[CANCER RESEARCH 64, 6892–6899, October 1, 2004]

Genetic Pathways to Glioblastoma: A Population-Based Study

Hiroko Ohgaki,[1] Pierre Dessen,[1] Benjamin Jourde,[1] Sonja Horstmann,[1] Tomofumi Nishikawa,[1] Pier-Luigi Di Patre,[1] Christoph Burkhard,[1] Danielle Schüler,[2] Nicole M. Probst-Hensch,[2] Paulo César Maiorka,[1] Nathalie Baeza,[1] Paola Pisani,[1] Yasuhiro Yonekawa,[4] M. Gazi Yasargil,[3] Urs M. Lütolf,[4] and Paul Kleihues[4]

[1]International Agency for Research on Cancer, Lyon, France; [2]Cancer Registry, Canton of Zurich, Zurich, Switzerland; [3]Department of Neurosurgery, College of Medicine, Little Rock, Arkansas; and [4]Departments of Neurosurgery, Radiology, and Pathology, University Hospital Zurich, Zurich, Switzerland

ABSTRACT

We conducted a population-based study on glioblastomas in the Canton of Zurich, Switzerland (population, 1.16 million) to determine the frequency of major genetic alterations and their effect on patient survival. Between 1980 and 1994, 715 glioblastomas were diagnosed. The incidence rate per 100,000 population/year, adjusted to the World Standard Population, was 3.32 in males and 2.24 in females. Observed survival rates were 42.4% at 6 months, 17.7% at 1 year, and 3.3% at 2 years. For all of the age groups, younger patients survived significantly longer, ranging from a median of 8.8 months (<50 years) to 1.6 months (>80 years). Loss of heterozygosity (LOH) 10q was the most frequent genetic alteration (69%), followed by *EGFR* amplification (34%), *TP53* mutations (31%), $p16^{INK4a}$ deletion (31%), and *PTEN* mutations (24%). LOH 10q occurred in association with any of the other genetic alterations and was predictive of shorter survival. Primary (*de novo*) glioblastomas prevailed (95%), whereas secondary glioblastomas that progressed from low-grade or anaplastic gliomas were rare (5%). Secondary glioblastomas were characterized by frequent LOH 10q (63%) and *TP53* mutations (65%). Of the *TP53* mutations in secondary glioblastomas, 57% were in hotspot codons 248 and 273, whereas in primary glioblastomas, mutations were more equally distributed. G:C→A:T mutations at CpG sites were more frequent in secondary than primary glioblastomas (56% *versus* 30%; $P = 0.0208$). This suggests that the acquisition of *TP53* mutations in these glioblastoma subtypes occurs through different mechanisms.

INTRODUCTION

Glioblastomas are the most frequent and malignant human brain tumors, and despite advances in surgical and clinical neuro-oncology, their prognosis remains poor. In a meta-analysis of 12 randomized clinical trials, the overall survival rate of high-grade gliomas (*e.g.*, glioblastomas and anaplastic astrocytomas) was 40% at 1 year and only slightly higher (46%) after combined radiotherapy and chemotherapy (1). Even these depressingly low survival rates may be overoptimistic because trial protocols contain admission criteria that often exclude patients with a particularly unfavorable clinical course. Therefore, this study assessed survival on a population-based level.

From a clinical and biological point of view, a distinction between primary and secondary glioblastoma is important. Primary (*de novo*) glioblastomas manifest rapidly, without evidence of less malignant precursor lesions, after a short clinical history. Secondary glioblastomas develop more slowly by progression from low-grade [World Health Organization (WHO) grade II] or anaplastic astrocytoma (WHO grade III; ref. 2). These glioblastoma subtypes affect patients at different ages and through different genetic pathways (2, 3). However, no data are available on the relative frequency of these glioblastoma subtypes at a population level.

Oncogenes (*EGFR*, *PDGF* and its receptors) and tumor suppressor genes ($p16^{INK4a}$, $p14^{ARF}$, *PTEN*, *RB1*, and *TP53*) are involved in the evolution of glioblastomas. Frequent loss of heterozygosity (LOH) at 1p, 10p, 10q, 19q, and 22q suggests the participation of additional tumor suppressor genes (2, 4–6). Whereas older age was shown to be predictive of poorer prognosis in several studies, the definition of genetic alterations predictive of response to therapy has been inconclusive, at least in part because of the usually small number of cases investigated (2, 7).

The present study is the first to examine key genetic alterations in glioblastomas and their impact on survival rates in a large population-based series of cases. It is based on 715 inhabitants of the Canton of Zurich, Switzerland who developed a glioblastoma during the period 1980 to 1994. We assessed the incidence of glioblastoma subtypes, survival rates, and key genetic alterations in this defined population.

MATERIALS AND METHODS

Patient Population. This study included 715 newly diagnosed cases of glioblastoma (International Classification of Diseases for Oncology 94403, 94413, and 94423; ref. 2) that occurred in the resident population of the Canton of Zurich, Switzerland (~1.16 million) during the 15-year period 1980 through 1994 (8). The incidence date was fixed as the date of the pathology report for patients who underwent surgery or the date of clinical diagnosis otherwise (including autopsied cases). Clinical diagnoses were based on CT or MRI. Survival time was computed as the time between the incidence date and the date of death, date of last contact if lost from follow-up evaluation, or December 31, 1999. Follow-up evaluation was complete for 99% of the cases with the mean follow-up time of 7.2 ± 7.6 months. Death certificates were collected at the Cantonal Cancer Registry. The mean age of patients was 61.3 ± 14.0 years. The age distribution of patients was as follows: <39 years, 6.9%; 40 through 49 years, 12.5%; 50 through 59 years, 21.1%; 60 through 69 years, 29.9%; 70 through 79 years, 22.1%; and >80 years, 7.6%.

Histology Review. The 715 glioblastomas included 8 giant-cell glioblastomas and 5 gliosarcomas. The majority of cases (571 cases, 80%) were histologically confirmed following surgical intervention (385 cases, 54%) or at autopsy (186 cases, 26%). The remaining 144 cases (20%) were clinically diagnosed only by CT or MRI, typically based on the presence of an irregularly shaped lesion of contrast enhancement with a central area of necrosis and perifocal edema (2). The original histologic specimens of 549 of 571 (96%) histologically diagnosed cases were reevaluated by two neuropathologists (P-L.D.P., P.K.) according to the new WHO classification of tumors of the nervous system (2).

Glioblastoma Subtypes. The following criteria were used to distinguish between glioblastoma subtypes: Tumors were considered primary (*de novo*) when the glioblastoma diagnosis was made at the first biopsy, without clinical or histopathologic evidence of a less malignant precursor lesion. The diagnosis of secondary glioblastoma was made only in cases with histopathologic evidence of preceding low-grade or anaplastic glioma.

Treatment. Approximately half of the patients (384 of 715; 54%) with glioblastomas underwent partial or complete surgical resection, usually at the Department of Neurosurgery, University Hospital, Zurich. The mean age of patients who underwent partial or complete surgical resection was significantly younger (56.1 ± 12.7 years) than those who did not undergo surgery (67.5 ± 12.8 years; $P < 0.0001$). Survival rates of patients who underwent

Received 4/27/04; revised 6/18/04; accepted 7/19/04.

Grant support: The Cancer League of the Canton of Zurich, the Federal Office of Public Health, Bern, Switzerland, and the Foundation for Promotion of Cancer Research, Japan.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Hiroko Ohgaki, International Agency for Research on Cancer, 150 cours Albert Thomas, 69372 Lyon Cedex 08, France. Phone: 33-472-73-85-34; Fax: 33-472-73-85-64; E-mail: ohgaki@iarc.fr.

©2004 American Association for Cancer Research.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/64/19/6892/2518559/zch01904006892.pdf by guest on 24 June 2024

partial or complete surgical resection were significantly longer (median, 7.9 ± 0.5 months) than those who did not undergo surgery (2.5 ± 0.1 months; $P < 0.001$). Information on radiotherapy was available for 494 cases (69%); of these, 307 (62%) received radiotherapy, usually with 2-Gy fractions and a total dose of 60 Gy. The mean age of patients who received radiotherapy was 54.6 ± 11.4 years, significantly younger than those who did not receive radiotherapy (68.4 ± 12.7 years; $P < 0.0001$). The survival rate of patients who received radiotherapy was significantly longer (median, 10 ± 1.2 months) than those who did not receive radiotherapy (2.0 ± 0.3 months; $P < 0.001$).

***TP53* Mutations.** DNA was extracted from paraffin sections as reported previously (9). Prescreening for mutations in exons 4 through 8 of the *TP53* gene by PCR–single-strand conformational polymorphism analysis was carried out as described previously (9). Primers for exon 4 were 5′-ACTGCTCTTT-TCACCCATCTAC-3′ (sense) and 5′-TCATGGAAGCCAGCCCCTCAG-3′ (antisense). Samples that showed mobility shifts in single-strand conformational polymorphism analysis were further analyzed by direct DNA sequencing on an automated sequencing system (ABI PRISM 3100 Genetic Analyzer; Applied Biosystems, Foster City, CA) using an ABI PRISM BigDye Terminator version 1.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems).

***EGFR* Amplification.** To detect *EGFR* amplification, differential PCR was performed as described previously (10) using the cystic fibrosis (*CF*) sequence as a reference. After PCR (26 to 30 cycles), PCR products were separated on 8% acrylamide gels using a Bio-Rad electrophoresis system (Bio-Rad, Hercules, CA). Gels were stained with ethidium bromide and digitalized using the Digital Science 1D analysis system (Kodak Digital Science, New Haven, CT). Quantitative analysis of the signal intensity was performed with the PhosphorImager 445 SI and the ImageQuant software (Molecular Dynamics, Urbana, IL). The mean *EGFR* to *CF* ratio of normal control DNA (peripheral blood of healthy adult donors) was 1.11 ± 0.05 (mean ± SD). The value of 2.36 (2× mean ± 3× SD) was regarded as the threshold for evidence of *EGFR* amplification (10).

Differential PCR for *p16^{INK4a}* Homozygous Deletion. To assess *p16^{INK4a}* homozygous deletion, differential PCR was carried out using a primer set located in exon 1α of the *p16^{INK4a}* gene and a β-actin sequence as a reference as reported previously (11). Briefly, DNA was amplified with 30 cycles of PCR, and the PCR products were separated on 7% acrylamide gels. Gels were photographed using a DC120 Zoom Digital Camera (Kodak, Rochester, NY), and densitometry of the PCR fragments was performed as described previously. Samples in which the *p16^{INK4a}* to β-actin ratio was $\leq$0.20 were considered as having homozygous deletion (11).

***PTEN* Mutations.** Prescreening for mutations in exons 1 through 9 of the *PTEN* gene by PCR–single-strand conformational polymorphism analysis was carried out as described previously (10). Samples that showed mobility shifts in single-strand conformational polymorphism analysis were further analyzed by direct DNA sequencing on an automated sequencing system as described previously (10).

Quantitative Microsatellite Analyses for LOH on Chromosome 10q. Quantitative microsatellite analysis was carried out using two microsatellite markers on chromosome 10q (12). The microsatellite markers are located within the commonly deleted regions of 10q23 (D10S536) and 10q25 (D10S1683). PCR reactions were performed in a total volume of 12.5 μL with 2× TaqMan Universal PCR Master Mix, 0.4 μmol/L of each primer, 60 nmol/L probe [21-bp oligomer complementary to the microsatellite CA repeat: 5′,6-carboxyfluorescein (FAM)-TGT GTG TGT GTG TGT GTG TGT-3′ 6-carboxy-tetramethylrhodamine], and ~10 ng DNA, with cycling parameters as reported previously (12). Primers, probe, and the TaqMan master mix were purchased from Proligo Primers and Probes (Paris, France), and PCR was carried out for each individual DNA in triplicate on a 96-well optical plate with an ABI 7900HT instrument (Applied Biosystems). The amplification of a reference pool of six reference loci served to normalize for differences in the amount of total input DNA as described previously (12). To calculate the average δCt [δCT (normal)], DNA was isolated from 10 formalin-fixed, paraffin-embedded normal tissues. The Ct, δCt [Ct (microsatellite) − Ct (reference pool)], δδCt [δCt (tumor) − δCt (normal)] values, the relative copy number ($2^{-\delta\delta Ct}$), and the tolerance interval (TI) with a confidence of 95% determined from the pooled SD of normal DNA for both microsatellite loci were calculated as reported previously (12). On the basis of this TI, copy numbers <1.42 were considered to represent losses, whereas those >2.81 were considered to be gains.

Statistical Analyses. The mean annual incidence rates per 100,000 population by sex were age-adjusted to the World Standard Population, the European Population, and to the United States standard of 2000 (13) and were calculated using 5-year age groups. Ninety-five percent confidence intervals (CIs) were calculated after the logarithm transformation. Student's *t* test was carried out to compare the mean age of patient groups. The log-rank test was carried out to assess univariate comparisons in survival rates. Multivariate Cox's regression was carried out to identify predictive factors of survival of glioblastoma patients. Logistic regression analysis was carried out to assess associations between different genetic alterations.

RESULTS

Incidence Rates. The incidence rates of glioblastomas for the years 1980 through 1994, age-adjusted to the World Standard Population, were 3.32 (CI, 2.69–4.09) for male cases and 2.24 (CI, 1.56–3.22) for female cases. The rates were higher when adjusted using the United States standard population, 4.63 (CI, 3.83–5.59) in male cases and 2.88 (CI, 2.18–3.79) in female cases.

Survival of Glioblastoma Patients. The median survival time of all of the glioblastoma patients after glioblastoma diagnosis was 4.9 months. Observed survival rates of glioblastoma patients were 42.4% at 6 months, 17.7% at 1 year, 3.3% at 2 years, and 1.2% at 3 years.

Age and Sex. Univariate and multivariate analyses showed that old age is a significant predictive factor for poor survival of glioblastoma patients (Fig. 1). Younger patients (<50 years) showed significantly longer survival (median, 8.8 months) than older patients (>50 years; median, 4.1 months; $P < 0.001$). Among patients >50 years, age was predictive of observed survival rates (Fig. 1). The male to female ratio of all of the glioblastoma patients was 1.28.

Glioblastoma Subtypes. In 38 glioblastomas (5.3% of all of the glioblastomas in this population), there was clinical and histopathologic evidence of progression from a less malignant precursor lesion, and these were diagnosed as secondary glioblastomas. The remaining 677 patients with glioblastoma (94.7%) showed neither clinical nor histopathologic evidence of evolution from a less malignant lesion and were classified as primary glioblastoma.

Primary glioblastomas developed in older patients (mean, 62 years), and secondary glioblastomas developed in middle-aged individuals (Figs. 2*A* and 3). Primary glioblastomas developed more frequently in male cases, and secondary glioblastomas were more frequently found in female cases (Fig. 3). Log-rank test showed that patients with primary glioblastomas had significantly shorter survival than those with secondary glioblastoma (Fig. 4), but age-adjusted multivariate analysis showed no significant difference.

Frequency of Genetic Alterations. The most frequent genetic alteration in glioblastomas was LOH 10q (69%), followed by *EGFR* amplification (34%), *TP53* mutations (31%), *p16^{INK4a}* homozygous deletion (31%), and *PTEN* mutations (24%). Similar frequencies of genetic alterations were observed in primary glioblastomas (Fig. 3). In secondary glioblastomas, *TP53* mutations and LOH 10q were frequent (65% and 63%), whereas the other alterations were infrequent (4 to 19%; Fig. 3). Univariate analysis showed that absence of *TP53* mutations and presence of LOH 10q were predictive of shorter survival of patients (Fig. 4). However, after age-adjustment (multivariate Cox's regression analysis), only the presence of LOH 10q was associated with shorter patient survival ($P = 0.067$). Patients with glioblastoma carrying a *TP53* codon 175 mutation showed shorter survival (median, 4.5 months) than those carrying the other mutation (7.8 months), but the difference was not statistically significant ($P = 0.17$).

Copresence of Genetic Alterations. For 240 glioblastomas, data were available on *TP53* mutations, *EGFR* amplification, *p16^{INK4a}* homozygous deletion, *PTEN* mutations, and LOH 10q. The most frequent combinations were LOH 10q and *EGFR* amplification

Downloaded from http://aacrjournals.org/cancerres/article-pdf/64/19/6892/2518559/zch01904006892.pdf by guest on 24 June 2024

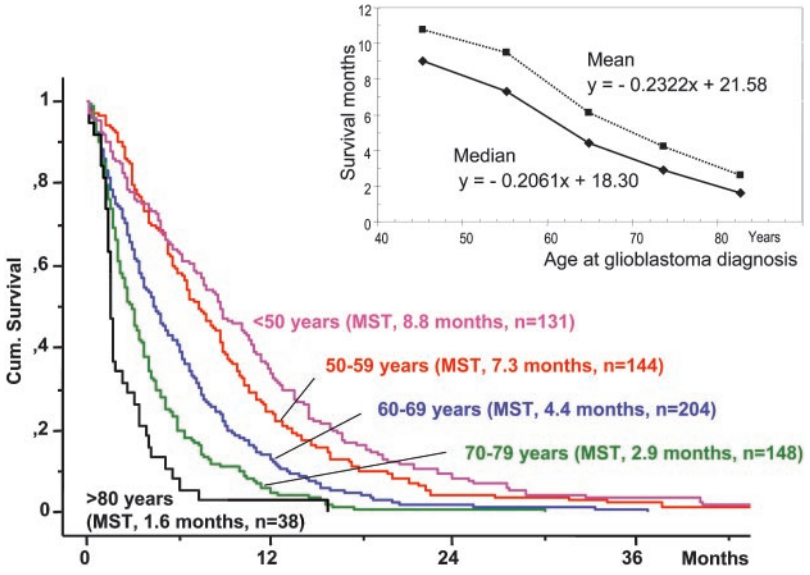

Fig. 1. Kaplan-Meier curves showing that younger age of patients with glioblastoma is predictive for longer survival (log-rank test: <50 years *versus* >50 years, $P < 0.001$; 50 to 59 years *versus* 60 to 69 years, $P < 0.001$; 60 to 69 years *versus* 70 to 79 years, $P < 0.001$; 70 to 79 years *versus* >80 years; $P = 0.0261$). On the basis of these data, formulae were established to calculate the median and mean survival time from the date of glioblastoma diagnosis (*right*).

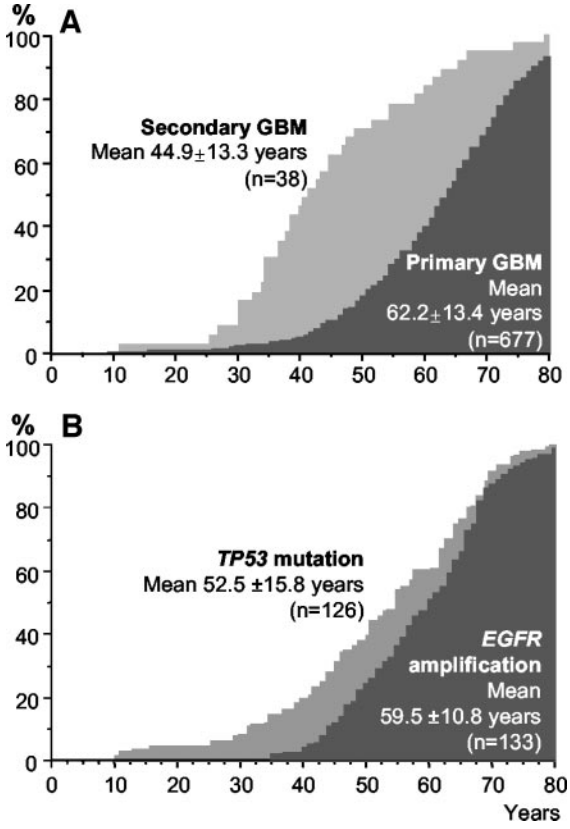

Fig. 2. *A*, cumulative age distribution of patients with primary and secondary glioblastomas. Secondary glioblastomas develop in younger patients than primary glioblastomas. *B*, cumulative age distribution of patients with glioblastomas with *TP53* mutations and those with *EGFR* amplification. *TP53* mutations are present in glioblastomas in all of the age groups of patients, whereas no glioblastomas in patients <35 years show *EGFR* amplification.

(25.8%), LOH 10q and *TP*53 mutations (23.7%), and LOH 10q and $p16^{INK4a}$ homozygous deletion (23.3%), followed by $p16^{INK4a}$ homozygous deletion and *EGFR* amplification (17.0%) and LOH 10q and *PTEN* mutations (16.2%), whereas other combinations of genetic alterations were infrequent (Fig. 5*A*).

Univariate and multivariate analyses showed that *EGFR* amplification and $p16^{INK4a}$ deletion tend to occur simultaneously (Fig. 5*A* and *B*). In contrast, *TP53* mutations, $p16^{INK4a}$ deletion, *EGFR* amplification, and *PTEN* mutations showed inverse associations with each other (Fig. 5*A* and *B*).

Type and Distribution of *TP53* Mutations and Polymorphisms. One hundred seventy *TP53* mutations were observed in 126 of 402 glioblastomas analyzed (31%). Double mutations were found in 26 cases; three mutations were found in 7 cases; and four mutations were found in 1 case. Of 170 mutations, 145 (85.3%) were missense mutations leading to amino acid change, 5 (2.9%) were nonsense mutations, 10 (5.9%) were deletions leading to stop codons, 1 (0.6%) was insertion leading to a stop codon, 5 (2.9%) were in-frame deletions, 1 (0.6%) was in-frame insertion, and 3 (1.8%) were splicing mutations. Fifty-three percent of deletions and insertions were located in codons 150 through 167.

In secondary glioblastomas, 57% of point mutations were in codons 248 and 273, whereas in primary glioblastomas, point mutations were

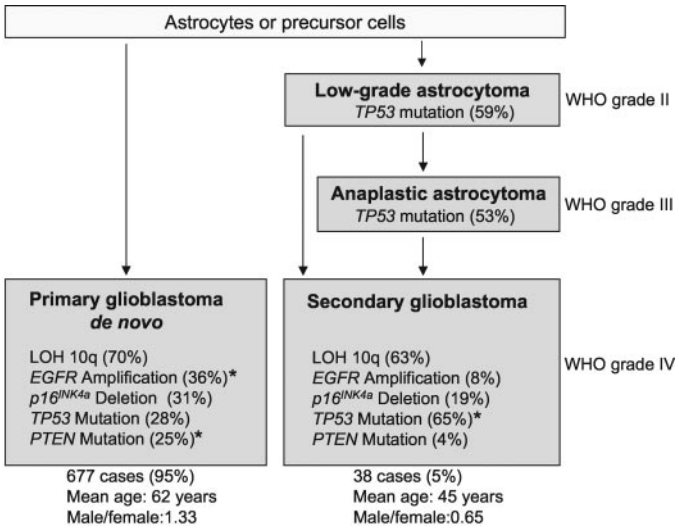

Fig. 3. Timing and frequency of genetic alterations during astrocytoma progression. The data of *TP53* mutations in low-grade astrocytomas (21) and anaplastic astrocytomas (unpublished data) are from the same population but are not necessarily from the same patients. Note that LOH 10q is frequent in primary and secondary glioblastomas, and *TP53* mutations are early and frequent genetic alterations in the pathway leading to secondary glioblastomas. ∗, Genetic alterations that are significantly different in frequency between primary and secondary glioblastomas.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/64/19/6892/2518559/zch01904006892.pdf by guest on 24 June 2024

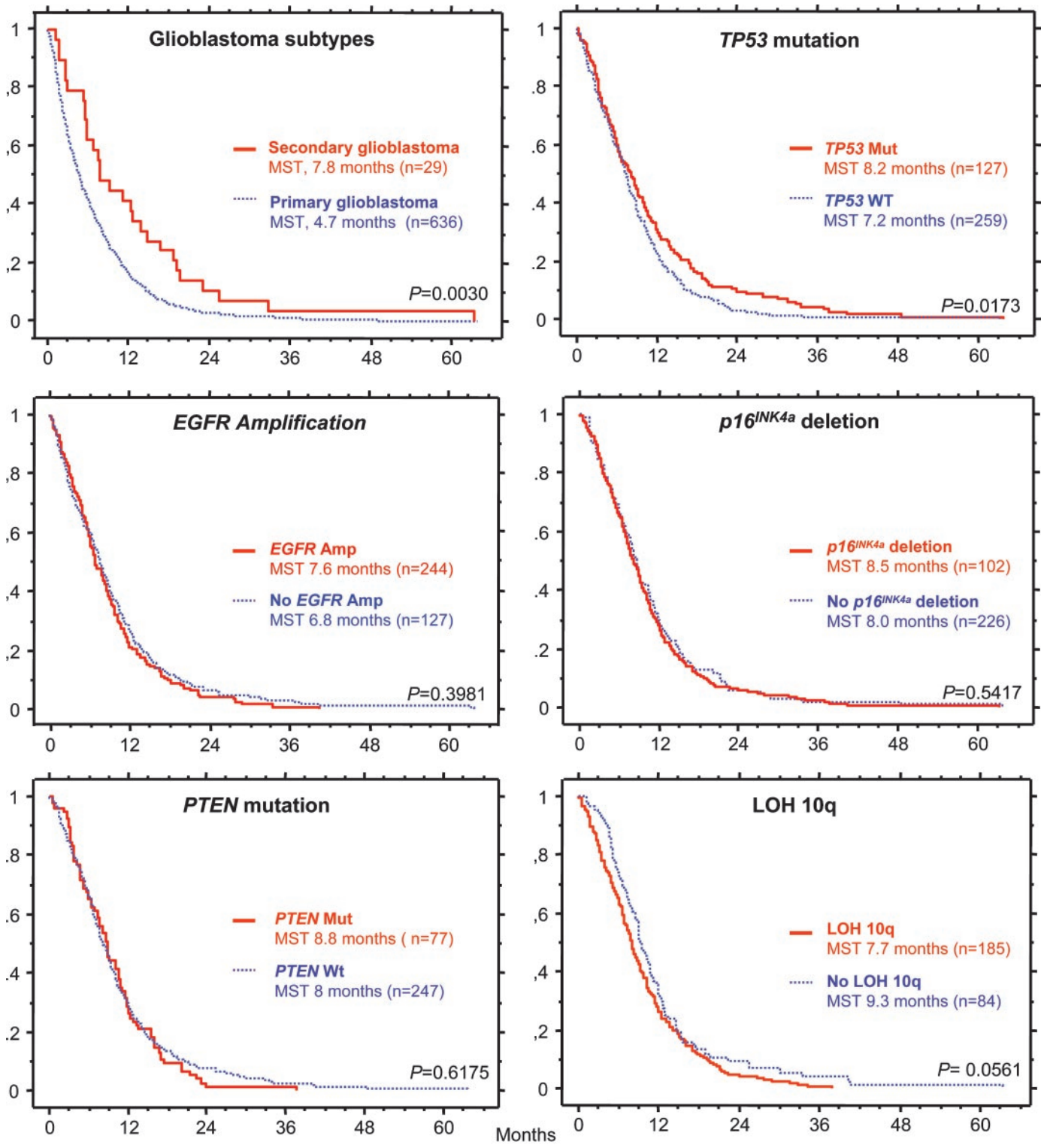

Fig. 4. Survival of glioblastoma patients. Kaplan-Meier curves show that survival of patients with secondary glioblastoma is significantly longer than that of those with primary glioblastoma (log-rank test, *top left*). Absence of *TP53* mutations and presence of LOH 10q are predictive of shorter survival. *EGFR* amplification, $p16^{INK4a}$ homozygous deletion, and *PTEN* mutations are not associated with prognosis of glioblastoma patients.

more equally distributed through exons (17% in codons 248 and 273; $P < 0.001$; Fig. 6). G:C→A:T mutations at CpG sites were significantly more frequent in secondary glioblastomas (56%) than in primary glioblastomas (30%).

The status of codon 72 polymorphism in glioblastomas was Arg/Arg, 58.4%; Arg/Pro, 33.8%; and Pro/Pro, 7.8%, which was similar to the allelic frequencies reported for healthy Caucasians (14).

Type and Distribution of *PTEN* Mutations. Seventy-eight *PTEN* mutations were observed in 77 glioblastomas (23.5%). Of these, 33.3% were missense mutations leading to amino acid change and preferentially located in exons 1 to 6 (*i.e.*, in the region homologous to tensin, auxilin, and dual-specificity phosphatases; Fig. 6). Nonsense mutations (12.8%) and deletions or insertions leading to stop codons (32.1%) were located more equally distributed throughout the exons

Downloaded from http://aacrjournals.org/cancerres/article-pdf/64/19/6892/2518559/zch01904006892.pdf by guest on 24 June 2024

A **Univariate analysis (chi-square test)**

	TP53	*p16^{INK4a}*	*EGFR*	*PTEN*
LOH 10q	**23.7%**	**23.3%**	**25.8%**	**16.2%**
TP53		**5.8%** ***P*=0.0011****	**7.5%** **P=0.015****	**7.9%**
p16^{INK4a}			**17.0%** ***P*<0.0001***	**6.0%**
EGFR				**5.8%** ***P*=0.085****

B **Multivariate analysis (Logistic regression)**

Dependent variable	Covariates: *TP53*	*p16^{INK4a}*	*EGFR*	*PTEN*
LOH 10q	**1.88***** (0.97-3.68)****** ***P*=0.063***	1.08 (0.58-2.03)	1.50 (0.80-2.82)	0.94 (0.48-1.83)
TP53		**0.40** (0.20-0.79) ***P*=0.009****	0.59 (0.31-1.14)	1.24 (0.63-2.46)
p16^{INK4a}			**2.74** (1.53-4.91) ***P*=0.001***	0.98 (0.49-1.95)
EGFR				**0.54** (0.27-1.10) ***P*=0.092****

Fig. 5. *A*, copresence of genetic alterations in glioblastomas. Percentages indicate fraction of cases showing two different genetic alterations simultaneously. Note that LOH 10q plus other genetic alterations (*TP53* mutations, *EGFR* amplification, *p16^{INK4a}* homozygous deletion, and *PTEN* mutations) are frequent. To assess association between genetic alterations, univariate analysis (*A*) and multivariate analysis (*B*) were carried out. Both analyses revealed an association between *EGFR* amplification and *p16^{INK4a}* deletion and an inverse correlation between *p16^{INK4a}* deletion and *TP53* mutations (*A* and *B*). In univariate analysis, inverse associations also were observed between *EGFR* amplification and *TP53* mutations, and *EGFR* amplification and *PTEN* mutations (*A*), and in multivariate analysis, a positive association was observed between LOH 10q and *TP53* mutations, and an inverse association between *PTEN* mutations and *EGFR* amplification (*B*). *Positive association. **Inverse association. ***Age-adjusted hazard ratio. ****95% CI.

(Fig. 6). The others included splicing mutations (11.5%), in-frame deletions (7.7%), and point mutations in 5′-untranslated region (2.6%).

DISCUSSION

This is the first population-based study on glioblastomas that includes incidence, survival rates, and key genetic alterations. It has a high degree of accuracy because the registry data were histologically verified in 80% of cases. Furthermore, we reviewed the histopathologic diagnosis in 96% of these cases according to new WHO Classification of Tumors of the Nervous System (2). The incidence rate of glioblastomas in the Canton of Zurich, adjusted to the World Standard Population per 100,000 population per year, was 3.32 in male cases and 2.24 for female cases, and if adjusted to the United States population, the rate was 4.63 in male cases and 2.88 in female cases. The incidence rate in male cases in Zurich was higher than the value of 3.69 recorded by the Central Brain Tumor Registry of the United States (ref. 13; 1992 through 1997, adjusted to the United States standard population), but the incidence rates in female cases were similar in Zurich and the United States.

Despite progress in surgery and radiotherapy and chemotherapy of brain tumors, the overall survival of patients with glioblastoma remains extremely poor. In the present population-based study, only 17.7% survived >1 year, 3.3% lived 2 years, and only 1.2% of patients were still alive 3 years after diagnosis. Similarly, a population-based study in Canada showed that after exclusion of neoplasms with a significant oligodendroglial component, only 15 of 689 glioblastoma patients (2.2%) diagnosed during 1975 to 1991 survived for 3 years (15). Clinical trials of patients with malignant glioma show a better outcome because they usually combine glioblastomas and anaplastic astrocytomas. A recent meta-analysis showed that 40% of patients with malignant glioma treated with radiotherapy and 46% treated with radiotherapy plus chemotherapy survived >1 year (1). However, clinical trials have a strong bias toward the recruitment of patients with higher preoperative Karnofsky performance score and of younger age (1). In this population-based study, 30% of patients were >70 years (see Materials and Methods). They typically have a low Karnofsky performance score and would not be eligible for a therapy trial, and they are less likely to be treated using surgery and radiotherapy (see Materials and Methods). This is the main reason for the low survival rates in the present population-based study when compared with results of clinical trials and underlines that even in a country with unrestricted access to a sophisticated health care system, the prognosis of older patients with glioblastoma is still depressingly poor.

Several therapy trials and hospital-based studies have shown that younger glioblastoma patients (<50 years) have a better prognosis than older patients (7, 16–18). The present study clearly showed that at the population level, age also was the most significant prognostic factor in univariate and multivariate analyses. Furthermore, this effect persisted through all of the age groups in a linear fashion, which allows calculation of the mean and median survival time from the date of glioblastoma diagnosis (Fig. 1).

Glioblastomas can be subdivided into primary and secondary neoplasms, which affect patients at different ages and through different genetic pathways (2, 3). We show here that at the population-based level, secondary glioblastomas are a rare disease, amounting to only 5% of all of the glioblastomas. This is consistent with the finding of Dropcho *et al.* (19), who observed that 19 of 392 (5%) patients with glioblastomas at the University of Alabama had histologically proven previous low-grade gliomas. However, in the population-based series of the California Cancer Registry, the number of newly diagnosed cases of low-grade and anaplastic astrocytomas was ~6% and 24% of incident glioblastoma cases (20). Similarly, in the present population-based study, the incidence rate of low-grade and anaplastic gliomas is approximately two or three times higher than that of secondary glioblastoma (21). The higher frequency of precursor lesions may be explained at least in part by the fact that a fraction of patients with low-grade or anaplastic astrocytoma die before progression to glioblastoma occurs. However, some cases with rapid progression from low-grade or anaplastic astrocytoma may have been misclassified as primary glioblastoma. Even considering this possibility, on a population-based level, secondary glioblastomas constitute a rare disease when compared with primary glioblastoma.

Univariate analysis revealed that patients with secondary glioblastoma survived significantly longer than those with primary glioblastoma, but this is likely because of the younger age of patients with secondary glioblastomas rather than a reflection of different biological behavior because the difference became nonsignificant in an age-adjusted multivariate analysis. We observed that secondary glioblastomas develop more frequently in women (male to female ratio, 0.65) than primary glioblastomas (1.33). This corroborates a previous finding that glioblastomas with *TP53* mutations (a genetic hallmark of

Downloaded from http://aacrjournals.org/cancerres/article-pdf/64/19/6892/2518559/zch01904006892.pdf by guest on 24 June 2024

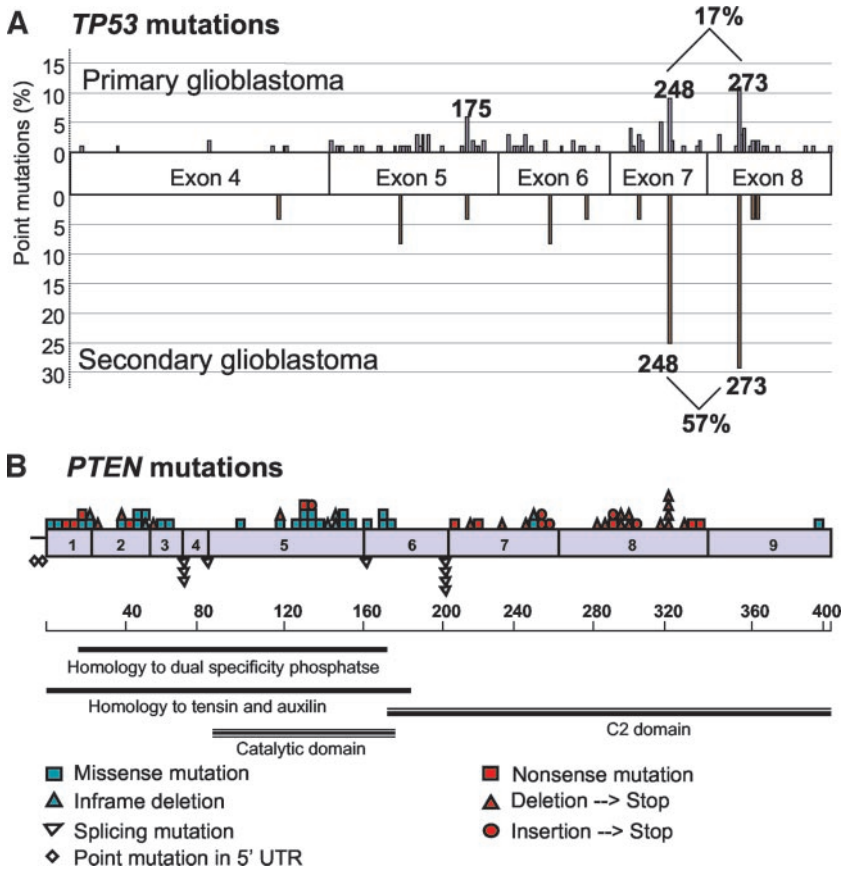

Fig. 6. *A*, distribution of *TP53* point mutations in primary and secondary glioblastomas. The majority (57%) of *TP53* mutations in secondary glioblastomas were located in codons 248 and 273, whereas *TP53* mutations in primary glioblastomas were more equally distributed across different codons with a slight peak at codons 248 and 273 (17%). *B*, distribution and type of *PTEN* mutations in glioblastomas. Note that mutations leading to protein truncation (nonsense mutation, deletion, insertion) are located throughout the exons, whereas missense mutations are preferentially located in exons 1 to 6 (*i.e.*, in the region homologous to tensin, auxilin, and dual-specificity phosphatases).

secondary glioblastomas) were more common in women (22). This is surprising because in hospital-based studies (2, 23) and in this population-based study, the incidence of low-grade or anaplastic gliomas in male patients was similar to or higher than in female patients (21). The possibility exists that gliomas progress more frequently or more rapidly to glioblastoma in female patients.

The present study is one of the largest genetic analyses in glioblastomas and the first carried out on a population. Although not all of the archival samples (in particular, autopsy cases) were suitable for PCR amplification, we were able to assess key genetic alterations in up to 71% of histologically diagnosed cases. The *TP53* gene encodes a protein that plays important roles in several cellular processes, including the cell cycle, response to DNA damage, apoptosis, cell differentiation, and neovascularization (24). Data on the predictive value of *TP53* mutations in glioblastomas are contradictory. Although some hospital-based studies showed no association between *TP53* status and outcome of glioblastoma patients (17, 18), Schmidt *et al.* (25) analyzed 97 glioblastoma cases and found that the presence of *TP53* mutations was a favorable prognostic factor. In the present population-based study, univariate analysis revealed that the presence of *TP53* mutations was predictive of longer survival (Fig. 4). However, age-adjusted multivariate analysis revealed no difference in survival between patients with and without *TP53* mutations. It has been reported that low-grade astrocytomas with a *TP53* mutation at codon 175 showed a significantly worse prognosis than morphologically similar tumors with mutations at other sites (26). In the present study, there was a tendency that patients with glioblastoma carrying a *TP53* codon 175 mutation had shorter survival times than those carrying other mutations, but the difference was not statistically significant.

It is of interest to note that the type and distribution of *TP53* mutations differed between glioblastoma subtypes. In secondary glioblastomas, 57% of mutations were located in the two hotspot codons, 248 and 273 (Fig. 6). In primary glioblastomas, mutations were more equally distributed through exons, only 17% occurring in codons 248 and 273 (Fig. 6). Furthermore, G:C→A:T transitions at CpG sites were significantly more frequent in secondary than in primary glioblastomas. The best-characterized mechanism of G:C→A:T transitions at CpG sites is deamination of 5-methylcytosine, which is clustered at CpG sites, resulting in substitution of 5-methylcytosine by thymine. This occurs spontaneously or is factor mediated (*e.g.*, through the action of oxygen radicals or by nitric oxide produced by nitric oxide synthase in conditions of chronic inflammation; ref. 27). Alternatively, our previous observation of a significant correlation between *TP53* mutations and promoter methylation of the O^6-methylguanine-DNA methyltransferase (*MGMT*) gene in low-grade astrocytomas and secondary glioblastomas suggests the possible involvement of endogenous or exogenous DNA alkylation at the O^6-position of guanine (28). In any case, G:C→A:T mutations at CpG sites, particularly in hotspot codons 248 and 273, seem to be an early event directly associated with malignant transformation in the pathway to secondary glioblastomas, whereas the less specific pattern of *TP53* mutations in primary glioblastomas may, in a majority of cases, constitute secondary events reflecting increased genomic instability during tumor progression.

Epidermal growth factor receptor (EGFR) is a transmembrane receptor that binds to extracellular ligands such as epidermal growth factor and transforming growth factor α and transduces a mitotic signal (29). *EGFR* amplification has been identified as a genetic hallmark of glioblastomas (2). The predictive value of *EGFR* amplification has been unclear. In previous studies (<40 cases), *EGFR* amplification was associated with poorer survival of glioblastoma patients (30, 31). One study of 97 patients showed a lack of predictive

Downloaded from http://aacrjournals.org/cancerres/article-pdf/64/19/6892/2518559/zch01904006892.pdf by guest on 24 June 2024

value of *EGFR* amplification (25); similarly, a meta-analysis of seven previous studies (total, 395 glioblastoma cases) did not detect a significant predictive value of *EGFR* amplification (32). Shinojima *et al.* (33) reported that *EGFR* amplification was a significant unfavorable predictor for overall survival in glioblastoma patients and that the *EGFR* gene status was a more significant prognostic factor in younger patients (<60 years). Other studies reported that *EGFR* amplification was a predictor of longer survival only in older glioblastoma patients (18, 34). Simmons *et al.* (17) reported that *EGFR* overexpression was associated with poorer survival of glioblastoma patients younger than the median age and that *EGFR* overexpression was negatively associated with survival in cases without the *TP53* mutation. The present population-based study indicates that the presence of *EGFR* amplification does not affect survival of glioblastoma patients at any age. The striking finding of *EGFR* amplification in the present study is the unusual age distribution. *EGFR* amplification closely reflects the age distribution of primary glioblastomas and was not detected in any glioblastomas of patients <35 years (Fig. 2*B*).

The $p16^{INK4a}$ gene binds to cyclin-dependent kinase 4 and inhibits the cyclin-dependent kinase 4–cyclin D1 complex (35, 36). This complex phosphorylates the RB1 protein, thereby inducing release of the E2F transcription factor that activates genes involved in the late G_1 and S phases (35, 36). In glioblastomas, disruption of the $p16^{INK4a}$ gene occurs through homozygous deletion (2). Findings regarding the predictive value of $p16^{INK4a}$ homozygous deletion have been inconsistent. In an analysis of 46 cases, Kamiryo *et al.* (37) reported that homozygous $p16^{INK4a}$ deletion was a significantly unfavorable criterion for survival of glioblastoma patients. Another study showed that homozygous $p16^{INK4a}$ deletion was associated with shorter survival only in a subgroup of glioblastoma patients >50 years of age (38). In the present population-based study, univariate and multivariate analyses failed to show any predictive value of homozygous $p16^{INK4a}$ deletion. We did observe a significant association between *EGFR* amplification and $p16^{INK4a}$ deletion, in agreement with the findings of previous hospital-based studies based on a small numbers of cases (39, 40).

PTEN gene locates on chromosome 10q23 and encodes a protein that plays important roles in the regulation of cell proliferation, apoptosis, and tumor invasion (41, 42). *PTEN* mutations have been reported in 15 to 40% of glioblastomas (42, 43). *PTEN* homozygous deletions may occur, but they are rare in glioblastomas (<2%; ref. 44). Promoter methylation may be alternative mechanisms of loss of *PTEN* expression, but the significance of *PTEN* methylation in the evolution of glioblastomas remains to be clarified (45). In several previous studies, *PTEN* mutations were not associated with prognosis of glioblastoma patients (18, 25, 46), and this was confirmed in the present population-based study. Interestingly, most missense mutations were located in exons 1 to 6, the region homologous to tensin, auxilin, and dual-specificity phosphatases, whereas nonsense mutations and deletions/insertions leading to stop codons and protein truncation were located more equally throughout the gene. This suggests that cells with *PTEN* truncation at any site or *PTEN* missense mutations in the region homologous to tensin/auxilin and dual-specificity phosphatases acquire transformed phenotype.

LOH 10 is the most frequent genetic alteration in glioblastomas and occurs in 60 to 80% of cases (47–50). Many glioblastomas seem to have lost one entire copy of chromosome 10. LOH occurs most frequently at three common loci (*i.e.*, 10p14-p15, 10q23–24, and 10q25-pter), suggesting the presence of several tumor suppressor genes (47–49, 51). We show here that LOH 10q is the most frequent genetic alteration in the pathways to primary and secondary glioblastomas. LOH 10q has been found to be associated with reduced survival of glioblastoma patients in previous studies (25, 52, 53), and this was confirmed at the population level. The presence of LOH 10q was the only genetic alteration associated with shorter survival.

Several previous studies focused on genetic alterations and their impact on survival of glioblastoma patients. However, most of these were based on small case numbers and usually on a single cancer-related gene. To obtain better understanding of effect of copresence of different genetic alterations, we carried out analyses of several key genetic alterations in a large number of glioblastomas. Our population-based study shows that LOH 10q was typically copresented with any of the other genetic alterations (Fig. 5). In contrast, *TP53* mutations, $p16^{INK4a}$ deletion, *EGFR* amplification, and *PTEN* mutations showed inverse associations with each other, except for a positive correlation between $p16^{INK4a}$ deletion and *EGFR* amplification (Fig. 5). This suggests that LOH 10q plus at least one other genetic alteration may be operative in the development of a majority of glioblastomas. LOH 10q25-qter distal to the *PTEN* seems to be associated with acquisition of the glioblastoma phenotype (54), suggesting that a tumor suppressor gene in this region may be crucial in the development of glioblastomas. Candidate genes include *DMBT1* (52, 55) and *FGFR2* (52). Identification and validation of such a gene would be an important advancement in our understanding of the pathogenesis of glioblastomas and in devising new strategies for the management of this most malignant brain tumor.

ACKNOWLEDGMENTS

We dedicate this work to the late Dr. Georges Schuler of the Cantonal Cancer Registry, Zurich, who was instrumental in the initiation of this project. We thank Ms. Anne-Marie Camus for technical assistance. We also thank the following colleagues in Switzerland for providing paraffin sections of glioblastoma cases: Dr. Renata Flury-Frei, Institute of Pathology, Cantonal Hospital Winterthur; Dr. Bernhard Stamm, Institute of Pathology, Cantonal Hospital Aarau; Dr. Robert Maurer, Institute of Pathology, City Hospital Triemli, Zurich; Dr. Werner Wüst, Institute of Pathology, Cantonal Hospital Chur; Dr. Robert C. Janzer, Institute of Pathology, University of Lausanne; and Dr. Alphonse Probst, Institute of Pathology, University of Basel.

REFERENCES

1. Stewart LA. Chemotherapy in adult high-grade glioma: a systematic review and meta-analysis of individual patient data from 12 randomised trials. Lancet 2002;359:1011–8.
2. Kleihues P, Cavenee WK, editors. WHO Classification of Tumors: Pathology and Genetics of Tumours of the Nervous System. Lyon: IARC Press; 2000.
3. Kleihues P, Ohgaki H. Phenotype vs genotype in the evolution of astrocytic brain tumors. Toxicol Pathol 2000;28:164–70.
4. Louis DN. A molecular genetic model of astrocytoma histopathology. Brain Pathol 1997;7:755–64.
5. Pietsch T, Wiestler OD. Molecular neuropathology of astrocytic brain tumors. J Neurooncol 1997;35:211–22.
6. Collins VP. Cellular mechanisms targeted during astrocytoma progression. Cancer Lett 2002;188:1–7.
7. Ohgaki H, Reifenberger G, Nomura K, Kleihues P. Gliomas. In: Gospodarowicz MK et al, editors. Prognostic Factors in Cancer. 2nd ed. UICC; 2001. p 725–43.
8. Parkin DM, Whelan SL, Feraly J, Teppo L, Thomas D. Cancer Incidence in Five Continents, vol. VIII. Lyon: International Agency for Research on Cancer; 2002.
9. Watanabe K, Tachibana O, Sato K, Yonekawa Y, Kleihues P, Ohgaki H. Overexpression of the EGF receptor and p53 mutations are mutually exclusive in the evolution of primary and secondary glioblastomas. Brain Pathol 1996;6:217–24.
10. Tohma Y, Gratas C, Biernat W, et al. PTEN (MMAC1) mutations are frequent in primary glioblastomas (de novo) but not in secondary glioblastomas. J Neuropathol Exp Neurol 1998;57:684–9.
11. Nakamura M, Watanabe T, Klangby U, et al. $P14^{Arf}$ deletion and methylation in genetic pathways to glioblastomas. Brain Pathol 2001;11:159–68.
12. Nigro JM, Takahashi MA, Ginzinger DG, et al. Detection of 1p and 19q loss in oligodendroglioma by quantitative microsatellite analysis, a real-time quantitative polymerase chain reaction assay. Am J Pathol 2001;158:1253–62.
13. Central Brain Tumor Registry of the United States (CBTRUS). Available at: http://www.cbtrus.org. Accessed 2003 Dec 1.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/64/19/6892/2518559/zch01904006892.pdf by guest on 24 June 2024

14. Matakidou A, Eisen T, Houlston RS. TP53 polymorphisms and lung cancer risk: a systematic review and meta-analysis. Mutagenesis 2003;18:377–85.
15. Scott JN, Rewcastle NB, Brasher PM, et al. Which glioblastoma multiforme patient will become a long-term survivor? A population-based study. Ann Neurol 1999;46: 183–8.
16. Burger PC, Green SB. Patient age, histologic features, and length of survival in patients with glioblastoma multiforme. Cancer 1987;59:1617–25.
17. Simmons ML, Lamborn KR, Takahashi M, et al. Analysis of complex relationships between age, p53, epidermal growth factor receptor, and survival in glioblastoma patients. Cancer Res 2001;61:1122–8.
18. Smith JS, Tachibana I, Passe SM, et al. PTEN mutation, EGFR amplification, and outcome in patients with anaplastic astrocytoma and glioblastoma multiforme. J Natl Cancer Inst 2001;93:1246–56.
19. Dropcho EJ, Soong SJ. The prognostic impact of prior low grade histology in patients with anaplastic gliomas: a case-control study. Neurology 1996;47:684–90.
20. Perkins CI, Cohen R, Young JL, Schlag R, Wright W. Cancer in California: Detailed Site and Histology, 1990–1994. Sacramento, CA: California Department of Health Services, Cancer Surveillance Section; 1977. p. 226–7.
21. Okamoto Y, Di Patre PL, Burkhard C, et al. Population-based study on incidence, survival rates, and genetic alterations of low-grade astrocytomas and oligodendrogliomas. Acta Neuropathol 2004;108:49–56.
22. Louis DN, von Deimling A, Chung RY, et al. Comparative study of p53 gene and protein alterations in human astrocytic tumors. J Neuropathol Exp Neurol 1993;52: 31–8.
23. Peraud A, Ansari H, Bise K, Reulen HJ. Clinical outcome of supratentorial astrocytoma WHO grade II. Acta Neurochir (Wien) 1998;140:1213–22.
24. Bogler O, Huang HJ, Kleihues P, Cavenee WK. The p53 gene and its role in human brain tumors. Glia 1995;15:308–27.
25. Schmidt MC, Antweiler S, Urban N, et al. Impact of genotype and morphology on the prognosis of glioblastoma. J Neuropathol Exp Neurol 2002;61:321–8.
26. Peraud A, Kreth FW, Wiestler OD, Kleihues P, Reulen HJ. Prognostic impact of TP53 mutations and P53 protein overexpression in supratentorial WHO grade II astrocytomas and oligoastrocytomas. Clin Cancer Res 2002;8:1117–24.
27. Ohshima H, Bartsch H. Chronic infections and inflammatory processes as cancer risk factors: possible role of nitric oxide in carcinogenesis. Mutat Res 1994;305:253–64.
28. Nakamura M, Watanabe T, Yonekawa Y, Kleihues P, Ohgaki H. Promoter hypermethylation of the DNA repair gene MGMT in astrocytomas is frequently associated with G:C->A:T mutations of the TP53 tumor suppressor gene. Carcinogenesis 2001;22:1715–9.
29. Arteaga CL. Epidermal growth factor receptor dependence in human tumors: more than just expression? Oncologist 2002;7(Suppl 4):31–9.
30. Hurtt MR, Moossy J, Donovan Peluso M, Locker J. Amplification of epidermal growth factor receptor gene in gliomas: histopathology and prognosis. J Neuropathol Exp Neurol 1992;51:84–90.
31. Torp SH, Helseth E, Dalen A, Unsgaard G. Relationships between Ki-67 labelling index, amplification of the epidermal growth factor receptor gene, and prognosis in human glioblastomas. Acta Neurochir (Wien) 1992;117:182–6.
32. Huncharek M, Kupelnick B. Epidermal growth factor receptor gene amplification as a prognostic marker in glioblastoma multiforme: results of a meta-analysis. Oncol Res 2000;12:107–12.
33. Shinojima N, Tada K, Shiraishi S, et al. Prognostic value of epidermal growth factor receptor in patients with glioblastoma multiforme. Cancer Res 2003;63:6962–70.
34. Batchelor TT, Betensky RA, Esposito JM, et al. Age-dependent prognostic effects of genetic alterations in glioblastoma. Clin Cancer Res 2004;10:228–33.
35. Huschtscha LI, Reddel RR. $p16^{INK4a}$ and the control of cellular proliferative life span. Carcinogenesis 1999;20:921–6.
36. Lowe SW, Sherr CJ. Tumor suppression by Ink4a-Arf: progress and puzzles. Curr Opin Genet Dev 2003;13:77–83.
37. Kamiryo T, Tada K, Shiraishi S, et al. Analysis of homozygous deletion of the p16 gene and correlation with survival in patients with glioblastoma multiforme. J Neurosurg 2002;96:815–22.
38. Labuhn M, Jones G, Speel EJ, et al. Quantitative real-time PCR does not show selective targeting of p14(ARF) but concomitant inactivation of both p16(INK4A) and p14(ARF) in 105 human primary gliomas. Oncogene 2001;20:1103–9.
39. Hegi ME, zur Hausen A, Ruedi D, Malin G, Kleihues P. Hemizygous or homozygous deletion of the chromosomal region containing the $p16^{INK4a}$ gene is associated with amplification of the EGF receptor gene in glioblastomas. Int J Cancer 1997;73:57–63.
40. Hayashi Y, Ueki K, Waha A, Wiestler OD, Louis DN, von Deimling A. Association of EGFR gene amplification and CDKN2 (p16/MTS1) gene deletion in glioblastoma multiforme. Brain Pathol 1997;7:871–5.
41. Simpson L, Parsons R. PTEN: life as a tumor suppressor. Exp Cell Res 2001;264: 29–41.
42. Dahia PL. PTEN, a unique tumor suppressor gene. Endocr Relat Cancer 2000;7:115–29.
43. Knobbe CB, Merlo A, Reifenberger G. Pten signaling in gliomas. Neuro-oncol 2002;4:196–211.
44. Knobbe CB, Reifenberger G. Genetic alterations and aberrant expression of genes related to the phosphatidyl-inositol-3′-kinase/protein kinase B (Akt) signal transduction pathway in glioblastomas. Brain Pathol 2003;13:507–18.
45. Baeza N, Weller M, Yonekawa Y, Kleihues P, Ohgaki H. PTEN methylation and expression in glioblastomas. Acta Neuropathol (Berl) 2003;106:479–85.
46. Backlund LM, Nilsson BR, Goike HM, et al. Short postoperative survival for glioblastoma patients with a dysfunctional Rb1 pathway in combination with no wild-type PTEN. Clin Cancer Res 2003;9:4151–8.
47. Rasheed BK, McLendon RE, Friedman HS, et al. Chromosome 10 deletion mapping in human gliomas: a common deletion region in 10q25. Oncogene 1995;10:2243–6.
48. Karlbom AE, James CD, Boethius J, et al. Loss of heterozygosity in malignant gliomas involves at least three distinct regions on chromosome 10. Hum Genet 1993;92:169–74.
49. Ichimura K, Schmidt EE, Miyakawa A, Goike HM, Collins VP. Distinct patterns of deletion on 10p and 10q suggest involvement of multiple tumor suppressor genes in the development of astrocytic gliomas of different malignancy grades. Genes Chromosomes Cancer 1998;22:9–15.
50. Ohgaki H, Schauble B, zur Hausen A, von Ammon K, Kleihues P. Genetic alterations associated with the evolution and progression of astrocytic brain tumours. Virchows Arch 1995;427:113–8.
51. Fults D, Pedone CA, Thompson GE, et al. Microsatellite deletion mapping on chromosome 10q and mutation analysis of MMAC1, FAS, and MXI1 in human glioblastoma multiforme. Int J Oncol 1998;12:905–10.
52. Tada K, Shiraishi S, Kamiryo T, et al. Analysis of loss of heterozygosity on chromosome 10 in patients with malignant astrocytic tumors: correlation with patient age and survival. J Neurosurg 2001;95:651–9.
53. Terada K, Tamiya T, Daido S, et al. Prognostic value of loss of heterozygosity around three candidate tumor suppressor genes on chromosome 10q in astrocytomas. J Neurooncol 2002;58:107–14.
54. Fujisawa H, Kurrer M, Reis RM, Yonekawa Y, Kleihues P, Ohgaki H. Acquisition of the glioblastoma phenotype during astrocytoma progression is associated with LOH on chromosome 10q25-qter. Am J Pathol 1999;155:387–94.
55. Kang W, Reid KB. DMBT1, a regulator of mucosal homeostasis through the linking of mucosal defense and regeneration? FEBS Lett 2003;540:21–5.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/64/19/6892/2518559/zch01904006892.pdf by guest on 24 June 2024