

Gene expression profile as a prognostic factor in high-grade gliomas

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Abstract. Some clinical factors have been useful in predicting prognosis in high-grade gliomas, however, unexpected differences in survival time have generated attempts to search for more precise parameters. It is clear that tumour behaviour depends mostly on gene alterations. Known single gene alterations failed to accurately define survival time, however, recently, the gene profiling based on microarray technology has raised hopes. Our aim was to assess whether the genetic predictor exceeds clinical parameters in the prognosis of malignant gliomas. We performed gene expression analysis of 28 gliomas (3 grade II, 10 grade III and 15 grade IV, according to WHO classification), and 5 control, normal brain samples, using Clontech oligonucleotide arrays with 3,757 known genes. The signal-to-noise statistics was used to separate classes, and the leave-one-out method was used to assess the smallest number of genes make it clear with a minimal cross-validation error. All gliomas, or only high-grade tumours, were clearly separated from the normal brain samples using 7 or 9 most differentially expressed genes. Hierarchical clustering failed, but the fuzzy c-means method was useful in high-grade gliomas to find a gene prediction model, which, with clinical factors, was assessed in survival analysis. Univariate analysis demonstrated that age, WHO grade (IV vs. III), radiation dose (≥ 50 Gy vs. 42 Gy), postoperative KPS score (100 points vs. others), neurological deficit as the first sign of the disease vs. others, and gene expression profile were significant predictors of survival. In multivariate analysis, the gene expression profile remained the only independent

predictor ($p=0.007$). Thus, our conclusion is that gene expression pattern predicts outcome in high-grade gliomas independently of other factors.

Introduction

Despite aggressive treatment with surgery, radio- and chemotherapy, prognosis for patients with malignant gliomas is still poor. The mean survival time in glioblastomas is <1 year, in anaplastic astrocytomas up to 3 years. However, survival does vary among patients (1-8). An unexpected clinical course reflects primarily biological differences between these tumors. A few clinical factors such as age, Karnofsky performance status, extent of tumour resection, have been identified as useful in assessing the prognosis (4,6,7,9-11). However, genetic heterogeneity of the tumours may exert the most significant influence on differences in the survival period (3,12). At present, our attempts have been focused on identifying those genetic alterations. Many studies have concentrated on genes such as EGFR, PTEN, TP53, CDKN2A, MDM2, and apoptotic and proliferation indices have been conducted; however, a single gene seems to be rarely predictive of survival, and opposing results have been reported. In other words, known differences in gene alterations, found in astrocytomas and glioblastomas, cannot be directly translated into knowledge of prognosis. For example, views on the influence of TP53 mutations on survival are divergent; some researchers advocate this association (8,13-15), others do not (9,12,16-26). Overexpression of MDM2 is also a poor predictor in some (3,24,27), but not in all studies (19,22). Losses of heterozygosity on 1p and 19q in tumours of oligodendroglial origin are an exception, when researchers agree as to their favourable impact on susceptibility to chemotherapy and survival time (26,28-32). However, this reverses to the previous stage of divergent results in the case of PTEN alterations. PTEN loss or mutations reduce survival in anaplastic astrocytomas and anaplastic oligodendrogliomas (8,26,30,31,33,34), but not always in glioblastomas (8,12,13,16,19,33-36). Similarly, an overexpression or

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Table I. Demographic and clinical data.

| Patient demographic and clinical data | All WHO grades (n=28) | | WHO grade III + IV (n=25) | |
|---------------------------------------|-----------------------|-------|---------------------------|-------|
| | n (%) | Range | n (%) | Range |
| Age (years) | | | | |
| <40 | 9 (32.1) | | 7 (28) | |
| ≥40 | 19 (67.9) | | 18 (72) | |
| <60 | 20 (71.4) | | 17 (68) | |
| ≥60 | 8 (28.6) | | 8 (32) | |
| Mean | 49.8 | 23-79 | 51.2 | 23-79 |
| Duration of symptoms (weeks) | | | | |
| Mean | 13.3 | | 13.6 | |
| Median | 5.5 | 2-112 | 6 | 2-112 |
| Sex | | | | |
| Male | 18 (64.3) | | 17 (68) | |
| Female | 10 (35.7) | | 25 (32) | |
| EOR | | | | |
| GTR | 14 (50) | | 12 (48) | |
| STR | 14 (50) | | 13 (52) | |
| Postoperative KPS | | | | |
| 100 points | 13 (46.4) | | 12 (48) | |
| ≤90 points | 15 (53.6) | | 13 (52) | |
| Radiation dose | | | | |
| Higher dose: 50 or 60 Gy | 17/27 (63) | | 16 (64) | |
| Lower dose: 42 Gy | 10/27 (37) | | 9 (36) | |
| Chemotherapy | | | | |
| Yes | 19 (67.9) | | 19 (76) | |
| No | 9 (32.1) | | 6 (24) | |
| First symptom of disease | | | | |
| Deficit | 6 (21.4) | | 6 (24) | |
| Epileptic seizure | 8 (28.6) | | 6 (24) | |

GTR, gross total resection; STR, subtotal resection; KPS, Karnofsky performance scale.

amplification of EGFR does not influence survival in glioblastomas (3,9,12,13,16,22,34-37), except in the elderly patients in whom it is related to a more favourable prognosis (8,9,25). Microarrays offer new opportunities in this area by permitting simultaneous evaluation of expression of thousands of genes. Successful attempts were made to find gene expression profiles correlated with prognosis in many tumours (38-41), recently, including gliomas (12,42-44). The aim of the present study was to identify, using microarray technology, a gene expression profile which might be prognostic in high-grade gliomas, and to compare its strength with known clinical factors.

Materials and methods

Patients samples. Tumour and normal brain samples were obtained at open-craniotomy surgery performed at Medical University of Warsaw, between 2002 and 2004; the samples were snap-frozen on dry-ice and stored at -80°C until use. The study was approved by the Institutional Review Board. All glioma samples were obtained at primary operation with no prior patient history of radio- or chemotherapy. Normal brain samples were obtained from patients operated on for long-lasting drug-resistant epilepsy. Histopathological diagnoses (according to the WHO classification) were made by two independent neuropathologists, with consensus reached in all cases. A total of 28 gliomas were assessed: 3 grade II tumours (2 fibrillary astrocytomas, 1 oligoastrocytoma), 10 grade III tumours (3 anaplastic oligodendrogliomas, 7 anaplastic astrocytomas) and 15 grade IV tumours (9 multiform glioblastomas, 3 giant cell glioblastomas, 3 gliosarcomas). Control tissues consisted of the white matter from resected temporal poles obtained from 5 patients with hippocampal sclerosis. All glioma patients, except one with a resected fibrillary astrocytoma (GTR), were irradiated with a dose of 42 - 62 Gy; 19 out of 25 patients with high-grade gliomas received also chemotherapy [CCNU (12), temozolomide (1), both agents, successively (4), PCV (1), PCV with temozolomide (1)]. Demographic and clinical data from our cohort of patients are shown in Table I. Median follow-up was 57.4 weeks, range: 14.4-137.1 weeks (median in high-grade subgroup: 53.7 weeks). By the end of the study 16 patients had died (high-grade gliomas); median follow-up in the deceased patients was 31.6 weeks; range: 14.4-71.4 weeks. In the survivors' group, the median follow-up was 100.4 weeks, in patients with high-grade gliomas 83.4 weeks; range in both groups: 55.6-137.1 weeks.

Isolation of total RNA. Total RNA was isolated by means of a modified method developed by Chomczynski and Sacchi (45). The frozen samples were placed on dry-ice, ground using a mortar and pestle, and subsequently, homogenized in TRIzol (Invitrogen, Carlsbad, USA) using a power homogenizer (IKA Labortechnik, Staufen, Germany). After a 5-min incubation with TRIzol and a further 2-min incubation with added chloroform (Sigma-Aldrich, St. Louis, MO, USA), the samples were centrifuged. The resulting upper aqueous phase was mixed with an equal amount of isopropyl alcohol (Sigma-Aldrich), and RNA was precipitated at -20°C overnight. On the subsequent day, the samples were centrifuged, the supernatant was removed, ice-cold 75% ethanol was added (Sigma-Aldrich), and the samples were centrifuged again. After removing the supernatant, air-dried RNA pellets were dissolved in deionised, RNAase free water. RNA integrity and purity were verified by electrophoresis on agarose gel (Gibco-BRL Life Technologies, Gaithersburg, USA) and by UV/Vis spectrophotometry (Perkin-Elmer, Überlingen, Germany) using RNA dissolved in TE (Applchem, Darmstadt, Germany). The presence of high-quality, undegraded RNA was established when intensity of 28S rRNA band was twice that of 18S rRNA with no streaking on the lower part of the sample lane, and the range of the absorbance A_{260}/A_{280} ratio was: 1.8-2.1.

cDNA microarray hybridization and scanning. Fluorescently labelled cDNA probes were generated using, at the most, 12 μ g of the total RNA by reverse transcription in the presence of aminoallyl-dUTP, subsequently, followed by a coupling reaction to Cy-3 dye (Amersham Pharmacia, Piscataway, USA) according to the manufacturer's protocol (Clontech # K-1860-1, BD Biosciences, Palo Alto, CA, USA). Probes were denatured and hybridized to glass microarrays with 3,757 known genes (Clontech # 7910-1, BD Biosciences) at 50°C overnight. The following day, the slides were washed four times successively: once, in a wash solution supplied by the manufacturer twice, in a mixture: 1/10 wash solution + 9/10 1X SSC (Sigma-Aldrich) and once in 1X SSC. Finally, the slides were spin-dried. The fluorescent intensity was assessed by scanning slides with a 5- μ m resolution at a light wavelength of 532 μ m, and a voltage of 650 PMT using a GenePix 4000B scanner, and the images were processed with GenePix Pro 3.0 software (Axon Instruments, Union City, USA).

Data analysis. Raw data as *.gpr format files were loaded to Gene Spring 6.1 (Silicon Genetics, Redwood City, USA) with background subtraction from signal intensities. Next, the values <0.01 were set to 0.01. Each measurement was divided by the median of all measurements in the sample marked as present or marginal; at the subsequent stage, each measurement for each gene in glioma samples was divided by the median of corresponding gene's measurements in the control samples. The data were next imported to MatLab ver. 6.5.0.180813a release 13 (MathWorks, Natick, USA) and normalized again by dividing gene measurements by the mean of a corresponding gene's measurements in all samples. Genes correlated with particular distinctions were identified using the signal-to-noise statistic: $d = (\mu_1 - \mu_2) / (\delta_1 + \delta_2)$; where μ and δ represent the mean and standard deviation of expression, for two comparable classes, respectively. The expression level of each gene relative to the mean expression level across all samples is represented by an appropriate colour. Red colour represents expression greater than the mean, and blue represents expression less than the mean. The intensity of each colour represents the magnitude of deviation from the mean. Value of these distinctions was confirmed by means of SVM with the leave-one-out cross-validation method, whereby a training set of all samples, but one, in that distinction, has been used to predict the class of a randomly withheld sample, and the accuracy rate was recorded. Starting from 30 genes (15 most over- and 15 most underexpressed) in a respective distinction, subtracting one gene by turn, we tried to find the minimum number of genes separating the two classes with good accuracy.

Survival prediction models were performed using two methods: hierarchical clustering and a modified fuzzy c-means. With the latter, on the basis of 200 most differentially expressed genes in high-grade gliomas in relation to the control group, the samples were divided into two sets according to membership function, in which any sample upon gene expression belongs to set A with probability x, and to set B with probability y, with an assumption that $x+y=1$. It is noteworthy that sets A and B were created upon gene expressions only. At the subsequent stage, the membership parameter was determined by the product of the survival

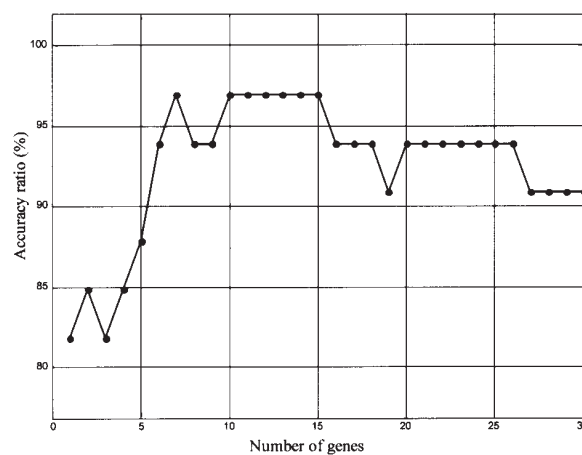


Figure 1. Accuracy ratio of prediction of probes to a proper group (gliomas or controls) using the leave-one-out method (LVSM, Kernel radial) depending on the number of genes used (range, 1-30). Error rate was the lowest with 7 genes used (4 most overexpressed, 3 most underexpressed).

time and the normalized probability value: $n_i = t_i [x_i / (x_i^2 + y_i^2)]$; in a few censored cases, their survival time was estimated according to the formula: $t_i' = (t_{\max} + t_i) / 2$, where t_{\max} is a maximum follow-up (may be censored) in set A or B, accordingly. Afterwards, the samples were sorted out according to the membership parameter, and divided to its median. Next, we built classifiers, and, using the leave-one-out cross-validation method with a different gene number we found a model with a minimal cross-validation error and the minimum number of genes.

Statistical analyses. Reference points for this study consisted of dates of surgical procedures. January, 2005 was the date of the last follow-up examination. Patients' deaths were end points. Survival time and other factors were analysed with Kaplan-Meier method. The following variables were tested: patient age (<40 years old vs. ≥ 40 , <60 years old vs. ≥ 60), sex, postoperative Karnofsky performance scale (100 vs. others), extent of resection (GTR vs. STR), WHO grade (III vs. IV), radiation dose [low (42 Gy) vs. high (50 Gy and above)], and survival prediction model. Cox's F test was used to analyze the difference between stratified groups, and Cox proportional hazard model, with all factors significant in univariate analysis, was used to find independent factors influencing survival time. Reciprocal associations between parameters were assessed by contingency tables and t-test. For all analyses $p < 0.05$ was accepted as significant. Statistical analysis was performed using Statistica 5.0 (StatSoft, Tulsa, USA).

Results

As expected, all the gliomas in the study were clearly separated from normal brain samples using the signal-to-noise statistic. On testing a variable number (1-30) of the topmost differentially expressed genes with the leave-one-out method, the cross-validation error rate was the lowest (0.03) when 7 genes (4 over- and 3 underexpressed) were used (Fig. 1). The 4

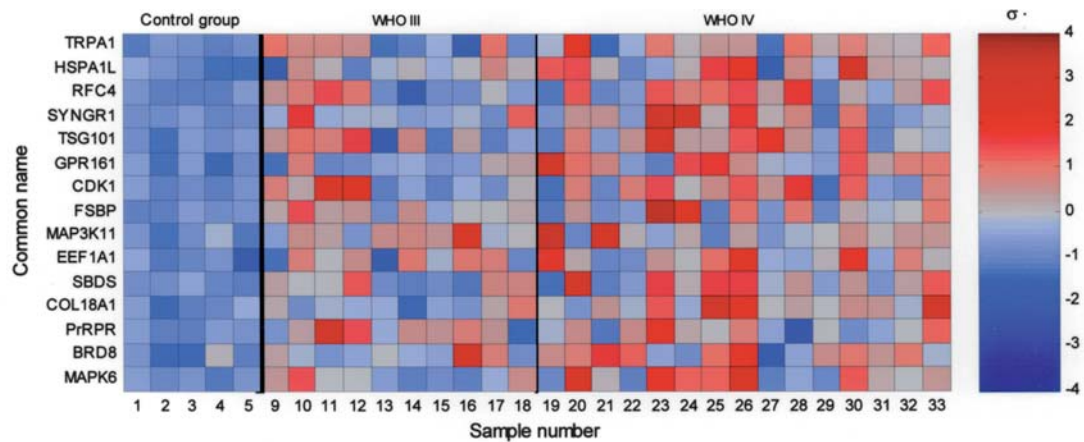


Figure 2. Genes overexpressed in high-grade gliomas in relation to controls, ranked by the signal-to-noise statistic. Colours depict high (red) and low (blue) relative expression to the mean.

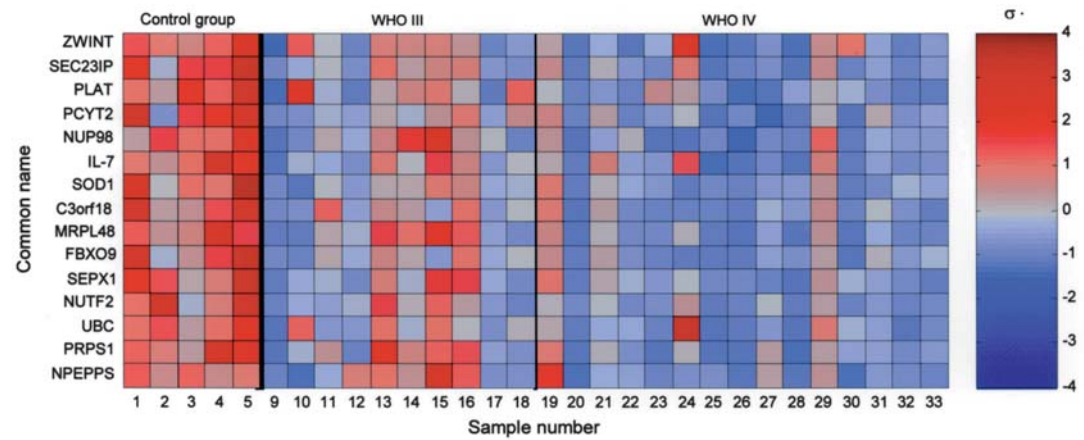


Figure 3. Genes underexpressed in high-grade gliomas in relation to controls, ranked by the signal-to-noise statistic. Colours depict high (red) and low (blue) relative expression to the mean.

most overexpressed genes in gliomas included: TRPA 1, acting as an ion channel, involved in signal transduction, mainly cold nociception (46,47), and recently discovered as a component of the mechanosensitive transduction channel of hair cell in the inner ear (48); HSPA1L, inhibiting apoptosis in gastric (49), prostatic (50), breast, gynaecological and bladder cancers (51), and influencing prognosis; RFC4, encoding replication factor C 37 kDa subunit required in elongation of primed DNA templates by DNA polymerase delta and epsilon (52); SYNGR1, whose product is a presynaptic membrane protein associated with presynaptic vesicles (53). Three genes, the most highly underexpressed in gliomas relative to the normal brain, included: ZWINT, playing a significant role in a normal centromere function, and, when depleted, causing an aberrant premature chromosome segregation (54-56); SEC23IP, encoding protein involved in the maintenance of the endoplasmic reticulum-Golgi intermediate compartment and Golgi structures (57); PLAT, encoding tissue-type plasminogen activator, and, when underexpressed in gliomas, may be associated with a hypercoagulated state in intratumoral vessels and its thrombosis (58). Of remarkable interest was also the fifth most overexpressed gene, i.e. CDK1, encoding kinase, which, acting in a complex with cyclin B1 as a mitosis

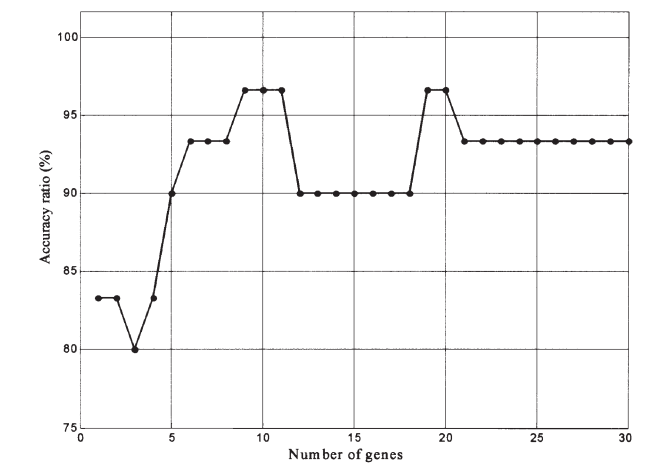


Figure 4. Accuracy ratio of prediction of probes to proper group (high-grade gliomas or controls) using the leave-one-out method (LVSM, Kernel radial) depending on the genes used (range,1-30). Error rate was the lowest with 9 genes used (5 most overexpressed, 4 most underexpressed).

promoting factor, plays the key role at the initial stage of mitosis (59).

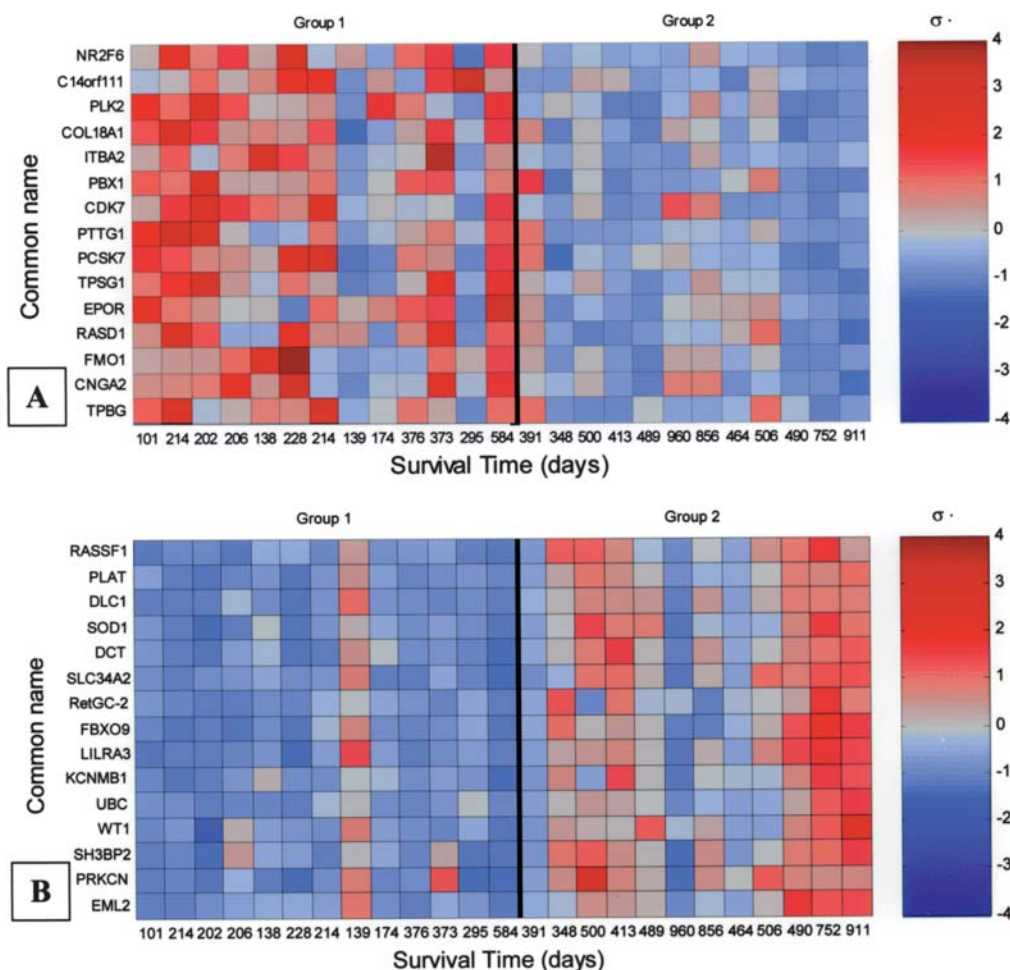


Figure 5. Genes differentially expressed between two groups of high-grade gliomas with a worse or better prognosis, i.e. group 1 and 2, respectively. The genes were ranked using the signal-to-noise statistic. Colours depict high (red) and low (blue) relative expression to the mean. A and B show 15 most over- and underexpressed genes in both groups, respectively.

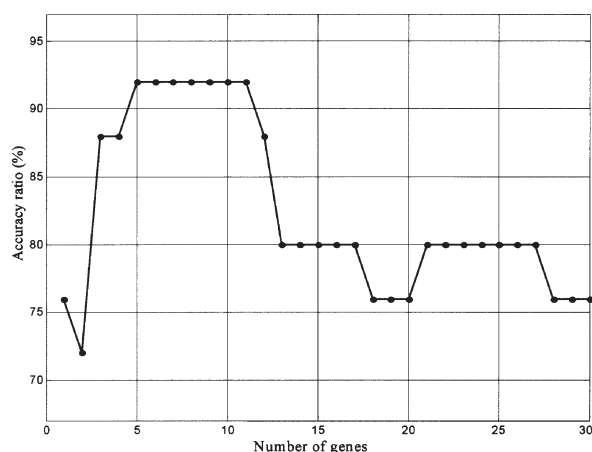


Figure 6. Accuracy ratio of prediction of probes to a proper group (a better or worse prognostic high-grade gliomas) using the leave-one-out method (LVSM, Kernel radial) with respect to the number of genes used (range: 1-30). The accuracy rate was the highest with 5-11 most differentially expressed genes.

The topmost differentially expressed genes in high-grade gliomas in relation to normal brain samples were very similar to those in all gliomas, with only few exceptions (Figs. 2 and 3). The cross-validation accuracy was 96.7% when 9 genes were applied (5 overexpressed and 4 underexpressed) (Fig. 4).

Apart from the 7 genes mentioned above, of importance in this distinction were also the TSG101 (the fifth most over-expressed gene), whose aberrant splicing or mutations are associated with breast cancer (60,61), and PCYT2 (the fourth most underexpressed gene), encoding an enzyme involved in membrane phospholipid synthesis (62).

Grade II tumours (according to WHO classification) were excluded from the survival analysis, since there were only three patients with a short follow-up in this group, and in view of a known, more favourable prognosis than in high-grade gliomas. In order to find genetic profiles correlated with survival, we first investigated whether hierarchical clustering might divide high-grade gliomas into two groups with different prognoses. Although tumours were clustered in two groups distinguishable by gene expression, the survival analysis showed no difference between them. After a failed unsupervised method, we used a partly supervised one known as 'fuzzy c-means' which combines information resulting from gene expression and that on survival. The most over- and underexpressed genes in the two resulting groups: the former with a worse, and the latter, with a better prognosis, are displayed in Fig. 5. Survival group 1 included 2 tumours grade III and 11 tumours grade IV, whereas survival group 2 consisted of 8 gliomas grade III and 4 gliomas grade IV. The accuracy ratio of prediction of withheld samples to a proper

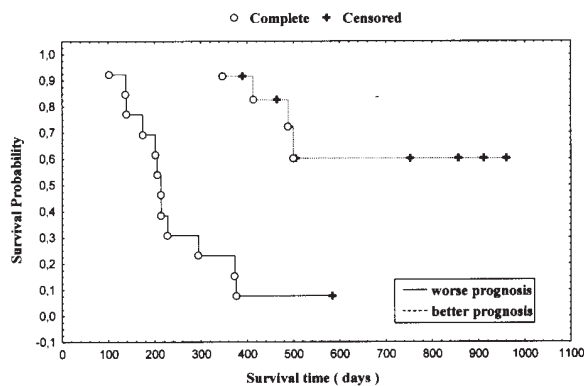


Figure 7. Kaplan-Meier curves for two patient groups divided according to gene expression profiles; Cox's F test: $p=0.0035$.

group (worse or better survival) using the leave-one-out method (LSVM, Kernel RBF) dependent on the number of genes used, is shown in Fig. 6. As can be seen, from 5 to 11 most differentially expressed genes represent the optimum number of genes with which the SVM classifier may predict a class of withheld samples with a high accuracy (92%).

Survival predictor based on gene expression was significant in the univariate survival analysis ($p=0.0035$); Kaplan-Meier curves displaying the difference in survival are shown in Fig. 7. Our further attempt was to find association between clinical parameters and survival in high-grade gliomas. Median survival was 498 days in tumours grade III, and 295 days in gliomas grade IV. The difference confirms that the WHO grade is an important predictor of survival ($p=0.0029$). A younger age (<40 years) was correlated with a longer survival time ($p=0.027$); the age ≥ 60 years showed the opposite ($p=0.0038$). Radiotherapy was used in all high-grade glioma patients. A higher postoperative radiation dose (50 Gy or 62 Gy) vs. a lower dose (42 Gy) was also associated with a more favourable prognosis ($p=0.0042$). Differences in survival time were also found in correlation with the first symptom or sign of disease (neurological deficit vs. other symptoms or signs; $p=0.018$), and in postoperative KPS score (100 points vs. others) ($p=0.043$). No other parameters were associated with survival: sex ($p=0.11$), extent of operation (GTR vs. STR; $p=0.14$), epileptic seizure as the first symptom of disease vs. other symptoms and signs ($p=0.4$), history duration dichotomized between median ($p=0.29$), use, or not, of chemotherapy ($p=0.35$).

Multivariate Cox regression analysis including a gene expression predictor and all significant clinical factors disclosed that all the other parameters, including WHO grading, become insignificant with gene expression profiling, and that the latter one remained a sole independent predictor of survival ($p=0.007$). It was also found that a higher dose of postoperative radiation ($p=0.098$) tends to exhibit a longer survival on multivariate analysis. Two groups of tumours divided with a gene expression pattern, except for WHO grading ($p=0.015$), did not differ by other factors: age, history duration, first symptom, extent of resection, postoperative KPS score, radiation dose, the use of chemotherapy. The better prognosis group included a higher number of WHO grade III gliomas, and in the worse survival group there were more WHO grade IV gliomas. Apart from a more

frequently used lower dose of radiation in older patients (>60 years), and more cases with GTR in WHO grade IV than WHO grade III, no significant associations were found among clinical parameters.

Discussion

A correct histopathological diagnosis is crucial in prognosis (6,29,36,63), and what is even more important, in susceptibility of some gliomas to chemotherapy (29,64). Results are frequently subjective, and there is significant disagreement among neuropathologists. The answer to the question how to best and accurately identify tumours, may be found in the domain of molecular technology (65-67). The challenge for molecular biology is to move the burden of histopathological assessment from classical techniques onto molecular methods. As shown by Nutt *et al* (68), classification of glioblastomas and nonclassic anaplastic oligodendrogliomas based on gene expression shows a significantly better correlation with survival than histological classification; due to this fact the former method may seem to be more robust. In our study, gene expression profiles clearly separated gliomas or high-grade gliomas from normal brain samples, and revealed that among the most overexpressed genes, many are involved in signal transduction and transcription or translation activity, e.g., TRPA1, RFC4, MAP3K11, BRD8, GPR161, PrRPR, EEF1A, MAP3K6. A better understanding of the significance of the most overexpressed, and also the most underexpressed genes in the pathogenesis of gliomas might enhance development of new treatment techniques. It is noteworthy that some of these overexpressed genes encode proteins involved in the G-protein signal transduction cascade, in which, activated G-proteins such as ras, are responsible for activation of serine-kinase kinases such as raf, and subsequently, for activation of MEK and MAPK, which, finally, results in alteration of gene transcription (69-72). An aberrant ras/raf/MEK/MAPK pathway plays an important role in malignant transformation, resistance to apoptosis, and enhanced glioma motility, and might be a target of anti-glioma therapy (69,71,72). Enhanced ras signaling is also partially responsible for radioresistance of some gliomas (30). Despite the absence of ras mutations in gliomas, contrary to mutations found in other cancers (69-72), overexpression of genes associated with ras signaling in our study results suggests its substantial role in gliomagenesis. HSPA1L, the second most overexpressed gene in gliomas and high-grade gliomas, apart from its known antiapoptotic influence in other cancers (49), has been recently discovered as expressed also in astrocytomas, and might be associated with resistance to apoptosis induced by chemotherapy, and, in this way, essential for prognosis (Tsogka S, 13th Congress of WFNS, 2005). ZWINT, the most underexpressed gene in our study, is essentially involved in a normal centromere function; its underexpression in tumours might cause an aberrant premature chromosome segregation resulting in aneuploidy in daughter cells (54-56). Oxidative stress damaging intracellular structures may be an initiating event in many diseases, including cancers. One of antioxidant enzymes is SOD1, mutated in amyotrophic lateral sclerosis (73), but also underexpressed in prostate cancers (74). Its underexpression in gliomas may be of significance in gliomagenesis. IL-7, a cytokine regulating B and T cell development,

was also underexpressed in our cohort study. IL-7 decreases tumorigenicity in mice gliomas (75), and positively influences TIL infiltration in human colorectal cancer (76). Therapeutic implications of these findings might be worth defining.

Despite a grave prognosis in all high-grade gliomas (1,4,6-8,21,36,63), some variability in survival has been noted (2,3,5). It is very likely that in the near future also prognosis in high-grade gliomas based on clinical factors and histopathological evaluation, will, at least partially, be replaced by prognosis based on gene expression (12,68). A vital issue in gliomas is to find out which of them will respond to therapy with their growth slackened, and which ones, despite surgery, radio- and chemotherapy will quickly progress, and the patient will die in a few months. Some factors, e.g., extension of resection and postoperative KPS score, will probably still have a prognostic influence, since they are independent of the biology of gliomas. It is difficult to imagine that the importance of gross total resection, in contrast to a partial resection or biopsy, will have completely disappeared, but other parameters related to tumour behaviour, e.g., age, sex, WHO grading, might be replaced by gene expression profile. At present, contrary to other cancers (77-80) there is no sole glioma marker associated with prognosis or useful in controlling disease progression. Some molecular alterations discovered so far, are associated with the survival time, i.e. LOH 10q, LOH 1p, LOH 19q, PTEN mutations (2,5,8,13,21,26,28-37) or those related to response to chemotherapy, i.e. CDKN2A loss, LOH 1p, LOH 19q (21,26,28-31), indicating that gene expression profiling of gliomas may be valuable in clinical practice. We have hypothesized that gene expression profiling in high-grade gliomas may allow distinguishing two groups of tumours with different prognoses. Results show that such grading is possible and prognosis of high-grade gliomas may be predictable based on the differences in gene expression. Among the 5-11 genes differentiating the worse and better survival groups with a good cross-validation accuracy, probably more interesting ones are those underexpressed in gliomas. In worse survival tumours, the most underexpressed gene relative to those with better survival ones was the tumor suppressor RASSF1, which normally interacts with cdc20, an activator of APC (anaphase promoting complex), resulting in inhibition of APC and stopping the cell cycle at the level of G1/S transition (81,82). RASSF1 negatively regulates cell cycle progression also by inhibiting accumulation of cyclin D1 protein (83). The second most underexpressed gene was PLAT, its underexpression is linked with necrosis and brain oedema in malignant gliomas (58). The third gene was tumour suppressor gene, DLC1. Its deletion or down-regulation have been observed in cancer of the breast (84), lung (85) prostate (86) and liver (87). SOD1 mentioned above, as well as DCT, the fifth most underexpressed gene in the worse survival, also shows an oxidoreductase activity apart from its involvement in melanin biosynthesis from tyrosine (88). Among the most overexpressed genes in the worse survival tumours, the first one was NR2F6 encoding a protein very similar to steroid and thyroid receptors, and involved in regulation of transcription (89); the third most overexpressed gene, PLK2, plays a role in cell cycle regulation and is involved in embryonic development (90).

Currently, clinical factors recognized as those affecting prognosis include WHO grade, age, extent of resection, and KPS status. WHO grading is the only axiom used at present (4,6), which was also confirmed in our study. The usefulness of other factors is questionable, with some disagreements in investigations. We have found a shorter survival for patients with neurological deficit as the first symptom of disease. In our cohort of patients, age was also found to be a significant prognostic factor, which is consistent with other studies (1,4,6,7,9-11). Gross total vs. subtotal resection did not influence prognosis, contrary to other reports (1,9,10). Previous studies on the effect of postoperative KPS score upon prognosis presented various conclusions. Some studies indicated an advantage of a better postoperative status on time of survival (1,4,6,7,10), whereas other authors did not report that relationship (9,11). Our patients with an excellent postoperative status (100 points in KPS), in contrast to patients showing symptoms or signs of disease, had a significantly longer survival time. Finally, a higher radiation dose showed an advantage in patient survival (4). No differences in survival related to sex, history duration, epileptic event as the first sign, and use of chemotherapy were found in univariate analysis, which was consistent with other reports (1,10,11). Multivariate analysis showed that only the gene predictor remained significant, whereas others, at the presence of gene expression profiling, became insignificant. The better survival in patients with high-grade gliomas is suggestive of potentially more differentiated tumours; not surprisingly, that group with better prognosis was found to have more grade III tumours than those of grade IV, contrary to a worse prognosis group in which more malignant according WHO grading tumours prevailed.

In conclusion, gene expression signatures distinguish gliomas or high-grade gliomas from a normal brain with good cross-validation error rates. Survival time in patients with high-grade gliomas is predictable from gene expression; clinical factors at the presence of the gene expression predictor become insignificant in multivariate analysis.

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