Incidence of the main genetic markers in glioblastoma multiforme is independent of tumor topology

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Received November 14, 2006

Glioblastoma multiforme (GBM) is the most common as well as the most aggressive type of primary brain tumor of astrocytic origin in adults. GBM is characterized by a high degree of intratumoral heterogeneity both in histomorphology and genetic changes. Trisomy/polysomy of chromosome 7, monosomy of chromosome 10, EGFR gene amplification and p53 deletion have been described as the typical genetic markers for tumor classification and prediction of possible response to therapy. Our work was based on detection of these four main genetic changes both in central and peripheral parts of the tumors to evaluate possible differences in the topological incidence of these genetic markers. Chromosomal abnormalities in tumor samples from a group of 21 patients surgically treated for GBM were characterized by means of the interphase-fluorescence *in situ* hybridization (I-FISH) technique using sets of centromere and locus-specific DNA probes. In addition, we performed a detailed analysis of one selected tumor sample using a genomic microarray system (GenoSensor Array 300) to characterize copy number changes of specific sequences and refine results obtained by I-FISH. However, the data show no significant differences in occurrence of the described genetic markers in either part of the tumor.

Key words: glioblastoma multiforme, interphase-FISH, array-CGH, trisomy/polysomy of chromosome 7, monosomy of chromosome 10, EGFR amplification, p53 deletion, molecular cytogenetics

Glioblastoma multiforme (GBM, WHO grade IV) is the most malignant type of brain tumor in adults, having an annual incidence of five to seven cases per 100,000 people. A higher incidence is observed in men (M) than women (W): the ratio of M:W is 3:2 [1]. Despite all available therapeutic approaches including surgery, chemotherapy and radiotherapy, curative treatment for GBM is in most cases not accomplished. Survival after diagnosis is usually not beyond one year [2, 3].

GBM belongs to the astrocytic gliomas, i.e. the tumorigenesis of GBM is believed to be a consequence of neoplastic transformation of a glial cell. According to the WHO classification, malignant astrocytomas (high-grade astrocytomas) are subdivided into two different histologically defined categories: anaplastic astrocytoma (WHO grade III), and glioblastoma multiforme (WHO grade IV). High-grade astrocytomas are characterized by intratumoral heterogeneity both in histomorphology and genetic changes [4].

The main cytogenetic alterations that have been found in GBM are partial or complete loss of chromosome 10 (detectable in 70-85% of all GBMs) [5–9], as well as polysomy of chromosome 7 (detectable in 50–80 % of all GBMs) [1,5,9–11]. Nevertheless, numerous other changes in chromosomes have been described in GBM tumors: gains of whole chromosomes 12, 13, 17, and 20, as well as losses of chromosomes 1, 8, 13, 14, 17, 19, 22 and Y [10–13]. The most common structural aberrations are deletions of 9p, 13q, 14q, 17p, 19q, and 22q, and gains of 3q, 4p, 7p, 7q, 12q, 19p, 20p, and 20q [1, 9, 11, 13–15].

Amplification of the gene for epidermal growth factor receptor (EGFR), which is localized in the 7p12 region, and mutation or loss of the p53 gene localized in 17p13.1 region seem to be the most important genetic changes with direct impact to GBM tumorigenesis. Mutation of the p53 gene is

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often found in patients of age 18-50 (44%) when compared with elderly patients (9%) [16–19].

Our study was aimed at the molecular cytogenetic analysis of the tumor samples from 21 patients with GBM diagnosis. Using interphase-fluorescence in situ hybridization (I-FISH), we examined numerical abnormalities of chromosomes 7 and 10, as well as for the specific alternations of the EGFR and p53 genes, which are known to be important for the classification of GBM subtypes (primary and secondary glioblastomas). To verify the results obtained by I-FISH, we employed the microarray-based comparative genomic hybridization assay (array-CGH) for whole-genomic screening of GBM abnormalities in one tumor sample, which was positive for both trisomy of chromosome 7 and monosomy of chromosome 10. However, the main goal of our study was to assess the possible discrepancies in occurrence of these cytogenetic markers in topologically different parts of the tumor.

Material and methods

Tumor samples. GBM samples from 21 adult patients were obtained from the Department of Neurosurgery, University Hospital in Brno. This research project was approved by the Ethics Committee of the University Hospital in Brno. All samples were histologically characterized according to WHO classification as astrocytoma grade III and IV. The central part of the tumor came from the geometric hub of the necrotic and cystic tumor tissue and the peripheral part was taken from a line dividing the pathologic and healthy tissue. Tumor samples were frozen in liquid nitrogen immediately after resection and were stored at -70°C. Portions of both sections of each tumor were used for tumor touch imprints. Briefly, a slice of the tumor was removed, and the surface made by the cutting was printed on a microscopic slide. These tumor touch imprints were utilized for I-FISH examination.

I-FISH method. After imprinting, the slides were dried and fixed in 100% methanol at 4°C for 20 minutes and then placed into a mixture of formic acid and methanol (1:3 v:v) at 4°C also for 20 minutes. The I-FISH hybridization procedure was performed according the manufacturer's protocol. Aquarius CEP7 / Spectrum Green and Aquarius CEP10 / Spectrum Red α-satellite centromeric probes (Cytocell) were chosen for determination of the number of chromosomes 7 and 10. Copies of the EGFR gene were detected using a LSI EGFR/CEP7 Dual Colour Probe (Abbott-Vysis). Possible deletion of the p53 gene was examined using a locus-specific probe LSI p53 / SpectrumOrange (Abbott-Vysis) hybridizing with the 17p13.1 region; this probe was combined with a centromeric probe CEP17 / Spectrum Green (Abbott-Vysis) hybridizing with α-satellite DNA of chromosome 17.

The slides were observed by fluorescent microscopy using an Olympus BX 61 with CCD camera COHU 4910. Fluorescent signals were evaluated by means of computer image analysis software LUCIA G 4.82-FISH (Laboratory Imaging s.r.o., Prague, Czech Republic). Approximately 100-200 interphase nuclei were assessed for each slide. The cut-off levels for detection of trisomy/polysomy of chromosome 7 or monosomy of chromosome 10 (3 % for both probes) and deletion of p53 gene (8% for the probe) were based on the levels of abnormal signals found in control slides (lymphocytes of peripheral blood obtained from 10 healthy donors) using the mean +3SD.

Array-CGH method. DNA from the central part of the tumor from a 69 year old patient was extracted from the frozen tissue using standard chloroform extraction and successfully hybridized to the specific genomic biochip containing 287 DNA clones (P1, PAC, BAC) relating to the known oncogenes or tumor suppressor genes (GenoSensor Array 300, Abbott-Vysis). Approximately 25 ng each of test (male) and normal reference (female) DNA were labeled with Cy 3-dCTP and Cy 5-dCTP fluorescent nucleotides (Perkin-Elmer, Foster City, CA, USA) using the Microarray Random Priming Kit according to the manufacturer's protocols. Briefly, DNA in the mixture with random primer solution in 1x final concentration was denatured at 100°C for 10 minutes. Subsequently, the mixture of DNA and 1x primer solution was moved to ice and cooled for an additional 10 minutes. In the next step, nucleotide mix, labeled nucleotides, and Klenow fragments were added, and the mixture was incubated in the dark at 37°C for 2 hours. After incubation, the sample was digested using a DNase (1:20) at 15°C for 60 min and the reaction was stopped by the stop buffer. Unincorporated nucleotides were then removed using MicroSpin S-200 HR columns Sephadex (Amersham Biosciences, Little Chalfont, UK). Probes were precipitated with 3M sodium acetate, then with 100% ethanol and were resuspended in 10 mM Tris buffer (pH 8.0). Aliquots of 2.5 µl of labeled test and reference DNA were mixed in a tube with Microarray Hybridization Buffer, then denatured at 80°C for 10 min and incubated at 37°C for 1 hour to block repetitive sequences. Subsequently, the hybridization mixture was hybridized with a commercially available GenoSensor Array 300 slide at 37°C for 60-66 h. This slide contains 287 genomic clones including telomeres, microdeletions, oncogenes, tumor suppressor genes and other selected loci representing each chromosome arm. After hybridization, the microarray slide was rinsed three times with 50% formamide in 2x SSC at 40°C and with 1x SSC at room temperature. The slide was then counterstained with DAPI at a concentration of 10 $\mu g.ml^{-1}$ and covered with a cover slip. Finally, the microarray was captured and analyzed using the GenoSensor Reader System (Abbott-Vysis); the normalized ratio of target indicated degree of gain or loss of copy number when compared with the sample's modal copy number. Targets with mean test-over-reference ratios <0.8 were considered as losses of DNA sequences, whereas those with ratios >1.2 were considered as gains of DNA sequences.

Results

Characterization of the patients. A group of 21 patients with histologically-verified GBM (WHO Grades IV or III–

IV) was selected for this study. The sex ratio in this group was determined as 13:8 (M:F) with an increased incidence in men (62%). The age of patients ranged from 29 to 77 years.

Frequency of the main cytogenetic markers of GBM studied by I-FISH. The obtained results clearly confirmed our preliminary results for a part of this group of patients [20]. Monosomy of chromosome 10 and trisomy/polysomy of chromosome 7 represented the most common genetic abnormalities in GBM cells. Monosomy of chromosome 10 was detected in 20 patients, i.e. in 95%. Trisomy/polysomy for chromosome 7 was found in 19 patients (90%). Only trisomy of chromosome 7 was present in 12 patients (63%). A heterogenic cell population consisting of both trisomic cells and cells with 4 to 6 copies of chromosome 7 was detected in 7 patients (37%). The simultaneous incidence of monosomy of chromosome 10 and trisomy/polysomy of chromosome 7 in the same nucleus was studied on samples from 16 patients; in 11 of them (69%) we found association of both chromosomal alterations. Amplification of the EGFR gene that is localized in the 7p12 region was found in 5 patients (24%). Finally, deletion of the p53 gene was found in 4 patients (19%).

Table 1. DNA copy number changes detected in glioblastoma multiforme sample using GenoSensor Array 300 Microarray.

Loci names	Cytogenetic location	DNA copy number changes
G31341	7ptel	gain
IL6	7p21	gain
EGFR	7p12.3-p12.1	amplification
ELN	7q11.23	gain
RFC2	7q11.23	gain
ABCB1(MDR1)	7q21.1	gain
CDK6	7q21-q22	gain
SERPINE1	7q21.3-q22	gain
MET	7q31	gain
TIF1	7q32-q34	gain
stSG48460	7qtel	gain
7QTEL20	7qtel	gain
10QTEL006	10p tel	normal
SHGC-44253	10p tel	normal
D10S249, D10S533	10p15	normal
GATA3	10p15	normal
WI-2389, D10S1260	10p14-p13	normal
BMI1	10p13	loss
D10S167	10p11-10q11	loss
EGR2	10q21.3	loss
PTEN	10q23.3	loss
FGFR2	10q26	loss
DMBT1	10q25.3-q26.1	loss
stSG27915	10qtel	loss

Detection of the GBM relevant genes by array-CGH analysis. To verify results on cytogenetic markers of GBM obtained by I-FISH method, we performed whole-genomic screening of GBM abnormalities with the use of array-CGH in one tumor sample, in which the trisomy of chromosome 7 and monosomy of chromosome 10 were proved using centromeric probes jointly in 72% of the nuclei. The results show that the loss of genetic material of chromosome 10 as detected by a centromeric probe using I-FISH is restricted to the region 10p11.1-p13 to 10q11.1-qter (genes BMI1, D10S167, EGR2, FGFR2, DMBT1, stSG27915, 10QTEL24). Furthermore, gain of genetic material in regions 7p11.1-pter and 7q11.1-qter (genes G31341, ELN, RFC2, CYLN2, ABCB1 (MDR1), CDK6, SERPINE1, MET, TIF1, stSG48460, 7QTEL20), as well as amplification of the EGFR gene (7p12.1-12) were found. However, array-CGH analysis did not find changes in copy number of specific sequences in the region 10p13-10pter. Thus, the terminal part of the short arm of chromosome 10 was not lost. The results obtained by the CGH-array technique for chromosomes 7 and 10 are summarized in Table 1.

Incidence of the main cytogenetic markers in different parts of the tumor. In all patients, we simultaneously examined samples taken from two different areas of each tumor: the central and peripheral region. Analysis of these pairs of samples suggested that the results were the same for both tumor regions in most patients. Nevertheless, monosomy of chromosome 10 in one patient and trisomy/polysomy of chromosome 7 in two patients were found in the central part of the tumor only. Similarly, deletion of the p53 gene was detected solely in the central part of the tumor in two patients. Overall results for all four cytogenetic markers are summarized in Table 2; detailed results of this topological analysis for each of the patients are given in Table 3.

Table 2. Comparison of genetic alterations in central and peripheral parts of the tumor.

Genetic alterations	The same genetic alterations observed in central and peripheral part of the tumor *)	Genetic alterations observed in peripheral part only *)	Genetic alterations observed in central part only *)
monosomy 10	19/20	0	1
polysomy 7	17/19	0	2
monosomy 10 and polysomy 7	10/11	0	1
EGFR amplification	5/5	0	0
p53 deletion	2/4	0	2

*) number of patients

Table 3. Detailed results obtained by FISH analysis

Patient number	Histology	Part of tumor	Polysomy chromosome 7[a]	Monosomy chromosome 10[b]	Simultaneous incidence of monosomy 10 and trisomy/polysomy 7[c]	EGFR amplification[d]	p53 deletion[e]
1	IV.	central	+	+	x[f]	NA	D
		peripheral	+	+	x	NA	ND
2	IV.	central	+	+	x	NA	ND
		peripheral	+	+	x	NA	ND
3	IV.	central	+	+	x	NA	D
		peripheral	+	+	x	NA	D
4	III.-IV.	central	+	+	x	A	D
		peripheral	+	+	x	A	ND
5	IV.	central	+	+	x	A	D
		peripheral	+	+	x	A	D
6	III.-IV.	central	+	+	+	NA	ND
		peripheral	+	+	+	NA	ND
7	IV.	central	+	+	+	NA	ND
		peripheral	+	+	+	NA	ND
8	III.-IV.	central	+	+	+	NA	ND
		peripheral	+	+	+	NA	ND
9	III.-IV.	central	–	+	–	NA	ND
		peripheral	–	–	–	NA	ND
10	IV.	central	+	+	–	NA	ND
		peripheral	–	+	–	NA	ND
11	IV.	central	+	+	+	NA	ND
		peripheral	+	+	+	NA	ND
12	IV.	central	+	+	+	NA	ND
		peripheral	+	+	+	NA	ND
13	IV.	central	+	+	–	A	ND
		peripheral	+	+	–	A	ND
14	IV.	central	+	+	+	NA	ND
		peripheral	+	+	+	NA	ND
15	IV.	central	+	+	+	NA	ND
		peripheral	+	+	+	NA	ND
16	IV.	central	+	+	+	NA	ND
		peripheral	+	+	+	NA	ND
17	IV.	central	–	+	–	A	ND
		peripheral	–	+	–	A	ND
18	IV.	central	+	+	+	A	ND
		peripheral	+	+	+	A	ND
19	IV.	central	+	+	+	NA	ND
		peripheral	+	+	+	NA	ND
20	IV.	central	+	–	–	NA	ND
		peripheral	+	–	–	NA	ND
21	IV.	central	+	+	+	NA	ND
		peripheral	–	+	–	NA	ND

[a] positive (+), negative (–) finding of trisomy/polysomy of chromosome 7
[b] positive (+), negative (–) finding of monosomy of chromosome 10
[c] positive (+), negative (–) simultaneous incidence of monosomy 10 and trisomy/polysomy 7
[d] NA – nonamplification, A – amplification
[e] ND – nondeletion, D – deletion
[f] no results

Discussion

Classification based on molecular cytogenetic analyses represents one of the most important tools in diagnosis of high-grade astrocytomas. Using molecular genetic and cytogenetic methods, we discovered many genetic abnormalities that may help to characterize different subtypes of GBM with potential impact on the choice of therapy. In our study, we focused on the detection of the main cytogenetic markers of GBM, i.e. trisomy/polysomy of chromosome 7, monosomy of chromosome 10, EFGR gene amplification and p53 gene deletion. In view of known technical problems associated with

in vitro cultivation of solid tumors, we employed the I-FISH technique that allowed genetic analysis of the individual tumor cells.

We summarized that our results clearly show the presence of the cell clones with trisomy or polysomy of chromosome 7 (detected in 93% of patients) and with monosomy of chromosome 10 (detected in 96% of patients) that were determined by other authors to be typical cytogenetic markers of GBM cells [1, 5–11].

The combined occurrence of chromosome 7 trisomy/polysomy and chromosome 10 monosomy in the same nucleus were observed in the majority of patients (69%). Both of these abnormalities were reported as a hallmark of chromosomal instability, i.e. high frequency of mitotic errors in the tumor tissue [5, 21–23]. The most often detected finding was the combination of the monosomy of chromosome 10 with the trisomy of chromosome 7 (in 86% of patients). Similar results for these aneuploidies were reported by Loeper and co-workers in their study [7]. Sprenger and Arslantas show in their studies that GBM subtype, which is characterized by incidence of aneuploidies +7/-10, is associated with the worst prognosis of the disease, as well as with a resistance to chemotherapy [9, 24]. Incidence of chromosome 7 in higher copy number (>5) is also connected with radioresistance and with lower survival rate of patients [12].

The loss of chromosome 10 causes deletions of some important tumor suppressor genes that are localized at this chromosome and may play an important role in pathogenesis of brain tumors, namely *PTEN* in the 10q23.3 region [8, 25–28] and *DMBT1* in the 10q25-26 region [28]. This is especially important since the loss of the DMBT1 gene correlates with initiation as well as with malignant progression of astrocytomas [29]. From the clinical point of view, the loss of chromosome 10 is usually associated with poor prognosis in elderly patients and with better prognosis in those younger than 45 years of age [30].

Genes localized on chromosome 7 are also known to participate in GBM progression, especially *EGFR* and other tumor suppressor genes. EGFR gene amplification is just one of the most frequent genetic abnormalities described in GBM [13]. This gene is localized in the 7p12 region and is involved in control mechanisms of cell proliferation. Its overexpression supports neovascularization, cell proliferation and resistance to cell death signaling. The occurrence of this abnormality in our group of patients (30%) is in accordance with published results on the frequency of this amplification [25].

In the past, amplification of *EGFR* has been one of the most studied genetic changes associated with the progression as well as with the prognosis of GBM in general [31, 32]. This aberration usually occurs in primary (de novo) glioblastomas and is associated with better prognosis for survival in elderly patients, whereas it seems to be an unfavorable hallmark in younger patients, especially if occurring together with standard p53 gene expression [2, 33]. The overexpression of EGFR is also in correlation with increased radioresistance of these tumors [34]. Published results clearly show that EGFR gene mutation has usually been detected in combination with monosomy of chromosome 10, whereas the presence of EGFR amplification and p53 deletion were reported only infrequently [2,4]. Our results are in accordance with these findings: the mutual incidence of EGFR amplification and monosomy of chromosome 10 was detected in five patients (100% of all patients with EGFR amplification), while the deletion of *p53* was observed only in two patients (40% of all patients with EGFR amplification).

Mutations or deletions of tumor suppressor genes play also an important role both in the neoplastic transformation process and in secondary GBM progression from low-grade astrocytomas [30, 35, 36]. The cell clones with p53 deletion were found in approximately 19% of patients. The presence of *p53* mutation correlates also with tumor malignancy, with better survival of patients [33, 37] as well as with higher sensitivity to radiotherapy [12].

To precise the described results obtained by I-FISH, we used the array-CGH technique for analysis of one tumor sample, in which the combined incidence of both numerical abnormalities of the chromosomes 7 and 10 was detected in the same cell nuclei, as mentioned above. Array-CGH has proven to be a specific, sensitive, and fast technique, which enables analysis of the whole genome in a single experiment. Using array-CGH, the loss of DNA sequences corresponding to the partial deletion of chromosome 10 in the 10p13-10qter region, as well as the gain of DNA sequences covering the whole of chromosome 7 were detected. In addition, the considerable amplification of *EGFR* was also proved in this sample. The successful application of the array-CGH technique has been performed in several studies of GBM and these results have helped to identify genes involved in the progression of glial tumors [38-42]. Similarly to our results, the GenoSenzor Array 300 method was used also for description of GBM subgroups that are characterized by abnormalities of chromosomes 7 and 10 [40, 43]. Moreover, genetic changes identified using array-CGH technique allow to predict the survival of the respective patient [44].

Nevertheless, when our results describing the occurrence of these genetic markers in topologically different samples of the same tumor were compared, there was no significant difference found between central and peripheral parts of the tumor: more than 90% of analyzed samples show identical genetic markers detected by the I-FISH method in both peripheral and central parts of the tumor. In some cases, the markers were detected in the central part of the tumor only, but this result is obviously caused by an unclear dividing line between healthy and neoplastic tissue in this specific type of tumor. The term "multiforme" refers to the high level of histomorphologic variability of GBM and should be used also for the description of genetic heterogeneity of this tumor. When microdissection and CGH techniques have been employed to characterize histologically different parts of the tumor, the presence of several cytogenetically heterogeneous cell subpopulations were de-

scribed in accordance with different histological characterization of the samples [7, 10, 45]. However, our samples were taken only on the basis of their localization in the tumor tissue (center vs. periphery of the tumor) and all of them were recognized as neoplastic tissue during the macroscopic per-operative examination. For this reason, the approximately identical clonal ratios in both the central and peripheral parts of the tumor tissue are not surprising.

To conclude, our results clearly confirmed similar frequency of the main genetic markers of GBM (monosomy of chromosome 10, trisomy/polysomy of chromosome 7, EGFR gene amplification and p53 gene deletion) both in samples taken from central and peripheral parts of the tumor, regardless of the histomorphological heterogeneity of the whole tumor. Nevertheless, the array-CGH based genomic screening of the GBM samples performed in combination with a detailed histopathological analysis of the examined samples would be helpful in future studies of GBM heterogeneity.

This study was supported by grants COST OC B19.001 and VZ MSM 0021622415. We thank Mrs. Iva Slamova for her technical assistance.

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