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Gain of Chromosome 7, as Detected by In Situ Hybridization, Strongly Correlates With Shorter Survival in Astrocytoma Grade 2

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The clinical course of astrocytoma grade 2 (A2) is highly variable and is not reflected by morphological characteristics. Earlier studies using small series of A2 cases suggest that in situ hybridization (ISH) with chromosome-specific DNA probes allows for frequent detection of aneusomy 1, trisomy 7, and monosomy 10. The role of trisomy 7 in astrocytoma carcinogenesis is disputed, however, because of its presence in non-neoplastic brain tissue, as detected by karyotyping. Our objective was to investigate whether there was a correlation between chromosomal aberrations and survival in a series of 47 cases of A2. All cases were evaluated for numerical aberrations of chromosomes 1, 7, and 10 by ISH. Chromosomal aberrations were detected in 68% of cases of A2. Trisomy/polysomy 7 was seen in 31 cases (66%), 22 of which (47%) had a high percentage of this numerical aberration. Only 11 of these 22 cases also showed aneusomy for 1 or 10. No cells or only a few 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 A2, correlating with the malignant progression of the disease. © 2002 Wiley-Liss, Inc.

INTRODUCTION

Genotypic characterization of astrocytoma grade 2 (A2) not only is important in understanding carcinogenesis but also might offer prognostic information. Such prognostic information may help resolve the controversy that exists with regard to the optimal treatment of A2. So far, only a few studies have investigated the correlation between cytogenetic aberrations and the clinical course of A2 (Kraus et al., 1994; Perry et al., 1997; Sallinen et al., 1997). Using classic karyotyping, Kimmel and colleagues (1992) showed that patients with astrocytomas with clonal abnormalities had shorter survival times compared with patients with astrocytomas without these abnormalities. Their group of cases of A2 was too small for separate analysis, however.

The most frequently reported genetic aberrations in A2 include mutation of *TP53*, loss of heterozygosity (LOH) of chromosome arm 17p (von Deimling et al., 1992), and trisomy 7 (Rey et al., 1987). A correlation between *TP53* mutations and survival of A2 patients seems unlikely (Kraus et al., 1994; Al-Sarraj and Bridges, 1995). Previous studies in small series of A2 cases suggest that in situ hybridization (ISH) and comparative genomic hybridization (CGH) allow for frequent detection of

chromosomal aberrations, such as gain of chromosomes 1 and 7, loss of chromosome 10, and hyperdiploidy (Table 1). Only two of these investigators correlated the cytogenetic results with the clinical course of patients with A2 (Perry et al., 1997; Sallinen et al., 1997). Sallinen et al. (1997) suggested the possible prognostic value of CGH in a series of 11 A2 cases. Perry et al. (1997) concluded that deletion of chromosome 10 was of prognostic value when studying the total spectrum of astrocytomas, while trisomy 7 showed no significant correlation with postoperative survival. The role of trisomy 7 in carcinogenesis of astrocytomas is controversial, given the fact that this aberration has been detected in cultured, histologically nonmalignant brain tissues (Heim et al., 1989; Moertel et al., 1993). In contrast, trisomy 7 has not been detected by ISH of normal and gliotic brain tissue (Arnoldus et al., 1992; Dalrymple et al., 1994). The objective

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TABLE I. Summary of Results in the Literature of In Situ Hybridization (ISH) and Comparative Genomic Hybridization (CGH) Studies in Adult Astrocytoma Grade 2 (A2)*

Method and chromosomes examined	Number of cases (A2)	Chromosome aberrations detected	Reference
ISH 10, 17	11	-10, -17, +17	Campomenosi et al., 1996
ISH 7, 10, X, Y	10	+7, -10	Liu et al., 1997
ISH 7, 10, 3	11	+7, -10	Perry et al., 1997
ISH 1, 7, 10, 17, X, Y, 1p36	4	+7, Aneuploidy	Rosso et al., 1997
ISH 7, 10	3	Tetrasomy 7 and 10	Steilen-Gimbel et al., 1996
ISH 1, 2, 7, 1p36	9	+1, -1p36, +7, +8q	Wernicke et al., 1997
CGH	9	-1p, +1pter, +7q, +8q,	Nishizaki et al., 1998
CGH	11	-10	Sallinen et al., 1997
CGH	10	+7, +7q, +8q	Schrock et al., 1994
CGH	10	-5p, +8q, +12p, +19p, -Xp	Weber et al., 1996

*For CGH, the most frequent aberrations are listed.

in this study was to reinvestigate the prognostic value of chromosome 7 aberration in A2. The ISH technique was applied to routinely processed, paraffin-embedded biopsy and resection samples, allowing for precise correlation of the genetic constitution of cells with their histologic features.

MATERIALS AND METHODS

Material from Patients

Forty-seven biopsy and resection samples diagnosed as supratentorial low-grade diffuse astrocytoma or A2 were collected from the files of the Departments of Pathology of the University Hospitals of Maastricht and Groningen and the Atrium Hospital in Heerlen. Astrocytomas diagnosed in patients under the age of 18 years were excluded. The samples were revised according to the World Health Organization (WHO) classification (Kleihues et al., 1993). Overall survival was assessed from the patients' records. As controls, 10 samples with non-neoplastic reactive gliosis were examined (Wessels et al., 2001). These samples were derived at post-mortem examination from patients who died from non-neoplastic neurological causes, that is, brain infarction (n = 5), hemorrhagic infarction (n = 1), traumatic hemorrhage (n = 2), and hypoxic encephalopathy (n = 2).

ISH Protocol

The most representative paraffin blocks were selected from the 47 A2 cases. Five-micron-thick sections were cut and pretreated according to a recently optimized protocol (Hopman and Ramaekers, 1998). After deparaffinization in xylol and dehydration in an ethanol series, the tissues were

pretreated in 85% formic acid containing 0.3% H₂O₂ for 20 min at room temperature. After dehydration in an acidified ethanol series, the specimens were incubated at 80°C in 1 M sodium thiocyanate. Proteolytic digestion was performed in 4 mg/mL pepsin (from porcine stomach; 2,500–3,500 U per 100 mg protein; Sigma, St. Louis, MO) for 10 min 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 min, followed by five subsequent washing steps in PBS and double-distilled water.

ISH was performed as described earlier (Hopman and Ramaekers, 1998). The DNA probes used were specific for the heterochromatin (sub-)centromere region of chromosome 1 (1q12, pUC 1.77; Cooke and Hindley, 1979), the alphoid region of chromosome 7 (p7t1; Waye et al., 1987), and the centromere region of chromosome 10 (D10Z1; Devilee et al., 1988). After denaturation at 80°C for 5 min, hybridization was performed overnight at 37°C under a coverslip in a mixture containing 2× standard saline citrate (SSC), 60% formamide, 10% dextran sulfate, and 0.2 mg/mL herring sperm DNA. Post-hybridization washes included two washes in 2× SSC/0.05% Tween at 40°C for 5 min, followed by two washes in 0.1× SSC at 60°C for 5 min. The biotin-labeled DNA probes were detected by an immunoperoxidase precipitation reaction. The slides were incubated for 30 min at room temperature with mouse-antibiotin monoclonal antibody, followed by biotinylated horse-anti-mouse immunoglobulin G and then with avidin-biotin-peroxidase complex (all reagents from Vector Laboratories, Burlingame, CA). Finally, dia-

minobenzidine (Sigma) in PBS containing 0.03% H_2O_2 was applied for visualization of the peroxidase activity. To improve identification of overlapping nuclei, we used a novel method that includes fluorescent DNA counterstaining with 4'6-diamino-2-phenylindole (DAPI; Sigma) and bright-field ISH. Microscopy was performed using a Leica-DMBRE microscope (Leica Mikroskopie und Systeme GmbH, Wetzlar, Germany) equipped with a filter set for DAPI.

Evaluation of ISH

The number of ISH signals per nucleus was counted in at least 200 nonoverlapping nuclei, following the criteria proposed by Hopman et al. (1992). Monosomy was defined as >25% of nuclei with no signal or one signal per nucleus. Trisomy/polysomy is defined as $\geq 5\%$ of nuclei containing three or more signals. In accordance with other studies, 20% of aberrant nuclei was used as the cutoff for a high-frequency aberration (Campomenosi et al., 1996; Perry et al., 1997). 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

Histologic revision of the 47 cases of A2 showed 42 (89%) cases of fibrillary astrocytoma, three (7%) cases of gemistocytic astrocytoma, and two (4%) cases of oligo-astrocytomas (all WHO grade 2). Forty-five cases were primary tumors, and two were recurrences from A2. No patients had been previously subjected to radiotherapy. Twenty-nine (62%) patients had undergone stereotactic biopsy, and in 18 (38%) patients the samples had been obtained by surgical resection. The follow-up period varied from 24 to 200 months. The median survival interval was 90 months (95% CI: 72–108 months).

Using probes for chromosomes 1, 7, and 10, the samples with reactive gliosis showed no cells or only sporadic cells with three ISH signals, and they were all classified as normal for all three chromosomes (Fig. 1a). Of the cases of A2, 31 (66%) showed aberrations for one or more of the chromosomes investigated (Table 2). The frequency of cells exhibiting numerical chromosomal aberrations exceeded 5% in 51% of the cases for chromosome 1, in 66% of the cases for chromosome 7 (Fig. 1b), and in 53% of the cases for chromosome 10. High-

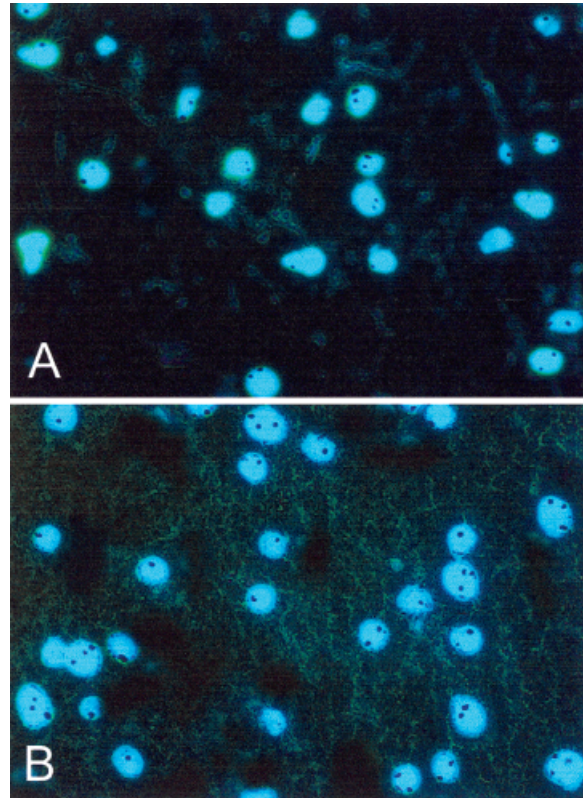


Figure 1. Bright-field microscopy of ISH for chromosome 7 combined with DAPI counterstaining of nuclei. **A:** A sample of reactive gliosis with no aberrations for chromosome 7. **B:** A2 showing many nuclei with three signals for chromosome 7. Magnification for both A and B $\times 800$.

TABLE 2. Summary of In Situ Hybridization Results in 47 Cases of Astrocytoma Grade 2*

Category	Chromosome 1	Chromosome 7	Chromosome 10
Normal ^a	23 (49%)	16 (34%)	22 (47%)
Low-aberrant ^b	21 (45%)	9 (19%)	15 (32%)
High-aberrant ^c	3 (6%)	22 (47%)	10 (21%)

^aNormal = <5% of cells per case with chromosomal aberrations.

^bLow-aberrant = 5–20% of cells per case with chromosomal aberrations.

^cHigh-aberrant = >20% of cells per case with chromosomal aberrations.

*The number (percentage) of cases with numerical chromosomal aberrations belonging to the indicated categories is presented.

frequency aberrations with increased copy numbers most often were detected for chromosome 7. In 47% 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

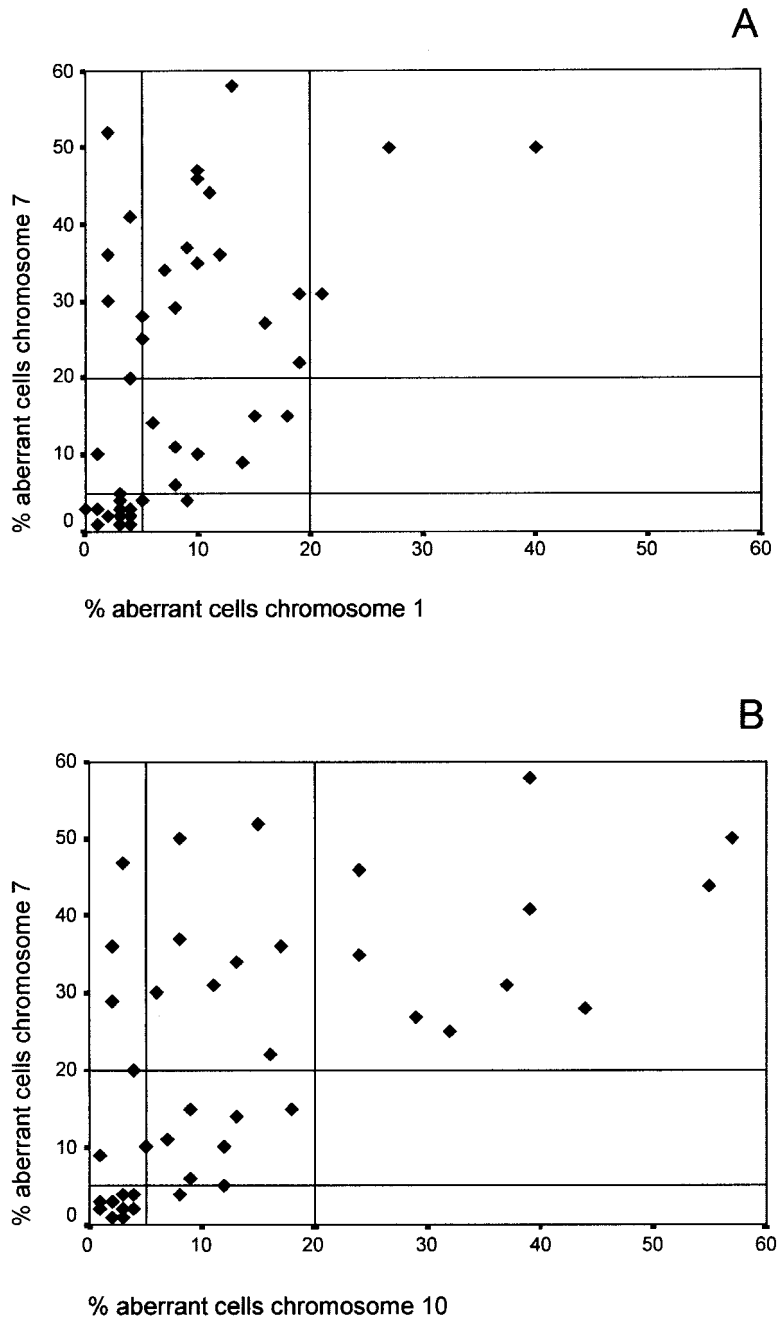


Figure 2. Scattergram showing the percentages of aberrant nuclei for chromosome 7 versus chromosome 7 (**A**) and for chromosome 7 versus chromosome 10 (**B**) for each of the individual cases of A 2 ($n = 47$).

showed higher copy numbers (three cases). All numerical aberrations of chromosomes 1 or 10 were 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 (Fig. 2a) and chromosomes 7 and 10 (Fig. 2b) have been depicted. As is shown, virtually all cases with aberrations of chromosomes 1 or 10 also exhibited high frequencies of

cells with aberrations of chromosome 7. The reverse, that is, cases with aberrations of chromosome 7 also showing chromosome 1 and 10 deviations, holds true only 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, correlated with shorter survival (log-rank test: $P = 0.028$). High-frequency aberrations of chromosome 7 (>20% of trisomic/polysomic cells as cutoff; high-aberrant group) cor-

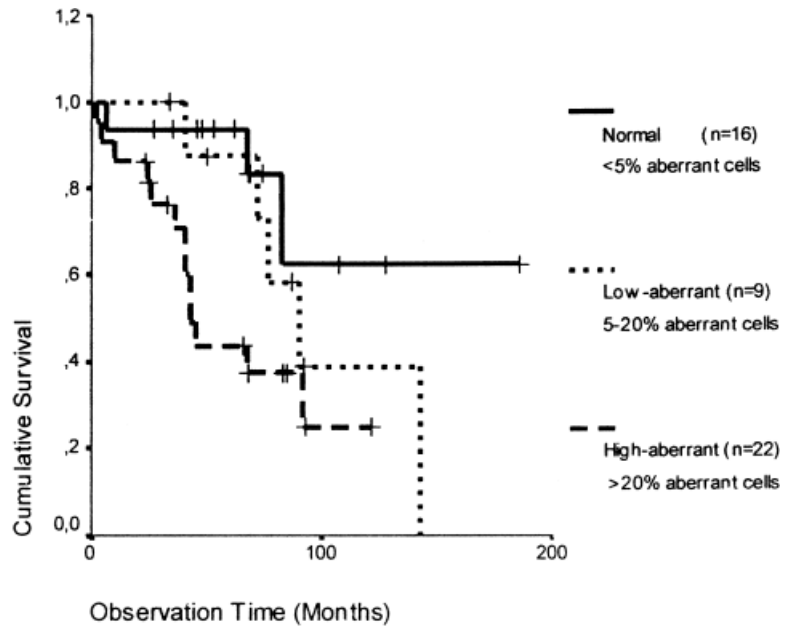


Figure 3. Survival of patients with A2 classified on the basis of aberrations of chromosome 7. High percentages (>20%) of aberrant nuclei for chromosome 7 correlate most strongly with shorter survival times (log-rank: $P = 0.028$).

related strongly with shorter survival times, compared with A2 with disomy or a low frequency of aberrations for chromosome 7 (normal and low-aberrant group taken together; log-rank test: $P = 0.008$; not shown in Fig. 3). The two tumors with monosomy 10 were associated with extremely short survival intervals, that is, 10 and 24 months.

DISCUSSION

The present study shows that numerical aberrations of chromosome 7 occur frequently in A2 and that their detection by ISH is 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 (Arnoldus et al., 1991; Dalrymple et al., 1994). This may suggest that trisomy 7, as detected by classic banding analysis in normal (Heim et al., 1989) and gliotic (Moertel et al., 1993) brain tissue, most probably is due to culturing artifacts. Another possible explanation is that the normal brain tissues in the study of Heim et al. (1989) contained malignant cells, because seven of their 11 patients were reported to have malignant brain tumors. Owing to limitations of the ISH technique, however, we cannot rule out the presence of small clones of cells with trisomy 7 in our gliotic samples.

Our ISH findings in A2 corroborate results of previous, smaller series (Table 1). Perry et al. (1997) described trisomy 7 in half of the A2 cases

they examined. In a double-labeled ISH experiment, Steilen-Gimbel et al. (1996) showed gains of both chromosomes 7 and 10. CGH studies also suggest that gain or amplification of chromosome 7 and arm 8q, in particular, is typical of A2 (Table 1). In this study we showed that high-frequency trisomy/polysomy of chromosome 7 is strongly correlated with shorter survival times, suggesting clonal expansion of this cell population during the malignant progression of A2.

Polysomy, in particular trisomy, of chromosome 7 not only is detected frequently in A2 but also is seen in other preneoplastic and neoplastic lesions. Trisomy 7 was shown to correlate with poor prognosis in prostate carcinoma (Bandyk et al., 1994). Furthermore, this aberration is present in the stem-cell compartment of colon carcinoma and has been suggested to be involved in the transition of colon adenoma to carcinoma (Herbergs et al., 1996). Thyroid hyperplasia with trisomy 7 is thought to progress more often to adenoma and carcinoma (Belge, 1994).

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 cannot support the suggestion that this represents a preneoplastic process (Moertel et al., 1993). Furthermore, the presence of trisomy/polysomy 7 did not correlate with the age of the patients in the series of A2 cases described here (unpublished

results), as was suggested for several other types of solid tumors by Broberg et al. (2001). We conclude that A2 frequently shows 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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