour localisations in adults exceeding 35% are indicated by *, whereas such differences in paediatric tumours are indicated by +. Differences between adult and paediatric ETs at the same site (posterior fossa or supratentorial) are indicated by #. Used abbreviations: n = number of analysed tumours.*

lower. In several LOH studies, LOH 22q in ETs ranged from 0% to 100% [5,9-11]. Differences between individual studies on ETs are also present for the involvement of NF-2 in ETs [5]. These discrepancies may reflect site- or age-related genetic pathways that lead to the formation of distinct subsets of ETs [5,12]. The common region of overlap of frequently detected aberrations most likely contains genes involved in the oncogenesis of ETs. Since Zheng et al. [6] and Knuutila et al. [13,14] already elaborated on this subject, we will not discuss this topic further.

Recently, two other CGH studies on ETs were published [2,15]. The results of these studies are not included in our overview of the authors' CGH findings in ETs. In one of these papers, a difference exists in the sensitivity of their CGH experiment using either the "standard" or "reverse" labelling technique (as shown by their Figure 2), which may result in an underestimation of the number of aberrations [2]. In the other paper, some chromosomal regions were excluded from analysis [15], as early CGH papers reported that false positive results may occur at 1p32-pter, 16, 19, and 22 [16]. However, after refining CGH, such artefacts are only occasionally detected [17] and partially depend on the quality of the metaphase chromosomes and of the two labelled DNAs [18]. Interestingly, in contrast to 1p32 pter, 16, and 19 which were excluded from analysis, chromosome 22 was analysed in their study and -22 and +22 was reported to be present in less than 10% and 23% of the 26 ETs, respectively [15]. A +22 was only detected in 2% and -22 in 53% of the 92 ETs analysed by Zheng et al. [6], Hirose et al. [7], and our group.

Comparison of ETs and other gliomas

Although one study reported resemblance at the molecular genetic level between ETs and astrocytic and oligodendroglial tumours (As and OTs) [9], others reported that aberrations involved in the oncogenesis of ATs were rarely detected in ETs [1,4,5,19]. OTs very frequently show loss of 1p and 19q [8,20-23]. Losses involving these regions were detected in 8 (40%) of the ETs analysed by us, but often a complete loss of chromosome 19 and a partial loss of 1p were detected. We did not find a correlation between aberrations of chromosome 1 or 19 in ETs and the presence of oligodendroglial features in these tumours at the histopathological level.

Genes involved in the two main cell-cycle regulatory pathways at the G1-S border, the RB1 pathway and the P53 pathway, are reported to be involved in ATs [24-28]. For the RB1 pathway it was shown that a loss of CDKN2A (9p21), amplifications of CDK4 (12q13.3-q14), and inactivation of RB1 (13q14.3) are alternatives for escape of growth control [24-27]. For the P53-pathway, TP53 (17p13) mutations or deletion, and amplification of mdm2 (12q13) appear to be alternatives for escape of growth control [28]. Our current results show that 13 of the 20 ETs harbour aberrations including +12q13 (CDK4), -9p21 (CDKN2A), and/or -13q14 (RB1), suggesting that the RB1

pathway is involved in the oncogenesis of a proportion of the ETs. Furthermore, 12 of the 20 ETs showed aberrations including -17p13 (TP53) and/or +12q13 (mdm2), indicating that the P53 pathway may also be involved in the oncogenesis of these tumours. No correlation was detected between the involvement of these pathways and age, tumour site, or histopathological diagnosis.

Histopathological classification
Two of the ETs included in the present study were diagnosed as GBM with focal ependymal differentiation in the form of perivascular pseudorosettes (N213 and N91) (Figure 1). A CGH study of 100 GBMs reported as most frequent aberrations -10q (77%), -10p (59%), +7 (75%), and -9p (59%) [29]. N213 contained none of these aberrations, whereas N91 contained +7, and -9p. These latter aberrations are also detected in lgEs. While -22 is the most frequently detected aberration in ETs, this aberration was not detected in the two GBM-Es. However, both GBM-Es are supratentorial localised and -22 was detected in only 33% of the supratentorial ETs in adults analysed by us, Zheng et al. [6], and Hirose et al. [7] (discussed below). Additionally, losses smaller than 10-12 MB are undetectable by CGH and might still be present on 22 [30]. It remains to be seen if such GBM-Es should indeed be separated from ordinary GBMs and included in the group of ETs as a grade IV tumour.

According to the WHO classification, mpEs are a distinct entity within the group of ETs. They are slow growing, grade I tumours with a generally favourable prognosis [1]. Since chromosomal imbalances accumulate during (malignant) progression of tumours, it was unexpected to find such an extensive array of genomic aberrations (13 in N273, 19 in N270). Both mpEs included in the present study showed clear similarities with regard to the chromosomal aberrations; -1pter-p31, +4p14-qter, +5p13-q31, -10, -14q, -16p, -19, and -22 were detected in both tumours, implicating that these chromosome arms probably contain genes that are important for the oncogenesis of mpEs in adults. As these aberrations were occasionally detected in the previously analysed paediatric mpEs [7], it remains to be established whether different genetic pathways exist for adult and paediatric mpEs.

LgEs may show malignant progression to aEs [1]. Although -22 was detected in 12 of the 13 lgEs, only 2 of the 4 aEs contained this aberration. Furthermore, although tumours generally show accumulation of genetic aberrations during malignant progression, the aEs contained fewer aberrations than the lgEs (an average of 5 in aEs, and 13 in lgEs), suggesting that different genetic pathways exist for lgEs and aEs. However, since all aEs were intracranially located, whereas approximately half of the lgEs occurred in the spinal canal, such differences between lgEs and aEs at the CGH level may also reflect site-dependent pathways.

Age- and site-related differences

As adult patients fare better than paediatric patients and LOH 22q was reported to be a common finding in adult (but not in paediatric) ETs, age-related genetic differences might exist [1,4]. Furthermore, the observations that patient age and tumour site are interrelated [1], that patients with neurofibromatosis type 2 (NF-2) disease develop spinal ETs, and that sporadic spinal ETs often show an altered NF-2 gene and LOH 22q [5,11], suggest a correlation between molecular genetic aberrations and tumour site. To identify age- or tumour site-dependent genetic pathways in ETs, we combined our results with those reported in the CGH study of Zheng et al. [6] and Hirose et al. [7]. Since overall only 3 paediatric spinal ETs were analysed, these tumours were not included. Additionally, as mpEs may be a distinct entity and it is not clear whether GBM-Es should be regarded as ETs, only the lgEs and aEs were analysed for this purpose.

The differences in the distribution of chromosomal imbalances for ETs in the supratentorial compartment, spinal canal and posterior fossa are illustrated in Figure 3. In ETs of adult patients, the following site-related differences in chromosomal imbalances as detected by CGH exceeded 35%: +2 in 38% of the spinal ETs; -2p in 40% of the posterior fossa ETs which never gained this chromosome; +7 in approximately in 60% of the spinal ETs, in 30% of the posterior fossa ETs, and occasionally in supratentorial ETs; -9 in 50% of the supratentorial ETs; +12 in approximately half of the spinal ETs, in one-third of the posterior fossa ETs, and occasionally in spinal ETs; -14q in 39% of the spinal ETs and -22 in 81% of the spinal ETs. Similarly, differences in distribution of chromosomal imbalances for intracranial paediatric ETs were detected: +1q and -6q are detected in 50% of the supratentorial and in 6% of the posterior fossa ETs. Overall these results suggest site-dependent genetic pathways for both adult and paediatric ETs.

Differences between adult and paediatric ETs of the same site were also noticed; -6 and +9 are detected in 53% of the adult posterior fossa ETs and in only 6% or less of the paediatric ETs at this localisation and -9q is detected in 50% of the adult supratentorial ETs and in 13% of the paediatric supratentorial ETs. Thus, this analysis offers evidence for the existence of genetic pathways for ETs that are not only site-dependent, but also age dependent.

In conclusion, our CGH study shows that the oncogenesis of ETs is rather complex and that -22 and -10q are the most frequently detected aberrations in these tumours. As in other gliomas, the RB1 and the P53 pathway seem to be involved in the oncogenesis of a proportion of the ETs. Since GBM-Es show some differences with ordinary GBMs at the CGH level, it remains to be seen if these tumours should be regarded as grade IV ETs rather than as ordinary GBMs. Combining our results with those of Zheng et

al. [6] and Hirose et al. [7], we have shown that different chromosomal imbalances are involved in the oncogenesis of ETs in adults that are correlated with tumour site; -22 was most frequently detected in infratentorial ETs, -9 was most frequently detected in the supratentorial ETs, whereas +2, +7, +12, and -14q were most frequently detected in spinal ETs. Furthermore, differences between posterior fossa or supratentorial ETs in children and adults suggest that age-related chromosomal imbalances exist. For further elucidation of the genetic background of Es, more tumours should be analysed with a large enough number in each subgroup as determined by patient age, tumour-site, and histopathological subtype.

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Chapter 4

Inverse correlation between genetic aberrations and malignancy grade in ependymal tumours: a paradox?

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Reprinted from: J Neurooncol 2004;66:111-6
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ABSTRACT

The goal of our study was to investigate the inverse correlation between number of genetic aberrations and malignancy grade in ependymal tumours (ETs) at the ploidy level. We examined 7 myxopapillary ependymomas (mpEs) (WHO grade I), 28 spinal and cerebral low-grade ependymomas (lgEs) (WHO grade II) and 18 cerebral anaplastic ependymomas (aEs) (WHO grade III) using image DNA cytometry. The ploidy status was correlated with clinicopathological characteristics and with the results obtained by comparative genomic hybridisation (CGH) analysis that we performed in about half of these tumours. MpEs were exclusively located in the spinal cord and aES in the cerebrum only, whereas lgEs were located in both the spinal cord and brain. We found aneuploidy or tetraploidy to be common in the group of mpEs (6 out of 7) and much less frequent in lgEs (6 out of 28) and aEs (4 out of 18). Three-year postoperative survival was 100% for mpEs, 100% for spinal low-grade Es, 92% for cerebral lgEs and 33% for aEs. Our CGH results in a selection of these tumours revealed the highest number of genetic aberrations in the mpEs (average 16; n=2), a lower number in lgEs (average 12; n=11) and the lowest number in aEs (average 5; n=6). Interestingly, in the group of low-grade Es and aEs, a high number of genetic aberrations as detected by CGH was not correlated with aneuploidy or tetraploidy. Three patients, all with mpEs, had local seeding. These results underline that mpEs are distinctly different from lgEs and aEs at the genetic level and that extensive genomic alterations and aneuploidy in ETs are not in itself an indicator of malignant behaviour.

INTRODUCTION

Ependymal tumours (ETs) are considered to originate from ependymal cells of the wall of the cerebral ventricles, the central canal of the spinal cord, and from ependymal remnants in the filum terminale. They constitute 3-9% of all neuroepithelial tumours, and 50-60% of spinal gliomas [1]. The World Health Organisation (WHO) classification of central nervous system tumours recognises various types of ETs; low-grade ependymomas (lgEs; WHO grade II), anaplastic ependymomas (aEs; WHO grade III), and myxopapillary ependymomas (mpEs; WHO grade I). MpEs occur predominantly in the conus-cauda region and are histopathologically characterised by tumour cells arranged in a papillary manner around vascularised stromal cores, with low or absent mitotic activity. Although mpEs generally do not progress to higher malignancy grades, they can disseminate in the subarachnoidal space [1,2]. Apart from perivascular pseudo-rosettes and true rosettes, lgEs are microscopically characterised by moderate cellularity, monomorphic nuclear morphology, and low or no mitotic activity. In contrast, aEs typically show high cellularity, brisk mitotic

activity, nuclear atypia, and in part of the cases florid microvascular proliferation and pseudopallisading necrosis.

Malignant progression of tumours is generally associated with accumulation of genetic aberrations in tumour cells [3]. Recently, several studies on ETs described a correlation between genomic alterations and tumour grade, localisation, patient age, and prognosis [4-7]. Surprisingly, some of these studies, including our own, revealed that lgEs carried on average more genomic alterations than aEs, while in mpEs the number of aberrations exceeded that in lgEs and aEs [5,6,8]. In order to further substantiate (and unravel) this inverse correlation, we performed Image DNA cytometry on a series of mpEs, lgEs, and aEs, and compared the results of the ploidy measurements with the clinico-pathological characteristics, as well as with the CGH results that we previously obtained in part of these tumours [6].

PATIENTS AND METHODS

Patients and tumours

Fifty-three ETs (42 primary, 10 recurrences, 1 metastasis) from 49 patients (25 males, 24 females, aged 1- 69 years) who were operated on between 1985 and 2000, were derived from the University Medical Centre St Radboud Nijmegen and the St Elisabeth Hospital Tilburg, The Netherlands. Table 1 summarises the age and sex of the patients, and the location, ploidy status and histological findings of the tumours. All lgEs and aEs were intraparenchymal, cerebral or spinal tumours, all mpEs intradural tumours were located in the lumbosacral region. The histopathological diagnosis was made on hematoxylin and eosin (H&E) stained histological sections according to the WHO 2000 criteria [1]. Follow-up data were available for at least three years after operation.

DNA ploidy assessment

Ploidy assessment was performed on all tumours using a Discovery Fluorbance system (software verrsion 2.61sp, Beckton Dickinson Cellular Imaging Systems, Leiden, The Netherlands). The cytospin specimens were scanned fully automatically with a 25 x objective (NA = 0.7) using a motorised stage and automatic focussing. Microscopic fields measuring 550 x 550 µm were digitised by a Xillix MicroImager 1400 monochromatic CCD camera (Xillix Technologies Corp, Vancouver, British Columbia, pixel size 6.8 x 6.8µm^2, 8 bit sampling), resulting in an effective resolving power of 0.54 x 0.54 µm^2. Images were digitised at λ = 580 nm for detection of nuclear Feulgen staining. At this wavelength the staining showed maximum absorbance. Detection of nuclei was performed in 3 steps:

1. By automated adjustment of the lamp voltage and by use of a shading correction algorithm the intensity of image points (pixels) belonging to the background (i.e. no staining is present) was kept at grey value of 200. Next, all pixels in the image with grey value below a threshold value were considered to be Feulgen-positive. This threshold value was set at 4 times the standard deviation of the background peak (calculated from a smooth histogram) below this peak.

2. Neighbouring (8-connected) image pixels that were positive for Feulgen were grouped together to form objects that possibly correspond to nuclei. Holes inside objects were filled.

3. For each object the area and the contour ratio were determined. Contour ratio is defined as 1000 x perimeter / (2 x $\sqrt{}$ (π x area)), describing the roughness of the contour of an object. Artefacts were removed by applying thresholds to these parameters. Parameter threshold values were determined for use in the entire study from manually selected, correctly recognised objects from a number of specimens. Objects with area over 150 pixels and contour ratio smaller than 1200 were used [9].

Objects found in this way were considered to represent correctly recognised, single (i.e. non-overlapping) nuclei. For all nuclei the integrated optical density (IOD) was calculated as a measure for the total amount of DNA. If 800 nuclei were detected before the entire specimen area was scanned, the measurement procedure was stopped.

Images of measured nuclei were displayed on the computer screen in a gallery display. For each specimen approximately 20 nuclei of reference cells and 200 nuclei of ET cells were manually selected. The IOD histogram of reference cells was visually inspected and a reference value was determined such that the reference cell peak in the calibrated IOD (cIOD) histogram had a value of 0.89. This number is found experimentally, to correct for the effects of diffraction and glare on small, dense objects on the IOD. Although others advise an upper-bound of 5% [10], in our study (probably due to the pre-processing of the specimens) this limit was too vigorous and cases with coefficient of variation (CV) of the reference cells smaller than 15% were accepted. Next, the cIOD histogram of each specimen was manually classified as either diploid or deviating from diploid by three observers. Consensus was reached for every case.

Comparative genomic hybridisation

Comparative genomic hybridisation (CGH) was performed on snap frozen tissue as previously described [6]. For logistical reasons, snap frozen tissue was only available

Location (number)	Ploidy status (number)	Gross total resection	Radiotherapy	Mean Age in years (range)	Sex	Supra(s)/ Infratentorial(i)	3-year survival % (number)
Spinal mpE (7)	Diploid (1)	0	0	46	f	n.a	100% (1)
	Aneuploid (4)	1	2	40 (33-46)	3f/1m	n.a	100% (4)
	Tetraploid (2)	1	1	42 (33-50)	2f	n.a	100% (2)
Spinal lgE (15)	Diploid (11)	4	3	36 (13-68)	7f/4m	n.a	100% (11)
	Aneuploid (1)	1	0	49	m	n.a	100% (1)
	Tetraploid (3)	3	0	44 (35-49)	3m	n.a	100% (3)
Cerebral lgE (13)	Diploid (11)	4	0	28 (2-59)	8f/3m	3s/8i	90% (10)
	Aneuploid (2)	2	2	11 (11-11)	1f/1m	1s/1i	100% (2)
Cerebral aE (18)	Diploid (14)	5	5	19 (1-60)	3f/11m	8s/6i	43% (6)
	Aneuploid (3)	0	1	16 (12-22)	1f/2m	2s/1i	0
	Tetraploid (1)	1	1	35	f	1s	0

Table 1 *Clinical characteristics and ploidy status. f: female; m; male; n.a.; not applicable.*

of 19 tumours. Briefly, normal DNA and tumour DNA were labelled with digoxigenin-dUTP (Boehringer Mannheim) and biotin-dUTP (Boehringer Mannheim) in a standard nick translation system, respectively. The pre-treated metaphase slides and the probes were denaturated simultaneously and hybridisation was performed for three days. After hybridisation, the slides were washed stringently and the biotinylated DNA was detected using streptavidin-FITC and the digoxigenin-labelled DNA with sheep-anti-digoxigenin-TRITC. Chromosomes were counter-stained with 4'-6-diamidino-2-phenylindole (DAPI) to identify the chromosome-banding pattern. A relative gain was scored when the mean tumour-reference ratio was > 1.2 and relative loss was scored when the ratio was < 0.8. Aberrations of 1.4 and 0.6 were called clear copy number gains or losses respectively. Aberrations > 1.6 were called high copy number gains. Adequate controls were included in all experiments as described by Jeuken et al. [6].

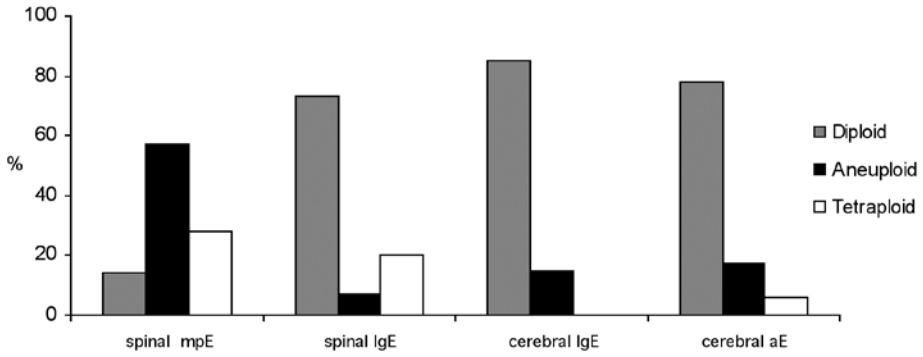

Figure 1 *Percentage of diploidy, aneuploidy, and tetraploidy.*

RESULTS

Survival

Survival data and patient characteristics are summarised in Table 1 and Figure 1. All patients with spinal ETs were alive after three years or died of unrelated causes. Eighty-six percent of patients with cerebral lgEs survived 3 years, against only one-third of patients with cerebral aEs.

Ploidy assessment

Ploidy assessment results are summarised in Table 1 and Figure 1. Of the 7 mpEs, only one was diploid. The rest was aneuploid (n = 4), or tetraploid (n = 2). One of the 14 spinal lgEs was aneuploid, 3 were tetraploid, and the rest (n = 11) were diploid.

Two of the 13 cerebral lgEs (15%) and 3 of the 18 cerebral aEs (16%) were aneuploid. Three patients, all with mpEs, had local seeding in the spinal canal. Two of these disseminating tumours were aneuploid. The number of patients who died due to their ET was too small to determine the influence of ploidy status on survival.

CGH

CGH results are shown in Table 2 and Figure 2. A wide range of genomic alterations was detected, the most frequently being loss of chromosome 22 (n = 14), loss of 17 (n = 5) and gain of 4q (n = 8). CGH showed less genomic alterations in the aEs (average 5) compared to the lgEs (average 12), while the mpEs showed the highest number of genomic alterations (average 16). No specific genomic alteration was correlated to histological grade or ploidy status.

DISCUSSION

In several tumour types the ploidy status was shown to carry prognostic information. For example, in cancer of bladder, prostate and ovary, aneuploidy correlates with a poor prognosis [11]. In colorectal tumours aneuploidy is associated with malignant progression [12]. On the other hand, diploid and near triploid neuroblastomas show poor and excellent clinical outcomes, respectively [13]. So far, in the limited number of DNA ploidy studies on ETs no correlation was found between tumour behaviour and ploidy status. However, most of the tumours examined were cerebral lgEs and aEs [14,15].

Our study shows a high percentage of mpEs with aneuploidy. This is in line with the findings of the interphase cytogenetics study of Scheil et al, in which an aneuploid karyotype was found in about half of the mpEs [8]. The percentage of spinal and cerebral lgEs/aEs with aneuploidy was much lower.

As others and we described previously, a wide range of genomic alterations was detected in ETs by CGH [4-8]. Interestingly, these studies showed that aEs had on average the lowest and mpEs the highest number of genetic aberrations [4-6,8]. Although there was no absolute relationship between the number of genomic alterations by CGH and ploidy assessment in our patients and the groups are small, the different groups differed clearly in this matter. AEs and cerebral lgEs were mostly diploid and aEs had fewer genomic alterations than the cerebral lgEs. Spinal lgEs showed less genomic alterations on average than mpEs, which were more often non-diploid than lgEs. A similar relationship between cytogenetic aberrations by CGH and ploidy status is found in other tumours [12,16,17].

Case	Sex/ Age	Histology/ Tumour site	CGH losses	CGH gains	Ploidy assessment
N270	30/m	MpE /spinal	1pter-p31, 1q, 2p16-24, 2q33-qter, 3p14-24, 6q23-qter, 6p, 10, 11, 14q, 16p, 17, 19, 22	4p14-qter, 5p14-q31, 9pter-q31, 12p12-q21, 18	Aneuploid
N273	32/f	MpE /spinal	1pter-p31, 4pter-p15, 8p, 10, 12p, 13q, 14q, 16p, 19, 22	4p14-qter, 5p13-q31, 7q21-31	Tetraploid
N222	56/m	IgE /spinal	16, 22	12	Tetraploid
N221	48/m	IgE /spinal	21^{C}, 22^{C}	7, 12	Aneuploid
N226	56/f	IgE /spinal	1, 2, 3, 6, 8, 10, 11, 13q, 14q, 16, 19, 20, 21, 22	4, 5, 7, 9, 18	Diploid
N229	39/m	IgE /spinal	2q, 6q, 13q, 22	19	Tetraploid
N231	45/f	IgE /spinal	1p, 4p, 5, 6p, 9, 10, 19, 20q, 21, 22	2, 3, 4q, 8q11-23, 13q, 14q	Diploid
N225	32/f	IgE /spinal	2, 3, 6, 10, 16, 17, 21, 22	4, 9, 13q, 14q	Diploid
N269	25/m	IgE /cerebral	1pter-p22, 2p, 2q32-qter, 6, 8p, 8q23-qter, 10, 16q, 21, 22, x	4q, 9	Diploid
N271	48/f	IgE /cerebral	1pter-p31, 10q23-qter, 14q, 16p, 19, 20, 21, 22	4q	Diploid
N267	24/f	IgE /cerebral	1p11-31, 2, 3, 4, 6, 7, 9, 10, 11, 12, 13q, 18, 21	1q, 5, 8, 19, X	Diploid
N266	10/f	IgE /cerebral	1, 2, 3, 6, 8, 10, 12, 13q, 14q, 16, 17, 21, 22	7, 9q, 11^{C}, 15q, 18C, 20	Diploid
N228	17/f	IgE /cerebral	2, 3, 6p, 10, 11pter-q13, 16, 17, 19, 20q, 21, 22	4	Diploid
N87	4/m	AE /cerebral	-	2pter-p22*, 11q14-22^{C}	Diploid
N227	2/m	AE /cerebral	10q, 16p, 22	X	Diploid
N224	1/m	AE /cerebral	17, 19, 20q, 22	X	Diploid
N91	60/m	AE** /cerebral	6q, 9p21-pter, 12p, 14q, 15q11-22	7, 12q13-15*	Diploid
N213	34/f	AE** /cerebral	21	12q, 12q11-21^{C}, 13q22-qter	Tetraploid
N76	25/m	AE /cerebral	3pter-q24, 5q, 10q, 11, 13q, 18, 21	9q	Aneuploid

Table 2 *CGH findings of 19 ETs. f: female; m: male; c: clear copy number; *: high copy number, ratio > 1.6, **: included features of glioblastoma multiforme.*

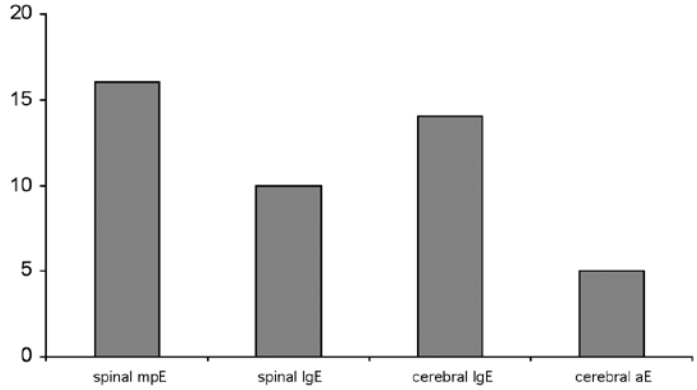

Figure 2 *Number of genetic alterations by CGH.*

MpEs are grouped together with lgEs and aEs in the category of ETs. The designation of respectively WHO grade I, II, and III to these tumours suggests tumours with different malignancy grades within the same spectrum. However, the typical histopathology and radiology [18] already indicate that mpEs form a separate entity. On the basis of their CGH results, Hirose et al. suggested that mpEs and spinal lgEs are different genetic sub-groups [5]. Also within the group of lgEs and aEs, different subgroups on the basis of localisation (spinal, posterior fossa, supratentorial) and age (paediatric versus adult population) have distinct genetic characteristics [5,6,19]. The results of our ploidy and CGH study support the idea that mpEs belong to an entity that is distinct from lgEs and aEs. Furthermore, it is entirely possible that in some diploid tumours relatively subtle aberrations strongly enhance malignant behaviour, while other tumours are aneuploid and harbour widespread genetic aberrations that do not lead to malignant behaviour. The result of this is that low-grade tumours can have more widespread genomic abnormalities as shown by CGH than high-grade tumours and can show a higher percentage of aneuploidy. Thus, this inverse correlation in ETs between the number of genetic aberrations and malignancy grade is caused by the following reasons. First, mpEs are not a precursor of higher-grade ETs but a separate entity. Second, genomic alterations are not necessarily an indication of malignant behaviour. Most patients with diploid aEs died, whereas the patients with the non-diploid mpEs were alive and well. Other examples of the latter are neuroblastomas with aneuploidy, which have a better prognosis than their diploid counterparts with fewer genetic aberrations [20].

Dissemination of mpEs usually occurs only within the subarachnoidal space and is probably due to their extramedullairy/ intradural location rather than to aggressive tumour behaviour. Seeding within the intradural space is distinctly different from metastases at distance, and thereby not a sign of aggressive tumour behaviour.

In conclusion, our results confirm that mpEs are genetically distinctly different from lgEs and aEs. Furthermore, it supports the idea that genomic imbalances and aneuploidy are not necessarily an indicator of malignant behaviour of a tumour, despite its potential for dissemination.

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Chapter 5

Differential patterns of insulin-like growth factors-I and -II mRNA expression in medulloblastoma

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Reprinted from: Neuropathol Appl Neurobiol 2004;30:503-12
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ABSTRACT

Insulin-like growth factors (IGFs) play an important role in tumour growth and development. We hypothesised that this is also the case for medulloblastomas, which are highly malignant cerebellar brain tumours usually occurring in children. In these tumours the expression patterns of IGF-I and II mRNA were studied. Tumour specimens obtained from 12 children and 2 adults at diagnosis were hybridised in situ with digoxigenin-labelled cRNA probes for hIGF-I and hIGF-II mRNAs. In all cases, tumour cells showed abundant expression of IGF-I mRNA. Nine of the 14 tumours showed variable but significant IGF-II expression. In these tumours, the hybridisation signal almost exclusively co-localised with a sub-population of Ki-M1P positive cells that were identified as ramified microglia (RM) cells. In the 5 tumours without IGF-II expression, microglia/brain macrophages with a more rounded amoeboid-like morphology predominated. RM cells in normal cerebellar tissues, residing abundantly in areas of the white and, to a lesser extent, in the grey matter, were IGF-II mRNA-negative. These RM cells showed a thinner and more extensively branched appearance and were more evenly distributed than those encountered in medulloblastoma. Probably, during the transformation from the resting ramified towards the amoeboid morphology (or vice versa) IGF-II mRNA expression is only temporarily induced. The physiological meaning of the induction of IGF-II mRNA expression by these cells in medulloblastoma remains unclear but any IGF-II peptide synthesised could exert unfavourable mitogenic and anti-apoptotic effects on adjacent tumour cells. However, in this relatively small number of cases we could not find any indications for a relationship between clinical characteristics of the various cases and the extent of IGF-II mRNA expression.

INTRODUCTION

Medulloblastomas (MBs) are primitive neuroectodermal tumours of the cerebellum. They represent the most common malignant paediatric central nervous system (CNS) tumours and account for approximately 20-25% of all malignant intra-cranial neoplasms in childhood [1]. MB is believed to originate from the undifferentiated external granule layer in the cerebellum, but the mechanism of tumorigenesis remains unknown [2,3]. Several reports suggest that the insulin-like growth factor (IGF) system contributes to the development of MB [4-8].

IGFs are small polypeptide mitogens that resemble pro-insulin in structure. Through binding to the IGF-I receptor, they elicit metabolic, growth promoting and anti-apoptotic effects in many types of cells, including oligodendroglia [4,9]. IGF-I is considered to mediate many of the growth promoting actions of growth hormone, whereas the

closely related IGF-II merely acts as an autocrine growth and motility factor during prenatal growth and development [10,11]. Evidence is accumulating that IGFs function as autocrine growth factors in a variety of tumours, including those of the CNS, where they are involved in the control of proliferation of previously transformed cells [4,9]. The IGF signal transduction system may even play a role in the process of transformation itself [4,9].

Del Valle et al. [8] showed that the majority of MBs examined by them (i.e. 16 of 20 cases) abundantly expressed the IGF-I receptor. In addition, the presence of the IGF-I receptor protein and IGF-I mRNA has been demonstrated in cell lines of human MB [6]. However, data on IGF-I mRNA expression in MB tissue are scarce.

At present, little is known about IGF-II mRNA expression in MB. Loss of imprinting of the maternal allele of the IGF-II gene, leading to biallelic expression, can occur in human MBs (and MB cell lines), although this does not necessarily lead to enhanced mRNA levels [12]. Since biallelic expression of IGF-II is also observed in foetal cerebellum, its occurrence in MB presumably merely reflects the tumour's embryonic nature.

In the present study we investigated both IGF-I and IGF-II mRNA expression in surgical resection specimens from 14 cases of MB by in situ hybridisation. This technique was used in order to address the question which cell types actually express IGF-I and IGF-II mRNA. Both normal human and porcine cerebellum samples were included as controls.

MATERIALS AND METHODS

Tissue specimens of patients and controls

Surgical resection specimens from 14 histologically proven and classified [1] cases of MB (all located in the posterior fossa) were investigated. Patient and tumour characteristics are summarised in Tables 1 and 2, respectively. Tissue samples of normal cerebellar tissues from subjects without neurological signs and symptoms (2 males, aged 1 and 48 years) were obtained at 5.5 hr post-mortem and used as controls. The Netherlands Brain Bank (Amsterdam, the Netherlands) provided these tissues. In all cases the rules of the Medical Ethical Committees have been observed. Because it was not possible to obtain normal human cerebellar tissue without post-mortem delay and the porcine IGF-system resembles that of humans in many aspects [13], we also included cerebellar tissues from healthy new-born piglets (n=5) as controls [14]. All tissue samples were directly fixed in PBS-buffered formalin 3.8% (v/v) for 24 hours. After dehydration in a series of ethanol and incubation in xylol, the

Patient number	Sex	Resection	Additional Therapy	Age (years)	Local Recurrence (years after operation)	Postoperative survival (years)
1	M	Partial	Cht + Rt	6	+ (8)	10, alive
2	M	Total	Cht + Rt	23	+ (2)	2
3	F	Total	Cht	1	-	11, alive
4	F	Partial	Cht + Rt	8	-	12, alive
5	F	Partial	Cht + Rt	4	+ (0.9)	1
6	M	Total	Cht + Rt	9	-	12, alive
7	F	Total	Cht	1	+ (0.1)	0,5
8	F	Total	Cht	1	-	14, alive
9	M	Total	Cht + Rt	5	+ (0.5)	2
10	M	Partial	Cht + Rt	9	-	10, alive
11	M	Partial	Cht + Rt	34	-	8, alive
12	M	Total	Cht + Rt	12	+ (3)	4
13	F	Total	Cht	2	+ (1)	1
14	M	Partial	Cht + Rt	5	-	11, alive

Table 1 *Patient's characteristics. M,male; F,female; ChT, chemotherapy; Rt, radiotherapy.*

Patient number	Histological subtype	Leptomeningeal seeding	IGF-II mRNA in situ hybridisation
1	Classic	yes	-
2	Classic	yes	+
3	Desmoplastic	no	+
4	Classic	yes	+
5	Classic	yes	-
6	Desmoplastic	no	-
7	Large cell	yes	+
8	Classic[1]	no	+
9	Desmoplastic	no	-
10	Classic	no	+
11	Classic	no	-
12	Classic[1]	yes	+
13	Classic[1]	yes	+
14	Desmoplastic	No	+

Table 2 *Characteristics of the MBs. [1] with local desmoplastic features.*

tissues were embedded in paraffin. Sections (8-10 µm) were cut and mounted onto glass slides pre-treated with 3-aminopropyltriethoxysilane (TESPA) (Sigma-Aldrich, St.Louis, MO, USA), and stored at 4ºC until analysis by in situ hybridisation (8-10-µm sections) and immunohistochemistry (4-µm sections).

In situ hybridisation

Digoxigenin(DIG)-labelled cRNA probes were prepared by standard RNA synthesis reactions with T7- or T3-RNA polymerase using DIG-UTP (Roche Molecular Biochemicals, Mannheim, Germany) as substrate [15]. Complementary DNAs encoding a part of hIGF-I (nucleotides 120-379) [16], hIGF-II (containing exons 7, 8, and part of 9 of the human IGF-II gene) [17], and human ribosomal 28 S RNA [18], were used as templates for the synthesis of anti-sense and sense DIG-labelled cRNA probes. The 28S ribosomal DIG-cRNA probes were used to evaluate the preservation of RNA in the various tissue specimens studied. In situ hybridisation was performed overnight at either 51°C (IGF-I mRNA), 63°C (IGF-II mRNA) or 55°C (28 S ribosomal RNA), following procedures described previously [19]. The DIG-labelled RNA-RNA hybrids were detected by sheep-anti-DIG Fab-fragments coupled to alkaline phosphatase and subsequent chromogenesis with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphatase [19]. This method yields a blue precipitate. Sections were counter-stained with nuclear fast red, dehydrated through a series of ethanol and mounted with Euparal (Waldeck GmbH and Co. KG, Münster, Germany).

Immunohistochemistry

Immunohistochemistry was performed using Ki-M1P as a primary antibody (kindly provided by Dr. M.R. Parwaresch; University of Kiel, Kiel, Germany). This mouse monoclonal antibody reliably recognises a formalin/ paraffin-resistant differentiation epitope (CD68) of microglia and brain macrophages [20,21]. Prior to immunohistochemistry, paraffin sections of tumours and normal cerebellum tissues were mounted on Superfrost microscope slides (Menzel-Glaser, Braunschweig, Germany), dewaxed, and re-hydrated. For antigen retrieval, sections were placed in a 0.05 M sodium citrate buffer (pH 6.0) in a microwave oven with a rotating plateau (Miele Supratronic M752) and heated for 30 min at 700 W. After heating the slides were allowed to cool in the retrieval solution for at least 20 min before further processing. Subsequently, the sections were treated with 0.3% H_2O_2 to block endogenous peroxidase activity and incubated with Ki-M1P monoclonal antibody (diluted 1:5000 in PBS, containing 1% bovine serum albumin) for 1 h. Sections were stained by using the PowerVision+ TM poly-HRP-anti-mouse immunoglobulin kit, according to the protocol supplied by the manufacturer (ImmunoVision Technologies, Co., Brisbane, CA, USA). Copper sulphate-enhanced 3,3-diaminobenzidine (Sigma-Aldrich, St. Louis, MO, USA) was used as a chromogenic substrate. All incubations were performed in a dark moist chamber at room temperature. Sections were counter-stained with Mayers' haematoxylin (Merck, Darmstadt, Germany) and mounted with Quick-D mounting medium (Klinipath, Duiven, the Netherlands).

Histological examinations

The various sections were examined by light microscopy using a Zeiss Axioscope microscope and photographed by a digital AxioCam HRc camera and AxioVision Reference 3.0 software for image processing (Carl Zeiss, Oberkochen, Germany). The abundance of IGF-I and IGF-II mRNA, and Ki-M1P immunoreactivity in each tissue section, as encountered by in situ hybridisation and immunohistochemistry, respectively, was judged to be negative (-) or positive (+) on blinded microscope slides by three independent investigators.

RESULTS

In situ hybridisation

All MBs, control human cerebellar tissues, and brain tissues obtained from new born piglets included in this study showed abundant presence of human ribosomal 28 S RNA (data not shown), pointing to the integrity of the RNA in the various tissue sections investigated. Subsequently, IGF-I and IGF-II mRNA expression patterns in the various MBs and control tissues were analysed by in situ hybridisation using anti-sense DIG-labelled hIGF-I and hIGF-II cRNA

probes, respectively. In all cases, sections hybridised with the corresponding sense probes were virtually negative.

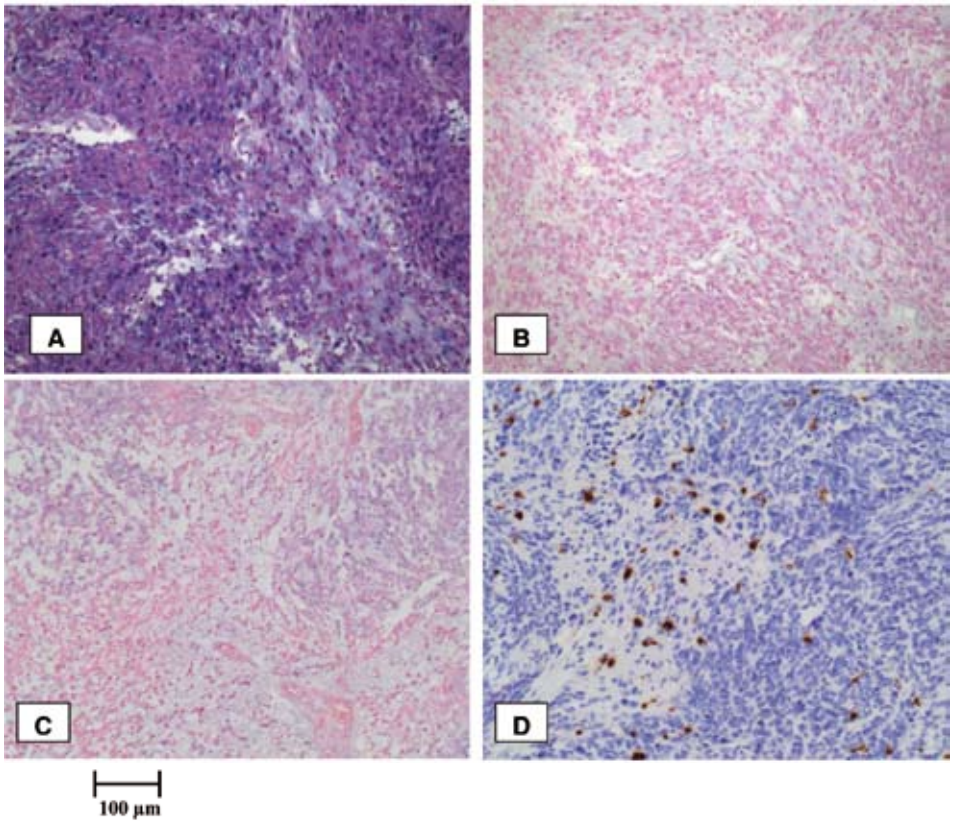

Figure 1 *Histochemical analysis of paraffin-embedded sections of a MB tumour specimen (i.e. tumour number 6) being representative for those tumours that do not show significant IGF-II mRNA expression. Sections were either hybridised with an anti-sense (A) or a sense digoxigenin-labelled (DIG) IGF-I cRNA probe (B). Purple or blue staining indicates the presence of IGF-I mRNA. C: in situ hybridisation with an anti-sense DIG cRNA specific for human IGF-II mRNA. D: immunohistochemistry was performed on successive paraffin tissue sections using a monoclonal antibody Ki-M1P. This antibody is considered to predominantly recognise cells belonging to the microglia / brain macrophage lineage (brown staining), in this case predominantly IGF-II mRNA negative more rounded amoeboid-shaped cells. Counter-staining was performed with haematoxylin. Original magnification for all panels, x 200.*

In all the 14 MBs investigated, small rounded tumour cells abundantly expressed IGF-I mRNA. Representative examples are shown in Figure 1, 2, and 3. IGF-I mRNA could also be detected in endothelial cells of blood vessels. The presence of IGF-I mRNA in microvessels, however, was difficult to assess, due to diffuse and strong staining of the tumour sections. In normal human (Figure 3) and porcine cerebellar tissue, only Purkinje cells were IGF-I mRNA positive. Nine of the 14 tumour specimens investigated showed variable but specific expression of the IGF-II gene. However, IGF-II mRNA could not be detected in tumour cells. The hybridisation signal appeared to be associated with dispersed cells exhibiting elongated nuclei, scanty cytoplasm, and fine polar or ramified processes (Figure 2 and 3). An exception was case 7, a 'large cell' MB [22], where IGF-II mRNA was also detected in relatively large cells with rounded morphology. It may be that the latter type of cells in fact represented tumour cells but this could not be established with certainty. In general, the IGF-II mRNA expressing microglia-like ramified cells showed a rather diffuse distribution pattern within the tumours (Figure 2) but tended to reach a higher density in perivascular spaces. In several cases of desmoplastic MBs and classic MBs with local desmoplastic features (i.e. tumours 3, 8, 13, and 14) containing reticulin-free nodules or 'pale islands' [23], IGF-II mRNA expressing ramified cells were abundantly present around, but not within these "pale islands" (Table 2 and Figure 2). In the sections of the two control human cerebellar tissues only the meninges were weakly positive for IGF-II mRNA (Figure 3). The same result was obtained for cerebellums from piglets (data not shown).

Immunohistochemistry

Because the morphology of the IGF-II mRNA positive cells in the various MBs resembled that of ramified microglia (RM) cells, this was verified immunohistochemically using Ki-M1P antibody that specifically recognises the microglia/ brain macrophage cell lineage. Careful, comprehensive comparison with successive sections stained for Ki-M1P revealed that in all relevant cases the IGF-II mRNA hybridisation signal strongly co-localised with Ki-M1P positive ramified cells (Figures 2 and 3). In fact, all tumour specimens showed pronounced staining with the Ki-M1P monoclonal antibody. Besides RM cells, also more rounded amoeboid-shaped cells were found to be Ki-M1P- positive. In most cases amoeboid-shaped cells predominated, except in cases 3 and 8. In tumours 1, 5, 6, and 11, showing virtually no IGF-II mRNA expression, RM cells contributed less than 20% of the total population of Ki-M1P positive cells (Figure 1). Both cell types were scattered among neoplastic cells and also present (especially RM cells) around capillaries, larger blood vessels and 'pale islands'. Usually, a few Ki-M1P positive amoeboid-shaped cells were found within 'pale islands' (Figure 2). Examination of the normal human cerebellum tissue revealed that Ki-M1P immunopositive RM cells by far predominated and resided abundantly in areas of the cerebellar white and, to a lesser extent, in the cerebellar grey matter (Figure 3). It was also noted that in general RM cells in the normal cerebellar tissues showed a thinner

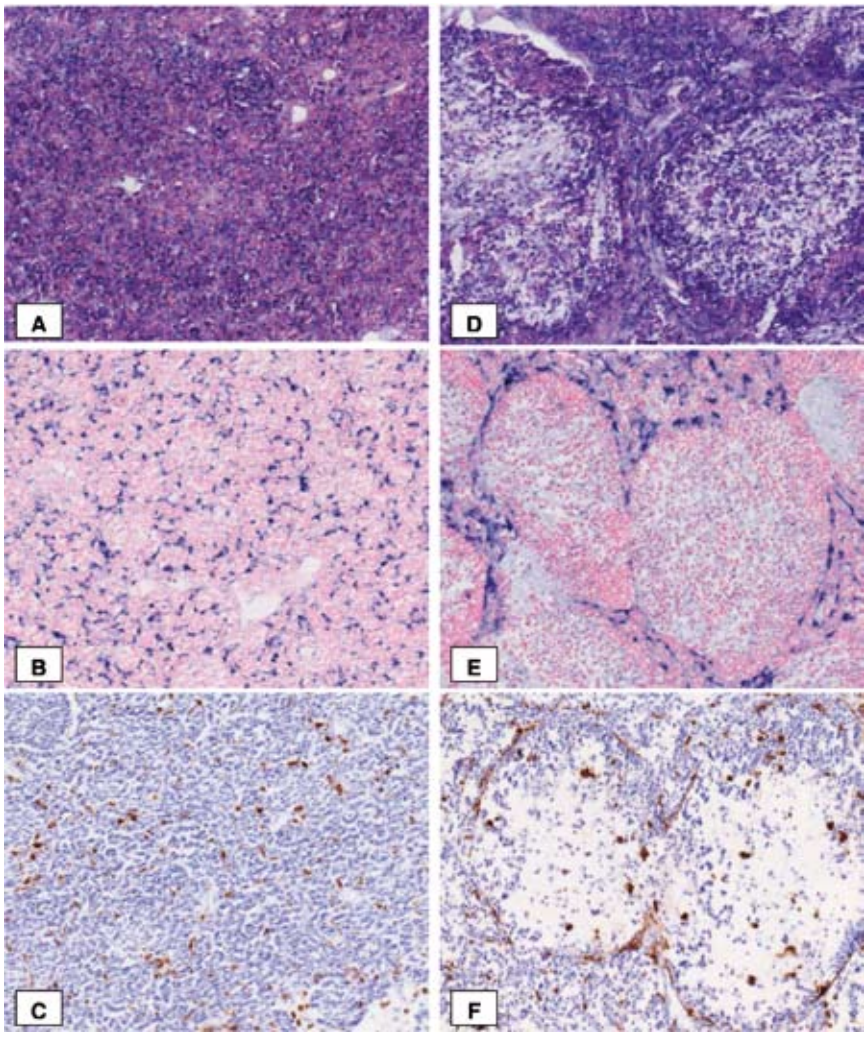

Figure 2 *Sections obtained from tumour specimen 8 (panels A-C). A: in situ hybridisation for mRNA expression of IGF-I; B: diffuse distribution pattern of IGF-II mRNA expressing ramified microglia (RM) cells being representative for various other MB tumours. C: immunohistochemical reactivity of monoclonal antibody Ki-M1P. IGF-II mRNA hybridisation signal strongly co-localised with Ki-M1P positive RM cells. Representative example (case number 3; panels D-F) of IGF-I mRNA (D) and IGF-II mRNA (E) expression in MB specimens containing areas with reticulin-free nodules ('pale islands'), being comprised of cells with round 'neurocytic' nuclei and abundant cytoplasm. IGF-II mRNA expressing RM react immunohistochemically with monoclonal antibody Ki-M1P (F), whereas the few Ki-M1P positive amoeboid-shaped cells within 'pale islands' were IGF-II mRNA negative. Original magnification for all panels: x 200.*

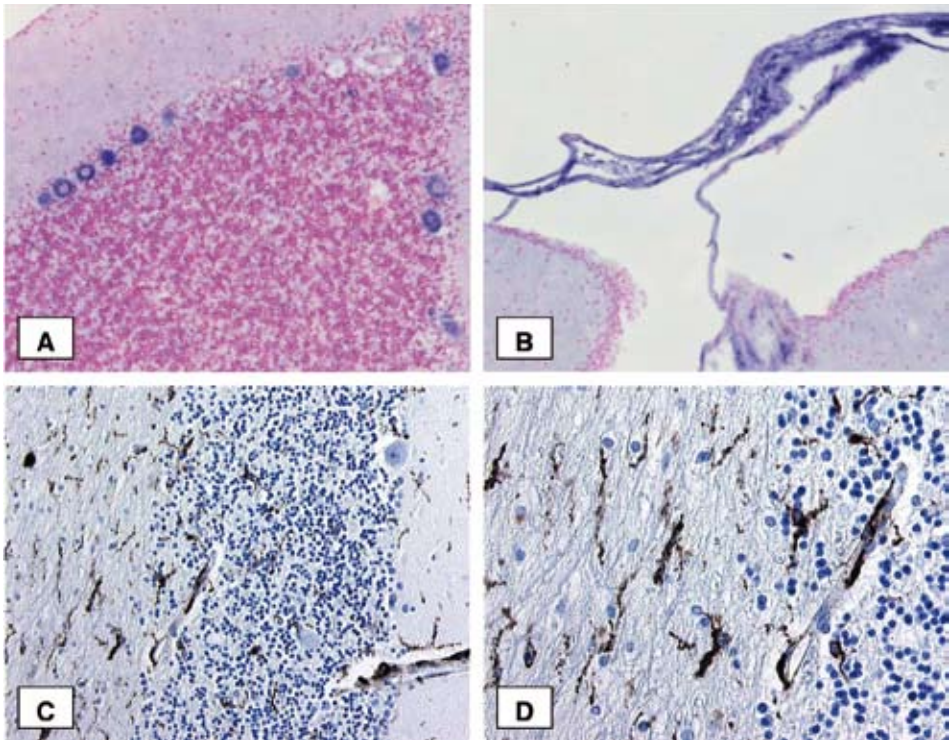

Figure 3 *Expression of IGF-I mRNA (A) and IGF-II mRNA (B) in Purkinje cells and meninges, respectively, of normal human cerebellum. C and D: a representative example of Ki-M1P positive RM cells in white matter of normal human cerebellum. Original magnification for panels A, B, and C: x 200; D: x 400.*

and more extensively branched appearance and were more evenly distributed than those encountered in MBs. Similar results for Ki-M1P immunohistochemistry were obtained for cerebellums of healthy piglets (data not shown).

DISCUSSION

IGFs are thought to play a pivotal role in the proliferation of MBs [9]. Several lines of evidence (mainly based on studies on the IGF-receptor and components involved in signal transduction) suggest that this occurs in an autocrine fashion. Patti et al. [6] reported that an autocrine loop involving the IGF-I receptor activates proliferation of a human MB cell line. Tumour cells in MB may exhibit relatively enhanced protein levels of the IGF-I receptor (when compared to e.g. cells of the granular layer and Purkinje cells in normal human cerebellum) and its major signal transducing substrate, insulin

related substrate-1 [7,8]. In addition, phosphorylated (i.e. activated) forms of several downstream signalling proteins such as Erk-1, Erk-2, and Akt/protein kinase B have been detected in MBs, but not in control cerebellar tissue [8].

In the present study we demonstrate that a large proportion of the small neoplastic cells in MB highly express IGF-I mRNA. This finding complements the results of the studies referred to above.

Several reports indicate that (at least in rodents), following brain injury, activated microglia also express the neuroprotective IGF-I [24]. Moreover, IGF-I may stimulate the proliferation of microglia [25]. Because of the extensive DIG staining of the various MB sections investigated by us (due to the abundant presence of tumour cells), we could not reliably identify individual Ki-M1P immunoreactive cells that were also IGF-I mRNA positive. In normal human cerebellar tissue IGF-I mRNA could only be detected in Purkinje cells, in agreement with previous reports on this subject [26].

IGF-II is considered to play an important role in the foetal development of the brain where it is abundantly expressed. Beyond the perinatal period, IGF-II expression in the human cerebellum has been shown to persist primarily in the meninges, the mRNA being transcribed from both parental alleles [27,28]. Our in situ hybridisation experiments on normal brain tissue of piglets and humans confirmed that IGF-II mRNA expression was indeed restricted to this region. Nine out of the 14 MBs analysed were found to be positive not only for IGF-I but for IGF-II mRNA as well. Surprisingly, and in contrast to the situation for IGF-I, in all cases (with the possible exception of the anaplastic/ large cell MB) neoplastic cells did not contain any histochemically detectable IGF-II mRNA. Instead, IGF-II mRNA expression appeared to be specifically associated with ramified Ki-M1P positive (i.e. CD68 antigen bearing) microglia. Albrecht et al. investigated the phenomenon of loss of imprinting (LOI) of the IGF-II gene in both MB cell lines and MBs [12]. LOI had occurred in one of four informative cell lines, although the particular cell line (MEB-MED-5) was derived from a tumour that originally exhibited imprinting of the IGF-II gene. The LOI of IGF-II in this cell line was not associated with increased expression of IGF-II mRNA, since two rounds of RT-PCR were required to generate enough cDNA-PCR product from the cell line. Three out of 7 informative tumours also showed LOI but again one could not establish a correlation between the presence and absence of imprinting of the IGF-II gene and IGF-II mRNA levels. Similar results were obtained by Zahn et al. for Ewing's sarcoma [29]. Using immunohistochemistry, Ogino et al. could not detect any IGF-II protein in the 5 MBs investigated by them [30]. The reason for this apparent discrepancy remains unresolved, although in their paper, Ogino et al. did not provide data on morphological features of the MBs investigated. It cannot be excluded that they have studied tumours, which predominantly contain the more rounded, IGF-II mRNA negative, microglia.

Cultured rat microglia (both resting and activated) have been shown to produce IGF-II that prevents (TNF)-alpha (derived from the same microglia) induced apoptotic death of oligodendrites in vitro [29]. The synthesis of IGF-II peptide by infiltrated microglia-like cells is also induced during wound repair, following hypoxic-ischemic injury in the developing rat brain [30]. In addition, IGF-II mRNA and peptide are also induced in macrophage-like cells after global ischaemia in adult rats [32]. Similarly, a high number of infiltrating microglia/ brain macrophages is found in most human brain tumours. A study by Roggendorf et al. [21] indicates that the distribution pattern of these Ki-M1P positive cells is distinct in various CNS neoplasms, underscoring the complex immunological function of the microglia/ macrophage cell system that expresses a wide variety of growth factors and cytokines. Indeed, in all MBs investigated by us these cells appeared to be abundantly present and exhibited, to a varying degree, either a ramified or a more rounded, amoeboid-like morphology. It is likely that the former type of cells represents microglia cells in a certain degree of a resting state. The exact function of these resting microglia is largely unknown but they may play a role in the surveillance, protection, and survival of neurons and oligodendrocytes [31-33]. RM cells were also found in the medulla and molecular layer of normal cerebellar tissues. However, in general their appearance seemed to be more slender and extensively ramified and virtually lacked IGF-II mRNA expression. Therefore, it cannot be excluded that these cells play another role when compared to the IGF-II mRNA positive cells encountered in the various tumour tissues. It is known that resting microglia respond to disturbances in their microenvironment by surrounding damaged neurons or other cells [33,34]. The outward sign of such activation is retraction of their branches leading to a more rounded amoeboid morphology. Depending on the severity of the tumour-induced injury, activated cells may either ultimately become phagocytic functioning as macrophages or transform back to a resting state [31,32]. Probably, during the transformation from the resting ramified towards the amoeboid morphology (or vice versa) IGF-II mRNA expression is only temporarily induced. The physiological meaning of the induction of IGF-II mRNA expression by these cells in MB remains unclear but one may speculate that any IGF-II peptide synthesised could exert unfavourable mitogenic and anti-apoptotic effects on adjacent tumour cells. However, although only a relatively low number of cases were investigated, we could not find any indications for a relationship between clinical characteristics of the various cases and the extent of IGF-II mRNA expression.

In summary, the results of the present study support the view that IGF-I is involved in the development and growth of MB and that the IGF system represents a potential target for future therapeutic approaches. Further studies should verify the expression of IGF-II by infiltrating microglia at the protein level and whether these cells express other growth factors and/or cytokines simultaneously.

ACKNOWLEDGEMENTS

We wish to thank Dr. J.A. Koedam (University Medical Centre Utrecht) and Prof. Dr. G.W.A.M. Padberg (University Medical Centre Nijmegen) for critical reading of the manuscript.

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Chapter 6

Genetic aberrations as detected by comparative genomic hybridisation and vascularisation in glioblastoma xenografts

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ABSTRACT

Angiogenesis is of vital importance for the growth of solid tumours and constitutes a target for anti-cancer therapy. Glioblastomas (GBMs) are histologically characterised by striking microvascular proliferation. The identification of the mechanism of angiogenesis is of major importance for the further development of anti-angiogenic therapy. Tumour angiogenesis might be the result of a combination of local tissue conditions (especially hypoxia) and specific genetic alterations acquired during oncogenesis. In order to investigate the relationship between genetic aberrations and tumour angiogenesis in GBM xenografts lines, the genetic alterations were examined by comparative genomic hybridisation (CGH). Two vascular phenotypes of GBM xenografts could be identified: a well vascularised and a poorly vascularised type. In this model, the poorly vascularised type had a greater number of genetic alterations. However, there was no unequivocal correlation between angiogenesis, growth rate and genetic alterations as detected by CGH.

INTRODUCTION

Angiogenesis is a critical process for the local growth of solid tumours and for metastasis [1,2]. Glioblastoma multiforme (GBM), the most frequent and most malignant of primary brain tumours, is characterised by aggressive intracerebral growth and the absence of metastases [3]. Hence, the prognosis of patients with GBMs is determined by local growth. At present no effective therapies are available [4,5]. GBMs are characterised by florid angiogenesis, which constitutes a target for new anti-cancer therapies [2,6,7]. Discovering the mechanisms involved in angiogenesis is essential for the development of therapeutic strategies aimed at neovascularisation.

Angiogenesis is regulated by angiogenic peptides, which appear to be produced in reaction to local hypoxia [8,9]. This is illustrated by the fact that Vascular Endothelial Growth Factor (VEGF), one of the main regulators of endothelial proliferation, is over-expressed in cells adjacent to hypoxic tissue [9,10]. Alternatively, tumour angiogenesis might be influenced by amplification of genes coding for factors that promote angiogenesis or loss of genes coding for endogenous inhibitors [11-13]. Thus, tumour angiogenesis might be facilitated and sustained by clonal expansion of tumour cells with specific genetic aberrations [12,13]. This is supported by the finding of induction of capillary-like tube formation in cultured endothelial cells by glioma cells in the absence of hypoxia [14,15]. Since GBMs acquire structural chromosomal alterations not present in less malignant diffuse astrocytic neoplasms, a direct relationship between genetic alterations and angiogenesis can be postulated [16]. GBMs show, in contrast to low-grade and anaplastic astrocytomas, both prominent glomeruloid

microvascular proliferation and classic angiogenesis [17]. The striking heterogeneity of the microvasculature in GBMs might be related to genetic heterogeneity within a GBM [17].

The aim of this study is to examine whether patterns of angiogenesis are related to genetic alterations as detected with comparave genomic hybridisation (CGH) in 10 GBM xenograft lines. With respect to tumour vascularisation, these lines have shown stable line-specific characteristics with striking differences between the lines, covering a range of well to poorly vascularised tumours [18]. In the present study, we quantitatively analysed the patterns of vascularisation and perfusion of the 10 xenografts lines as described by Bernsen et al. [18]. The parameters measure the process of neoplastic vascularisation. In our opinion, this is the best quantitaitve measure of angiogenesis as it represents the functional end-result of the many factors and procesesses involved in angiogenesis. CGH is the ideal technique for studying genetic changes in tumours, as it can detect losses and gains in tumours without the need for specific probes or previous knowledge of chromosomal aberrations present [19]. In this study the relationship of these patterns in comparison to angiogenesis is investigated by CGH.

MATERIALS AND METHODS

Patients and xenografts

Xenografts were derived from supratentorial GBMs of 10 patients (4 female, 6 male, age 33-70 years) (Table 1). A neuropathologist (PW) diagnosed the original tumours according to the criteria of the World Health Organisation [4]. The pathologic specimens were obtained by subtotal resection and 7 representative samples were used for implantation [18]. Tumours were passaged subcutaneously in nude mice (BALB/c nu/nu mouse) as described by Bernsen et al. [19]. Tumour volume was calculated by the formula: Volume = length x width x height x π/6 [19]. Growth rate was measured weekly by an experienced observer and expressed as mm^3/week. Growth rate was arbitrarily divided in fast (more than 30 mm^3/week), moderate (between 10 and 30 mm^3/week) and slow (less than 10 mm^3/per week). Tumours were harvested before reaching a volume of 1 cm^3. The growth rate and vascularisation fro all xenografts were stable during passaging and line-specific [18].

Quantification of angiogenesis and hypoxia

Athymic mice bearing a subcutaneous human GBM in the right flank were injected intravenously with 2 mg of the hypoxic marker pimonidazole-hydrochloride in PBS and with 15 mg kg^{-1} of the perfusion marker Hoechst 33342 (Sigma, St Louis, MO, USA) in PBS 30 minutes and a few minutes before the animals were sacrificed,

No	Sex/age (years)	RVA mean(SD)	PF mean(SD)	Hypoxia	Growth rate mm3/week	CGH loss	CGH gain
Well vascularised							
E18	F/47	0.11 (0.05)	0.63 (0.15)	Negative	fast	9p, 10, 11q23-qter, 12q21-qter,13q22-qter 16q, I8q, Xp^{cc},Xq	1p, 5p, 7pter-q11
E80	M/70	0.13 (0.04)	0.62 (0.15)	Not done	fast	6q22-qter, 9p22-13, 10	7pter-q11, 7p15-21^{hc}, 19
E102	M/51	0.18 (0.08)	0.52 (0.09)	Negative	fast	10p-q21, 11q14-qter, 14q 11-23	7, 20
E110	M/56	0.13 (0.03)	0,56 (0.12)	Not done	fast	6, 9p21-24, 10, 11, 12q 13q, 15q, 17pter-q21	1p34- pter, 7, 7p11-13^{hc}, 8q23-24.1^{hc},16, 17q22-qter, 9, 20p-q11
E120	F/67	0.14 (0.02)	0.44 (0.08)	Negative	slow	4q, 10, 13q, 16, X	7pter-q21, 9q32-qter, 17, 18,19, 20
Mean(SD)		0.14 (0.03)	0.55 (0.08)				
Poorly vascularised							
E2	M/33	0.08 (0.02)	0.58 (0.16)	Strongly positive	slow	2q31-qter, 4p, 5q11-23, 8pter-q21, 9p, 10q23-qter, 11pter-p13, 12q14-22, 16p, 17q22-qter, 20p	1q11-25^{cc}, 2p, 2p12-16^{cc}, 4q11-28, 5p, 7pter-p21, 7q^{hc}, 12p13^{cc}, 14q12-21, Xpter 11.3^{hc}
E34	M/70	0.08 (0.02)	0.65 (0.15)	Strongly positive	fast	1p, 2q33-qter, 4, 6q15-23, 7q22-qter, 8p^{cc}, 9p, I0q23-qter^{cc}, 14q22-31, I9q	1q, 2p, 5pter-q31, 6pter-q14, 7pter-q21, 8q^{cc}, 13q31-qter, 18, 20p, 21
E49	F/58	0.07 (0.02)	0.63 (0.18)	Positive	moderate	3pter-q25, 5q, 6, 9p21, 10, 11q, 13q, 14q, 15q, 17p	1q25-qter, 4pter-q31, 7, 9q, 12, 17q, 18
E98	M/69	0.09 (0.04)	0.62 (0.26)	Positive	moderate	2q12-14, 4q21-25, 8q23-qter, 9p, 10q22-qter, 13q11-31, 21	7, 19p
E106	F/58	0.07 (0.03)	0.64 (0.2)	Positive	fast	8pter-q22, 10, 11, 21, X	2, 4q11-13^{hc}, 7, 8q22-qter, 8q23-24^{hc}, 9q, 12q13-15^{hc}
Mean (SD)		0.08(0.008)	0.62 (0.03)				

Table 1 *Sex/age (years): sex and age of the patient from which xenograft was derived; F = female; M = male; RVA = relative vascular area; PF = perfusion fraction; SD = Standard deviation; Growth rate: fast = more than 30 mm3/week, moderate = between 10 and 30 mm3/week, slow = less than 10mm3/week. CGH = Comparative genomic hybridisation; CC = clear copy number changes,ratio more than 1.4 or less than 0.6; HC = high copy amplification, ratio 1.6 or more.*

respectively [18,20,21]. Tumours were snap-frozen and stored in liquid nitrogen. From each sample, a total of four frozen sections were taken, containing both central and peripheral regions. The sections were air dried and fixed in acetone for 3 min after which they were washed 10 min in PBS. To visualise the vasculature, sections were first incubated with the monoclonal rat anti-mouse endothelium antibody ME 9F1 [22] after fixation for 10 minutes in acetone. The sections were incubated for 45 minutes at room temperature and then rinsed in PBS. Sections were subsequently incubated for 30 min at RT with a TRITC-labelled goat anti-rat antibody (Jackson ImmunoResearch Lab. Inc., West Grove, PA, USA) and rinsed in PBS and then incubated with a second TRITC-labelled antibody (donkey anti-goat, Jackson Immuno Research Lab. Inc.) resulting in a bright fluorescent signal of the vascular pattern. Tumour sections were scanned by a computer-controlled procedure [23]. Each tumour section was scanned separately for the perfusion marker and the vessel staining. After processing, the scanned sections were reconstructed into two composite images: one of the vascular structures and another of the perfused areas (Hoechst). When both images were combined the overlapping structures represented those vascular structures which were perfused by Hoechst at the time of injection (perfused vascular area) (Figure 1). The relative vascular area (RVA) was expressed as the ratio between the surface area of all vascular structures and the total tumour surface [17,23]. The perfused fraction (PF) was calculated as the total area (pixels) of perfused vascular structures divided by the total area of all vascular structures [23].

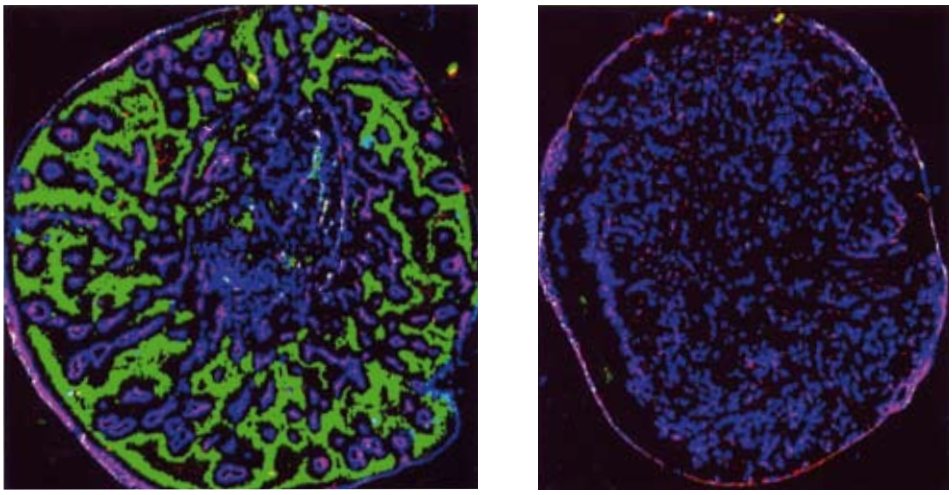

Figure 1 *Digitised images of sections of tumour E106 (left panel) and E102 (right panel), stained immunohistochemically for vessels (red) and hypoxia (green). The blue colour represents the perfusion marker Hoechst 33342. The E106 tumor shows extensive hypoxia complementary to the Hoechst dye in contrast to the E102 tumour where hypoxia is not present. Magnification 100x.*

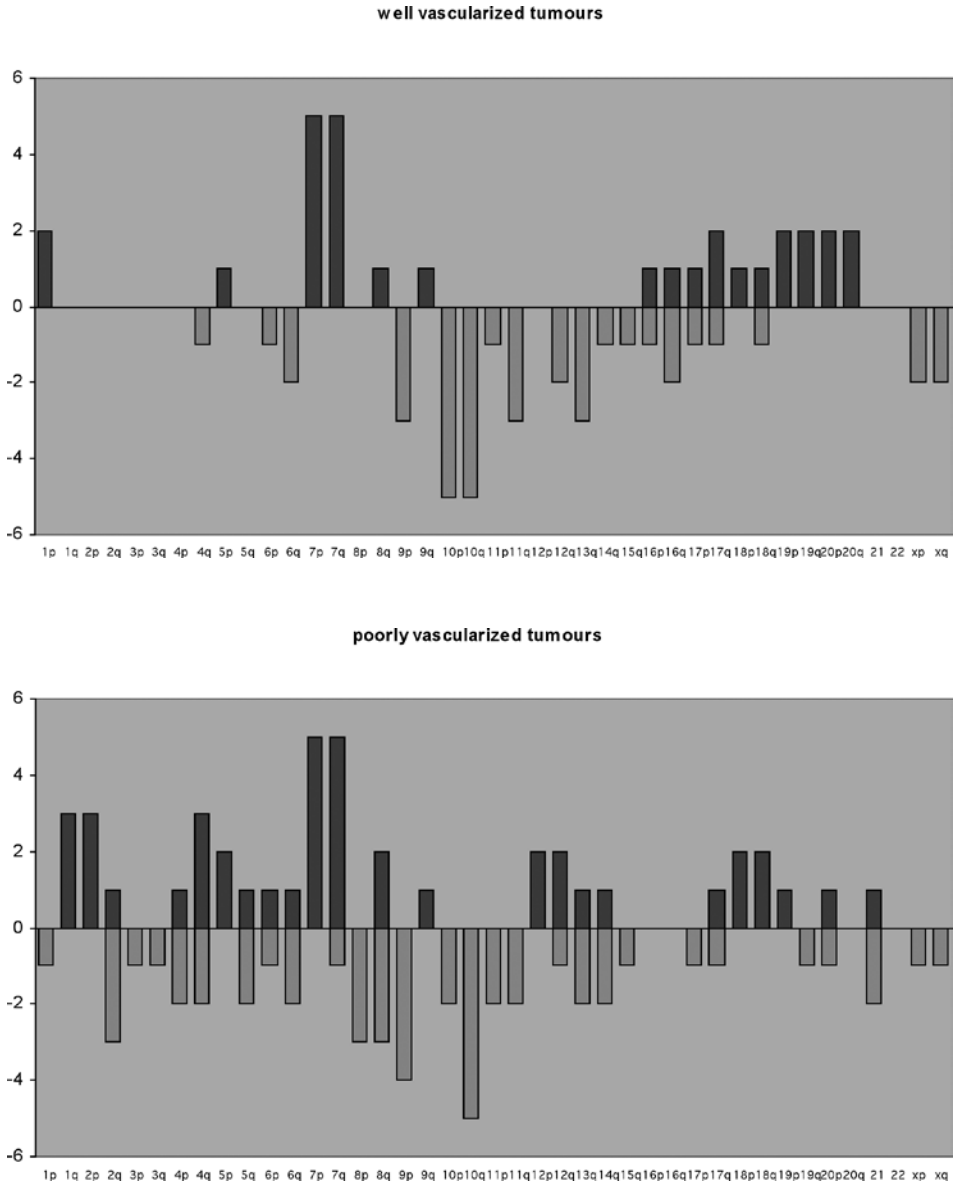

Figure 2 *Summary of the total number of genetic alterations for the well vascularised and poorly vascularised group. Open bars represent losses, closed bars represent gains. The vertical line indicates the number of losses or gains in each group. The chromosome arms are indicated at the bottom of the figure. In acrocentic chromosomes (13,14,15,21,22) only the q arms can be analysed by CGH, the Y chromosome is not analysed by CGH.*

After scanning the vascularisation and perfusion in the xenografts, the same sections were incubated overnight at 4°C to detect hypoxia with Rabbit-anti-pimonidazole (supplied by J. Raleigh, Department Radiation Oncology and Toxicology, UNC School of Medicine, Chapel Hill, USA), diluted 1:2000 in PBS + 1% BSA. The next day the sections were washed three times in PBS and incubated for 2 hours at room temperature with donkey-anti-rabbit-FITC in PBS-B (Jackson Immuno Research Lab. Inc) diluted 1:100. Staining for hypoxia was assessed semi-quantitively as strongly positive, positive or negative. For technical reasons, hypoxia could not be rated in E80 and E110.

Comparative genomic hybridisation

CGH was performed as previously described [24]. Briefly, normal DNA and tumour DNA were labelled with digoxigenin-dUTP (Boehringer Mannheim) and biotin-dUTP (Boehringer Mannheim) in a standard nick translation system, respectively. The pre-treated metaphase slides and the probes were denatured simultaneously and hybridisation was performed for three days. After hybridisation, the slides were washed stringently and the biotinylated DNA was detected using streptavidin-FITC and the digoxigenin-labelled DNA with sheep-anti-digoxigenin-TRITC. Chromosomes were counter stained with 4'-6-diamidino-2-phenylindole (DAPI) to identify the chromosome-banding pattern. Adequate controls were included in all experiments [24].

RESULTS

The results of the assessment of angiogenesis, quantification of perfusion, the degree of hypoxia, growth rate and genetic changes in the 10 xenografts are summarised in Table 1. The RVA values measured on 8 sections from each tumour ranged from 0.07 (E49) to 0.18 (E102). The perfusion rate ranged between 0.44 and 0.65. The tumours showed major differences in the degree of hypoxia (Table 1). Examples of typical patterns are shown in Figure 1: xenograft E106 shows extensive hypoxia whereas it is absent in the well vascularised E102.

Growth rate was constant within each line and passage, but showed major differences between the lines. Tumours E2 and E120 had slow growth rates; E49 and E98 had intermediate growth rates; E18, E34, E80, E102, E106 and E110 were fast growing tumours. The growth rates were not related to the RVA or PF (Table 1).

The profiles of genetic alterations are summarised in Table 1 and are described in detail by Jeuken et al. [15]. Briefly, all tumours had loss of the whole or parts of chromosome 10 and gain of chromosome 7 or 7p. Furthermore, amplification of whole chromosome 19 or 19p and loss of chromosome 13 as well as loss of chromosome

11 and gain of chromosome 8q were frequently found. E34 showed additional genetic alterations (loss of 1p and 19q) that are frequently found in oligodendroglial tumours [24], but these were not related to a specific vascularisation type.

On the basis of the RVA, two different vascularisation types of xenografts can be recognised (Table 1 and Figure 1). The poorly vascularised (pv) type has an RVA < 0.10 (E2, E34, E49, E98, E106). The well vascularised (wv) type has an RVA > 0.10 (E18, E80, E102, E110 and E120). The pv type shows hypoxia whereas the wv tumours that could be evaluated are negative for the hypoxic marker. Mean RVA for the wv group was 0.14 (SD 0.03) and for the pv group 0.08 (SD 0.008). There was a significant difference in RVA between the pv and wv group ($P < 0.01$, Wilcoxon test). Interestingly, the PF was not significantly different between the wv and pv group (0.55, SD 0.08 and 0.62, SD 0,03 respectively). Figure 2 shows the sum of genetic alterations for each chromosome arm of the wv and the pv group. The pattern of genetic alterations was not clearly different between the pv type and wv type, but the pv group had a higher number of aberrations (88) than in the wv group (70). Growth rate was not related to the vascular type.

DISCUSSION

In diffuse astrocytic tumours, florid angiogenesis is associated with high-grade malignancy [4,16]. This angiogenic process is induced by hypoxia [8-10]. In addition, tumour angiogenesis may be upregulated by genes resulting from the genetic changes acquired during tumour progression [13].

Our previous study [19] showed that the chromosomal imbalances in these xenografted GBMs are similar to the original GBMs, as well as GBMs described by others [25,26], indicating that the GBM xenigraft lines are valid models from a genetic point of view. The CGH analysis of different passages indicated that xenografted GBMs, like original GBMs, show intratumoural genetic heterogeneity.

By quantitative microscopic analysis, we found two patterns of vascularisation in GBM xenografts: well vascularised (wv) and poorly vascularised (pv). The pv/hypoxic xenograft group is marked by more numerous genomic aberrations (Figure 2). This pattern might represent either genetic heterogeneity between the xenografts or genetic instability induced by hypoxia [27]. No consistent single chromosome aberration separating the groups was observed, however. An association between single genetic aberrations and vascularisation were sought by scanning the human genomic database for chromosomal localisation of factors involved in angiogenesis;e. g. platelet-derived growth factor-A (7p22) and B (22q12.3-13.1), platelet-derived growth

factor receptor (5q31-32), fibroblast growth factor 1 (5q31), 2 (4q25-27), 6 (12p13), 9 (13q11-12), fibroblast growth factor receptor 3 and 4 (4p16.3, 5q35.1-ter), fibroblast growth factor beta receptor (1p33-32), transforming growth factor B1 (19q13.1-13.3) and B2 (1q41), VEGF-A (6p21-12), VEGF-B (11q13) and VEGF-C (4q34). By CGH analysis no correlation was found between genetic alterations in these chromosomal regions and angiogenic type in these xenografts, with the exception of gain on 1p (fibroblast growth factor beta receptor) and 19q (transforming growth factor B1) in two and three xenografts in the wv group respectively. In particular regions carrying the VEGF-A, VEGF-B and VEGF-C genes did not differ between the two types. Amplification of 6p21-12 (VEGF-A region) was seen in only one tumour. However, the extensive genomic alterations in the end phase of malignant progression make it difficult to find a pattern of genomic alterations. Also CGH has limitations with regard to sensitivity and detects only copy number changes larger than 2Mb [28]. CGH does not detect translocations or inversions, which are other mechanisms by which gene expression can be altered [29].

The existence of different vascular phenotypes of GBM xenograft lines can be explained in various ways. Firstly, GBMs show a major heterogeneity of the vascularisation within the tumour [17], and the wv xenograft lines could represent samples derived from foci of efficient angiogenesis. Secondly, the process of serial passaging of xenografts may result in the selection of angiogenic cell clones. However, the observation of line-specific and stable characteristics of vascularisation does not support this explanation [17]. Thirdly, the different vascular phenotypes of xenografts lines may represent differences between neoplastic cells in response to angiogenic factors, such as hypoxia.

In our opinion, the xenograft model with quantitative image analysis is a valuable model to study angiogenesis in GBMs as it quantifies the end-result of this process; i.e. the formation of capillaries by activated endothelial cells [2]. Angiogenesis in GBMs might be related to genetic aberrations, but requires further investigations. This can be accomplished by studying the xenografts in more detail. A study using area-specific CGH has revealed focal genetic micro-heterogeneity in GBMs [30]. CGH can be used on microdissected tumour cells taken from areas with different angiogenic capacity. Furthermore, glioma cell clones can be analysed for their properties to induce angiogenesis [15]. Clones showing a strong capacity to induce angiogenesis can then be analysed by CGH and compared to clones without these properties for differences in genetic alteratons. The use techniques with a higher resolution like fluorescence in situ hybridisation (FISH) and single stranded conformation polymorphism (SSCP) for specific groups of genes is a strategy to further elucidate genomic alterations. These techniques could be used in addition to CGH, as they do not provide an overview of genomic alterations.

In conclusion, two vascular phenotypes of xenografted GBMs were identified with respect to RVA and degree of hypoxia. Poorly vascularised tumours had more genomic alterations, but no unequivocal correlation between angiogenesis and patterns of genetic alterations as detected by CGH was established.

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Chapter 7

Three-dimensional (3D) reconstruction and quantitative analysis of the microvasculature in medulloblastoma and ependymoma subtypes

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Accepted for publication by Angiogenesis

Pieter Wesseling is supported by grant KUN 2003-2975 of the KWF/Dutch Cancer Society.

ABSTRACT

In the World Health Organisation (WHO) classification of tumours of the nervous system, four main histopathological subtypes of medulloblastomas (MB), classic MB, desmoplastic MB, MB with extensive nodularity and advanced neuronal differentiation, and large cell/ anaplastic MB, as well as of ependymal tumours (ET), low-grade ependymoma, anaplastic ependymoma, myxopapillary ependymoma, and subependymoma, are recognised. Under the hypothesis that the microvascular architecture of tumours is a reflection of the histopathological subtype, we performed three-dimensional (3D) reconstructions of the microvasculature in MB and ET subtypes using computerised image analysis. In addition, we quantitatively assessed three microvascular parameters (vessel number, vessel area, vessel perimeter). 3D reconstructions showed a dense pattern of irregular vessels in classic and large cell MB. In desmoplastic MB and MB with extensive nodularity, the vessels were more unevenly distributed and organised around the nodular areas. Classic MB and large cell MB had on average the largest vessel area and perimeter. The highest number of vessels was seen in classic MB and MB with extensive nodularity. 3D analysis of ETs showed that low-grade ependymoma had larger but fewer vessels compared to anaplastic ependymoma, while myxopapillary ependymoma had a complex, heterogeneous pattern of vessels and subependymoma few but regular vessels. In ETs, the highest values for vessel number, vessel area, and vessel perimeter were found in anaplastic ependymoma and the lowest values in subependymoma. We conclude that our 3D reconstructions shed unprecedented light on the tumour vasculature in MBs and ETs and expect that such reconstructions are helpful tools for further studies on angiogenesis.

INTRODUCTION

Medulloblastomas (MBs) and intracranial ependymal tumours (ETs) are neuroepithelial tumours, mainly occurring in individuals younger than 16 years old. Spinal ETs occur in all age groups [1,2,3]. The World Health Organisation (WHO) classification of tumours of the nervous system distinguishes four main histopathological subtypes of MBs and of ETs. MB variants are classic MB, desmoplastic MB, MB with extensive nodularity and advanced neuronal differentiation, and large cell/anaplastic MB. ET variants are low-grade ependymoma (lgE; WHO grade II), anaplastic ependymoma (aE; WHO grade III), myxopapillary ependymoma (mpE; WHO grade I), and subependymoma (subE; WHO grade I) [3]. The MB subtypes mentioned above are all designated by the WHO as high-grade (grade IV) malignancies and so far are considered as belonging to the same spectrum. In the ET group, on the other hand, subE and mpE are different entities rather than belonging to the spectrum of lgE and aE.

Treatment for MBs and intracranial lgEs and aEs is not yet based on histopathological features. However, some recent studies show that children with a large cell/ anaplastic MB have a poorer outcome [4,5], whereas children with a MB with extensive nodularity tend to fare better than children with other MB subtypes [6]. The prognostic significance of histological malignancy grade in lgEs and aEs is unclear [3]. MpEs generally have an indolent behaviour and do not need additional therapy after gross total resection. SubEs are often coincidental findings during autopsy, and only sporadically cause clinical symptoms [3,7].

There is a huge interest in the tumour vasculature as a potential target for anti-tumour therapy [8,9,10]. So far, however, knowledge about the tumour microvasculature in MBs and ETs is limited. Accurate visualisation of the microvascular network is important for a better understanding of tumour angiogenesis. We therefore performed three-dimensional (3D) reconstruction as well as quantitative analysis of 3 vascular parameters (vessel number, vessel area, vessel perimeter) in the main histopathological subtypes of MBs and ETs under the hypothesis that the histopathological subtype is reflected by the microvascular architecture. For 3D reconstruction, a recently developed technique was applied [11]. The 3D images of the tumour microvasculature were compared to those of normal cerebral neocortex, cerebral white matter, and cerebellar cortex and white matter.

MATERIALS AND METHODS

Tissues

Representative biopsy samples of a paraffin embedded classic MB, desmoplastic MB, MB with extensive nodularity, large cell MB, supratentorial lgE, posterior fossa aE, spinal mpE, and supratentorial subE, as well autopsy material of adult normal cerebral neocortex, cerebral white matter, cerebellar cortex, and cerebellar white matter were selected from the archives of the Department of Pathology, Radboud University Nijmegen Medical Centre. A special method was used to allow for cutting up to 60 serial sections of constant thickness and with minimal loss or distortion of tissue [11]. For 3D visualisation in this study, we investigated 45-60 serial 4 µm sections. The serial sections were stained for endothelial cells with a monoclonal antibody to CD34 (mouse monoclonal, 1: 750, Neomarkers, Fremont, USA). Immunostaining was performed using the avidin-biotin-complex method; the chromagen used to highlight vessels was 3.3'- Diaminebenzidine tetrahydrochloride (DAB), resulting in a brown product. In order to improve the signal to noise ratio during image acquisition, no counterstaining was used.

3D visualisation

Computerised 3D visualisation of the vascular bed was performed as recently described by our group [11]. In short, 45-60 images of corresponding microscopic fields in subsequent tissue sections were digitally stored. A microscopic field (10 x objective, field size 0.74 mm^2) was aligned as closely as possible with a rotating microscope stage, using an image overlay on the computer screen depicting the vascular profiles of the previous tissue section. After storage of an entire series of images, automated recognition of vascular profiles (image segmentation) was performed based on positive immunohistochemical staining of endothelial cells. Results of segmentation for each microscopic field were shown as an overlay in the original image on a computer screen for visual inspection and, if necessary, interactive correction. Image registration was fine-tuned automatically using an affine image transformer, which corrects for shifts, rotations and stretching in x and y direction. Finally, 3D visualisation was performed using a marching cube algorithm, resulting in static images of the 3D reconstruction. Of each tumour, two representative microscopic areas were analysed in this way. Cerebral neocortex, cerebral white matter, cerebellar cortex, and cerebellar white matter of a normal human brain obtained by autopsy, served as control tissues.

Quantitative analysis of vasculature

Quantitative analysis of the vasculature was performed for each tumour in a single tissue section halfway the series of CD34-stained serial sections following the method we described earlier [12]. In addition, the same was done on normal cerebellar and cerebral cortex and white matter. In the section used for quantitative analysis 8-20 microscopic fields (10 x objective, field size 0.74 mm^2) were analysed and in these fields the vessel number, vessel area, and vessel perimeter were calculated.

RESULTS

Figure 1 shows a compilation of haematoxylin and eosin (H&E) stained sections (first row), CD34 stained areas highlighting vasculature (second and fourth row) and 3D reconstructions of the microvasculature in these areas (third and fifth row) in four subtypes of MB and ET. For comparison, similar images of normal cerebral neocortex, cerebral white matter, cerebellar cortex, and cerebellar white matter are included. Figures of normal cerebral neocortex and white matter were obtained with kind permission from Gijtenbeek et. al [11]. Figure 2 shows the results of quantitative assessment of vessel number, vessel area, and vessel perimeter of the cerebral neocortex, cerebral white matter, cerebellar cortex, and cerebellar white matter, MBs and ETs, that were used for 3D reconstruction of the microvasculature.

Normal cerebral and cerebellar tissue

3D reconstruction of the cerebral neocortex, cerebral white matter, cerebellar cortex, and cerebellar white matter revealed a regular vascular pattern with little variation in the vessel diameter. Compared to normal cerebral and cerebellar white matter, the cerebral neocortex and the cerebellar cortex had a more intense, somewhat more delicate, but regular vascular pattern (Figure 1). The microvascular parameters in cerebral neocortex and cerebellar cortex were in the same range for vessel area and perimeter, while vessel number was higher in cerebellar cortex. Compared to normal cerebral and cerebellar white matter, these parameters in cerebral neocortex and cerebellar cortex were more variable. In cerebral and cerebellar white matter the vessel number, vessel area, and vessel perimeter were comparable and relatively low, with little variation from field to field (Figure 2).

MBs

With 3D reconstruction, classic MB had a dense, more or less evenly distributed microvascular pattern, the vessels showing a variable diameter. In desmoplastic and extensive nodularity MBs, the blood vessels showed an uneven distribution with organisation around the nodular ('pale island') zones. Large cell MB blood vessels showed an uneven distribution and the formation of tufts. Compared to normal cerebellar cortex and white matter (i.e. the tissue in which MBs occur), more variation was present for all microvascular parameters in all MB subtypes. However, the values for the parameters were generally still in the range of normal cerebellar cortex or white

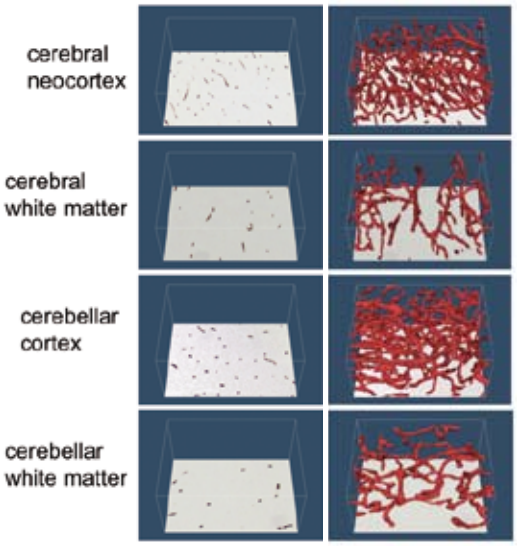

Figure 1

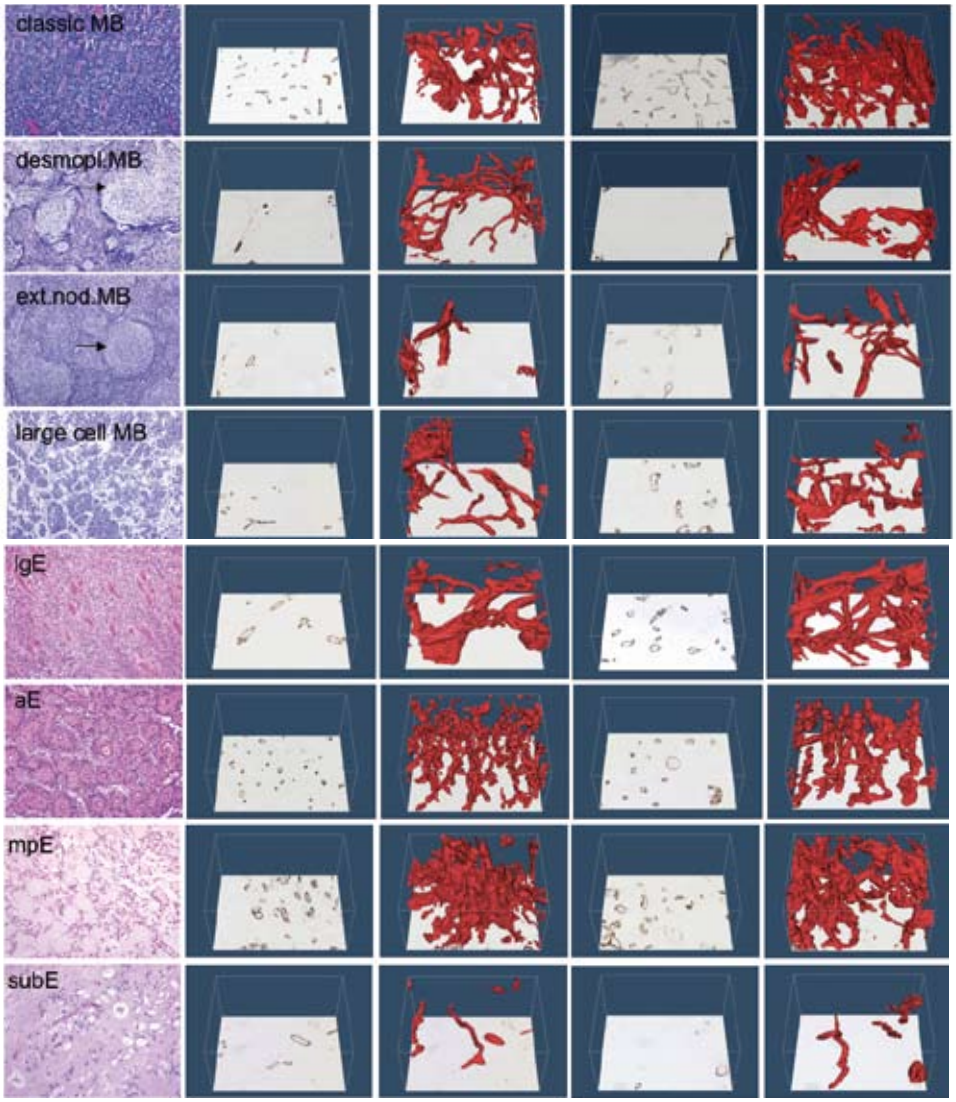

Figure 1 *Haematoxylin and eosin (H&E) stained histological sections (original magnification x 100), 2-dimensional images (immunohistochemical (CD34) staining for endothelial cells) and 3D reconstructions of the vasculature of cerebral neocortex, cerebral white matter, cerebellar cortex, cerebellar white matter, classic MB, desmoplastic MB, MB with extensive nodularity, large cell MB, supratentorial lgE, posterior fossa aE, spinal mpE, and supratentorial subE. The arrows in the H&E stained sections of desmoplastic MB and MB with extensive nodularity indicate nodular ('pale island') zones.*

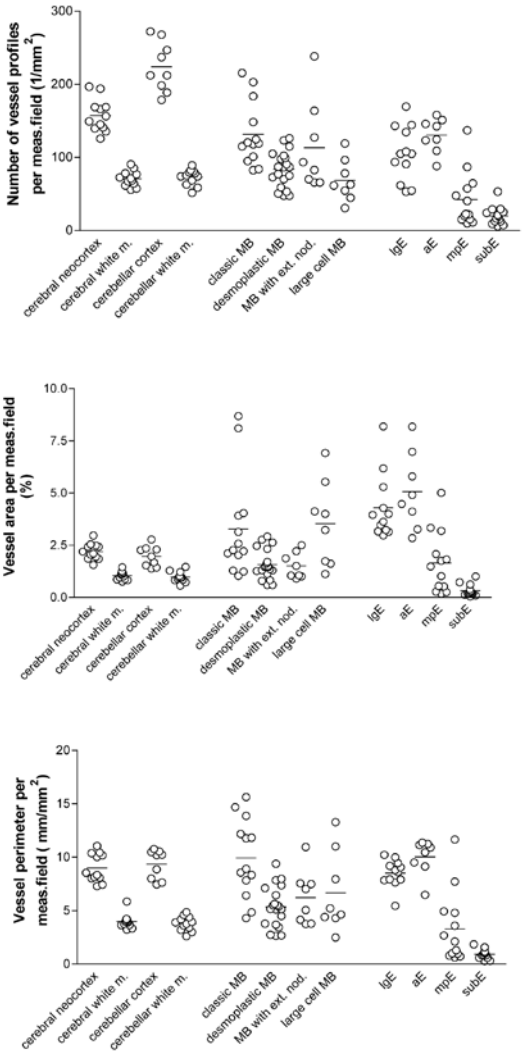

Figure 2 *Vessel number, vessel area, vessel perimeter of cerebral neocortex, cerebral white matter, cerebellar cortex, cerebellar white matter, classic MB, desmoplastic MB, MB with extensive nodularity, large cell MB, supratentorial lgE, posterior fossa aE, spinal mpE, supratentorial subE. Vessel number - number of complete vascular profiles in a field; each vascular profile demarcated from the surrounding tissue by a continuous basement membrane was considered as a separate blood vessel. Vessel number is the vascular density of the field. Vessel area - sum of areas of all individual vascular profiles in a field in mm². Vessel perimeter - sum of perimeters of all individual vascular profiles in a field in mm [11]. Abbreviations; ext. nod. = extensive nodularity, m. = matter, meas. = measured.*

matter. In occasional microscopic fields, the vessel number in large cell MB was lower than in normal cerebellar white matter, while in some fields the vessel area and vessel perimeter in classic and large cell MB exceeded those of normal cerebellar cortex. Median vessel area and perimeter were higher in classic and large cell MB than in desmoplastic and extensive nodularity MB, while median vessel number were higher for classic and extensive nodularity MB. Variation for vessel area and perimeter was most extensive in classic and large cell MB. Variation for vessel number was largest in classic MB and MB with extensive nodularity.

ETs

3D reconstructions of the ETs showed that lgE had fewer but larger vessels as compared to aE. MpE contained a complex, irregular and partly dense vascular pattern, while subE showed relatively few but regular vessels. The vascular parameters in low grade and anaplastic ET were comparable, showed much more variation than in normal cerebral and cerebellar tissue, and while vessel number and perimeter were in the range of normal brain tissue, the vessel area was found to be much higher. In contrast, in subE all three vascular parameters were lower than in normal cerebral and cerebellar white matter. The mpE showed a relatively high variation for vascular parameters over the different fields, in some of them the values for these parameters were in the range of subE, while in others the value for vascular area and perimeter (but not for vessel number) exceeded that of normal cerebral neocortex and cerebellar cortex.

DISCUSSION

Studies on angiogenesis in tumours of the CNS generally focussed on astroglial tumours (for recent review, see [10]), and relatively little attention has been given to this process in MBs and ETs. Some studies in the latter tumours investigated angiogenesis as a prognostic factor for survival, or as a possible target for therapy [13-18]. Thus far, the correlation between survival and vascular pattern is unclear for MBs and ETs. In a study of 23 patients with MBs, Ozer et al. [16] found high vessel density to be a significant unfavourable prognostic factor. This contrasts with another study of 78 children with MBs by Grotzer et al. [17], in which vessel density was not predictive of outcome. Nevertheless, these authors considered MBs as good candidates for anti-angiogenic strategies [17]. In a study of 112 cerebral ETs, progression-free survival time was found to be significantly shorter for VEGF and EGFR positive lgEs, as compared to VEGF and EGFR negative lgEs [14]. In another study of 100 intracranial ETs, markers for hypoxia (carbonic anhydrase 9, hypoxia-inducible factor 1 alpha, and VEGF) were not related to outcome [18]. In a study of supratentorial primitive neuroectodermal tumours and MBs, the spatial organisation of tumour vessels was

described to allow for differentiation between different prognostic subtypes [19].
Our study is the first on 3D reconstruction of microvasculature in MBs and ETs. We conducted this study with the hypothesis that the histopathology of different subtypes is reflected by a difference in the tumour microvasculature and compared the architecture of the tumour vessels with that of normal cerebral and cerebellar tissue. For MBs, classic and large cell MBs had a denser vasculature pattern than desmoplastic MB and MB with extensive nodularity. In the latter two MB types we found vessels to be organised around the nodular, reticulin-free zones. In fact, this is what one would expect knowing that the nodules in these subtypes are classically described as reticulin-free 'pale islands' while the wall of blood vessels does contain reticulin. The vasculature of ETs varied widely. The most dense pattern was seen in aE, while at the other end of the spectrum subE only showed few but regular vessels. The vasculature of mpE, which is a WHO grade I tumour, had a distinct irregular and partly dense pattern. While mpEs are classified together with lgEs and aEs in the category of ETs, their typical histopathology, radiology, and genetic characteristics indicate that mpEs form a separate entity [19,20]. Although variation for the vascular parameters was high in MBs, these parameters were generally in the range of normal cerebellar cortex and white matter. Of the vascular parameters of ETs, only mean vessel area was larger for lgE, aE, and mpE, as compared to normal cerebral and cerebellar tissue. Variation of vascular parameters was high for all ETs, except for subE. The subE had less vessels than normal cerebral tissue.

For several reasons, our present 3D and quantitative results should be interpreted with some caution. First, in the one representative tumour that was investigated in each category, only two microscopic areas were used for 3D reconstruction and 8-20 microscopic fields were used for quantitative analysis. It would be overly optimistic to expect that this study fully accounts for intra- and intertumoural heterogeneity. Furthermore, it is important to realise that, dependent on the growth pattern of the (brain) tumour studied, the intratumoural vessels may be newly formed vessels, incorporated pre-existent vessels, or a combination of both [21]. Especially in infiltrative tumours like MBs, the majority of the tumour vessels may in fact be incorporated vessels, while in ETs (that generally show a more expansive growth pattern) the vast majority of the tumour vasculature may well be derived from neovascularisation, especially so in the subE which typically grows as a solid, exophytic tumour in the ventricular system. Whether brain tumours with vascular parameters within the range of normal cerebral and cerebellar tissue are good candidates for anti-angiogenic therapies depends on the actual degree of neovascularisation. Anti-angiogenic therapies are considered effective only if newly formed vessels are targeted [22]. Even in the absence of newly formed vessels, however, microenvironmental factors may cause vascular changes like vasodilatation. In the quantitative part of our study the latter would result in (areas with) increased vessel area and vessel perimeter without an increase in vessel number.

Interestingly, our quantitative analysis revealed a somewhat higher vessel number in normal cerebellar cortex than in normal cerebral neocortex. This finding might be explained by the fact that the areas selected for quantitative analysis of cerebellar cortex were all located in the granular layer. As this layer contains an extremely high number of (small) neurons, one can imagine that the demand for oxygen and nutrients (and therefore the vascular density) is especially high in this zone.

In conclusion, 3D reconstructions contribute to a better understanding of the true aspect of tumour vasculature [11]. We found a spectrum of patterns in the 3D reconstructions of the microvasculature in MB en ET subtypes, ranging from intensely to sparsely vascularised. Our results clearly show the disorganised and tortuous nature of the vasculature of some MB and ET subtypes, aspects that are more difficult to appreciate in 2-dimensional sections or by quantitative analysis. In the future, 3D reconstruction of the tumour microvasculature in combination with markers identifying newly formed vessels will be of help to determine the degree of angiogenesis dependency of tumours. Unfortunately, unequivocal markers for such newly formed tumour vessels are not yet available. In animal models, 3D reconstructions can also be used to monitor the effects of anti-angiogenic treatment and to test hypotheses concerning the pathobiological significance of different stimuli or inhibitors of this process.

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Chapter 8

Radiotherapy for partially resected spinal ependymomas: a retrospective study of 60 cases

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ABSTRACT

Low-grade ependymomas (lgEs) and myxopapillary ependymomas (mpEs) are the most common primary tumours of the spinal cord. Recurrence-free survival depends on local control of the tumour. The value of additional radiotherapy (RT) is still a matter of debate. The aim of this retrospective study was to analyse radiotherapy, surgery and the preoperative state with regard to recurrence rate and long-term neurological outcome. Sixty patients with spinal lgEs (40) and spinal mpEs (20) were included. According to local policy, 14 patients who underwent total resection and 20 patients with incomplete resection were irradiated postoperatively. Total resection was achieved in 34 of the 60 tumours. Preoperative state and long-term outcome was assessed according to a previously published scale. When postoperative RT was applied after partial resection, only 3 of 11 lgEs and 1 of 9 mpEs recurred. All partially resected non-radiated lgEs (n = 3) and 2 of the 3 partially resected non-radiated mpEs recurred. There was no recurrence after total resection. Only one of 6 patients with disseminated mpEs had clinical symptoms caused by the disseminated tumour. Long-term neurological outcome was related to preoperative conditions with no difference between partially and totally resected tumours. Our study shows that RT is only beneficial for partially resected lgEs and mpEs. Local recurrence-free survival of spinal lgEs and mpEs is obtained by total resection. Long-term neurological outcome is related to preoperative conditions. Seeding is seen in mpEs and does not cause clinical symptoms in most of the patients.

INTRODUCTION

Although ependymomas are relatively rare, they are the most common primary tumours of the central nervous system arising in the spinal cord and comprise 60% of spinal primary tumours [1]. They originate from the ependymal cells lining the ventricular system and the central canal in the spinal cord. Whereas 69% of the intracranial ependymomas occur in children, most spinal ependymomas (96%) are seen in adults [2]. The vast majority of spinal cord ependymomas belong to the cellular low-grade ependymomas (lgEs) (WHO grade II) or myxopapillary (mpEs) (WHO grade I) histologic subtype. LgEs are found throughout the spinal canal and can be situated both intra- and extramedullary. MpEs are located extradurally in the sacral coccygeal region, and intradurally in the conus-cauda region and are classified as extramedullary tumours [2]. Spinal ependymomas are generally tumours of low-grade malignancy with little infiltrative potential [3-8]. However, mpEs have a potential for dissemination within the spinal canal [9]. An unknown percentage of mpEs tend to disseminate within the spinal canal despite the low malignancy grade [9-11]. No

agreement exists on the management of these disseminated tumours as it is not known if they cause clinical symptoms.

For spinal ependymomas, recurrence-free survival depends on local control of the tumour [1,8,12,13]. Subtotal removal results in local recurrence in up to 50-70% [13,14]. Total resection provides the best long-term local control and reduces the risk of recurrence to 5-10% [6,8,12]. However, complete removal of the tumour is not possible in 57-76% of the cases because of the risk of serious neurological deficits [6,15,16]. Whether radiotherapy (RT) following subtotal resection of primary low-grade spinal ependymomas is beneficial, is a matter of debate [17]. Although accumulated data suggest that irradiation may be beneficial, most series are limited by a small number of patients, lack of clinical follow-up and the absence of a trial with matched controls treated without irradiation [18-22].

We performed a retrospective study of 60 patients with 40 primary spinal lgEs and 20 primary spinal mpEs. We determined the effects of postoperative RT, surgical results (total versus partial resection) and preoperative state on local recurrence and long-term neurological functional outcome. In addition, the management of intraspinal seeding of mpEs is discussed.

PATIENTS AND METHODS

Patient population and demographics

Sixty-three patients with 72 tumours from 2 hospitals (University Medical Centre St Radboud, Nijmegen, The Netherlands; St Elisabeth Hospital, Tilburg, The Netherlands) who were treated for spinal ependymomas between 1985 and 2000 were analysed retrospectively. The subependymomas, anaplastic ependymomas (n = 3) and recurrences (n = 9) were excluded from analysis. Information was obtained from the medical records about clinical presentation, imaging, operative procedures, neuropathological diagnosis and long-term neurological outcome. Preoperative state and long-term neurological outcome was assessed according to the McCormick scale (Table 1) [8]. Diagnosis was made according to the WHO classification [10]. The mean age at diagnosis was 42 years for the lgE patients (range 13-82 years, 25 males, and 15 females) and 30 years (range 14-57 years, 9 males, and 11 females) for the mpE patients. Preoperative diagnosis was made by MRI (n = 45) and CT-myelo (n = 15). The time between onset of symptoms and diagnosis of the primary tumours varied from a few weeks to 10 years for both lgEs and mpEs. Forty (66%) of the primary ependymomas were classified as lgEs (WHO grade II) and 20 (33%) as mpEs. All mpEs were located extramedullary in the conus-cauda equina and filum

LgEs

Total resection						Partial resection					
		Long-term functioning						Long-term functioning			
Preoperative functioning	Number of patients	I	II	III	IV	Preoperative functioning	Number of patients	I	II	III	IV
I	13	13				I	7	7			
II	7	4	2	1		II	4	3	1		
III	5	1	2	2		III	3		1	1	1
IV	1				1	IV					

MpEs

Total resection						Partial resection					
		Long-term functioning						Long-term functioning			
Preoperative functioning	Number of patients	I	II	III	IV	Preoperative functioning	Number of patients	I	II	III	IV
I	6	6				I	8	7	1		
II						II	1		1		
III	2	2				III	2		1		1
IV						IV	1				1

Table 1 *Preoperative and long-term functional status according to the clinical/functional classification scheme of McCormick [8]. Grade I: neurologically normal; mild focal deficit not significantly affecting function of involved limbs; mild spasticity or reflex abnormality; normal gait. II: presence of sensomotor deficit affecting function of involved limb; mild to moderate gait difficulty; severe pain or dysesthetic syndrome impairing patient's quality of life; still functions and walks independently. III: more severe neurological deficit requires cane/brace for walking or significant bilateral upper extremity impairment: may or may not function independently. IV: severe deficit; requires wheelchair or cane/brace with bilateral upper extremity impairment; usually not independent.*

terminale. Table 2 gives an overview of the site of the primary spinal lgEs and mpEs. The follow-up ranged from 3 to 15 years (mean 7 years).

Location	LgEs (Number of patients)		MpEs (Number of patients)	
	Total resection	Partial resection	Total resection	Partial resection
Cervical	4	4		
Cervicothoracic	2	3		
Thoracic	6	1		
Thoracolumbar	3	3	4	8
Lumbosacral	11	3	4	4

Table 2 *Location of primary spinal lgEs and mpEs.*

Course of treatment

Patients were treated with an attempted total resection of the ependymoma through a posterior approach. Total resection was defined as macroscopic complete resection. Participating hospitals differed with regard to the indication for radiotherapy (RT). One centre offered RT to all patients, whereas the other centre only irradiated some of the patients with partially resected ependymomas. Decisions to irradiate in the latter group were made on individual grounds. As some physicians were not convinced of the beneficial effects of RT, some patients with partially resected tumours were not irradiated postoperatively. RT was given in a dose of 45 to 50 Gy locally with a wide margin in daily fractions of 1,8-2,25 Gy. Elective whole spinal cord radiation was not given. All recurrences were local and in the radiation field. Treatment consisted of resection and RT. In one case treatment was omitted due to the poor condition of the patient.

RESULTS

Of the 14 lgEs cases with partial resection, 11 received RT. All three non-irradiated partially resected lgEs recurred, against 3 of the 11 radiated partially resected lgEs (27%) ($p < 0.001$). Of the 9 irradiated partially resected mpEs only one recurred, against 2 of the 3 non-irradiated partially resected mpEs ($P < 0.001$) (Table 3). No recurrences occurred after macroscopic total resection.

Total resection of the primary tumour was achieved in 26 of the 40 lgEs (65%) and in 8 of the 20 (40%) mpEs.

	LgEs (n = 40)				MpEs (n = 20)			
	Total resection (n = 26)		Partial resection (n = 14)		Total resection (n = 8)		Partial resection (n = 12)	
Male/ Female	17/ 9		8/ 6		3/ 5		6/ 6	
Age (years)	45		38		36		27	
-Range	13-82		17-68		14-57		16-40	
Radiotherapy (+/-)	10+	16-	11+	3-	4+	4-	9+	3-
Recurrences (number/ %)	0	0	3 (27%)*	3 (100%)*	0	0	1 (11%)*	2 (66%)*
Mean time to recurrence (years)			7	10			10	8

Table 3 *Patients' and treatment characteristics for the primary lgEs and mpEs,* (P < 0.001).*

The long-term neurological morbidity was related to the preoperative neurological condition. Patients with no or minor neurological deficits preoperatively rarely deteriorated after surgery. The extent of resection did not have an impact on neurological status (Table1). One patient with a total resected tumour suffered from myelomalacia due to radionecrosis one year after being irradiated with 28 fractions of 1,80 Gy.

Six patients with primary mpEs had dissemination of the tumour within the spinal canal. For two of these patients in whom the original diagnosis was made by CT-myelo, this was found by MRI during the follow-up. These disseminated tumours caused clinical symptoms (a radicular syndrome) in only one patient. He had undergone a partially resection without additional RT 6 years before, and had clinical symptoms from the local recurrence as well as the disseminated tumour.

DISCUSSION

On the basis of small, largely uncontrolled studies the value of RT after partial resection of spinal lgEs and mpEs is difficult to establish [9,21,22]. Despite these limitations, the accumulated data suggest that irradiation may be beneficial [14,20,21]. In our study, RT significantly reduces local recurrence both for lgEs and mpEs. No recurrences occurred after total resection, which is in concordance with other studies [6,8]. We conclude that RT is only indicated for partially resected lgEs and mpEs, but not after macroscopic gross total resection. Because these patients survive for many years, they are at risk of late complications of RT. As recurrences of these tumours usually occur at the primary site within the radiation field, craniospinal irradiation is not considered useful [22].

The role of surgery in the management of spinal lgEs and mpEs has been well documented. It is widely accepted that surgical resection is the treatment of choice and that radical removal alone controls the disease. Complete removal of the tumour, however, is often impossible because of the risk of serious neurological deficits such as paralysis or severe sensory disturbances (6,15,16). Most studies claim a total resection rate for spinal lgEs and mpEs of 57-76% [3,9,12,23].

In our study, the strongest predictor of long-term functional outcome for both lgEs and mpEs was preoperative functional status. Patients with severe neurological deficits were the least likely to improve postoperatively. The latter is in accordance with previous reports [6,8]. Patients with a partial resection were on average not worse off as compared to those with completely resected tumours.

Interestingly, six patients with mpEs (30% of mpEs) had intradural seeding, causing clinical symptoms in only one patient. Although mpEs have a low proliferation rate and are designated as WHO grade I, they do have a significant potential for dissemination through the spinal canal [9]. This might be partly caused by their extramedullary location, favouring seeding of loose tumour cells, and is not considered a sign of aggressive tumour behaviour. In the past, seeding of mpEs has been reported sporadically, most often within the spinal canal [11]. Due to the use of MRI in the majority of patients, we found a higher incidence of intraspinal seeding. They are difficult to operate on due to their small size and usually stay unchanged even after years of follow-up. When showing symptoms, total resection is the treatment of choice.

In conclusion, our study shows that recurrence-free survival of spinal lgEs and mpEs is determined by total resection, and that RT has a beneficial effect on recurrence-free survival only in partially resected tumours. Furthermore, postoperative clinical outcome is largely determined by the preoperative morbidity and is not different for patients with totally resected tumours as compared to those with partially resected tumours. Local seeding is almost exclusively seen in mpEs. In mpEs, this phenomenon is relatively frequent but does not cause clinical symptoms in the majority of patients and treatment of these seeded tumours is indicated only when they cause clinical symptoms.

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Summary and general discussion

Tumours of the central nervous system (CNS) are the leading cause of cancer related death in patients under 16 years. These tumours differ in histopathological spectrum, localisation and behaviour from adult CNS tumours. In addition, the treatment requires special attention with regard to the need to preserve a normal cognitive and physical development of the child. Medulloblastomas (MBs) and ependymal tumours (ETs) account for 21-42% of CNS tumours in children. In this thesis, some novel molecular, morphological, and prognostic aspects of MBs and ETs are presented.

In chapter 1, an overview is given of MBs and ETs. Epidemiologic data, clinical symptoms, radiological, histopathological and molecular diagnostic features are summarised. The different histopathological subtypes of MBs and ETs and their clinical behaviour are described. The most common cytogenetic abnormalities as detected by karyotyping, loss of heterozygosity (LOH), comparative genomic hybridisation (CGH), and ploidy studies in MBs and ETs are presented. In all patients, surgery is the initial step with gross total resection as its aim. For both MBs and ETs, gross total resection is the most important prognostic marker for disease-free survival. Further therapeutic options for patients with MBs, based on stratification of patients into 'average-risk' and 'high-risk-groups' are presented. For patients with ETs, the role of radiotherapy (RT) and chemotherapy (ChT) is discussed. The treatment of very young children (under the age of three) with MB and ET remains a major challenge as craniospinal irradiation is avoided because of late adverse neurocognitive and neuroendocrine sequelae. The outcome after a recurrence of MBs and intracranial ETs is generally poor, and no standard treatment at this point is available yet. About one-third of ETs is localised in the spinal cord; these tumours generally carry a good prognosis.

In chapter 2 we describe genetic alterations as detected by CGH in MBs. Ten MB biopsies and one MB cell line were examined to investigate if genetic alterations can be used as a prognostic factor. One amplification site on chromosome 8q24 was detected in the cell line corresponding to the known amplification of the MYCC gene in this cell line. The gain of 2p21-24 in two tumours was shown to represent amplification of the MYCN gene by Southern blot hybridisation and fluorescence in situ hybridisation. No specific pattern of genetic alterations was associated with the clinical behaviour of the 10 tumours. Other investigators (describing a total of 150 MBs) also found a spectrum of non-random genomic losses and gains. Our results were not completely concordant with cytogenetic analysis, probably due to inherent weaknesses of both procedures. Cytogenetic analysis of solid tumours evaluates only a limited number of cells. In addition, CGH detects only changes involving loss or gain of DNA, implicating that translocations, inversions, and multiploidy cannot be detected by CGH as equal amounts of tumour and control DNA are used in the experiments.

Chapter 3 contains a study correlating distinct genetic subtypes of ETs with patient age and tumour localisation. So far, clinical studies have revealed that postoperative survival depends on age, tumour localisation, and extent of tumour resection. However, it is well established that tumour localisation and age are interrelated. Patients with spinal ETs are on average 40 years, whereas ETs in the posterior fossa are predominantly found in children. The genome of 20 ETs was screened for chromosomal imbalances by CGH. Loss of chromosome 22q (75%), 10q (65%), 21 (50%), and 16p (50%) were the most frequently detected aberrations. In adults, spinal ETs relatively often showed gain of chromosome 2, 7, 12, and loss of 14q, posterior fossa ETs loss of chromosome 22, and supratentorial ETs loss of chromosome 9. Preference of some chromosomal imbalances for either paediatric or adult ETs were seen, the most striking being gain of chromosome 9 and loss of chromosome 6 in adult posterior fossa ETs. The genetic background of ETs is complex and partly correlated with tumour localisation, histopathological subtype and age of the patient.

In the study described in chapter 4, we investigated the inverse correlation we observed before between number of genetic aberrations (determined by CGH) and WHO malignancy grade in ET at the ploidy level. We found aneuploidy or tetraploidy to be common in the group of myxopapillary ependymomas (mpEs) and much less frequent in low-grade ependymal tumours (lgEs) and anaplastic ependymomas (aEs). Tumour material for CGH was available in about half of these tumours, and revealed the highest number of genetic aberrations in the mpEs, a lower number in lgEs and the lowest number in aEs. Interestingly, aEs contained fewer aberrations than the lgEs. Generally, malignant progression involves accumulation of genetic aberrations. Our findings, however, suggest that different genetic pathways exist for lgEs and aEs. Similarly, the mpEs, defined as a grade I malignancy according to the WHO classification, showed an extensive array of genomic aberrations. These results confirm that mpEs are distinctly different from lgEs and aEs at the genetic level and that extensive genomic alterations and aneuploidy in ETs are not in themselves an indicator of malignant behaviour, c.q. adverse prognosis.

In chapter 5, we describe a study about the relevance of insulin-like growth factors (IGFs) in MBs. IGFs are small polypeptide mitogens that resemble pro-insulin in structure. Through binding to the IGF-I receptor, they elicit growth promoting and anti-apoptotic effects. IGF-I is considered to mediate many of the growth promoting actions of growth hormone, whereas IGF-II acts as an autocrine growth and motility factor during prenatal growth and development. The expression patterns of IGF-I and -II mRNA in 14 MBs of 12 children and 2 adults were studied. In all cases, tumour cells showed abundant expression of IGF-I mRNA. Nine tumours showed significant IGF-II expression. In these tumours, the hybridisation signal almost exclusively co-localised with a sub-population of Ki-M1P positive cells that were identified as microglia cells

with a ramified morphology. Probably, during the transformation from the resting ramified towards the amoeboid morphology (or vice versa) IGF-II mRNA expression is temporarily induced. The induction of IGF-II mRNA expression by these cells in MBs could theoretically exert mitogenic and anti-apoptotic effects on adjacent tumour cells, but the exact meaning of this phenomenon needs further elucidation. These results support the view that IGF-I and -II are involved in the development and growth of MB, and that the IGF system is a potential target for therapy.

Chapter 6 and 7 are studies on tumour vasculature. Angiogenesis is considered of vital importance for the growth of solid tumours and metastases, and may therefore constitute a target for anti-cancer therapy. The knowledge of the mechanisms of angiogenesis is of importance for the further exploration of anti-angiogenic therapy. Tumour angiogenesis occurs as a result of both local tissue conditions (especially hypoxia) and specific genetic alterations acquired during oncogenesis.

Chapter 6 describes an investigation into the correlation between vasculature and genetic aberrations (as detected by CGH) in subcutaneous glioblastoma multiforme (GBM) xenograft lines, growing in mice. The prognosis of patients with a GBM is determined by local growth of their tumour. GBMs are histologically characterised by striking microvascular proliferation, which constitutes a target for new anti-cancer therapies. Angiogenesis is thought to be regulated by peptides, produced by tumour cells in reaction to local hypoxia. Alternatively, tumour angiogenesis may be caused by specific genetic alterations acquired during oncogenesis. The striking heterogeneity of the microvasculature in GBMs might be related to genetic heterogeneity within a GBM. To analyse the relationship between genetic aberrations and tumour angiogenesis, 10 GBM xenografts were analysed by CGH. The 10 xenograft lines differed in vascular pattern, ranging from well to poorly vascularised. In this model, there was a trend of poorly vascularised tumours having more genetic alterations. However, there was no unequivocal correlation between vasculature, growth rate and patterns of genetic alterations in this tumour type.

Chapter 7 is a study describing the three-dimensional (3D) reconstruction of the microvasculature in MB (classic MB, desmoplastic MB, MB with extensive nodularity, large cell MB) and ET histopathological subtypes Low-grade ependymoma (lgE), anaplastic ependymoma (aE), myxopapillary ependymoma (mpE), subependymoma (subE). It concerns a pilot study in which one sample of each histopathological subtype is examined. 3D reconstructions were performed by applying a new technique using computerised image analysis and reconstruction. In addition, we assessed vessel number, area, and perimeter, in each of these tumours. 3D reconstruction of the vasculature of MBs showed a dense pattern of irregular vessels in classic and large cell MB, whereas in desmoplastic MB and MB with extensive nodularity the

microvasculature was more unevenly distributed and organised around the nodular areas. Median vessel number, area, and perimeter were in the range of cerebellar cortex and white matter, with the exception of a higher vessel area in some areas of classic MB and large cell MB. 3D analysis of ETs showed that lgE had fewer, but larger vessels as compared to aE, while mpE had a complex, irregular pattern of vessels. SubE had few but regular vessels. In ETs, the highest values for vessel number, area, and perimeter were found in aE and the lowest values in subE. 3D vasculature reconstructions give additional insight in the tumour microvasculature and gives direction for further research.

In chapter 8, a retrospective clinical study on the value of additional RT for spinal lgEs and mpEs in adults and children is presented. Recurrence-free survival depends on local control of the tumour. The value of additional RT after surgery is still a matter of debate. The aim of this retrospective study was to analyse the preoperative state, surgery and RT, with regard to recurrence rate and long-term neurological outcome. Forty patients with spinal lgEs and 20 patients with mpEs were analysed. According to local policy in different hospitals, 14 of 34 patients who underwent total resection and 20 of 26 patients with incomplete resection were irradiated postoperatively. There was no recurrence after total resection, with or without RT. All partially resected non-radiated lgEs and 2 of the 3 partially resected non-radiated mpEs recurred. When postoperative RT was applied after partial resection, only 3 of 11 lgEs and 1 of 9 mpEs recurred. We concluded that local recurrence-free survival is obtained by total resection, that RT is only beneficial for partially resected tumours, and that long-term neurological outcome is related to preoperative conditions.

Implications for future research

Progress in the treatment of patients with MBs and ETs can only be achieved by a system of stratification according to prognostic and predictive factors of these tumours. Such a classification consists of a better combination of clinical, histopathological, molecular, genetic, and vascular features. The ultimate goal is ‘tailor-made’ treatment, in which patients with the most aggressive tumours receive the most intense therapy, while children with less aggressive tumours are treated as effectively but will be spared the late sequelae related to extensive treatment. It is clear that it is of utmost importance to determine reliable, reproducible prognostic factors. Gradually, more prognostic and predictive factors are becoming available to help us find a robust subdivision in the seemingly chaotic heterogeneous pool of MBs and ETs. Recent trials have shown that the large cell/ anaplastic histopathological variant of MB is associated with a poor prognosis [1]. At the molecular level, loss of 17p13.3, and/ or MYCC/MYCN amplification in MBs are also associated with a poorer outcome [1]. IGF-I and -II in MBs are of importance for tumour growth, but their prognostic

value needs further investigation [2]. So far, no study on ETs has provided prognostic molecular markers that reliably predict survival or tumour recurrence [3]. Large CGH studies suggest an association between gain of 1q and a worse clinical outcome [4,5]. The studies in this thesis have shown that histopathological ET subtypes differ with regard to genetics, ploidy, and vascular pattern. Larger groups of patients with ETs are needed to further improve stratification, optimising treatment accordingly.

One way to further investigate the associations between histopathological subtype, growth rate of the tumours, vasculature and genetic alterations is to use xenograft models. However, care should be taken that such models mimic the human intracerebral or intracerebellar situation as much as possible. In such models, the impact of new pharmacological, radiation, and anti-angiogenic therapies on vasculature, growth rate, and relapse rate of the tumour can be investigated.

The successful translation of angiogenesis inhibitors to clinical application depends on our understanding of the angiogenetic process [6]. Our studies have corroborated the importance of further studies of the vasculature in MBs and ETs, as it appears that specific patterns are associated with subtypes of tumours. Our findings therefore urge at further studies on the interaction of the tumours and bloodvessels at the level of metabolism and genetics. Furthermore, 3D reconstruction of the vasculature with markers for new versus pre-existent vessels could help to determine which vessels are actually a target for anti-angionetic therapy. Such studies will pave the way to pharmacotherapeutical trials aimed at reduction of tumour growth.

In conclusion, the findings of the studies presented here have shed some new light on the pathophysiology of MBs and ETs, and can serve as a basis for further exploration of their molecular, genetic and vascular characteristics. This will hopefully ultimately lead to more ‘tailor made’ therapies, optimising therapeutic effect and minimising damage to the cognitive and physical development of children with such tumours.

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Samenvatting en discussie

Tumoren van het centrale zenuwstelsel (CZS) zijn de belangrijkste doodsoorzaak bij kinderen met kanker. Deze tumoren verschillen in histopathologisch spectrum, localisatie en beloop van tumoren op de volwassen leeftijd. Medulloblastomas (MBs) en ependymale tumoren (ETs) vormen in deze groep het belangrijkste deel (21-42%). De behandeling van deze CZS tumoren is moeilijk omdat de mentale en lichamelijke ontwikkeling nog niet voltooid is en beschadigd kan worden door de therapieën. In dit proefschrift worden nieuwe moleculaire, morfologische en prognostische aspecten van MBs en ETs besproken.

In hoofdstuk 1 wordt een overzicht gegeven van MBs en ETs. Epidemiologische gegevens, klinische symptomen, radiologische, histopathologische en moleculair diagnostische procedures worden samengevat. De verschillende histopathologische subtypes van MBs en ETs en hun klinische kenmerken worden beschreven. De meest voorkomende cytogenetische afwijkingen zoals die gedetecteerd kunnen worden door 'loss of heterozygosity' (LOH), 'comparative genomic hybridisation' (CGH), en ploïdie studies, worden besproken. Bij alle patiënten is chirurgie met als doel totale extirpatie, de eerste stap. Voor zowel MBs als ETs is totale extirpatie de belangrijkste prognostische marker voor de overleving. Daarna volgen verdere therapeutische opties. Voor patiënten met MBs zijn deze gebaseerd op een verdeling in 'gemiddeld risico' en 'hoog risico' groepen. Voor patiënten met ETs wordt de rol van chemotherapie (ChT) en radiotherapie (RT) besproken. De behandeling van kinderen jonger dan drie jaar met een MB of ET blijft een grote uitdaging aangezien RT vermeden moet worden om latere cognitieve en endocriene schade te voorkomen. De uitkomst na een recidief MB of ET is slecht, en op dit moment zijn er geen standaard behandelingen voorhanden. Ongeveer eenderde van de ETs is gelokaliseerd in het ruggenmerg, deze tumoren hebben doorgaans een goede prognose.

In hoofdstuk 2 worden genetische veranderingen, gedetecteerd door comparative genomic hybridisation (CGH), in MBs beschreven. Tien MB biopten en één MB cel lijn werden onderzocht om na te gaan of bepaalde genetische afwijkingen prognostische relevantie hebben. Een amplificatie op chromosoom 8q24 kwam overeen met de bekende MYCC gen amplificatie van deze cellijn. De amplificatie op 2p21-24 van het MYCN gen in twee tumoren werd bevestigd door Southern blot hybridisation en FISH. Er kon geen specifiek patroon van genetische veranderingen geassocieerd worden met bepaalde klinische karakteristieken van de tumoren. Overige onderzoekers, met in totaal 150 MBs vonden eveneens een aspecifiek spectrum van genetische afwijkingen. Onze CGH resultaten kwamen niet geheel overeen met de cytogenetische analyse, waarschijnlijk ten gevolge van inherente zwakheden van beide procedures. Cytogenetische analyse onderzoekt slechts een gedeelte van de tumor cellen. CGH detecteert alleen winst of verlies van DNA, dus translocaties, inversies en multiploïdie

worden niet gedetecteerd aangezien er voor de procedure gelijke hoeveelheden tumor en controle DNA worden gebruikt.

Hoofdstuk 3 is een studie die specifieke genetische subtypes van ET correleert met de leeftijd van de patiënt en de lokalisatie van de tumor. Tot dusver hebben klinische studies aangetoond dat postoperatieve overleving afhankelijk is van leeftijd, lokalisatie van de tumor en de uitgebreidheid van de resectie. Echter, tumor lokalisatie en leeftijd zijn gerelateerd. Patiënten met spinale ETs zijn gemiddeld 40 jaar, terwijl daarentegen ETs in de fossa posterior voornamelijk voorkomen bij kinderen. Het genoom van 20 ETs werd middels CGH gescreend op chromosomale afwijkingen. De meest frequent gevonden afwijkingen waren verlies van chromosome 22q (75%), 10q (65%), 21 (50%), en 16p (50%). Bij de volwassenen hadden de spinale ETs relatief vaak winst van chromosome 22, fossa posterior ETs verlies van chromosome 22 en supratentorie'le ETs verlies van chromosoom 9. Specifieke chromosomale afwijkingen voor ETs op kinder of volwassen leeftijd werd gevonden, met als belangrijkste bevinding winst van chromosoom 9 en verlies van chromosoom 6 bij volwassen fossa posterior ETs. De genetische achtergrond van ETs is complex en deels verband houdend met tumor lokalisatie, histopathologische subtype en leeftijd van de patiënt.

Het verband tussen het aantal genetische afwijkingen (gedetecteerd door CGH en ploïdie meting) en de WHO maligniteitsgraad bij ETs werd onderzocht in hoofdstuk 4. Wij vonden dat aneuploidy of tetraploidy vaker voorkwam in de groep van myxopapillaire ependymomen (mpEs) en minder frequent bij laaggradige ependymale tumoren (lgEs) en anaplastische ependymomen (aEs). Tumorweefsel voor CGH was beschikbaar voor ongeveer de helft van de tumoren, en toonden het hoogste aantal genetische afwijkingen aan in de mpEs, een lager aantal in lgEs en het laagste aantal in aEs. De aEs hadden dus verrassend genoeg minder genetische afwijkingen dan de lgEs. In het algemeen behelst maligne progressie een toename van genetische afwijkingen. Onze bevindingen suggereren dat lgEs en aEs een ander genetisch patroon hebben. Hetzelfde geldt voor mpEs die uitgebreide genetische afwijkingen vertoonden en gedefinieerd worden als een graad I maligniteit volgens de WHO classificatie. Deze bevindingen bevestigen dat mpEs wezenlijk verschillen van lgEs en aEs op genetisch niveau, en dat uitgebreide genetische afwijkingen en aneuploïdie op zich geen tekenen zijn van maligne klinisch gedrag, of duiden op een slechte prognose.

Hoofdstuk 5 betreft een studie over de relevantie van insuline-like growth factors (IGF's) in MBs. IGFs zijn kleine polypeptide mitogens die lijken op pro-insuline qua structuur. Door binding aan de IGF-I receptor veroorzaken ze groei en anti-apoptotische effecten. IGF-I stimuleert het groei hormoon terwijl IGF-II als een autocriene groei en motiliteitsfactor werkt gedurende de prenatale groei en ontwikkeling. Het expressiepatroon van IGF-I en IGF-II mRNA in 14 MBs van 12 kinderen en twee

volwassenen werd bestudeerd. In alle gevallen vertoonden de tumorcellen volop expressie van IGF-I mRNA. Negen tumoren vertoonden IGF-II expressie. In deze tumoren was het hybridisatie signaal bijna uitsluitend gelokaliseerd in een subpopulatie van Ki-M1P positieve cellen die werden geïdentificeerd als microglia cellen met een 'ramified' (vertakte) morfologie. Waarschijnlijk wordt tijdens de transformatie van de rustende 'ramified' naar de amoeboide morfologie (of vice versa) de IGF-II mRNA expressie geïnduceerd. De inductie van IGF-II mRNA expressie in deze cellen in MBs zou theoretisch gezien mitogene en anti-apoptotische effecten kunnen geven op ernaast gelegen tumor cellen, doch de exacte betekenis hiervan behoeft verder verduidelijking. Onze bevindingen steunen het idee dat IGF-I en -II betrokken zijn bij de ontwikkeling en groei van MBs, en dat het IGF systeem een potentieel therapeutische doelwit is.

Hoofdstuk 6 en 7 zijn studies over tumor vascularisatie. Angiogenesis wordt van vitaal belang geacht voor de groei van solide tumoren en metastasen, en vormt daarmee een doel voor therapie. Het identificeren van het mechanisme van angiogenesis is van belang voor het ontwikkelen van anti-angiogenetische therapie. Tumor angiogenesis is zowel het resultaat van lokale weefsel condities (vooral hypoxie) als van specifieke genetische veranderingen, geaccumuleerd tijdens de oncogenesis.

Hoofdstuk 6 beschrijft een onderzoek naar het verband tussen vasculaire patronen en genetische afwijkingen (gedetecteerd door CGH) in subcutaan geïmplanteerde, glioblastomas multiforme (GBM) xenograft lijnen. De prognose van patiënten met een GBM wordt bepaald door de lokale groei van de tumor. GBMs worden histologisch gekarakteriseerd door een zeer intensieve vascularisatie, welke een doelwit kan zijn voor nieuwe therapieën. Angiogenesis wordt verondersteld gereguleerd te worden door peptides, geproduceerd door tumor cellen in reactie op hypoxie. Anderzijds wordt de angiogenesis mogelijk veroorzaakt door specifieke genetische veranderingen ontstaan tijdens de oncogenesis. De heterogeniteit van de vasculatuur in GBMs is mogelijk gerelateerd aan genetische heterogeniteit binnen een GBM. Om de relatie tussen de genetische afwijkingen en de angiogenesis te analyseren werden tien GBM xenograften genetisch geanalyseerd middels CGH. De tien xenograft lijnen verschilden in vasculair patroon, variërend van goed tot slecht gevasculariseerd. In dit model werd een trend gezien waarbij de slecht gevascullariseerde tumoren meer genetische afwijkingen hadden. Er was echter geen strikte correlatie tussen vasculatuur, groeisnelheid en bepaalde genetische patronen.

Hoofdstuk 7 is een studie over drie-dimensionale (3D) reconstructies van het vaatstelsel van de vier histopathologische subtypes van MBs (classic MB, desmoplastisch MB, MB with extensive nodularity and advanced neuronal differentitation, large cell/ anaplastic MB) en ETs (Low-grade ependymoma (lgE), anaplastic ependymoma (aE),

myxopapillary ependymoma (mpE), subependymoma (subE)). Het betreft een pilot study waarbij van elk subtype één exemplaar werd onderzocht. 3D reconstructies werden verricht door het toepassen van nieuwe beeldanalyse technieken. Daarnaast keken we naar vaat aantallen, vaatoppervlakte en vaatperimeter in elk tumor subtype. De 3D reconstructies van de vaten van de MBs toonden een vol, dicht patroon van irreguliere vaten in de classic en large cell MBs, terwijl in het desmoplastic MB en het MB met extensive nodularity, de vaten meer ongelijk verdeeld waren, en georganiseerd waren rondom de nodulaire gebieden. Het gemiddelde met betrekking tot vaat aantallen, oppervlakte en perimeter kwam overeen met dat van cerebellaire cortex en witte stof, met uitzondering van een groter vaatoppervlakte in sommige gebieden van het classic en large cell MB. De 3D reconstructies van de vaten van de ETs toonden dat lgE minder doch grotere vaten had in vergelijking met aE, terwijl het vaatpatroon van mpE complex en irregulier was. Het subE had slecht enkele, doch regelmatige vaten. De hoogste waarden voor vaat aantallen, oppervlakte en perimeter werden gezien bij het aE, en de laagste waarden bij het subE. 3D vaatreconstructies hebben een toegevoegde waarde voor het inzicht in de tumor vasculatuur en zijn een waardevol instrument voor toekomstige studies naar angiogenesis.

In hoofdstuk 8, wordt een retrospectieve klinische studie over de waarde van RT voor spinale lgEs en mpEs bij volwassenen en kinderen, beschreven. Ziektevrije overleving is afhankelijk van lokale controle van de tumor. De waarde van radiotherapie na operatie staat nog steeds ter discussie. Het doel van deze retrospectieve studie was om de preoperatieve staat, de chirurgische uitkomst en het wel of niet geven radiotherapie te onderzoeken, met betrekking tot het vóórkomen van recidieven en neurologische uitkomst op langere termijn. Veertig patiënten met een lgEs en 20 patiënten met mpEs werden retrospectief onderzocht. Volgens het lokale beleid in verschillende ziekenhuizen, werden 14 van de 34 patiënten die een totale resectie ondergingen en 20 van de 26 die een incomplete resectie ondergingen, postoperatief bestraald. Recidieven traden niet op na een totale resectie, met of zonder radiotherapie. Alle patiënten met partieël geresecteerde, niet bestraalde lgEs (n=3) en twee van de drie patiënten met partieël geresecteerde, niet bestraalde mpEs kregen een recidief. Bij postoperatieve RT na een partieël resectie, hadden drie van de 11 patiënten met een lgEs en één van de negen mpE patiënten, een recidief. Onze studie toonde aan dat RT alleen zinvol is bij partieel geresecteerde lgEs en mpEs. Lokale controle van spinale lgEs en mpEs wordt bereikt door totale resectie. De neurologische uitkomst op lange termijn is gerelateerd aan de preoperatieve conditie.

Implicaties voor verdere studie

Voortgang in de behandeling van MBs en ETs kan alleen bereikt worden door een stratificatie systeem gebaseerd op prognostische en voorspellende factoren van

deze tumoren. Een dergelijke classificatie systeem bestaat uit een combinatie van klinische, histopathologische, moleculair genetische en vasculaire kenmerken. Het uiteindelijke doel is 'op maat' therapie, waarbij de patiënten met de meest agressieve tumoren de intensiefste behandeling krijgen, terwijl kinderen met minder agressieve tumoren zo effectief mogelijk behandeld worden, doch de late gevolgen van intensievere behandelingen bespaard blijven. Het is derhalve van het grootste belang om betrouwbare en reproduceerbare prognostische factoren te bepalen. Geleidelijk aan komen er meer prognostische en voorspellende factoren beschikbaar die een betere verdeling geven in ogenschijnlijke chaotisch heterogene pool van MBs en ETs. Recente trials hebben aangetoond dat de histopathologische large cell/ anaplastic MB variant geassocieerd is met een slechte prognose [1]. Op moleculair niveau zijn verlies van 17p13.3, en/ of MYCC/MYCN amplificatie in MBs geassocieerd met een slechtere uitkomst [1]. IGF-I en -II zijn van belang voor tumor groei in MBs, doch hun prognostische relevantie moet nog verder onderzocht worden [2]. Tot dus ver zijn er geen studies in ETs die betrouwbare moleculair prognostische markers hebben opgeleverd [3]. Grotere CGH studies suggereren een associatie tussen winst van 1q en een slechtere klinische uitkomst [4,5]. De studies in dit proefschrift hebben aangetoond dat de histopathologische ET subtypes verschillen met betrekking tot genetica, ploïdie, en vasculair patroon. Grotere groepen van ET patiënten zijn nodig voor het verder verbeteren van het stratificatie systeem om daarmee behandelingen te kunnen optimaliseren.

Een van de manieren om de associatie tussen histopathologische subtypes, groei snelheid van de tumoren, vasculatuur en genetische veranderingen verder te onderzoeken is het gebruiken van xenograft modellen. Echter, het is zaak om er op te letten dat deze modellen zoveel mogelijk de intracerebrale en intracerebellaire situatie nabootsen. Met dergelijk modellen kunnen dan de gevolgen van nieuwe farmacologische, radiotherapeutische en anti-angiogenetische therapieën op de vasculatuur, groei snelheid en recidief kans van de tumoren onderzocht worden.

Het succesvol toepassen van angiogenesis remmers in de klinische praktijk hangt af van ons begrip van het angiogenetisch proces [6]. Onze studies hebben het belang van toekomstige onderzoek naar de vasculatuur van MBs en ETs onderstreept, aangezien specifieke vaatpatronen geassocieerd lijken met bepaalde tumor subtypes. Deze bevindingen geven derhalve aanleiding tot toekomstige studies naar de interactie van tumoren en bloedvaten op metabool en genetisch niveau. 3D reconstructies van de vasculatuur met markers voor nieuwe versus preëxistente vaten kunnen ons verder helpen bepalen welke vaten een potentieel doelwit zijn voor anti-angiogenetische therapie. Dergelijke studies kunnen een voorbereiding zijn voor farmacotherapeutische trials gericht op reductie van tumorgroei bij patiënten.

Tenslotte, de uitkomsten van de studies hier besproken hebben een nieuw licht geworpen op pathofysiologie van MBs en ETs, en kunnen dienen als een basis voor verder onderzoek naar de moleculaire, genetische en vasculaire kenmerken van deze tumoren. Dit zal hopelijk leiden tot meer therapieën op maat, hiermee het therapeutisch effect maximaliseren, en de schade aan de cognitieve en lichamelijke ontwikkeling van kinderen met dergelijke tumoren zoveel mogelijk beperken.

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Dankwoord

Dit proefschrift is tot stand gekomen door de steun, inzet en expertise van velen. Graag wil ik op deze wijze alle mensen bedanken die de afgelopen jaren een bijdrage hebben geleverd. In het bijzonder wil ik de volgende mensen noemen.

- Prof. Dr. GWAM Padberg, beste George, mijn dank voor de fantastische opleidingstijd en de mogelijkheid na mijn assistentschap verder te gaan met wetenschappelijk onderzoek. De betrokkenheid en het commentaar op de concept artikelen waren van essentieel belang.

- Dr. J Pomp, beste Jacqueline, Dankzij jouw scherpe, strenge en humoristische commentaar kwamen de laatste drie artikelen van dit proefschrift tot stand. Mijn erkentelijkheid hiervoor is groot.

- Dr. R Jenkins, dear Bob, thanks to you and your staff I had the opportunity to work in what is considered one of the best clinics and research institutes of the world. My time in the Mayo clinic was a great inspriration for the rest of my career, and served as an excellent start for my work on molecular genetics.

- Dr. RH Boerman, beste Dolf, jij bent degene geweest die dit onderzoek geïnitieerd heeft. Mijn dank voor je enthousiasme en expertise op het gebied van de moleculaire genetica, zonder welke dit proefschrift nooit tot stand was gekomen.

- Dr. P Wesseling, beste Pieter, Nog altijd is de pathologie de ‘gouden standaard’. Jouw bijdrage was dan ook elke keer onmisbaar. Mijn dank voor de vruchtbare samenwerking en de support van de afgelopen jaren.

- Dr. AC Kappelle, beste Arnoud, mijn dank voor de begeleiding de afgelopen jaren en het helpen doorzetten als het op sommige momenten tegenzat. Ik heb de samenwerking altijd als zeer prettig ervaren.

- Dr. JAWM van der Laak, beste Jeroen, een aantal artikelen van dit proefschrift zijn dankzij jouw expertise op het gebied van software en digitale plaatjes tot stand gekomen. Mijn dank hiervoor.

- Fiona Oostrom, beste Fiona, dank voor de secretarieële ondersteuning bij het voorbereiden van het manuscript.

Curriculum vitae

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In 1992 and 1993 he worked as a resident in the neurology department of Medisch Centrum Alkmaar and cooperated on a project concerning depression in Alzheimer and Parkinson's disease (The Netherlands Brain Institute, Amsterdam). In 1994, he started his training as a neurologist at the University Medical Center St Radboud, Nijmegen (Prof. Dr. GWAM Padberg). Two years later, thanks to a grant by the Dutch Cancer Society, his doctoral research project on translational research in medulloblastomas and ependymal tumours started. For a year, he worked in the department of laboratory medicine and pathology (Dr. R Jenkins Mayo clinic, MN, USA), and in the department of pathology (Prof. Dr. D Ruiter, University Medical Center St Radboud, Nijmegen), after which he continued his residency. Since 2001, he works as a neurologist at the Reinier de Graaf hospital, Delft.