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 in 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 (p16INK4a, p14ARF, 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 autopsy 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., F.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
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′-ACTGCTTCTTT-TCACCCATCTAC-3′ (sense) and 5′-TCATGGAGCCAGCCCTCAG-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 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 visualized using a Typhoon 9410 Imaging System (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 ± 2 × SD) was regarded as the threshold for evidence of EGFR amplification (10).

**Differential PCR for p16INK4a Homozygous Deletion.** To assess p16INK4a homozygous deletion, differential PCR was carried out using a primer set located in exon 1 of the p16INK4a gene and a β-actin sequence as an 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 p16INK4a to β-actin ratio was ≤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 μM of each primer, 60 mM Mg/λ probe [21-bp oligomer complementary to the microsatellite CA repeat: 5′-6-carboxyfluorescein (FAM)-TGT GTG TGT GTG TGT TGT-3′] and 10 ng DNA, with cycling parameters as reported previously (12). Primers, probe, and the TaqMan master mix were purchased from Prologio 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–δδCt), 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.
(25.8%), LOH 10q and TP53 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. 5A).

Univariate and multivariate analyses showed that EGFR amplification and p16 INK4a deletion tend to occur simultaneously (Fig. 5A and B). In contrast, TP53 mutations, p16 INK4a deletion, EGFR amplification, and PTEN mutations showed inverse associations with each other (Fig. 5A 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...
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 \textit{PTEN} Mutations. Seventy-eight \textit{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.
The incidence rate in male cases in Zurich was higher than the value in the United States, the rate was 4.63 in male cases and 2.88 in female cases. The incidence rate in male cases in the United States (ref. 13; 1992 through 1997, adjusted to the United States population) was 3.32 in male cases and 2.46 in female cases. The incidence rate in male cases 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 this is likely because of the younger age of patients with secondary glioblastomas than those with primary glioblastomas.

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 Droppo 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.

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 gliodendroglial 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.

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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 (1:3.3). This corroborates a previous finding that glioblastomas with TP53 mutations (a genetic hallmark of...
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 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).

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%).
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. 2B).

The p16INK4a 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 G1 and S phases (35, 36). In glioblastomas, disruption of the p16INK4a gene occurs through homozygous deletion (2). Findings regarding the predictive value of p16INK4a homozygous deletion have been inconsistent. In an analysis of 46 cases, Kamiroyo et al. (37) reported that homozygous p16INK4a deletion was a significantly unfavorable criterion for survival of glioblastoma patients. Another study showed that homozygous p16INK4a 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 p16INK4a deletion. We did observe a significant association between EGFR amplification and p16INK4a 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, p16INK4a deletion, EGFR amplification, and PTEN mutations showed inverse associations with each other, except for a positive correlation between p16INK4a 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-pter 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.

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