Clinical applications of molecular cytogenetics in astrocytoma grade II

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CLINICAL APPLICATIONS OF MOLECULAR
CYTOGENETICS IN ASTROCYTOMA GRADE II

CLINICAL APPLICATIONS OF MOLECULAR CYTOGENETICS IN ASTROCYTOMA GRADE II

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan
de Universiteit Maastricht,
op gezag van de Rector Magnificus,
Prof. Dr. A.C. Nieuwenhuijzen Kruseman
volgens het besluit van het College van Decanen,
te verdedigen
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Abbreviations

A II	Astrocytoma grade II (low- grade diffuse astrocytoma)
A III	Astrocytoma grade III (anaplastic astrocytoma)
A IV	Astrocytoma grade IV (glioblastoma multiforme)
CGH	Comparitive genomic hybridization
CT-scanning	Computed tomography- scanning
DMBT-gene	Deleted in malignant brain tumor- gene
EGFR-gene	Epidermal growth factor receptor- gene
EORTC	European organization for research and treatment of cancer
FISH	Fluorescence in situ hybridization
ISH	In situ hybridization
Ki-67 LI	Ki-67 Labeling index
LOH	Loss of heterozygosity
MRI	Magnetic resonance imaging
PTEN-gene	Phosphatase and tensin homology-gene
p-arm	Short arm of chromosome
q-arm	Long arm of chromosome

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Introduction

Gliomas are the most frequent primary intracranial neoplasms with an estimated incidence of 5-7 per 100.000 per year.¹ On basis of the predominant cell type gliomas are classified as astrocytomas, oligodendrogliomas, ependymomas and mixed types (e.g. oligo-astrocytomas).² Astrocytomas constitute about 80%-90% of gliomas.³ Based on histological characteristics astrocytomas are subdivided into four grades with increasing malignancy.^{2,4}

Astrocytomas grade II (A II) or low-grade diffuse astrocytomas and more specifically the supratentorial subtype occurring in adult patients, are the main subject of this thesis.

The main biological features of supratentorial adult A II comprise a diffuse infiltration pattern into the adjacent normal brain and an inherent tendency to transform into high-grade astrocytomas, i.e. astrocytoma grade III (AIII), and astrocytoma grade IV (A IV). This malignant transformation of astrocytomas is accompanied by an accumulation of genetic aberrations.

Controversy exists upon optimal treatment of patients with A II, which may vary between gross tumor resection followed by immediate radiotherapy on the one hand, and a 'wait and see' policy on the other. This controversy is in particular caused by the unpredictable clinical course of A II.

The presence of cytogenetic aberrations in oligodendrogliomas and oligo-astrocytomas was shown to predict the sensitivity to chemotherapy of these tumors.⁵ In astrocytomas most cytogenetic studies have focused on A III and A IV. In A II fewer cytogenetic studies have been performed and their clinical relevance is until now not clear.

The main focus of this thesis was to evaluate the clinical application of molecular cytogenetic approaches in A II. For this purpose we used the in situ hybridization (ISH)-assay, that allows detection of chromosomal loss or gain in single cells, as well as immunohistochemical assays, both in routinely processed brain tumors.⁶

Aim of the studies

In CHAPTER 1 an introduction of A II is given and factors that interfere with optimal treatment of A II are discussed, followed by a review of cytogenetic alterations that are associated with development of A II and of malignant progression to A III/ A IV. Possible applications of cytogenetic techniques in clinical practice of A II are suggested.

In CHAPTER 2 we assess whether the detection of chromosomal aberrations enhances the diagnostic sensitivity of stereotactic biopsy specimens. Tissues with an inconclusive histological diagnosis are analyzed by ISH, and these cytogenetic findings are compared with the findings in reactive gliosis and in A II.

In CHAPTER 3 the prognostic value of numerical aberrations for chromosome 7, frequently implicated in astrocytoma carcinogenesis, is investigated in a group of forty-seven A II patients.

The prognostic value of the cytogenetic parameters in this group of A II is compared with the prognostic value of clinical and biological parameters in CHAPTER 4.

In CHAPTER 5 we evaluate A II and A IV for aberrations of the centromere region of chromosome 10 and of the 10q25.3 region, where a tumor suppressor gene is located that may play a role in initiation of A II and/or malignant transformation to A III/ A IV.

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2

The dilemma of astrocytoma grade II: biological parameters and clinical course

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Abstract

Because of its unpredictable clinical course, treatment strategies for astrocytoma grade II (A II) may vary between 'wait and see' on the one hand, and gross tumour resection followed by immediate radiotherapy on the other. A survey of the clinical studies on astrocytoma grade II shows a 5-year survival ranging between 27-85% and a lack of consistent prognostic parameters, except for patient age and the presence of neurological deficit. At present no unequivocal guide exists for the choice of therapy for patients with an A II, also because of the shortcomings of histological classification systems. Routine microscopy tends to underestimate malignancy grading of astrocytomas and in most cases cannot distinguish between indolent and progressive subtypes. Recent studies suggest that with proliferation and genetic markers subgroups of A II with a rapid progressive clinical course can be identified. Therefore these markers should be included in ongoing and future clinical trials of patients with A II.

Incidence of astrocytoma grade II and causal factors

Astrocytoma grade II (A II), the most common variant of low-grade glioma, has an estimated incidence between 0.5-1.0 per 100.000 persons/ year.¹ A II have a peak incidence in early adulthood with the mean age of patients at time of diagnosis between 35 and 40 years. Only a small percentage of A II occur in patients before 18 or after 65 years of age. For unknown reasons there is a slight predominance in men (ratio 1.2).⁷ Most A II occur sporadically and rarely as a manifestation of a familial tumour syndrome. An example of the latter is the Li-Fraumeni syndrome, caused by an inherited mutation of the p53 tumour suppressor gene, in which astrocytomas may co-exist with other solid tumours.⁸ The only well-documented environmental risk factor for astrocytoma of all grades is skull irradiation in young patients with haematological malignancies.⁹ Although working in certain chemical industries e.g. synthetic rubber processing, petrochemical refineries and pesticide and fertilization manufacturing, was shown to be associated with an increased risk for brain tumours, causative agents have not been identified.¹⁰ Also there is little evidence that the use of cellular telephones enhances the risk for astrocytoma.¹¹ Furthermore the low variation of incidence rates for central nervous system tumours across Europe argues against specific environmental factors causing these malignancies.^{1,12} Epidemiological studies focussing on specific causal factors specific for A II have so far not been presented in the literature.

Presenting symptoms and imaging of astrocytoma grade II

Epileptic seizure is the most common presenting symptom of A II and occurs in approximately 80% of the patients.¹³⁻¹⁵ This is probably due to the fact that A II show often a superficial localisation and low growth rate.¹⁶ Less common are focal neurological deficit (30%) and mental changes (10-30%). Uncommon are presenting symptoms due to raised intra-cranial pressure such as headache, vomiting and papilledema (10%).¹³⁻¹⁵

In the pre-computed-tomography (CT)- era focal neurological deficit and raised intra-cranial pressure were reported in a higher percentage of patients with A II.¹⁷ The decline of these percentages in recent series is due to the use of modern neuro-imaging techniques and stereotactic-guided biopsies, allowing diagnosis of A II at an earlier stage.¹⁸

FIGURE 1. MRI of a astrocytoma grade II in the right frontal lobe. The lesion is hypo-intense in the T1-weighted image (A) and hyper-intense in the T2-weighted MRI image (B).

A II may arise anywhere in the hemispheres, but shows a preference for the frontal and temporal lobes.^{17,19} On CT-scans A II are typically hypodense, poorly demarcated and non-enhancing lesions. CT-scans tend to underestimate the size of A II. Magnetic resonance (MR) scanning is more sensitive than CT in detecting A II.²⁰ Upon MR-scanning A II are hypo-intense on T1-, and hyper-intense on T2-weighted scans (*Figure 1*). T2-weighted images provide a more accurate estimation of tumour size and infiltration by neoplastic astrocytes.²¹

Calcifications and cysts are sometimes present.²² Although fairly typical, these neuro-imaging techniques are by no means diagnostic for A II. For example, contrast-enhancement is occasionally observed in these malignancies.²² On the other hand, lesions with a typical A II appearance may well turn out to be high-grade gliomas, since one third of these more progressed malignancies show no contrast-enhancement.²³⁻²⁵

In the near future Positron emission tomography (PET) and proton magnetic resonance spectroscopy (MRS) may provide additional diagnostic precision in A II. PET with ¹⁸F-fluorodeoxyglucose (FDG) may predict malignant transformation of low-grade glioma²⁶ and PET with the amino acid tracer ¹¹C-methionine has a possible correlation with survival of low-grade glioma patients.²⁷ PET appears to have potential in detection of glioma recurrence; stable or decreasing uptake of ¹¹C methionine after radiotherapy during follow-up is a favourable sign.²⁸ MRS, measuring levels of lactate and choline in brain tumours enables discrimination between radionecrosis and tumour recurrence.²⁹

Study design	Reference	Number of A II analyzed	Median patient age	Follow-up data			Potential prognostic parameters						
				5-year survival (%)	10-year survival (%)	Anaplastic change (number reoperated)	Patient age	Neurological deficit	Performance score	Tumour volume	Contrast enhancement	Extent of resection	Radiation therapy
	Shaw et al., 1989	78	30	51	23		N					NS	P
	Whitton et al., 1990	60	36	36	26		N					NS	NS
	North et al., 1990	77		55	43		N	N	P			P	
	McCormack et al., 1992	53	35	64		86% (7)	N	N#	P		N		
	Phillipon et al., 1993	179		60	40	72% (25)	N		P		NS	P	NS
	Shibamoto et al., 1993	119	35	60	41		N			N	NS	NS	P
	Rajan et al., 1994	82		52	39		N	NS	P			P#	
	Firsching et al., 1994	65		50	19	35% (40)	NS		P		NS	NS	
	Berger et al., 1994	53	37				NS					P	NS
	Janny et al., 1994	49	37	54	34	57% (14)	NS	NS	P#	NS	NS	P#	NS
	Nicolato et al., 1995	76		38	22	89% (9)	N	NA	P			P	NA
	Touboul et al., 1995	90	37	51	20		NS		P			P	P#
	Piepmeyer et al., 1996	55	30	85	70	65% (17)	NS	N		NS	NS	P	NS
	Bahary et al., 1996	63	33	67	33		N					P	

Retrospective	Leighton et al.,	87	40	62	39	51% (37)	N	N	P	NS	P	P#
	Kreth et al., 1997	197	35	60		42% (64)	N	N#	P	N	N#	
	SchliSchuurman et al., 1997	46	40	41			N	N			N	
	Lote et al., 1997	258	37		42	45% (67)	N	N#	P		N	NS
	Van Veelen et al., 1998	90	41	27	14	70% (36)	N	N	P			P
	Peraud et al., 1998	75	37				NS		P		N	
	Bauman et al., 1999 ^a	401		65 ^b	40 ^b		N	N	NS			P
	Nakamura et al., 2000	88	41	78	21		NS			NS		P#
	Arienti et al., 2001	49	45	47	24		N#	N#	P			
												NS
Prospective	Eyre et al., 1993	54	38	50 ^b	38 ^b		N	N	P#	N		P
	Karim et al., 1996	343	38	47/50 ^c			N	N				
	Karim et al., 2002	290		63/66 ^d						P#	N	NS
	Shaw et al., 2002	211		72/64 ^e			N					NS

TABLE 1. A summary of clinical features and potential prognostic parameters in retrospective and prospective studies of A II patients.

Abbreviations and symbols:
P = positive association with survival (p<0.05); # = only associated in univariate analysis
NS = no significant association with survival of Philippon et al (1993) and Leighton et al (1997)
a = study also including patient populations of Philippon et al (1993) and Leighton et al (1997)
b = estimated from Kaplan-Meier curves; c = low- (45 Gy) versus high-dose (59.4 Gy) radiotherapy (EORTC I-trial)
d = none versus post-operative (54 Gy) radiotherapy (EORTC II-trial); e = low- (50 Gy) versus high-dose (64.8 Gy) radiotherapy (US-trial)

Clinical course and prognostic parameters

Although several clinical studies have tried to identify possible prognostic parameters for A II (see *Table 1*), its clinical behaviour is still largely unpredictable for several reasons. First, the proliferative and progressive behaviour of A II is highly variable.³⁰ Second, studies on A II are difficult to interpret, as they are mostly retrospective in nature and include other subgroups of low-grade gliomas. Furthermore, the use of different treatment strategies may be an important confounder in most studies.

Table 1 summarises recent studies in which CT and MR have been applied to predict the clinical course, and which have attempted to describe prognostic parameters of A II.^{7,13-15,19,31-52}

The 5- and 10-year survival rate of patients in A II studies varies largely, i.e. between 27%-85% and 14-70%, respectively. Only four prospective randomised trials have been conducted in low-grade glioma patients, two of which have only recently been published.^{49,52} All other studies are retrospective, and often include not only A II, but also other variants of low-grade gliomas, such as pilocytic astrocytomas, oligodendrogliomas and oligo-astrocytomas, which have a better prognosis than A II.^{15,17,31,44,50}

Apart from study inclusion heterogeneity, another source of bias lies in the variance of treatment strategies. Few studies describe patient selection for different treatment strategies, e.g. biopsy followed by a "wait and see" strategy, or gross tumour resection with immediate post-operative radiotherapy. Because of this lack of information the impact of timing and type of interventions on the clinical course of A II is difficult to assess.

Patient age is the most consistent prognostic parameter in these studies. Patient age under 35 years^{7,48} or under 40 years in other series^{46,53} is associated with increased survival. In most studies epilepsy as presenting symptom and high performance status are also significantly associated with a more favourable clinical course.

Other factors with a less clear association are tumour volume, extent of tumour resection, contrast enhancement, as well as timing and dose of radiation therapy (*Table 1*).

An analysis of prognostic parameters from the two intervention studies conducted by the European Organization for Research and Treatment of Cancer (EORTC), was recently reported (see also: treatment strategies). In this report: age over 40 years, presence of neurologic deficits, astrocytoma histology, tumour crossing the midline and tumor diameter larger than 6cm were associated with shorter survival of low-grade glioma patients.⁵³ Diffuse infiltration of tumour cells into normal brain makes total resection of A II often impossible, ultimately leading to disease progression, either

caused by expansive tumour growth or by malignant transformation into A III or A IV. The percentage of A II with progression to high-grade astrocytoma (A III/A IV) is reported to vary between 35% and 89%. However, documentation of histological diagnoses at second biopsy, at resection or at autopsy is limited (Table 1).^{13-15,17,33,38,39,44,54} A review from the pre-CT- era demonstrated that two thirds of low-grade astrocytomas exhibited a higher malignancy grade at time of re-operation.⁵⁵

On basis of their clinical course mainly two different subgroups of A II can be defined, i.e. A II that remain indolent for years causing chronic epilepsy (indolent A II), or A II that cause progressive neurological deficit (progressive A II). Indolent A II are more often located in the grey matter, whereas progressive A II are more often located in the white matter.^{43,54} This is illustrated by the favourable outcome in patients with cortical located A II, who underwent (incomplete) resection of these tumours because of intractable seizures.⁵⁶ This suggests that the cellular origin of A II may be an important factor determining its clinical course. Piepmeyer and colleagues⁵⁴ found support for this hypothesis showing a difference in clinical behaviour, i.e. localisation and duration of symptoms, amongst tumours arising from different astrocyte lineages.

Treatment strategies

Patient age is a decisive parameter in current treatment strategies of A II. In fact, A II occurring in older patients often behave more malignantly than high-grade astrocytomas in younger patients. When grading patient age most studies use 40 years and some others 35 years as cut-off points. There seems to be consensus that patients over 35-40 years of age should undergo an aggressive treatment, i.e. maximal tumour resection followed by radiation therapy.^{7,57}

The same applies for the consensus on surgery for younger patients with increased intracranial pressure, neurological deficits related to mass effect and uncontrollable seizures. There is less consensus on the role of surgery in A II without significant mass-effect in young patients with medically controllable seizures (For a review see Keles et al, reference 53). However, for A II in non-eloquent areas, tumour resection is often preferred because of association with better prognosis in most retrospective studies of A II. These positive effects of tumour resection may, however be explained by selection bias,^{17,37,38} since tumour resection may have a (temporary) beneficial effect by reducing the space-occupying mass of A II. An influence of tumour resection on the pace and rate of malignant transformation has so far not been demonstrated.

Timing and dose of radiation therapy is also under debate. The first randomised EORTC- (European Organisation for Research and Treatment of Cancer) trial found no differences in outcome between low-grade glioma patients who underwent low-dose (45 Gy) versus high-dose (59.4 Gy) post-operative radiation therapy ('the believers trial').⁵⁰ This was confirmed by the recently published trial of the American Radiation Therapy Oncology groups ('the US trial'), which also found no difference in survival between low (50.4 Gy) and high-dose (64.8 Gy) localised radiation therapy.⁵² In the second EORTC-trial, immediate post-operative radiation therapy was compared to postponed radiation therapy at the time of disease progression ('the non-believers trial').⁵¹ In this trial immediate post-operative radiation of 54 Gy resulted in longer progression-free survival, but had no effect on overall survival.⁵¹

The results of these trials are difficult to extrapolate to young A II patients, because separate analysis of different age- or histological subgroups has not been reported so far.

In fact, interpretation of these trials is hampered by apparent histological heterogeneity of the tumours studied. In the US trial the majority (68%) of the low-grade glioma comprised oligodendrogliomas or oligo-astrocytomas.⁵² In the second EORTC trial 22% of the tumours were reclassified as A III/A IV after histo-pathological review.⁵¹

In view of its dubious positive effects one also has to take into account the possible side-effects of radiation therapy, especially the risk for radiation-induced encephalopathy. A recent Dutch multi-centre study⁵⁸ showed that encephalopathy occurs when repeated high-fraction doses (> 2 Gy) are used. The authors concluded that otherwise cognitive dysfunction in low-grade glioma patients is mainly due to the malignancy itself and to possible side-effects of anti-epileptic drugs.⁵⁸

Routine chemotherapy for A II patients is not indicated.⁴⁹ The only prospective trial reported to date revealed no difference between low-grade glioma patients treated with radiotherapy plus CCNU versus radiotherapy alone.⁴⁹ A trial by the Radiation Oncology Group (RTOG 98-02) in which patients with unfavourable low-grade glioma, defined as patient age > 40 years and/or incomplete resection, were randomised to radiation therapy plus PCV chemotherapy versus radiation therapy alone, was recently closed. Analysis of the data is not yet concluded. The efficacy of the oral chemotherapeutic drug imatinib mesylate in recurrent low-grade gliomas is currently tested in a phase II trial (EORTC coordinator Dr MJ van den Bent, Erasmus Medical Centre, Rotterdam; E-mail: bent@neuh.azr.nl). From a recent phase II trial it is concluded that the oral chemotherapeutic drug temozolomide may be active in progressive low-grade gliomas⁵⁹, which will be investigated in future phase III trials.⁶⁰

As there is no clear-cut evidence to guide treatment choices in young patients with A II, one can also question the necessity of histopathological diagnosis in these cases.^{5,57,61} Histological confirmation does not always alter treatment strategy and a stereotactic biopsy is not without risks. A retrospective study showed that patients with transient neurological symptoms and radiological suspicion of a low-grade glioma had a median survival that was unaltered when postponing biopsy and other interventions until disease progression.⁶¹ One can argue that this 'wait and see' policy, with its uncertainty about the diagnosis, negatively influences the quality of life of patients with suspected low-grade glioma. This was, however, not confirmed in a recent study, where no difference in quality of life was found between patients with suspected and with proven low-grade gliomas.⁶²

Histological grading systems

At present astrocytomas are either classified according to the St. Anne/Mayo grading system⁴ or according to the World Health Organisation (WHO) protocol.² In both systems tumors are graded in the area with most anaplastic features using the following histopathological features: nuclear atypia, mitotic activity, endothelial proliferation and necrosis (*Table 2*).

The St. Anne/Mayo system divides astrocytomas into four grades. However in practice this is a three-grade system because grade 1 astrocytomas are extremely rare. The most prominent feature of St. Anne/Mayo grade 2 is nuclear atypia. The absence or presence of mitotic figures differentiates between grades 2 and 3. The St. Anne/Mayo astrocytoma grade 2 is comparable to 'astrocytoma grade II' or 'diffuse astrocytoma' in the WHO definition, i.e. a tumour with a moderately increased cellularity of astrocytes and occasional nuclear atypia. Mitotic activity is generally absent.² A difference between the two classification systems concerning A II lies therefore in the fact that WHO allows for the detection of a single mitosis.² On basis of the type of astrocyte that is predominantly present three variants of A II can be distinguished, i.e. the fibrillary, the gemistocytic and the protoplasmic A II. Fibrillary A II is by far the most common variant, exhibiting an extensive network of neuroglial fibrils extending between the

St. Anne-Mayo (1988)	
Grade	Definition
1	No histological features present
2	One histological feature present, usually nuclear atypia
3	Two features present, typically nuclear atypia and mitoses
4	Three of four features present, i.e. nuclear atypia, mitoses and microvascular proliferation and/ or necrosis

World Heath Organisation- WHO (2000)	
Grade	Definition
I	Pilocytic astrocytoma: Circumscribed tumour especially occurring in children
II	Diffuse astrocytoma : Moderate hypercellularity and occasional nuclear atypia. Mitosis absent or in single cell
III	Anaplastic astrocytoma: Hypercellularity and distinct nuclear atypia. Marked mitotic activity
IV	Glioblastoma multiforme: Marked nuclear atypia and brisk mitotic activity. Prominent microvascular proliferation and/ or tumor necrosis

TABLE 2 . Histopathological classification systems of astrocytoma.

astrocytes (*Figure 2*). Gemistocytic A II is characterised by large astrocytes with eccentric nuclei. Although these tumours are all classified as WHO grade II, the presence of a high percentage (>20%) of gemistocytes is associated with shorter survival.⁶³ Some authors, therefore consider gemistocytic astrocytoma as a variant of anaplastic astrocytoma (WHO grade III)⁶⁴. The very rare protoplasmic A II, characterised by mucoid degeneration and microcyst formation, often has an indolent clinical course.⁶⁵

In both grading systems increasing malignancy grade is associated with a less favourable prognosis.^{4,66,67} This is clearly shown in *Figure 3*, depicting the survival curves from the study of Daumas-Duport et al⁴ for astrocytomas graded according to the St. Anne-Mayo system. From these data it may be appreciated that for the individual patients these histological classification systems are less useful, as survival within one grade may vary widely. Conversely, survival between different grades may show significant overlap, as is the case between A II and A III.⁶⁸ Histological heterogeneity within the tumour is another major source of confusion when the diagnosis is based on fragments

FIGURE 2. Fibrillary A II with an increased cellularity and size of the astrocytes.

FIGURE 3. Survival curves for patients with astrocytomas classified according to the St. Anne/Mayo grading system (Cancer, Vol. 62, No. 10, 1988, page 2157. Reprinted by permission of Wiley-Liss.)

of the malignancy.⁶⁹ Diagnosis on stereotactic biopsy samples underestimates the histological grade in 10-25% of astrocytomas, as compared to resection specimens of

the same tumour.⁷⁰⁻⁷² This risk of underestimation is reduced by taking a larger number (>6) of stereotactic biopsies per tumour.⁷²

The differentiation between 'pure' astrocytoma and mixed oligo-astrocytomas is of clinical relevance, because the presence of an oligodendroglial component increases the chance of chemo-sensitivity.⁷³ However this distinction may be difficult because tumour cells may often combine astrocytic and oligodendroglial features, while also the histological diagnosis is influenced by heterogeneity within the specimen.⁷⁴

Finally, a problem in clinical practice of stereotactic biopsy specimens is the differentiation between A II and reactive gliosis, a proliferation of glial cells in response to neural tissue damage. Both tissue types may only show a mild increase in astrocyte cellularity and some nuclear atypia using routine microscopy and histological diagnosis often remains inconclusive in such cases.⁷⁵

Biological aspects of astrocytoma grade II

Differences in clinical behaviour and genotypic constitution have been shown to underlie the variable clinical course of individual cases of AII. The development of astrocytoma is associated with genetic instability and an imbalance between proliferation and programmed cell death (apoptosis) of astrocytes. Recent studies have suggested that markers for proliferation activity^{76,77} and certain cytogenetic changes^{78,79} may predict the malignant transformation from A II to A III/A IV.

Proliferation markers in astrocytoma grade II

An established histological method to estimate proliferative activity in tumours is to count the percentage of cells with mitotic figures. However, since in A II mitoses are absent by definition, other procedures for the determination of its proliferative capacity have been sought, amongst which the bromodeoxyuridine (BrdU)^{80,81} incorporation assay, or immunostaining of the Proliferating Cell Nuclear Antigen (PCNA)⁸² and the Ki-67-antigen.⁷⁷ Although the two former protocols showed prognostic value the latter has shown to provide the most reliable estimate of the proliferative ratio in brain tumours and to be easily applicable in routine pathology.^{83,84} The Ki-67 antigen is present in all active phases of the cell cycle, but absent in G₀ phase. Specific

antibodies allow its detection in routinely processed material of gliomas.⁸² The percentage of Ki-67 positive cells, expressed as the Ki-67-labeling index (LI) has a positive correlation with histological grade in astrocytomas. In a series of grade II astrocytomas a Ki-67 LI of more than 2% was predictive of shorter survival, independent of the patient's age.⁷⁶

Molecular cytogenetics of astrocytomas

Different molecular techniques, including karyotyping,⁸⁵ mutation analysis,⁸⁶ allelotyping,⁸⁷ in situ hybridization (ISH),⁶ comparative genomic hybridisation (CGH)⁸⁸ and expression profiling⁸⁹ have been used to study astrocytomas.

In particular the in situ hybridization (ISH) and the comparative genomic hybridization (CGH) technique are well suited for the analyses of numerical and structural chromosomal aberrations. The ISH technique uses chromosome specific DNA probes that allow the detection of chromosomal imbalances, e.g. losses, gains and amplifications, in individual cells of paraffin-embedded brain tumour samples.^{6,90,91} The ISH assay is particularly well suited for the study of A II, which is often surrounded by reactive, non-neoplastic, cells, and from which frequently only small stereotactic biopsy samples with a limited number of cells are available. An typical example of a FISH-assay for chromosome 1 and 7 in an A II is shown in *Figure 4*.

When relatively large and more homogeneous samples are available the CGH assay allows detection of gains and losses of genetic material across the entire tumour genome.⁹² In CGH differently fluorescent labelled tumour DNA and normal reference DNA are hybridised simultaneously to normal metaphase chromosomes. Digital analysis of the fluorescence intensity ratios enables to detect chromosomal gains or losses of 2 Megabases (Mb) or more, as exemplified in *Figure 5*.

Table 3 summarizes frequent chromosomal alterations that have been found in the different grades of astrocytomas (Table compiled on basis of references^{2,93-95}). From this table it is obvious that only few of the target genes that are either enhanced or lost through the chromosomal changes have been identified.

Typical chromosomal aberrations in A II include loss or mutation of the p53 tumour suppressor gene and trisomy for chromosome 7.

In A IV two distinct genetic subtypes exist, namely primary (or de novo) and secondary (or progressive) A IV.⁹⁶ In primary A IV no prior evidence for a low-grade precursor lesion exists. Typical aberrations in these tumours include amplification of the epidermal growth factor receptor (EGFR)-gene,⁹⁷ located on chromosome 7, and loss of chromosome 10,⁹⁸ where the PTEN/MMAC1 tumour suppressor gene is located.⁸⁵

FIGURE 4. Double-target fluorescence in situ hybridization (FISH) demonstrating trisomy for chromosome 7 (green signals) and a normal copy number for chromosome 1 (red signals) in an astrocytoma grade II. The nuclei are counterstained with DAPI (blue).

FIGURE 5 Comparative genomic hybridization (CGH) analysis of an astrocytoma grade IV in which gain for chromosome 7, and losses of chromosomes 10,6, 9 and 14q are clearly visible.

A) Fluorescent ratio image; tumour DNA (green) and normal reference DNA (red) are hybridized to a normal metaphase. B) Average ratio profile calculated from 10 metaphases. The red bar indicates chromosomal loss while the green bar indicates gain of chromosomal regions. (Image courtesy of Dr EJM Speel and Mrs SMH Claessen, Department of Molecular Cell Biology, University of Maastricht, The Netherlands)

Tumor subtype	Chromosome aberration	Frequency of aberrations	Gene involved (locus)	Altered protein expression
A II	-17p +7 +8q -22q -10p -10q	60-70 % 10-50 % 0-40 % 15-20 % 0-20% 0-20%	P53 (17p13.1) EGFR (7p12-14) c-MYC (8q22) NF2 (22q12) Unknown (10p14) DMBT (10q25) PDGFR α (4q11-12)	PDGFR α $\uparrow$
A III	-9p +12q -13q -19q	20-30 % 10-20 % 20-25 % 40-50 %	Ink4A (9p21) CDK4 (12q13-14) RB(13q14) Unknown (19q13)	
Secondary AIV	-17p -10q -19q	60-70 % 70-80 % 50-60 %	P53 (17p13.1) DMBT (10q25) Unknown (19q13) DCC (18q21)	DCC $\downarrow$
Primary A IV	-10 +7 -9p +12q	70-80 % 50-80 % 30-60 % 10%	PTEN (10q23.3) EGFR (7p12-13) Ink4a/ARF (9p21) MDM2 (12q14-15)	EGFR $\uparrow$ MDM2 $\uparrow$

TABLE 3. A summary of common genetic abnormalities reported for the different grades of astrocytoma.

Abbreviations: PDGFR= platelet derived growth factor receptor, EGFR= epidermal growth factor receptor, NF=neurofibromatosis, DMBT= deleted in malignant brain tumour, CDK= cycline dependant kinase, Rb=retinoblastoma, PTEN= phosphatase tyrosine gene, MDM= murine double-minute, DCC=deleted in colon carcinoma.

An example of a primary A IV with gain of chromosome 7 and loss of chromosome 10, detected by CGH, is shown in figure 5.

In both A II and secondary A IV, losses of chromosome arm 17p with mutation of the p53 tumour suppressor gene are often present. Other genetic alterations are less frequent in A II as compared to A IV. The cytogenetic alterations that are associated with two important aspects of A II i.e. initiation of A II and malignant progression of A II to A III or secondary A IV, are discussed below.

Initiation of astrocytoma grade II

In about two thirds of the cases of A II studied mutation or loss of the p53 tumour-suppressor-gene on chromosome 17p13.1 is present.⁹⁹ Several protein regions of P53 play different roles in cellular processes, one of the most important being the regulation of gene transcription. Absence of functional P53 protein leads to a deregulation of the cell cycle and absence of induction of the normal process of apoptosis, thereby also leading to genomic instability. Although loss or mutation of the p53-gene have been suggested as early lesions in the process of initiation of astrocytoma, additional genetic alterations are necessary for astrocytoma carcinogenesis. An important mechanism for growth stimulation of astrocytomas is the simultaneous overexpression of growth factors receptors and their receptors, leading to an autocrine stimulation of the RAS-signalling pathway.¹⁰⁰ A frequently overexpressed growth factor in A II is the platelet derived growth factor receptor alpha (PDGFR- α) subunit.¹⁰¹ A close correlation has been found between overexpression of PDGFR- α and LOH of 17p, the locus of the p53 gene, which suggests that growth stimulation and p53 mutation may have synergistic effect in the initiation of AII.

Recent ISH and CGH studies have shown that other chromosomal aberrations also often occur in A II, i.e. gains of chromosome 7 or 7q, 8q and general polyploidisation.^{79,102-105}

A typical example of gain of chromosome 7 in an A II, as detected by fluorescence-ISH (FISH) is shown in *Figure 4*. As a target on chromosome 7p the Epidermal Growth Factor Receptor (EGFR) gene has been suggested, but amplifications of this gene are very rare in A II.¹⁰⁶ Loss of regions on 22q has also been observed in A II. The neurofibromatosis 2 (NF2) gene located in this region has however been eliminated as a candidate tumour suppressor gene by mutation analysis.¹⁰⁷ Furthermore deletions of regions of chromosome 10, i.e. 10p14-15 and 10q25-26, have been found in A II.¹⁰⁸

Malignant progression of A II to A III or secondary A IV

An important role in malignant progression of A II has been suggested for cell cycle regulator genes involved in the Ink4A-CDK4-Rb pathway (see Table 3). Ink4A (or p16) and CDK4 code for proteins that regulate phosphorylation of the retinoblastoma (Rb) protein, which in turn regulates transition from the G₁ to the S phase of the cell cycle. In approximately half of the A III tumours either one of these three genes is mutated (see Table 3), which is an explanation for uncontrolled cell proliferation.^{109,110} Another frequent alteration in both A III and secondary A IV is chromosomal loss of 19q where an unidentified tumour suppressor gene is located that might play a role in A II progression.¹¹¹

Molecular allelotyping studies suggest that (secondary) A IV that have progressed from A II have a common deletion of the 10q25-26 region.¹¹² One of the candidate tumor suppressor genes in this region is the Deleted in Malignant Brain Tumor (DMBT1) gene, the product of which has been linked to processes of cell differentiation and migration of epithelial cells.¹¹³ Major hallmarks of primary A IV are loss of chromosome 10 with PTEN/MMAC1 mutation and amplification and/or overexpression of the EGFR gene.⁹⁵ In primary A IV the P53 pathway is also disrupted, through loss of ARF, or less frequently through amplification of MDM2.

Clinical application of molecular markers in A II

With regard to the prognostic value of molecular cytogenetics in A II it can be stated that a higher number of genetic alterations, as detected by CGH, is associated with a more rapid malignant progression.⁷⁸ Using ISH it was shown that trisomy of chromosome 7 is associated with shorter survival of A II patients.⁷⁹

A possible association of p53 mutations with survival has been suggested by some studies¹¹⁴, but contradicted by others.¹¹⁵ A recent study suggest that mutation of codon 175 ('a hot spot codon') of the p53 gene is associated with a shorter patient survival in A II.¹¹⁶

Molecular analyses are also of potential interest when a (stereotactic) sample error is suspected. At one side the detection of trisomy for chromosome 7 might help to discriminate between non-neoplastic reactive gliosis and A II in case of an inconclusive histological diagnosis.¹¹⁷ On the other hand the detection of monosomy for chromosome 10, distinctly uncommon in A II, may help to discriminate between A II and A IV.¹⁰³

Furthermore demonstration of loss of chromosomes 1p and 19q is already of value in predicting chemosensitivity of oligodendroglioma and mixed oligo-astrocytomas. Similar losses have also been observed in a minority of A II^{105,107,118}, suggesting that the detection of these alterations may also predict chemo-sensitivity of a subgroup of (histological) A II with oligodendroglial genotype.^{5,119}

The first cDNA expression array study, showing up- and down-regulation of different genes that have not been implicated in astrocytoma carcinogenesis before, confirms the complexity of genetic alterations in A II.⁸⁹ From a cDNA-array study in high-grade glioma the potential use of this technique toward a molecular, instead of a histological classification of gliomas, is suggested.¹²⁰

Conclusions

Managing patients with A II poses a therapeutic dilemma. Because of its unpredictable clinical course, treatment strategies may vary between gross tumour resection followed by immediate radiotherapy on the one hand, and a 'wait and see' approach on the other hand. At present the best prognostic parameter is patient age, while other clinical parameters correlating with prognosis are the presence of neurological deficit and a low performance score at time of presentation.

In particular for young patients (under 35 or 40 years of age) with A II that only cause seizures, the efficacy of surgical intervention and early radiotherapy has never been proven. Since histological confirmation through stereotactic biopsy does not alter the treatment strategy a 'wait and see' policy is often recommended for these patients.

For A II that occur in patients over 40 years of age and A II that have a mass effect or progressive neurological deficit, gross total tumour resection improves survival. The role of post-operative radiotherapy is still under debate, however if applied the fraction dose should not exceed 2 Gy and a total dose of 45 Gy is probably as effective as 59.4 Gy. Routine treatment with chemotherapy is not indicated for A II patients, and at present serves only as a salvage therapy for recurrent A II.

Recent data suggests that biological and genetic parameters closely correlate with the highly variable clinical course of A II. Of these the Ki-67 proliferation marker should be included in routine pathology. Analysis of these tumours could include genetic alterations, in particular gain of chromosome 7 (monitored by ISH) and losses of 1p/19q (monitored by CGH), or altered gene expression as determined by cDNA expression arrays. These markers are promising candidates to serve as prognostic

indicators in A II and the analysis of tumour biopsies should therefore be imperatively integrated into clinical trials to prospectively identify outcome predictors as tools for improvement of diagnosis and treatment in the future.

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3

Chromosome aberrations as detected by in situ hybridization differentiate between reactive gliosis and astrocytoma grade II

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Abstract

Histopathological diagnoses of stereotactic biopsy specimens underestimate tumor grade in approximately thirty percent of astrocytomas. In particular the diagnosis of samples from patients with astrocytoma grade II (A II) may be inconclusive due to extensive histopathological heterogeneity in these specimens. Our objective was to examine whether in situ hybridization (ISH) using chromosome specific DNA probes can be of additional value in this respect by detecting chromosomal aberrations in A II and thereby distinguish these from non-neoplastic reactive gliosis in stereotactic biopsy samples. The patient material consisted of 10 samples containing non-neoplastic reactive gliosis, 20 cases of A II, and 13 cases of stereotactic samples in which the differentiation between reactive gliosis and A II was inconclusive. Numerical chromosomal aberrations for the chromosomes 1, 7, and 10 were evaluated by ISH applied to paraffin sections. No numerical chromosomal aberrations were detected in samples containing reactive gliosis. In the A II 16 out of 20 samples showed numerical chromosomal aberrations for one or more of the chromosomes investigated. In 4 out of 13 samples with an initially inconclusive tissue diagnosis the primary biopsy revealed numerical chromosomal aberrations. In three of these high-grade astrocytoma was diagnosed at a later stage.

We conclude that the detection of numerical chromosomal aberrations by ISH is a valuable additional tool in the distinction between A II and reactive gliosis.

Introduction

Due to small size and histopathological heterogeneity the tumor grade of diffuse astrocytoma is often underestimated in stereotactic biopsy specimens. Stereotactic biopsy samples are not representative of the tumor in 30% of astrocytomas as compared to gross resection specimens of astrocytomas.^{1,2} In particular in stereotactic biopsy samples from patients with astrocytoma grade II tissue diagnosis may be inconclusive.³ Astrocytoma grade II (A II) are frequently mixtures of neoplastic astrocytes and reactive gliosis, a proliferation of glioma cells, microglia and astrocytes in response to neural tissue damage. In an attempt to morphologically differentiate between a non-neoplastic or a neoplastic cause of astrocytic proliferation different methods have so far been applied, such as the intra-operative cytological wet smear technique, acridine orange staining, the AgNOR-technique, and additional immunohistochemical staining for P53- and Ki-67- antigens.⁴⁻⁸

In most samples with reactive gliosis P53 immunoreactivity is absent, and Ki-67 labeling index is less than 1 percent. However, 10 % of these non-malignant lesions show P53 immunoreactivity, and occasionally exhibit a Ki-67 labeling index higher than 3 %.^{7,8} Cytogenetic procedures can be of additional value in the differentiation between reactive gliosis and astrocytoma. However with most cytogenetic techniques only sporadic chromosomal aberrations are detected in A II besides loss of 17p and mutations of p53-gene.⁹ The in situ hybridization (ISH)-technique allows a more frequent detection of chromosomal aberrations in tumors while normal and non-neoplastic tissues are disomic.¹⁰⁻¹³

The purpose of this study was therefore to determine whether the ISH technique offers additional diagnostic value by detecting tumor cells in areas of astrocytomas which are not typical enough to allow a morphological diagnosis. ISH analysis in these inconclusive cases was compared with samples containing reactive gliosis on the one hand, and cases with evident A II on the other hand.

All samples were investigated with (sub-)centromere probes for the chromosomes 1, 7 and 10. Chromosome 1 has been shown to present a sensitive general marker for aneusomy¹⁴, while gain of chromosome 7 and loss of chromosome 10 are frequent events in astrocytomas.¹⁵⁻¹⁶ The ISH results were compared with the (tumor) cell proliferation (Ki-67) index, which is considered to have additional value in the differentiation between non-neoplastic and neoplastic astrocytic proliferation.

Material and methods

Patient material and clinical data

The formalin fixed, paraffin- embedded archival material consisted of three groups of tissue samples:

1. Reactive gliosis from post-mortem examination in patients who died from a neurological non-neoplastic cause (n=10), i.e. brain infarction (n=5), hemorrhagic infarction (n=1), traumatic hemorrhage (n=2), and hypoxic encephalopathy (n=2).
2. Astrocytoma grade II (A II; n=20), according to the WHO classification.¹⁷
3. Stereotactic biopsy samples (n=13) with an inconclusive tissue diagnosis, i.e. no differentiation between non-neoplastic reactive gliosis and A II.

Material was retrieved from the files of the Departments of Pathology from the University Hospitals of Maastricht and Groningen, the Atrium Hospital in Heerlen, and the Medical Spectrum Twente in Enschede, The Netherlands. Clinical data with regard to interval of tumor progression, defined as histological or radiological evidence of progression to higher grade astrocytoma, were available.

In situ hybridization: procedure, probes and evaluation.

Pretreatment of the sections

ISH was performed using a recently optimized protocol.¹⁸ Five-micron thick sections from selected paraffin blocks were deparaffinised in xylol, dehydrated in an ascending ethanol series, and pretreated in 85% formic acid containing 0.3% H₂O₂ for 20 minutes at room temperature. After dehydration in 0.01 M HCl in 70% ethanol, followed by washings in following steps of 70%, 90%, and 100% ethanol, the specimen were incubated at 80° C in 1 M sodium thiocyanate (NaSCN). Proteolytic digestion was done in 4mg/ml pepsin (from porcine stomach: 2500-3500 units per 100 mg protein: Sigma, St Louis, USA) for 10 minutes at 37° C in 0.02 M HCl to increase tissue permeability. After dehydration in acidified ethanol series, the tissues were fixated in 1% formaldehyde in phosphate-buffered saline (PBS) for 15 minutes.

Probes

The probes were specific for the heterochromatin (sub)centromere region of chromosome 1 (1q12, pUC 1.77; Cooke and Hindley¹⁴), the alphoid region of

chromosome 7 (p7t1, Waye et al¹⁹), and the pericentromere region of chromosome 10 (D10Z1, Devilee et al²⁰).

In situ hybridization (ISH) procedure

The probes were hybridized under a coverslip in a mixture containing 2 x standard saline citrate (SSC), 60% formamide, 10% dextran sulphate, and 0.2 mg/ml herring sperm DNA. After denaturation at 80° C for 5 minutes the slides were placed in a moist chamber and incubated overnight at 37° C. The slides were then washed twice in 2 X SSC/ 0.05% Tween at 45° C, and in 4 X SSC/ 0.05% Tween at room temperature.

The biotin-labeled DNA probes were indirectly detected by an immunoperoxidase enzymatic precipitation reaction. The slides were incubated for 30 minutes at room temperature with mouse-antibiotin monoclonal antibody, followed by biotinylated horse-anti-mouse, and finally with avidin-biotin-peroxidase complex (all reagents from Vector Laboratories, Burlingame, CA, USA). Finally 0.1 M diaminobenzidine (DAB, Sigma, St Louis, MO, USA) in PBS containing 0.03% H₂O₂ was applied for visualisation of the peroxidase activity. Nuclear counterstaining was done with hematoxylin or alternatively with 4,6-diamino-phenylindol (DAPI, Sigma, St.Louis, MO, USA).

Signal evaluation and interpretation

The number of signals per nucleus was counted in at least 200 non-overlapping nuclei, following criteria proposed by Hopman et al.²¹ Due to truncation of cell nuclei the percentage of cells with no or one ISH signal differs between experiments, but has a maximum of 25% in tissue sections.²¹ Therefore monosomy was defined as > 25% of nuclei with no or one signal per nucleus.

All histological areas classified as genetically disomic contained only sporadic nuclei exhibiting more than 2 signals. We could not determine whether or not the rare aneusomic cells should be considered as genetically aberrant or result from nuclear overlap. In normal tissue this total fraction of nuclei does not exceed 5%.^{22,23} Therefore nuclei were only classified trisomic if > 5% of nuclei contained three or more signals. Nuclei containing four and more than four signals were defined as tetrasomic and polysomic, respectively.

Brightfield microscopy was performed using a Leica-DMBRE microscope equipped with a filterset for DAPI. Images were captured and stored with the Metasystem Image Pro System (Heidelberg, Germany), using a charge-coupled device (CCD) camera.

Ki-67 immunostaining

In all samples the proliferation status was assessed as the Ki-67 immunostaining index by using the mouse monoclonal Mib-1 antibody. Endogenous peroxidase was blocked with 0.3 % H₂O₂ in methanol for 20 minutes. Antigen retrieval was achieved by treatment with 10mM citrate buffer (pH 6.0) in a domestic microwave oven for 10 minutes at 700W. The first layer consisted of Mib-1 antibody 1:12 (Immunotech S.A. Marseille, France) in PBS containing 1% bovine serum albumin. The second layer antibody was biotin-labeled horse antimouse IgG 1:200 in PBS/Tween containing 4% normal goat serum. The third layer consisted of avidin-biotin complex applied for 1 hour (Vector Laboratories, Burlingame, CA, USA). Signals were developed using diaminobenzidine/H₂O₂. The Mib-1 positive cells were counted in at least 200 nuclei in the areas with the highest immunopositivity.

Results

Reactive gliosis

Neuropathological revision of these 10 cases showed an increase in astrocytic cellularity without neoplastic features. With ISH, using probes for the chromosomes 1, 7 and 10, all evaluable (87%) tests revealed disomy. Very sporadic nuclei with three signals were seen, but none of the samples fulfilled the criteria for trisomy. In one sample with reactive gliosis the Ki-67 labeling index was 3.5%, while in the other samples it did not exceed 1%.

Diagnosis	N	Number of samples with numerical chromosomal aberrations (% Total)			Aberrant for one or more chromosomes	Ki-67 LI (%cells/range)
		Chr.1	Chr.7	Chr.10		
Gliosis	10	0	0	0	0	<1 (<1-3)
A II	20	12 (60%)	13 (65%)	10 (50%)	16 (80%)	2.1 (<1-9)
Inconclusive	13	3 (23%)	4 (31%)	1 (8%)	4 (31%)	2 (<1-10)

TABLE 1. Summarized results of numerical chromosomal aberrations and Ki-67 labeling index (LI) for the three investigated patient groups.

FIGURE 1. Routine microscopy (A) and in situ hybridization (B) with a probe for chromosome 7 in a sample with an inconclusive tissue diagnosis showing frequent nuclei with three or more signals

Astrocytoma grade II

Revision revealed 18 fibrillary and 2 gemistocytic A II. The mean age of the patients was 39.9 years (range: 24-68 years) and the median interval to tumor progression was 35 months. The ISH results are summarized in *Table 1*. Numerical chromosomal aberrations for one or more of the chromosomes investigated were present in 80% of the A II: for chromosome 1 in 60%, for chromosome 7 in 65%, and chromosome 10 in 56% of the samples. Only one sample (case 4) showed monosomy for the chromosomes 1 and 7. All other aberrations comprised trisomy, tetrasomy and polysomy. Case 1 showed focal areas with clustering of nuclei with trisomy and tetrasomy. In all other samples nuclei with abnormal hybridization signals were scattered throughout the preparation. The mean Ki-67 labeling index in the astrocytomas was 2.1% (range <1-9).

Cases with inconclusive diagnosis

All samples with inconclusive tissue diagnosis were stereotactic biopsy specimens in which it was not possible to distinguish between reactive gliosis and A II on basis

of routine histology. Revision of one sample, derived from a patient with a suspected recurrent astrocytoma after radiotherapy, revealed endothelial cell proliferation and necrosis. This we considered non-neoplastic radiation damage. In all other samples, in which microscopy revealed a mild increase in astrocyte cellularity and pleiomorphy, neuropathological revision agreed with the initial inconclusive diagnosis. ISH results and the Ki-67 labeling index are shown in *Table 2*.

According to follow-up data the samples could be divided into three groups:

1. Samples in which astrocytoma was diagnosed at a later stage (cases 1-7): In 3 of these 7 samples the primary biopsy already showed numerical chromosomal aberrations (*Figure 1*). Ki-67 labeling indices of these samples were 1, 10, and 5% respectively. In case 7 no tissue confirmation of a malignancy was obtained but astrocytoma grade IV (A IV) was diagnosed by combination of histopathology and radiological progression of the space-occupying lesion.
2. Samples in which the follow-up period of the patients was too short and where second biopsy or resection was deferred (cases 8-11): In case 8 the primary biopsy showed numerical chromosomal aberrations for the chromosomes 1 and 7. The Ki-67 labeling index of this sample was less than 1%.
3. Samples in which a non-neoplastic diagnosis was made at a later stage (cases 12-13): Case 12 showed no numerical chromosomal aberrations and case 13 was not evaluable for ISH due to cell lysis.

Discussion

In the underlying study we show that the ISH technique, applying chromosome specific probes to routine processed brain tissue samples, allows the efficient distinction of reactive gliosis from A II. Since the separation of these two histomorphological entities is often difficult this finding is of clinical relevance.

No samples with reactive gliosis showed chromosomal aberrations, while 80% of the samples containing evident A II were classified as genetically aberrant for the chromosomes 1, 7 and / or 10. The normal findings in the non-neoplastic reactive gliosis group coincide with the few previous ISH studies concerning normal²³ and gliotic brain tissue.²⁴ With classical karyotyping trisomy 7 was reported to occur apparently normal brain cells probably due to a culture artifact.²⁵

Case	Numerical chromosome aberrations (% Nuclei)			Ki-67 LI (% cells)	Follow-up
	Chromosome 1	Chromosome 7	Chromosome 10		
1	Tri (10%)	Tri (10%)	Tri (5%)	5	<i>Malignant progression</i> Post-mortem: A IV, 10 months Resection: A III, 5 months Second biopsy: A IV, 2 months Resection: AA, 4 months Second biopsy: A IV, 1 month Second biopsy: A IV, 40 months MRI, 35 months
2	Tri (21%)	Tri (19%) Tetra (11%) Poly (1%)	D	1	
3	D	Tri (25%)	D	10	
4	D	D	D	<1	
5	D	D	D	<1	
6	D	D	NE	<1	
7	D	D	D	4	
8	Tri (10%)	Tri (32%) Tetra (9%)	NE	<1	<i>No progression in time followed</i> 4 months 20 months 6 months 22 months
9	D	D	NE	<1	
10	D	D	D	<1	
11	D	D	D	<1	
12	D	D	D	<1	<i>Non-malignant diagnosis</i> Multiple sclerosis Radiation necrosis
13	NE	NE	NE	NE	

TABLE 2. In situ hybridization results and Ki-67 labeling index in samples with uncertain tissue diagnosis. Abbreviations and symbols: D = Disomy; Tri = Trisomy; Tetra = Tetrasomy; Poly = Polysomy; NE = Not evaluable; A III= astrocytoma grade III; A IV= astrocytoma grade IV; MRI = Progression on MRI; Ki-67 LI = Ki-67 Labeling Index

Genetic studies concerning chromosomal aberrations in A II involve mainly classical karyotyping¹⁵⁻¹⁶ and flow cytometry and mostly show diploid cells in A II.²⁶ In cell lines of these malignancies low-grade diffuse astrocytomas chromosomal aberrations were however found, such as trisomy for chromosome 7 and loss of one of the sex chromosomes. The aberrations are similar to those found in high-grade astrocytomas but occur in a very low frequency in A II.¹⁵⁻¹⁶ With molecular genetic techniques, such as loss of heterozygosity (LOH)- single-strand conformation polymorphism (SSCP)-studies, and micro-satellite-analyses, again genotypic alterations in A II are sporadically detected, while occurring at a higher frequency in astrocytomas grade III and IV.²⁷ With the ISH-technique chromosomal aberrations can be found more efficiently as compared to classical karyotyping or LOH studies because both metaphasic and interphasic cells are evaluated. Earlier ISH-studies on small series of A II already revealed hyperdiploid cells, gain of chromosome 1 and 7, loss of chromosome 10, and aberrations of chromosome 17.¹⁰⁻¹³ By using three chromosome probes with single-target ISH analysis we could detect high percentages of aneusomic cells in most A II. In four samples with an initial inconclusive tissue diagnosis aneusomies were detected by ISH. Of these four cases three showed rapid progression to A IV. This may however also suggest that in these cases, where the differential tissue diagnosis was to choose between reactive gliosis and A II, the patients most probably suffered from A IV, which were not detected at first diagnosis due to sampling errors. In the fourth case no progression was seen after 4 months of follow-up. Because the high frequency of genetic aberrations in this case the patient is clinically monitored on a regular basis. Our findings indicate that the detectability of genotypic alterations extends well beyond the microscopical border of astrocytomas. ISH for detection of chromosome aberrations also seems more sensitive than Ki-67 immunocytochemistry. In 2 of the 4 inconclusive cases high Ki-67 labeling indices were found, which supports the high-malignant character of these lesions. However in the 2 other lesions Ki-67 levels were low, suggesting that this marker has a lower sensitivity in the detection of lesions with a malignant clinical course as compared to the ISH-technique. We conclude that numerical chromosomal aberrations for the chromosomes 1, 7 and 10 detected by ISH, can distinguish between non-neoplastic tissue proliferation and astrocytoma. The ISH procedure therefore has additional value in the differential diagnosis of reactive gliosis versus A II.

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4

Gain of chromosome 7 as detected
by in situ hybridization correlates
with shorter survival in
astrocytoma grade II

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Abstract

The clinical course of astrocytoma grade II is highly variable and not reflected by morphological characteristics. Earlier studies in small series of astrocytoma grade II (A II) suggest that in situ hybridization (ISH) with chromosome specific DNA probes allows a frequent detection of aneusomy 1, trisomy 7, and monosomy 10. However, the role of trisomy 7 in astrocytoma carcinogenesis is disputed because of its presence in non-neoplastic brain tissue as detected by karyotyping. Our objective was to investigate if a correlation between chromosomal aberrations and survival was present in a series of 47 cases of A II. The tissues were evaluated for numerical aberrations of chromosomes 1, 7 and 10 by ISH. Chromosomal aberrations were detected in 68% of A II. Trisomy/polysomy 7 was observed in 31 cases (66%), and in 22 of these (47%) a high percentage of this numerical aberration was seen. Only 11 of these 22 cases also revealed aberrations for 1 and/or 10. No, or only very low frequencies, of cells with aberrations were detected in non-neoplastic control samples. Using Kaplan Meier analysis, trisomy/polysomy 7 correlated significantly with shorter survival. Hence, as determined by ISH, trisomy/polysomy 7 is absent in non-neoplastic brain tissue and is frequently detected in A II, correlating with the malignant progression of the disease.

Introduction

Genotypic characterization of astrocytoma grade II (A II) is not only important in understanding carcinogenesis, but might also offer prognostic information. Such prognostic information may help to resolve the controversy that exists with regard to the optimal treatment of A II. So far, only few studies investigated the correlation between cytogenetic aberrations and the clinical course of A II.¹⁻³ Using classical karyotyping Kimmel et al⁴ showed that astrocytomas with clonal abnormalities had a shorter survival compared to astrocytomas without these abnormalities, however their group of A II was too small for separate analysis.

The most frequently reported genetic aberrations in A II include mutation of the p53-gene, loss of heterozygosity (LOH) of chromosome arm 17p and trisomy 7.⁵⁻⁶ No correlation between p53-gene mutations and survival of A II patients was found.¹ Previous studies in small series of A II suggest that in situ hybridization (ISH) and comparative genomic hybridization (CGH) allow a frequent detection of chromosomal aberrations, such as gain of chromosomes 1 and 7, loss of chromosome 10, and hyperdiploidy (Table 1).^{2-3, 7-14} Only two of these investigators correlated the cytogenetic results with the clinical course of A II.²⁻³ Sallinen et al³ suggested a possible prognostic value of CGH in a series of 11 A II. Perry et al² concluded that a deletion of chromosome 10 was of prognostic value when studying the total spectrum of

Method and chromosomes examined	Number of A II cases	Chromosome aberrations detected	Reference
ISH 10,17	11	-10,-17,+17	Campomenosi et al ⁷
ISH 7,10,X,Y	10	+7,-10	Liu et al ⁸
ISH 7,10,3	11	+7,-10	Perry et al ²
ISH 1,7,10,17,X,Y,1p36	4	+7, Aneuploidy	Rosso et al ⁹
ISH 7,10	3	Tetrasomy 7 and 10	Steilen-Gimbel et al ¹⁰
ISH 1,2,7,1p36	9	+1,-1p36, +7,	Wernicke et al ¹¹
CGH	9	+8q	Nishizaki et al ¹²
CGH	11	-1p,+1pter,+7q,+8q,-10	Sallinen et al ¹³
CGH	10	+7, +7q, +8q	Schrock et al ¹⁴
CGH	10	-5p,+8q,+12p,+19p,-Xp	Weber et al ¹⁵

TABLE 1. Summary of results in the literature of in situ hybridization (ISH) and comparative genomic hybridization (CGH) studies in adult astrocytoma grade II. For CGH the most frequent aberrations are listed.

astrocytomas, while trisomy 7 showed no significant correlation with post-operative survival.

The role of trisomy 7 in carcinogenesis of astrocytomas is controversial, given the finding that this aberration has been detected in cultured, histologically non-malignant, brain tissues.¹⁵⁻¹⁶ In contrast, trisomy 7 has not been detected in normal and gliotic brain tissues by ISH.¹⁷⁻¹⁸

The objective of this study was to investigate the prognostic value of chromosome 7 aberrations in A II. The ISH technique was applied to routinely processed, paraffin-embedded, biopsy and resection samples, allowing a precise correlation of the genetic constitution of cells with their histological features.

Material and methods

Patient material

Forty-seven biopsy and resection samples diagnosed as astrocytoma grade II (A II), or supratentorial low-grade diffuse astrocytoma, were collected from the files of the Departments of Pathology of the University Hospitals of Maastricht and Groningen, and the Alrijde Hospital in Altrecht. Astrocytoma in patients under the age of 18 years were excluded. The samples were revised according to the WHO classification.¹⁹ From the patient records the overall survival was assessed. As controls, ten samples with non-neoplastic reactive gliosis were examined (see also reference 20). These samples were derived at post-mortem examination from patients who died from a non-neoplastic neurological cause, i.e. brain infarction (n=5), hemorrhagic infarction (n=1), traumatic hemorrhage (n=2), and hypoxic encephalopathy (n=2).

In situ hybridization protocol

The most representative paraffin blocks were selected from the 47 A II. Five micron thick sections were cut and pretreated according to a recently optimized protocol.²¹ After deparaffinization in xylol, and dehydration in ethanol series, the tissues were pretreated in 85% formic acid containing 0.3% H₂O₂ 20 minutes at room temperature. After dehydration in an acidified ethanol series, the specimens were incubated at 80° C in 1M sodium thiocyanate (NaSCN). Proteolytic digestion was performed in 4mg/ml pepsin (from porcine stomach: 2500-3500 units per 100 mg protein: Sigma, St Louis, USA) for 10 minutes at 37° C in 0.02 M HCl. After dehydration in an acidified ethanol series the tissues were fixed in 1% formaldehyde in phosphate-buffered saline (PBS) for 15 minutes, followed by five subsequent washing steps in PBS and double-distilled

water. In situ hybridization (ISH) was performed as described earlier (Hopman et al., 1998). The DNA probes used were specific for the heterochromatin (sub)-centromere region of chromosome 1 (1q12, pUC 1.77; Cooke and Hindley²²), the alphoid region of chromosome 7 (p7t1; Waye et al²³), and the pericentromere region of chromosome 10 (D10Z1; Devilee et al²⁴). After denaturation at 80° C for 5 minutes hybridization was performed overnight at 37° C under a coverslip in a mixture containing 2 x standard saline citrate (SSC), 60% formamide, 10% dextran sulphate, and 0.2 mg/ml herring sperm DNA. Post-hybridization washes included two washes in 2 X SSC/ 0.05% Tween at 40° C for 5 minutes followed by two washes in 0.1 X SSC at 60° for 5 minutes. The biotin-labeled DNA probes were detected by an immunoperoxidase precipitation reaction. The slides were incubated for 30 minutes at room temperature with mouse-antibiotin monoclonal antibody, followed by biotinylated horse-anti-mouse IgG, and finally with avidin-biotin-peroxidase complex (all reagents from Vector Laboratories, Burlingame, CA, USA). Finally diaminobenzidine (DAB, Sigma, St Louis, MO, USA) in PBS containing 0.03% H₂O₂ was applied for visualisation of the peroxidase activity. To improve identification of overlapping nuclei, a novel method using fluorescent DNA counterstaining with 4,6-diamino-phenylindol (DAPI, Sigma, St.Louis, MO, USA) and brightfield ISH was used. Microscopy was performed using a Leica-DMBRE microscope (Leica Mikroskopie & Systeme GmbH, Wetzlar, Germany) equipped with a filterset for DAPI.

Evaluation of In Situ Hybridization

The number of ISH signals per nucleus was counted in at least 200 non-overlapping nuclei, following the criteria proposed by Hopman et al.²⁵ Monosomy was defined as >25% of nuclei with no or one signal per nucleus. Trisomy/ polysomy is defined as ≥5% of nuclei containing three or more signals. In accordance with other studies, 20% of aberrant nuclei was used as cut-off for a high-frequent aberration.^{2, 7} The ISH results were categorized as 'normal' (<5% aberrant nuclei), 'low-aberrant' (5-20% aberrant nuclei), and 'high- aberrant' (>20% aberrant nuclei). The prognostic value of these numerical chromosome aberrations was analyzed using log-rank tests on Kaplan-Meier curves.

Results

Histological revision of the forty-seven cases of A II revealed 44 (93%) cases of fibrillary astrocytoma and three (7%) cases of gemistocytic astrocytomas (all WHO grade II). Two

Category	Chromosome 1	Chromosome 7	Chromosome 10
Normal	23 (48%)	17 (35%)	22 (46%)
Low-aberrant	22 (46%)	9 (19%)	16 (33%)
High-aberrant	3 (6%)	22 (46%)	10 (21%)

TABLE 2. Summary of ISH Results in 47 cases of astrocytoma grade II. The number (percentage) of cases with numerical chromosomal aberrations are listed according to the following categories:
Normal = <5% of cells per case with chromosomal aberrations
Low-aberrant = 5-20% of cells per case with chromosomal aberrations
High -aberrant = >20% of cells per case with chromosomal aberrations

of the A II samples showed a oligodendroglial component, which was however less than 20% of the nuclei. Forty-five cases were primary tumors, and two were recurrences from A II.

No patients had been subjected to prior radiotherapy. Twenty-nine (62%) patients had undergone a stereotactic biopsy, and in 18 (38%) patients the samples had been obtained by surgical resection. The follow-up period varied between 24 and 200 months. The median survival interval was 90 months (95%-CI: 72-108 months).

Using probes for the chromosomes 1, 7 and 10 the samples with reactive gliosis showed no or only sporadic cells with three ISH signals, and were all classified as normal for all three chromosomes (*Figure 1A*). Of the astrocytomas grade II, 31 cases (66%) revealed aberrations for one or more of the chromosomes investigated (*Table 2*). The frequency of cells exhibiting numerical chromosomal aberrations exceeded 5% in 52% of the cases for chromosome 1, in 65% of the cases for chromosome 7 (*Figure 1B*), and in 54% of the cases of chromosome 10. High frequent aberrations with increased copy numbers were most frequently detected for chromosome 7. In 46% of the samples the frequency of cells with aberrations exceeded 20%. A much more limited number of cases falling into the "high-aberrant" category were detected with probes for chromosomes 1 and 10 (*Table 2*). The most frequently detected numerical aberrations of chromosome 7 consisted of trisomies (28 cases), but a few cases showed higher copy numbers (3 cases). All numerical aberrations of chromosomes 1 or 10 comprised increases in copy numbers, except for two cases with monosomy 10.

In *Figure 2* the individual cases and the correlation between the percentages of aberrant nuclei for chromosomes 7 and 1 (*Figure 2A*) and chromosomes 7 and 10 (*Figure 2B*) have been depicted. As seen virtually all cases with aberrations of chromosomes 1

FIGURE 1. Brightfield microscopy of ISH for chromosome 7, with DAPI nuclear counterstaining. (A) A sample of reactive gliosis with no aberrations for chromosome 7. (B) Astrocytoma grade II showing frequent nuclei with three signals for chromosome 7. Magnification: (A B) x 800.

FIGURE 2. Scatter plot showing the percentages of aberrant nuclei for chromosome 7 versus chromosome 7 (A), and for chromosome 7 versus chromosome 10 (B) for each of the individual cases of astrocytoma grade II (n=47).

Figure 3. Survival of patients with astrocytoma grade 2 classified on the basis of aberrations of chromosome 7. High percentages (>20%) of aberrant nuclei for chromosome 7 correlate most strongly with shorter survival (log-rank; p-value 0.028).

and/or 10 also exhibit (high) frequencies of cells with aberrations of chromosome 7. The reverse, i.e. cases with aberrations for chromosome 7 also showing chromosome 1 and 10 deviations, only holds true for a limited number of cases.

The results of the Kaplan Meier analyses are shown in *Figure 3*. Trisomy/polysomy 7, according to the different categories, correlates with shorter survival (log-rank; $p=0.028$). High-frequent aberrations of chromosome 7 (>20% of trisomic/polysomic cells as cut-off; high-aberrant group) correlated strongly with shorter survival as compared to A II with disomy or a low frequency of aberrations for chromosome 7 (normal and low-aberrant group taken together; log-rank; $p=0.008$; not shown in *Figure 3*). The two tumors with monosomy 10 showed extremely short survival intervals, i.e. 10 and 24 months.

Discussion

The present study shows that numerical aberrations of chromosome 7 are frequently occurring in astrocytoma grade II (A II) and that their detection by in situ hybridization (ISH) are of prognostic value. The absence or extremely low frequency of numerical

chromosome 7 aberrations in the control samples with non-neoplastic reactive gliosis is in accordance with the results of earlier ISH studies in non-neoplastic brain tissue.^{17, 18} This may suggest that trisomy 7, as detected by classical banding analysis in normal and gliotic brain tissue, most probably is due to culturing artifacts.^{15, 16} Another possible explanation is that the normal brain tissues in the study of Heim et al.¹⁵ contained malignant cells, because seven of their 11 patients were reported to have malignant brain tumors. However, due to limitations of the ISH technique, we cannot rule out the presence of small clones of cells with trisomy 7 in our gliotic samples.

Our ISH findings in A II corroborate with results of previous, smaller series (Table 1). Trisomy 7 was described by Perry et al.² in half of the A II cases examined. In a double-label ISH experiment, Steilen-Gimbel et al.¹⁰ showed gains of both chromosomes 7 and 10. CGH studies also suggest that gain or amplification of in particular chromosomes 7 and/ of 8q is typical for A II (Table 1). In this study we show that high-frequent trisomy/polysomy of chromosome 7 is strongly correlated with shorter survival, suggesting a clonal expansion of this cell population during the malignant progression of A II.

Polysomy, in particular trisomy, of chromosome 7 is not only frequently detected in A II but is also seen in other preneoplastic and neoplastic lesions. Trisomy 7 was already shown to correlate with poor prognosis in prostate carcinoma.²⁶ Furthermore, this aberration is present in the stemcell compartment of colon carcinoma and has been suggested to be involved in the transition of colon adenoma to carcinoma.²⁷ Thyroid hyperplasia with trisomy 7 is supposed to progress more often to adenoma and carcinoma.²⁸

These studies and our present findings strongly support the involvement of trisomy/polysomy 7 in carcinogenesis and tumor progression. We found no strong indications that trisomy/polysomy 7 is associated with reactive gliosis, and therefore can not support the suggestion that this represents a preneoplastic process.¹⁶ Furthermore, the presence of trisomy/polysomy 7 did not correlate with the age of the patients in the series of A II described here (unpublished), as was suggested for several other types of solid tumors by Broberg et al.²⁹

We conclude that astrocytoma grade II frequently contains a considerable number of tumor cells with trisomy/polysomy 7. The detection of this numerical chromosomal aberration by ISH may thus be of prognostic value in this type of brain tumor.

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5

Proliferation and aneusomy strongly predict survival of young patients with astrocytoma grade II.

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Abstract

The clinical course of astrocytoma grade II (AII) is highly variable and not reflected by histological characteristics. As one of the best prognostic factors higher age identifies rapid progressive A II. For patients over 35 years of age an aggressive treatment is normally advised. For patients under 35 years there is no clear guidance for treatment choices, and therefore also the necessity of histopathological diagnosis is often questioned. We studied the additional prognostic value of the proliferation index and the detection of genetic aberrations for patients with A II. The tumour samples were obtained by stereotactic biopsy or tumour resection and divided into two age groups, i.e. 18-34 year (n=19) and ≥ 35 year (n=28). Factors tested included the proliferation (Ki-67) index, and numerical aberrations for chromosomes 1, 7 and 10, as detected by in situ hybridization (ISH). The results show that age is a prognostic indicator when studied in the total patient group, with patients above 35 years showing a relatively poor prognosis. Increased proliferation index in the presence of aneusomy appears to identify a subgroup of patients with poor prognosis more accurately than predicted by proliferation index alone. We conclude that histologically classified cases of A II comprise a heterogeneous group of tumours with different biological and genetic constitution, that exhibit a highly variable clinical course. Immunostaining for Ki-67 in combination with the detection of aneusomy by ISH allows the identification of a subgroup of patients with rapidly progressive A II. This is an argument not to defer stereotactic biopsy in young patients with radiological suspicion of A II.

Introduction

Controversy exists with respect to optimal treatment protocols for low-grade diffuse astrocytoma (astrocytoma WHO grade II; A II) because prospective studies comparing treatment strategies are rare.¹⁻³ Another reason for this controversy is that the interval after which malignant progression of A II occurs is difficult to predict. Clinical factors that may correlate with survival include those related to patient age, presenting symptoms, duration of symptoms, performance status, tumour volume, extent of resection, and timing and dose of radiotherapy.^{4,5} Patient age is the single consistent prognostic factor in these retrospective studies. Patient age under 35 years,^{6,7} or under 40 years in other series⁸ is often associated with a prolonged survival. Because the benefits of early treatment have to be balanced against the possibility of long-term side effects from radiation therapy, patient age is decisive in current treatment protocols for A II. For patients under 35 years of age the benefit of early and aggressive treatment has never been proven sufficiently and therefore treatment is often deferred.⁹ As a result in young patients also a controversy exists concerning the usefulness of an immediate histological diagnosis, involving stereotactic biopsy.¹⁰

Ultimately the majority of A II progresses to high-grade astrocytomas (astrocytoma WHO grade III and grade IV; A III/A IV), which is characterized by an increase in proliferation activity and an accumulation of genetic abnormalities. Proliferation and cytogenetic markers may therefore identify rapid progressive A II. Increased proliferation activity correlates with shorter survival in most series of astrocytomas, although the number of A II was often too small for separate analyses.^{11,12} Numerical chromosomal aberrations have been reported in astrocytomas, such as aneuploidy 1, trisomy 7 and monosomy 10. It has been shown that trisomy 7 correlates with shorter survival of A II patients.¹³

In the underlying study the prognostic value of the proliferation (Ki-67) index and the detection of numerical aberrations for the chromosomes 1, 7 and 10 was evaluated. The correlation of these parameters with survival analysis was performed for patients aged 18-34 years and ≥ 35 years to assess whether these parameters allow the identification of rapid progressive A II in young patients.

Material and methods

Patient material

Tissue specimens from 47 adult patients diagnosed with supratentorial astrocytoma grade II (A II) were collected from the data files of the departments of Pathology of the University Hospitals of Maastricht and Groningen, and the Atrium Hospital in Heerlen, The Netherlands. Histopathological revision according to the WHO classification¹⁴ revealed 44 fibrillary astrocytomas and three gemistocytic astrocytomas (all WHO grade II). Astrocytomas with mitotic figures were not included in this series. In order to diminish the probability of sampling error A II in which the neuro-radiologists suspected high-grade astrocytoma and which showed radiological characteristics for tumor bleeding as well as extensive contrast enhancement, were not included in this series. Patient records were examined with regard to the first neurological symptoms, radiological findings, neurosurgical procedure, dose and timing of radiotherapy, and survival interval.

Mean and median age was respectively 38 and 41 years (range: 18 to 69 years). The study included 22 women (47%) and 25 men (53%). Seizures were the most frequent presenting symptom (77%), followed by focal neurological deficit (26%), mental changes (15%) and signs of raised intra-cranial pressure (17%). The median duration of pre-operative symptoms was 3 months (range: 1 week to 157 months). Most tumours were located in the frontal (51%) and temporal/parietal (45%) lobes and less frequently in the occipital lobe (4%). The majority of patients (n=41) underwent neurosurgery immediately after coming to medical attention and had their first neuro-imaging test. In six patients neurosurgical intervention was extended for 36 to 150 months. No association was found, using Pearson's correlation coefficient, between pre-operative interval and proliferation index or chromosomal status. For this reason all cases were included in this study.

Neurosurgical procedures consisted of a biopsy in 29 (62%) and resection in 18 (38%) patients. Radiotherapy was given immediately post-operative in 28 (60%) patients, delayed in 10 (21%) patients, and nine (19%) patients were still not irradiated at the last follow-up.

Proliferation index: Ki-67 immunohistochemistry

Five micron thick paraffin sections were pre-incubated in methanol with 0.3 % H₂O₂. Tissues known to be negative and positive for Ki-67 were used as controls. Antigen retrieval was achieved by incubation with 10mM citrate buffer (pH 6.0) in a domestic

microwave oven at 700W for ten minutes. The sections were incubated with the mouse monoclonal antibody MIB-1 directed against Ki-67 (Immunotech S.A. Marseille, France) at a 1:12 solution in PBS containing 4% normal goat serum for 60 minutes. Subsequently, biotin-labelled horse anti-mouse antibody at a 1:200 dilution and avidin-biotin peroxidase complex (Vector Laboratories, Burlingame, CA, US) were applied for 60 and 45 minutes, respectively. Peroxidase activity was detected using diaminobenzidine in PBS/ imidazol buffer with 0.02% H_2O_2 . Nuclei were scored for positivity in 500 cells in each sample in the areas with highest immunopositivity. The mean proliferation (Ki-67) index was 2.7% (range 0.0%-9.2%).

In situ hybridization

In situ hybridization (ISH) was performed as described earlier.¹⁵ Five-micron thick paraffin sections were deparaffinized and pre-treated in 85% formic acid/ 0.3% H_2O_2 for 20 minutes. Thereafter the slides were incubated in 1M sodium-thiocyanate at 80° C for 10 minutes. Subsequently proteolytic digestion was performed by 4mg/ml pepsin (Sigma Chemical Co, St.Louis, MO) for 10 minutes at 37° C in 0.02 M HCl. Then the slides were fixed in 1 % formaldehyde in phosphate buffered saline (PBS) for 15 minutes, and rinsed in PBS and double distilled water.

The biotin-labeled DNA probes used in this study were specific for the centromeric region of chromosome 1 (1q12, pUC 1.77), the alphoid region of chromosome 7 (p7t1) and the pericentromeric region of chromosome 10 (D10Z1). The probes were hybridised to the target-DNA in a mixture containing 2 x standard saline citrate (SSC), 60% formamide, 10% dextran sulphate, and 0.2 mg/ml herring sperm DNA. After denaturation at 80° C for 5 minutes the slides were incubated overnight at 37° C. Subsequently, the slides were washed twice in 2 X SSC/ 0.05% Tween at 45° C, and in 4 X SSC/ 0.05% Tween at room temperature. To detect probe hybridization the slides were incubated for 30 minutes at room temperature with mouse-antibiotin monoclonal antibody, followed by biotinylated horse-anti-mouse, and finally with the avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA). Finally 0.1M diaminobenzidine (Sigma) in PBS containing 0.03% H_2O_2 was applied for visualisation of the peroxides activity. To improve identification of the individual nuclei bright-field microscopy was combined with fluorescent nuclear counterstaining using 4,6-diaminophenylindol (DAPI, Sigma). The samples were evaluated with a Leica DMBRE microscope (Leica Mikroskopie & Systeme GmbH, Wetzlar, Germany). The number of signals per nucleus was counted in at least 200 non-overlapping nuclei. Trisomy/ polysomy (gain) was defined as >5% of nuclei containing three or more signals, and monosomy (loss) as >25% of nuclei with none or one signal per nucleus. The tumours were classified as

aneusomic when gain or loss of at least one of the chromosomes was detected. The other A II were classified as disomic. As detected by in situ hybridization 32 (68%) of the tumours showed aneuploidy for one or more of the chromosomes investigated. The most frequent aberration was aneuploidy for chromosome 7 in 31 (66%) of A II. Aneuploidy for chromosome 1 was detected in 24 (51%) and aneuploidy for chromosome 10 in 25 (53%) of the samples. Monosomy 10 was only detected in two samples (4%), which both also showed trisomy/polysomy 7. The data of the individual chromosomes are described in detail elsewhere.¹³

Statistical analyses

The following factors were considered as possible prognostic parameters for survival: patient age, sex, presenting symptoms, pre-operative duration of symptoms, tumour location, neuro-surgical procedure, timing of radiotherapy, proliferation index and chromosomal status.

The influence of these factors on survival was tested by univariate analysis using log-rank tests. Possible prognostic factors from the univariate analyses with p-values less than 0.10 (according to recommendation in the literature¹⁶) were entered into the multivariate analyses using a forward stepwise method in order to identify independent prognostic parameters.¹⁷ Univariate and multivariate associations between factors and survival were assessed with a Cox regression model. Subsequently tumour-related factors were used for analysis in age-stratified groups.

Results

The median survival interval for patients with astrocytoma grade II as estimated by the Kaplan-Meier method was 90 months (95% confidence interval 72 to 108 months). When determining the influence of age on the period of survival after the first diagnosis of A II it becomes obvious that the group aged ≥ 35 years at the time of diagnosis exhibits a significantly shorter survival period as compared to the 18-34-aged patient group (Figure 1; Log-rank; p-value=0.05). Other factors significantly associated with shorter survival were focal neurological deficit at presentation, proliferation (Ki-67) index $>1\%$, and aneuploidy.

Figure 1. Association between patient age (18-34 vs. ≥ 35 years) and survival in A II.
(Kaplan-Meier, log-rank test, p -value = 0.05)

FIGURE 2 High proliferation (Ki-67) index in A II is associated with shorter survival in (A) patients aged 18-34 (p -value=0.02) and (B) patients ≥ 35 years (p -value = 0.03).
(Kaplan-Meier; pooled log-rank test, p -value=0.002)

..... Proliferation index $\leq 1\%$ (n=18)
 — Proliferation index $> 1\%$ (n=29)

FIGURE 3

Relation of aneusomy with shorter survival in (A) A II patients aged 18-34 (p -value=0.36) and (B) patients ≥ 35 years (p -value = 0.09). (Kaplan-Meier; pooled log-rank test, $p=0.05$)

..... Disomy (n=15)
 — Aneusomy (n=32)

FIGURE 4

The combination of both high proliferation (Ki-67) index and aneusomy is strongly associated with a shorter survival in A II patients (A) aged 18-34 ($p=0.01$) and (B) aged ≥ 35 years ($p=0.001$). (Kaplan-Meier; pooled log-rank test, $p<0.0001$)

..... Proliferation index $\leq 1\%$ or Disomy (n=26)
 — Proliferation index $>1\%$ and Aneusomy (n=21)

Univariate analysis

PARAMETER		HAZARD RATIO (95% CI) ^a	P-VALUE
Proliferation index >1%	(n=29)	3.63 (1.22-10.82)	0.02
Focal neurological deficit	(n=13)	2.78 (1.12-6.90)	0.03
Aneusomy	(n=32)	3.29 (0.97-11.20)	0.06
Patient age ≥35 years	(n=28)	2.52 (0.97-6.58)	0.06

Multivariate analysis

PARAMETER		HAZARD RATIO (95% CI) ^a	P-VALUE
Proliferation index >1%	(n=29)	4.81 (1.50-15.38)	0.01
Patient age ≥35 years	(n=28)	3.01 (1.13-7.99)	0.03
Aneusomy	(n=32)	3.66 (1.06-12.62)	0.04

TABLE 1. Unfavourable prognostic factors for patients with astrocytoma grade II.

Upon multivariate analysis proliferation index >1%, patient age ≥35 years, and aneusomy were independently correlated with shorter survival (*Table 1*). To investigate the interactions between the proliferation index and aneusomy with age we analysed the influence of these two factors on survival stratified for age. When correlating the proliferation index to survival in the two age groups separately, it appears that in the group of 18-34 year old patients a clear-cut distinction can be made between those with a proliferation index ≤1%, showing long-term survival, and those with a proliferation index >1%, showing more progressive A II (*Figure 2A*; Log-rank; p-value =0.02). Also in the ≥35 year old patients the proliferation index proved to have additional prognostic value, although less apparent as compared to the younger group (*Figure 2B*; p-value=0.03).

Using the in situ hybridization protocol with probes for chromosomes 1, 7 and 10, cases of A II with an apparently normal (disomic) chromosomal content can be separated from aneusomic cases. In the whole group of patients with A II the detection of aneusomy has additional value in distinguishing between rapid and slow progressive A II. In the stratified age groups an aberrant chromosomal constitution is not associated with shorter survival in both the 18-34 year (*Figure 3A*; p-value=0.36) and the ≥35year (*Figure 3B*; p-value=0.09) old group.

However, the combination of a high proliferation index and aneusomy very accurately identified patients with an unfavourable outcome in both the 18-34 year (*Figure 4A*; p-value=0.01) and the ≥35 year-old patient groups (*Figure 4B*; p-value=0.001). The

combination of both factors reclassified three (27%) of 11 patients of 18-34 years into the group with relative good prognosis (Compare *Figure 4A* to *2A*). In five of 18 patients in the ≥ 35 years group the high proliferation index with disomy now correlated with a more favourable course (compare *Figure 4B* to *Figure 2B*).

Figure 5 illustrates a case of A II, localised in the frontal lobe of a 25-year patient with epileptic seizures as sole manifestation. The proliferation (Ki-67) index was 4.5% and a high-percentage (35%) of nuclei with trisomy for chromosome 7 was detected by ISH. Despite the favourable clinical features (young age, no focal deficit) the patient rapidly progressed to astrocytoma grade IV, and the survival interval after histological diagnosis was only 36 months.

FIGURE 5. Astrocytoma grade II in a patient aged 25 years with high proliferation (Ki-67) index (A), and frequent nuclei with trisomy for chromosome 7, detected by in situ hybridization (B). Counter-staining with DAPI in B. Magnifications X800

Discussion

A 'wait and see' policy has been propagated for patients under 35 years of age, suffering from epileptic seizures and radiological suspicion of astrocytoma grade II (A II).⁹ An important argument for not performing a stereotactic biopsy in these patients is that the histological diagnosis of A II does not alter treatment strategy. In the current study we demonstrate that the detection of a high proliferative activity in combination with chromosomal aberrations identifies a subgroup of young A II patients with a rapid malignant course.

Our study confirms previous studies of A II showing the prognostic value of patient age and symptoms at presentation.^{5,8} In multivariate analyses only the age of patients at diagnosis remained significantly associated with survival as an independent clinical factor, when comparing patient groups under and over 35 years of age. However, when including biological factors describing the proliferative capacity and genomic constitution of the lesion, subgroups of patients with a significantly shorter survival could be distinguished within these two separate age groups. In our study immunostaining for the proliferation marker Ki-67 in over 1 % of tumour cells showed the strongest association with survival of patients with A II. The proliferation (Ki-67) index has been described to increase with the grade of astrocytomas although overlap between grades exists.^{11,12} The additional value of the Ki-67 labelling protocol in A II was also demonstrated in two recent studies, although in these studies no stratification for patient age was performed.^{18,19}

We show a significant correlation of proliferation (Ki-67) index with survival for patients under 35 years, separating a group of young patients with a high proliferation index and a prognosis similar to that of patients >35 years. Immunolabeling of lesions from patients ≥ 35 years of age identified a subgroup with a low proliferation index, correlating with a somewhat better prognosis. In the multivariate analysis a proliferation (Ki-67) index >1% proved to be a strong independent prognosticator. This corroborates with a bromodeoxyuridine-(BrdU)- proliferation marker analysis of A II that also showed cell cycle activity in >1% of tumour cells to be associated with shorter survival.²⁰ A possible drawback of the proliferation (Ki-67) index as prognosticator in A II is reflected by the wide variation of cut-off levels (between 2% and 10%) used in different studies.^{18,19,21} In our series the discriminative power of the proliferation (Ki-67) index diminished when using a higher cut-off level than 1% (data not shown). These differences in cut-off levels, amongst different studies, may be explained by differences in the immunohistochemical procedures applied such as the method of

antigen retrieval, the immunolabelling protocol, and by differences in the scoring criteria of (clustered) Ki-67 positive cells. Therefore, an additional, but independent prognosticator is needed.

The detection of numerical genomic aberrations has such additional prognostic value to the proliferation (Ki-67) index. For astrocytomas of all grades it appears that certain genetic changes are associated with an unfavourable clinical course. Astrocytomas with chromosomal abnormalities as detected by karyotyping cause a shorter survival as compared to astrocytomas without these abnormalities.²² An in situ hybridization study showed that monosomy for chromosome 10, harbouring the tumour suppressor gene PTEN/MMAC1, results in shorter survival.²³ However, in these series the majority of tumours represented high-grade astrocytoma (grades III and IV). In A II the most frequently reported genetic aberration is loss or mutation of the p53-gene, which shows however no association with clinical course.^{24,25}

One of the very few additional studies correlating genetic aberrations and clinical course includes a comparative genomic hybridization (CGH) analysis which showed that A II with rapid malignant progression exhibit a significantly higher number of chromosomal aberrations as compared to A II with an indolent behaviour.²⁶ However, the sensitivity of this CGH technique is relatively low due to contamination with normal/reactive cells, which are often present in A II.²⁷

In contrast, the in situ hybridization (ISH) procedure is a routinely applicable to paraffin embedded samples of central nervous system tumours.²⁸ Our study demonstrates that the detection of chromosomal aberrations by ISH, using a panel of probes for chromosomes 1, 7 and 10, offers an extra independent predictor for survival of patients with A II. After stratification for age the presence of chromosomal aberrations alone is not significantly associated with shorter survival, which may be caused by the small size of both groups. However, in both age groups the detection of aneusomy by ISH adds value to the proliferation index, in that increased proliferation in the presence of chromosomal aberrations is associated with a poor prognosis.

A correlation between aneusomy and proliferative activity was demonstrated in astrocytomas of all grades, containing aneusomies for chromosome 7 and 10 particularly in Ki-67- positive cells.²⁹ This suggests that an accumulation of chromosomal aberrations in proliferating cells plays an important role in the early stages of astrocytoma progression. This also suggests that the combination of both these parameters is therefore very useful in identifying A II with a rapidly malignant clinical course.

Although imaging studies were used as adjunct to histology, also A III/IV may present as non-enhancing lesions.³⁰ Therefore one could argue that in the stereotactic specimen

sampling error from high-grade astrocytomas may have biased the results. This is contradicted, however by the relatively long median survival of 90 months, also compared to other A II studies.^{31,32} Another argument against underscoring of the tumours is given by the fact that only one of the biopsy specimens exhibited monosomy 10, which is characteristic for low-grade areas in high-grade astrocytomas.³³

The additional value of genetic and biological parameters to the current histological WHO classification was also seen in the three gemistocytic variants of A II. Previous studies suggest that A II with high percentages (>60%) of gemistocytes, in fact behave more similar to A III.³⁴ Although two of our gemistocytic samples contained lower percentages of gemistocytes, the increased proliferation activity and the presence of aneusomy in all three samples was associated with a relatively short survival (range 41-43 months).

We postulate that identification of subtypes of astrocytomas on basis of genetic and biological factors has additional prognostic value to the current histological classification. Tissue should be obtained in all patients with A II in order to assess the proliferation activity and, if possible, the presence of trisomy for chromosome 7. In the future these markers may help optimising treatment strategy, in particular in young patients with astrocytoma grade II, for whom optimal treatment is now controversial.

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10q25.3 (DMBT1) copy number changes in astrocytoma grade II and IV

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Abstract

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In the literature it has been suggested that loss of the 10q25-26 region, including the DMBT1-gene (10q25.3), is correlated with initiation and/or malignant progression of astrocytomas, although the results of the LOH-studies that led to this assumption are not unequivocal. For this reason we compared copy number changes of 10q25.3 to those of the pericentromeric region (10q12) by double-target fluorescence in situ hybridization (FISH) in ten cases of astrocytoma grade II and grade IV each. The same specimens were analysed for copy number changes of chromosome 1, as a marker for polyploidy, and chromosome 7, which is often gained in astrocytomas. Our results show that selective loss of the 10q25.3 region was present in 2 of 10 specimens in both astrocytoma grade II and grade IV, and occurred only in tumors with polysomy for 10q12. Furthermore, astrocytoma grade II often showed polyploidy for chromosomes 1, 7 and 10 (8 of 10 specimens). In addition, astrocytoma grade IV frequently exhibited losses of chromosome 10 in a high percentage of nuclei. Although based on a small number of cases the results show that loss of the 10q25.3 region is uncommon in astrocytoma grade II and mostly coincident with loss of chromosome 10 in grade IV tumors. These data indicate that selective loss of the 10q25.3 region, including the DMBT1-gene, is not an initiating event in the carcinogenesis of astrocytoma.

Introduction

Astrocytoma is the most frequently occurring primary brain tumor and is classified according to the World Health Organization (WHO) criteria into grades with increasing malignant behavior.¹ Astrocytoma WHO grade II (A II), or diffuse astrocytoma, occurs in younger patients and often presents with epileptic seizures. Astrocytoma WHO grade IV (A IV), or glioblastoma multiforme, more often causes progressive neurological deficit or raised intracranial pressure, and generally occurs in older patients.

In A II frequent genetic alterations include loss or mutation of the p53 gene² and trisomy for chromosome 7.^{3,4} We recently reported that gain of chromosome 7 is associated with shorter survival of patients with A II.⁵ In A IV the most frequent genetic alteration, occurring in approximately 90% of cases, involves deletions of chromosome 10. Most A IV have lost an entire copy of chromosome 10, but partial deletions also occur.⁶ Two of these commonly deleted regions in A IV comprise 10q23, containing the PTEN/MMAC1-gene^{7,8}, and 10q25-26, containing the Deleted in Malignant Brain Tumor 1 (DMBT1)-gene.⁹

Monosomy for chromosome 10 and selective loss of 10q23 are uncommon in A II.¹⁰⁻¹³ However, molecular allelotyping has indicated that loss of heterozygosity (LOH) within the 10q25-26 region may be associated with initiation of A II and/or malignant progression of A II to A IV.^{11, 14-16}

The role of the 10q25-26 region in astrocytoma carcinogenesis is, however, questionable. In *Table 1* studies in A II and A IV are listed that pay special attention to loss of chromosome 10 and 10q25-26.^{9,11-22} This overview demonstrates that selective loss of 10q25-26 is only detected in a small percentage (<10%) of A II. Since these studies were based on microsatellite analyses we cannot exclude that they underestimate the actual frequency of 10q25-26 loss, because A II tissue often consists of non-homogenous areas of tumor and non-neoplastic cells.²³

The objective of the underlying study was therefore to investigate whether or not physical loss of the 10q25.3 region can be detected in these tumors by the in situ hybridization (ISH) procedure, and if it this provides a marker for rapid malignant progression of A II to A IV.

For this purpose cell suspensions were prepared from frozen tissue of A II and A IV, and analysed by double-target FISH, using probes for the 10q25.3 and the pericentromeric region 10q12, allowing the detection of selective 10q25.3 loss at a single cell level.

TABLE 1. A summary of molecular cytogenetic studies concerning chromosome 10 with special attention to the 10q25-26 region in astrocytoma grade II and IV of adult patients. Only the two most extensive FISH studies are listed.

Deletions of chromosome 10		Method	Reference
A II *	A IV *		
Monosomy	0/72	ISH	Hilton et al ¹²
Monosomy	49/64	FISH	Amalfitano et al ¹⁷
Monosomy	0/3	MA	Karlsson et al. ¹⁸
q-arm	0/3		
p-arm	0/3		
Monosomy	7/30	MA	Fults et al ¹⁹
p-arm	2/30 ^a		
q25-qter	6/30		
Monosomy	0/33	MA	Rasheed et al ¹⁵
q25.1	1/33		
Monosomy	0/7	MA	Maier et al ^{11,20}
q-arm	1/7		
q25-26	1/7		
Monosomy	3/23	MA	Ichimura et al ²¹
q-arm	0/23		
p-arm	5/23		
Monosomy	0/8 ^b	MA	Fujisawa et al ¹⁴
10q	5/8		
10q25-qter	2/8		
Monosomy	0/4	MA	Steck et al ¹⁶
q-arm	2/4		
q25-26	2/4		
DMBT1	0/5	Q-PCR	Mollenhauer et al ⁹
DMBT1	0/18	Q-PCR	von Deimling et al ¹³
DMBT1	8/21	PCR	Somerville et al ²²

Symbols and abbreviations;

* = frequency of chromosome 10 deletions in astrocytoma grades II (A II) and IV (A IV);

a = partial deletions of both 10p and 10q; b = high-grade foci in five secondary glioblastoma;

MA = micro-satellite analysis ; Q-PCR = quantitative- polymerase chain reaction

Material and methods

Tumor specimens

Frozen specimens of 10 astrocytoma grade II (A II; cases 1 to 10) and 10 astrocytoma grade IV (A IV; cases 11 to 20) were collected from the files of the Pathology Departments of the University Hospitals Maastricht (Netherlands) and Leuven (Belgium). The tumors were derived through stereotactic biopsy (65%) or resection (35%) in the period between 1993 and 2001. The A II series consisted of nine fibrillary and one gemistocytic astrocytoma. The group of A IV consisted of nine primary and one secondary (progressive) glioblastoma. Sections with representative tumor tissue were selected by the neuro-pathologist (B.K.) according to the World Health Organization (WHO)- classification.¹ Progression free and overall survival were assessed from the patients' records.

Ki-67 and P53 immunohistochemistry

Of the same specimen 5 micron thick paraffin-embedded tissue sections were deparaffinized and pre-incubated in methanol with 0.3 % H_2O_2 . Antigen retrieval was achieved by incubation with 10mM citrate buffer (pH 6.0) in a microwave oven at 700W for ten minutes. The sections were incubated with the mouse monoclonal antibody MIB-1 directed against Ki-67 (Immunotech S.A. Marseille, France) at a 1:12 dilution, or with the mouse monoclonal antibody DO-7, directed against human P53 protein (Dako A/S, Glostrup, Denmark), at a 1:25 dilution in phosphate buffered saline (PBS) containing 4% normal goat serum for 60 minutes. After a washing step, biotin-labeled horse anti-mouse antibody at a 1:200 dilution and avidin-biotin peroxidase complex (Vector Laboratories, Burlingame, CA, US) were applied for 60 and 45 minutes, subsequently. Peroxidase activity was detected using diaminobenzidine in PBS/ imidazole buffer with 0.02% H_2O_2 . Tissues known to be negative for Ki-67 and P53 protein were used as control. Positive nuclei were scored in 500 cells in each sample in the areas with the highest immunopositivity. The Ki-67 labeling index (LI) was expressed as the percentage of immunopositive cells from the total cells. Scoring for nuclear P53 staining was done on a four point scale: 0 indicating no staining, + indicating <5% positive nuclei, ++ indicating 5-20% positive nuclei, and +++ indicating >20% of nuclei with positive staining.

Double-target FISH

Isolation of nuclei

Tumor nuclei were isolated from 30 μ thick sections as described previously.²⁴ The sections were digested in 1 ml of 100 μ g/ml pepsin (from porcine stomach: 800 U/ mg protein; Sigma St. Louis, MO, USA) in 0.01 M HCl for 20 minutes at 37° C. In order to obtain single nuclei the tissue material was resuspended through a thin needle (21 G). 200 μ l of the cell suspension was cytocentrifuged for 5 minutes at 1200 rpm onto a poly-L-lysine coated slide. The slides were subsequently dehydrated in 70%, 96% and 100% ethanol.

DNA probes

Double-color fluorescence in situ hybridization (FISH) was performed using a combination of DNA probes for the 10q25.3 and the 10q12 regions. The PAC probe mapping to 10q25.3 (D10S209; references 9, 20, 25) was labeled with biotin by nick translation. The 10q12 probe (D10Z1; reference 26) was labeled with digoxigenin. The specificity of these probes were validated on human metaphase preparations, as is shown in Figure 1.

FISH analysis was also performed with a combination of probes for the (sub)-centromeric region of chromosome 1 (1q12,pUC 1.77; reference 27) , as a marker for ploidy status, and for the alphoid region of chromosome 7 (p7t1; reference 28). All probes were hybridized in a mixture containing 2x standard saline citrate (SSC, pH 7.0), 50% formamide, 10% dextran sulfate and 0.2 mg/ml herring sperm DNA (hybridization mixture).

Fluorescence in situ hybridization

Ten microliters of the hybridization mixture was added to the slides under a coverslip. Denaturation was performed at 75° C for 4 minutes, followed by hybridization overnight at 37° C. The following day the slides were washed twice for 5 min in 50% formamide, 2 X SSC/ 0.05% Tween pH 7.0, at 45° C, and thereafter twice for 5 min in 2 X SSC/ 0.05% Tween pH 7.0, at 42° C, and once for 5 min in 4 X SSC/ 0.05% Tween pH7.0, at room temperature.

The biotinylated probe was detected by rhodamine conjugated to avidin. If necessary this signal was intensified by subsequent incubation with biotinylated goat-anti-avidine and avidine-rhodamine (Vector Laboratories, Burlingame, CA, US). The digoxigenin-

labeled probe was detected by mouse anti-digoxin monoclonal antibody (Sigma), and (if necessary) the signal was intensified by rabbit-anti-mouse, followed by swine-anti-rabbit antibodies both conjugated to FITC (Vector Laboratories). The nuclei were counterstained with 4'6-diamino-2-phenylindole (DAPI; Sigma). Fluorescence microscopy was performed using a Leica-DMRE microscope (Leica Mikroskopie und Systeme GmbH, Wetzlar, Germany) with the appropriate filter sets. All slides were evaluated by two investigators (PW, AH), blinded for the clinico-pathological data. At least 100 nuclei were analysed and aberrations were only scored if present in >5% of the nuclei.^{5,29} Clustered cells or multinucleated cells were not analysed. Microphotographs were recorded with the In Situ Image System (Metasystems GmbH, Altussheim, Germany) using a black and white CCD camera.

Statistical evaluation

Patient age and Ki-67 labeling index were correlated to tumor grade using student-T-tests. The prognostic value of tumor grade on survival was analyzed using log-rank tests on Kaplan-Meier curves.

Results

Clinical data and immunohistochemistry

The clinical data and immunohistochemical findings are summarized in *Table 2*. The mean age of A II patients was 40 years (range: 25-54 years) and of A IV patients 59 years (range: 31-75 years) (student T-test, $p < 0.01$). Fourteen patients were male, and six were female. The mean progression free interval for A II patients was 59 months (range 7- 80 months) and the mean survival interval of A IV patients 8 months (range 2- 33 months) (Log-rank; $p = 0.002$).

Immunohistochemical analysis showed overexpression of P53, with > 5% positive nuclei, in 7 grade II and in 4 grade IV tumors. The mean Ki-67 labeling index (LI) in A II was 2.9% (range 0-7%) and in the A IV 18.7% (range 9-33%) (T-test, $p < 0.01$). The differences in patient age, clinical course and Ki-67 LI further validate the histological classification of A II and A IV samples.

FISH data

All tumors were analyzed by FISH for copy number changes of the (peri-)centromeric regions of chromosomes 1 (1C), 7 (7C) and 10 (10C) and 10q25.3.

TABLE 2. Clinical data, immunohistochemical data for P53 and Ki-67, and FISH results in astrocytoma grade II (cases 1-10) and grade IV (cases 11-20).

	case	age	survival	IHC		FISH			
				P53	Ki-67	1C	7C	10C	10q25
A II	1	25	N80	+++	<1%				
	2	29	N14	++	2%				
	3	30	28	-	3%				
	4	54	N10	++	6%				
	5	44	N25	-	3%				
	6	48	N6	++	7%	a	a	a	a
	7	50	39	+++	2%	a	a	a	a
	8 ^b	35	LFU	+	5%				
	9	41	7	++	<1%				
A IV									
	10	49	N49	++	<1%				
	11 ^c	75	33	-	11%				
	12	74	2	+++	31%	a	a	a	a
	13	31	8	+++	33%				
	14	70	3	-	9%				
	15	68	15	++	15%				
	16	36	8	+	13%				
	17	59	4	-	12%				
	18	60	11	+++	11%				
	19	74	2	-	25%				
	20	49	8	+	18%				

Symbols and abbreviations:

disomy (two signals) polysomy (three or more signals) monosomy (one signal)

a = six signals for chromosome 7, four signals for chromosome 10

b = gemistocytic astrocytoma grade II; c = secondary glioblastoma

N = no tumour progression at follow-up; LFU = lost to follow-up;

On average 52% (range 34-100%) of the nuclei in A II showed normal (disomic) copy number for all three targets, while this was the case for only 33% (range 12-52%) of the nuclei in A IV (T-test, $p=0.04$). Table 2 shows that all tumors, with the exception of one A II (case 1), contained one or more populations of nuclei with aberrant chromosome copy numbers.

Polysomies, in particular tri- and tetrasomies for all investigated chromosomes were frequently present in A II (8 out of 10 cases). In these 8 A II specimens major cell

FIGURE 1. Double-FISH on a human metaphase with a 10q23 probe (red) and 10q12 (green) pericentromeric (C10) probe, clearly showing the specificity of both probes.

Illustrative cases of different genetic alterations, in:

(A-C) Astrocytomas grade II:

(A) Polysomy for both 10q25.3 and 10q12 in case 8.

(B) Relative loss of 10q25.3 as compared to 10q12 in case 9.

(C) Three different alterations in the same tumor: polysomy of 10q12 and 10q25.3, monosomy of both regions and selective loss of 10q25.3 in case 10.

(D) Astrocytoma grade IV with nuclei showing monosomy for 10q12 and 10q25.3 in case 19.

FIGURE 2. Bar graphs showing chromosome 10C and 10q25.3 copy number distributions of the corresponding cases of figure 1. The copy-number of 10q25.3 is shown on the X-axis, of 10C on the Y-axis and the percentage of nuclei on the Z-axis. The bars represent:

□ disomy ■ monosomy 10
 ■ polysomy ■ relative loss of 10q25.3

populations showed similar copy numbers for 10q25.3 and 10C, indicating maintenance of the 10q25.3 region. As a typical example of this A II tumor cell population the double-target FISH for 10q25.3 and 10C (*Figure 1A*) and the corresponding frequency distribution of spot numbers (*Figure 2A*) are illustrated for case 8.

In two A II, i.e. cases 9 and 10 in Table 2, cells with selective loss of 10q25.3 were detected, besides the major cell population with polysomic cells (*Figure 1B and 2B*).

In one of these specimens, i.e. case 10, loss of both 10C and 10q25.3 was noticed in 10% of the nuclei (*Figure 1C and 2C*). In 6 of 10 A IV (i.e. cases 14 to 20) loss of both 10C and 10q25.3, interpreted as monosomy for chromosome 10, was present. In 5 of these 6 specimens major fractions of cells with monosomy for chromosome 10 and minor fractions with disomic cells were observed (*Figure 1D and 2D*). Selective loss of 10q25.3 was present in two A IV specimens, i.e. cases 14 and 20, that also contained major cell populations with either polysomy or monosomy for chromosome 10, respectively. The only secondary glioblastoma in this series, i.e. case 11, exhibited a tetraploid clone, without selective loss of 10q25.3.

Since selective loss of 10q25.3 has been associated with malignant progression of A II, it is noticeable that both A II with selective 10q25.3 loss (i.e. cases 9 and 10) show a low proliferative activity and overexpression of P53. One of these patients (case 9) showed tumor progression within 7 months, while in the other patient (case 10) no tumor progression had occurred after 49 months of follow-up.

Discussion

In the underlying study we analyzed astrocytomas grade II (A II) and IV (A IV) for 10q25.3/10q12 and chromosome 1 and 7 copy number differences. So far the 10q25.3 region in astrocytomas has only been studied by molecular allelotyping, which has resulted in contradictory reports in the literature (see below). Therefore we applied the FISH technology, which allows for the detection of copy number changes for this chromosome region at the single cell level. Our results show that the majority of A II often contain gain of all investigated chromosomes, indicating overall polyploidy. A IV often contain loss of chromosome 10, gain of chromosome 7 and/or overall polyploidy. Copy number differences between 10q25.3 and 10C, indicating selective loss of 10q25.3, were detected in only a minority of both low- and high-grade astrocytomas.

A deletion mapping study in a large series of A II showed no partial deletions in 10q.²¹ Maier et al.^{11,20} reported partial deletions exclusively targeting to the 10q25-26 region

in 3 of 8 low-grade gliomas, but these lesions comprised two oligodendroglial tumors and only one A II. Finally, Steck et al.¹⁶ found losses of chromosome 10q in all four cases of A II in their series, which was in two cases limited to 10q25-26. The two latter research groups propose an initiating role for loss of the 10q25-26 region in the development of astrocytomas.^{11,16} However the combined results of the deletion mapping studies listed in *Table 1* show that selective loss of this subtelomeric region can only be detected in a limited number (<10%) of A II cases. A possible explanation for the higher frequency at which we detected a selective loss of 10q25.3 in A II (20% of the cases) as compared to the previously discussed literature, may be that disomic (normal) cells often constitute a considerable fraction in A II. This admixture with normal cells could explain the underestimation of 10q25-26 loss by molecular deletion mapping procedures.

In both A II with selective loss of 10q25.3 concomitant major fractions of cells with polysomic copy numbers for the pericentromeric chromosome 10q12 (10C) probe were present. In A IV the results of our FISH analyses are comparable to previous deletion mapping studies. From *Table 1* it is obvious that complete loss of chromosome 10 is more frequently detected in A IV than selective loss of 10q25-26. In accord with these observations we found monosomy for chromosome 10 in 6 out of 10 A IV and selective loss of 10q25.3 in only 2 out of 10 A IV. Because of this relatively low frequency of isolated loss of 10q25.3 as compared to the much higher frequency of the other chromosomal changes occurring in astrocytomas, we suggest that loss of 10q25.3 with the DMBT1 gene occurs as a secondary genetic event in astrocytomas and does not act as an initiating factor in A II carcinogenesis.

However, for two reasons the data should be interpreted with caution. First, small deletions and intragenic mutations could be missed by FISH analysis. Second, very recent data suggest that another tumor suppressor gene on 10q25.3-26.1, encoding an orphan G-protein-coupled receptor GPR26, might be epigenetically silenced in gliomas of different grades.³⁰

Some research groups have also suggested that loss of 10q25-26 can be regarded as a marker for progression of A II to A IV (secondary glioblastoma).^{14,29} Hulsebos et al.³¹ found that loss of heterozygosity (LOH) of 10q25-26 had occurred in 2 of 7 low-grade astrocytomas that had progressed to A IV, and in 3 of 15 recurrences of A IV. Fujisawa et al.¹⁴ reported LOH of 10q25-qter in 7 of 8 A IV foci, an alteration that was still not present in the low-grade area of the same tumors. In accord with this assumption we found that one of the A II cases with 10q25.3 loss (case 9) showed rapid malignant progression. In the other A II with 10q25.3 loss (case 10) no tumor progression has occurred after 49 months of follow-up. However, only in this specimen, derived through

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7

Summary

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Summary

Astrocytoma grade II (A II), or low-grade diffuse astrocytoma, is a primary brain tumor that in particular occurs in young adults with an estimated incidence between 0,5-1,0 per 100.00 persons/year. A II are histological characterized by an increased cellularity and nuclear atypia of astrocytes, the star-shaped supporter cells of the central nervous system. A II often have an indolent behavior only causing medical controllable epileptic seizures. However, because of the diffuse infiltration of normal brain which makes total resection impossible, the majority will ultimately progress to high-grade astrocytoma, i.e. astrocytoma grade III and IV (A III/ A IV). The interval to malignant progression of A II is highly variable and not reflected by histological features. Prognostic parameters to identify the subgroup of rapid progressive A II are therefore essential for optimizing treatment strategy.

Malignant progression of astrocytomas is accompanied by an accumulation of genetic alterations in the tumor-DNA and an increased proliferation activity. On bases of cytogenetic studies different 'genetic pathways' have been described, one pathway leads to the formation of primary (or de novo) A IV and a second pathway leading to A II and ultimately to secondary (or progressive) A IV.

Cytogenetic studies in astrocytomas have in particular been focused on grade III and IV astrocytomas, and less frequently on A II. The most frequent reported alteration in A II is loss or mutation of the p53 tumor suppressor gene, which probably does not correlate with the clinical behavior of the tumor. Other cytogenetic alterations have been less frequently reported in A II, due to limitations of the cytogenetic techniques used and due to contamination of normal cells that are often present in A II. Moreover the clinical relevance of cytogenetic analysis in A II is still unclear.

The in situ hybridization (ISH) -assay allows the detection of structural chromosomal aberrations at a single cell level and is therefore particularly suited for analysis of A II. We applied this technique on routinely processed tissue samples and determined whether the detection of chromosomal aberrations is of potential diagnostic and prognostic value in A II.

CHAPTER 1 contains a general introduction on gliomas and astrocytomas and in CHAPTER 2 the studies on prognostic parameters and current treatment strategies in astrocytoma grade II are reviewed. Most of these studies are retrospective and difficult to interpret due to selection bias and because most studies also include other variants of low-grade gliomas. From these studies patient age at diagnosis appears as the single

consistent unfavourable prognostic parameter, and patient age is therefore decisive for current treatment strategies. For patients over 35 years of age an aggressive treatment, comprising tumor resection and radiotherapy, is propagated. For patients under 35 years of age the effectiveness of such an approach has not been proven and for these patients the usefulness of histological confirmation by stereotactic biopsy is often doubted. A major cause for controversy with regard of optimal treatment of A II are limitations of the histological classification of these tumors. First, routine histology tends to underestimate tumor grade due to histological heterogeneity of astrocytomas. Particularly in small stereotactic samples differentiation between A II and reactive gliosis, a proliferation of astrocytes in response to non-neoplastic neurological diseases, is difficult. Second, slow and fast-progressive A II cannot be distinguished on bases of their histological features. Previous studies suggest that the Ki-67 proliferation marker may predict malignant progression of A II, however the prognostic value of cytogenetic markers is largely unknown.

CHAPTER 3 describes the use of the molecular cytogenetic technique *in situ* hybridisation (ISH) for the detection of chromosomal aberrations in small stereotactic samples with an inconclusive histological diagnosis, in which the neuro-pathologist was unable to differentiate between astrocytoma and reactive gliosis. ISH analysis in thirteen of these inconclusive cases was performed with probes for chromosome 1, 7 and 10. The probe for chromosome 1 is a general marker for aneuploidy, while gain of chromosome 7 and loss of chromosome 10 have often been implicated in astrocytoma carcinogenesis. In four of the inconclusive cases chromosomal aberrations were detected, in particular gain of chromosome 7. Similar aberrations were present in samples with proven A II and absent in samples with non-neoplastic reactive gliosis. Three of the four cases with chromosomal aberrations in the initial biopsy showed rapid malignant progression. Our data indicate that ISH has additional diagnostic value to detect neoplastic cells in stereotactical biopsies of patients suspected of diffuse astrocytomas in which routine microscopy is inconclusive.

CHAPTER 4 describes the prognostic value of chromosomal aberrations in A II, as detected by ISH. A series of 47 A II were investigated for numerical aberrations of chromosomes 1, 7 and 10. Our result show that more than half of the specimen contained aneusomies, in particular tri and tetrasomies. These chromosomal aberrations always involved chromosome 7. A clear correlation was present between the presence of nuclei with aneusomy for chromosome 7 and shorter survival of A II patients. The fact that similar aberrations for chromosome 7 were not detected in

reactive gliosis, contradicts the findings of previous karyotyping studies with trisomy 7 in non-neoplastic brain tissue. In this series monosomy for chromosome 10 was present in two of the samples. These samples showed a rapid malignant clinical course, which suggests that the presence of monosomy 10 in histological A II is due to a sampling error from A IV.

CHAPTER 5 compares the prognostic value of cytogenetic and biological parameters versus the prognostic value of clinical parameters in the same series of A II. The median survival interval for patients with A II was 90 months. On multivariate analysis independent prognostic parameters for shorter survival were patient age > 35 years of age, a Ki-67 proliferation index > 1% and the presence of chromosomal aberrations as detected by ISH. The median survival interval for A II with both a high Ki-67 proliferation index and aneusomy was 40 months, as compared to 140 months for tumors without this unfavorable combination. In particular in young patients, for whom treatment strategy is controversial, the detection of these parameters can clearly identify A II with rapid malignant progression. This is in our view an argument not to defer biopsy in young patients with radiological suspicion of A II.

CHAPTER 6 focuses on the 10q25.3 region, where the Deleted in Malignant Brain Tumor (DMBT1)- candidate tumor suppressor gene is located that might be important in development and/ or malignant progression of A II. The role of this region has been suggested by loss of heterozygosity (LOH)-analysis, however the evidence that led to this assumption is questionable. A series of A II and A IV were investigated by ISH with probes for the 10q25.3 and the pericentromeric region of chromosome 10. Only in a minority of both A II and A IV relative loss of the 10q25.3 region was detected. These samples always showed other chromosomal aberrations, in particular overall aneuploidy. These data indicate that loss of the 10q25.3 region is not an initiating event in astrocytoma carcinogenesis.

IN CONCLUSION, in situ hybridization has additional diagnostic and prognostic value to routine microscopy of A II. Chromosomal aberrations such as trisomy of chromosome 7 and aneuploidy are present in the majority of A II, and correlate with the clinical course. In future the detection of these and other cytogenetic alterations will help optimizing treatment strategy for astrocytoma patients.

Samenvatting

Gliomen zijn de meest frequent voorkomende primaire hersentumoren, en worden al naar gelang celtype onderverdeeld in astrocytomen, oligodendrogliomen en ependymomen. Astrocytoma graad II (A II), ook wel laaggradig diffuus astrocytoma genoemd, is een hersentumor die met name voorkomt bij jong volwassen patiënten met een incidentie van 0.5-1.0 per 100.000 personen per jaar. Een A II wordt gekenmerkt door een toegenomen celdichtheid en kernatypie van astrocyten (de stervormige steuncellen van het centraal zenuwstelsel). Doordat het A II zich diffuus uitbreidt tussen het gezonde hersenweefsel is complete neurochirurgische resectie zelden mogelijk. Dit is een van de oorzaken voor de huidige behandelingscontroverse voor patiënten met A II.

Een deel van de A II vertoont geen of nauwelijks groei en kan zo jarenlang geen klachten veroorzaken, of alleen medicamenteus te behandelen epileptische insulten. Echter, A II kan ook een snelle, maligne progressie doormaken naar het hooggradig astrocytoma (astrocytoma graad III, A III, of graad IV, A IV). Vanwege dit onvoorspelbare beloop is de behandelingsstrategie bij A II discutabel, en is het zoeken naar prognostische parameters van belang om de behandeling in de toekomst te kunnen optimaliseren.

De maligne progressie van A II gaat gepaard met een toename van celproliferatie en een opeenstapeling van genetische afwijkingen in het tumor-DNA. In tegenstelling tot A IV, zijn bij A II weinig studies naar deze cytogenetische afwijkingen verricht. De enige afwijking die vaak in A II gerapporteerd wordt is verlies of mutatie van het p53-gen, een afwijking die waarschijnlijk niet gecorreleerd is aan het klinisch beloop. Verder zijn weinig genetische afwijkingen in A II beschreven, hetgeen verklaard kan worden door de beperkingen van de gehanteerde cytogenetische technieken en doordat in A II vaak normale, niet-maligne, cellen voorkomen die de gevoeligheid van cytogenetische analyse negatief kunnen beïnvloeden.

De in situ hybridisatie (ISH) techniek is zeer geschikt voor het aantonen van chromosomale afwijkingen in A II, omdat deze techniek niet wordt beïnvloed door de aanwezigheid van normale cellen. Daarom werd onderzocht of de detectie van chromosomale afwijkingen in A II met ISH diagnostische en prognostische waarde heeft, hetgeen uiteindelijk kan leiden tot een optimalere behandeling van A II patiënten.

In HOOFDSTUK 1 wordt een korte inleiding over gliomen en astrocytomen gegeven, waarna in HOOFDSTUK 2 een overzicht van literatuurstudies wordt gepresenteerd die

de prognostische parameters in A II hebben onderzocht. De meeste van deze studies zijn retrospectief en moeilijk te interpreteren ten gevolge van selectiebias en doordat deze studies naast A II vaak ook andere varianten van laaggradige gliomen bevatten. Uit deze studies blijkt wel dat de leeftijd van de patiënt ten tijde van de diagnose de enige consistente prognostische parameter was voor het beloop van A II. Daarom is de leeftijd van de patiënt ook bepalend voor de huidige behandelingsstrategieën. Voor patiënten ouder dan 35 jaar wordt een intensieve behandeling, bestaande uit tumorresectie gevolgd door radiotherapie, geadviseerd. Voor jongere patiënten is de effectiviteit van deze interventies nooit bewezen. Ook bestaat er ernstige twijfel of het nemen van een stereotactische biopsie wel zinvol is bij jonge patiënten met een radiologische verdenking voor A II, aangezien histologische bevestiging het beleid toch niet verandert.

De behandelingscontroverse voor jonge patiënten met A II wordt veroorzaakt door beperkingen in de histologische gradering. Ten eerste wordt de tumorgraad van astrocytomen vaak te laag ingeschat door de histologische heterogeniteit van astrocytomen. Daardoor is vooral in een kleine biopsie het onderscheid tussen A II en reactieve gliose, een verlittekening die bij tal van niet-maligne neurologische aandoeningen kan voorkomen, vaak niet te maken. Ten tweede kan op basis van het histologisch beeld geen onderscheid worden gemaakt tussen langzaam-groeiend en snel-groeiende progressieve A II. Eerdere studies hebben gesuggereerd dat aanvullende immuunhistochemische kleuring voor de proliferatiemarkers Ki-67, de maligne progressie van A II wel kan voorspellen. Of het aantonen van specifieke genetische afwijkingen in A II ook prognostische waarde heeft, is nooit uitgebreid onderzocht.

HOOFDSTUK 3 beschrijft het nut van de in situ hybridisatie (ISH) techniek met behulp van het aantonen van chromosomale afwijkingen in stereotactisch biopsie-materiaal met een niet-conclusieve histologische diagnose, waarbij de patholoog niet kon differentiëren tussen A II en reactieve gliose. Dertien van deze gevallen werden onderzocht met ISH, waarbij gebruik werd gemaakt van probes voor de chromosomen 1, 7 en 10. Chromosoom 1 is een algemene marker voor ploïdie-status, terwijl winst van chromosoom 7 en verlies van chromosoom 10 worden verondersteld een rol te spelen bij de maligne progressie van astrocytomen. In 4 van de 13 niet-conclusieve gevallen waren chromosomale afwijkingen aanwezig, met name winst voor chromosoom 7. Deze afwijkingen waren aanwezig in controlemateriaal met eenduidige A II, maar afwezig in reactieve gliose. Deze studie toont het nut van cytogenetische analyse middels ISH van stereotactisch biopsiemateriaal met een niet-conclusieve histologische diagnose.

In HOOFDSTUK 4 wordt de prognostische waarde van chromosomale afwijkingen, gedetecteerd met ISH, bestudeerd. Hiervoor is een serie van 47 gevallen met A II onderzocht met probes voor de chromosomen 1, 7 en 10. In meer dan de helft van de tumoren waren chromosomale afwijkingen aanwezig, waarbij in alle gevallen chromosoom 7 numerieke afwijkingen vertoonde. Een duidelijke correlatie werd gevonden tussen een hoog percentage kernen met afwijkingen voor chromosoom 7 en een kortere overleving van de A II patiënten. Twee tumoren met verlies van chromosoom 10 vertoonden de snelste maligne onttaarding.

In HOOFDSTUK 5 is de prognostische waarde van deze cytogenetische parameters vergeleken met de prognostische waarde van klinische en biologische parameters in dezelfde A II serie. De mediane overleving van A II patiënten was 90 maanden. Na multivariate analyse bleken 3 onafhankelijke prognostische parameters te wijzen op een kortere overleving: 1) leeftijd van de patiënt > 35 jaar, 2) de Ki-67 proliferatie-index > 1% en 3) de aanwezigheid van chromosomale afwijkingen. De mediane overleving van patiënten met een hoge Ki-67 index en chromosomale afwijkingen was 40 maanden, vergeleken met een mediane overleving van 140 maanden van patiënten met A II zonder deze ongunstige combinatie. Vooral bij jonge patiënten blijkt dat met behulp van de proliferatie en genetische markers een subgroep snel-progressief verlopende A II kan worden geïdentificeerd. Wij concluderen dat dit een argument kan zijn om een stereotactische biopsie bij jonge patiënten met een radiologische verdenking van A II niet uit te stellen.

In HOOFDSTUK 6 wordt de 10q25.3 regio bestudeerd, waar het DMBT-1 gen is gelokaliseerd. Dit gen speelt mogelijk een rol bij het ontstaan en/ of de maligne progressie van A II. Deze rol is gesuggereerd na genetische analyse middels de loss of heterozygosity (LOH)-techniek, waarvan de resultaten echter niet eenduidig waren. Daarom onderzochten wij zowel A II als A IV met een probe voor de 10q25.3 regio, gecombineerd met een probe voor de centromeer regio van chromosoom 10, beiden gedetecteerd met ISH, hetgeen als voordeel heeft dat de analyse op individuele tumorcellen uitgevoerd kan worden. Relatief verlies van de 10q25.3 werd slechts in enkele gevallen van A II en A IV gevonden. Deze tumoren bevatten ook altijd andere genetische afwijkingen, met name polyploidie, hetgeen suggereert dat verlies van de 10q25.3 regio geen initiërende rol in de carcinogenese van A II speelt.

CONCLUSIE: de cytogenetische ISH-techniek heeft een aanvullende diagnostische en prognostische waarde in de routine diagnostiek van A II. Chromosomale afwijkingen

zoals trisomie van chromosoom 7 en polyploidie komen vaak voor in A II, en lijken te correleren met het klinisch beloop. Het implementeren van cytogenetische technieken in de routine diagnostiek zal in de toekomst kunnen bijdragen tot het verbeteren van de behandeling voor A II patiënten.

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Curriculum vitae

De schrijver van dit proefschrift werd geboren op 11 juni 1970 te Bilthoven. In 1988 werd het VWO van het Sint Bonifatiuscollege in Utrecht gevolgd door de studie Geneeskunde aan de Rijksuniversiteit Groningen. Tijdens het doctoraalexamen, afgerond in 1993, werd een keuzeonderzoek verricht getiteld 'Spierkrachtmeting bij kinderen met de handdynamometer', onder supervisie van Prof. Dr. H.J.G.H. Oosterhuis, neuroloog. Nadien werden de co-assistentenschappen gevolgd in het Sint Elisabeth hospitaal, Curaçao, Nederlandse Antillen. Na het artsexamen in 1995 werkte hij als arts-assistent neurologie in het academisch ziekenhuis Groningen en het Sint Clara ziekenhuis te Rotterdam, en als Senior House Officer Cardiology in het Freeman hospital in Newcastle upon Tyne, UK. In januari 1997 trad hij in dienst als AGNIO Neurologie in het academisch ziekenhuis te Maastricht, waar onder begeleiding van Dr. A. Twijnstra werd gestart met dit onderzoek. In november 1998 werd begonnen met de opleiding neurologie in het Maasland ziekenhuis te Sittard (B-opleider: Dr. H.W.A.M. Anten), waarna vanaf november 1999 de opleiding werd voortgezet in Maastricht (opleider: Prof. Dr J. Troost). Gedurende 1 jaar (mei 2000-2001) was hij werkzaam op het in situ hybridisatie laboratorium (Dr. A.H.N. Hopman) van de vakgroep Moleculaire Celbiologie (Prof. Dr F. C.S. Ramaekers), wat mogelijk werd gemaakt door een beurs van het Koningin Wilhelmina Fonds. In november 2004 hoopt hij de opleiding tot neuroloog af te ronden.