

# Screening of genomic imbalances in glioblastoma multiforme using high-resolution comparative genomic hybridization

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**Abstract.** Comparative genomic hybridization (CGH) is a molecular cytogenetic technique that allows the genome-wide analysis of DNA sequence copy number differences. We applied conventional CGH and the recently developed high-resolution CGH (HR-CGH) to tumour samples from 18 patients with glioblastoma multiforme (GBM) in order to compare the sensitivity of CGH and HR-CGH in the screening of chromosomal abnormalities. The abnormalities were studied in topologically different central and peripheral tumour parts. A total of 78 different changes were observed using CGH (0-16 per tumour, median 3.5) and 154 using HR-CGH (0-21 per tumour, median 6). Using HR-CGH, losses were more frequent than gains. The representation of the most prominent changes revealed by both methods was similar and was comprised of the amplification of 7q12 and 12q13-q15, the gain of 7, 3q and 19, and the loss of 10, 9p, and 13q. However, HR-CGH detected certain other abnormalities (the loss of 6, 14q, 15q and 18q, and the gain of 19), which were rarely revealed by CGH. Using HR-CGH, the numbers and types of chromosomal changes detected in the central and peripheral parts of GBM were almost the same. The loss of chromosomes 10 and 9p and the gain of chromosomes 7

and 19 were the most frequent chromosomal alterations in both tumour parts. Our results from the GBM analysis show that HR-CGH technology can reveal new, recurrent genetic alterations involving the genes known to participate in tumorigenesis and in the progression of several human malignancies, thus allowing for a more accurate genetic characterization of these tumours.

## Introduction

Glioblastoma multiforme (GBM) (WHO grade IV) is the most malignant type of brain tumour of astrocytic origin (1). GBM is usually divided into two groups: (i) Primary or *de novo* GBM is clinically aggressive, occurs in older patients and is characterized by a more complex karyotype, (ii) secondary GBM, developed from pre-existing low-grade astrocytomas, occurs in younger patients and has a better prognosis (2). The tumorigenesis of GBM is the consequence of the accumulation of genetic events that confer a growth advantage to a glial cell. Genetic alterations occur at multiple sites, and the most frequent of these include the loss of chromosomes 10 and 9p and the gain of the entire chromosome 7 with a 7p12 (*EGFR*) amplification (3,4). Different chromosomal aberrations and their combinations can provide useful information with respect to tumour classification (5,6), prognosis (7,8) and response to therapy (9,10).

Various improvements in molecular cytogenetic techniques allow for a more precise determination of chromosomal changes. Chromosome comparative genomic hybridization (CGH) represents the possibility of obtaining genome-wide analyses of DNA sequence copy number differences (11) and has been successfully applied to GMB studies (12,13). This method detects changes that are present in  $\geq 50\%$  of the specimen cells, with the affected region exceeding 5-10 Mb. However, many chromosomal aberrations are present in an abundance lower than 50% and this may result in problems with their identification. High-resolution CGH (HR-CGH), developed by Kirchoff *et al* (14), is primarily suited for the detection and localization of genomic changes down to 3-5 Mb and/or those with lower aberrant clone prevalence (15). HR-CGH was successfully used in clinical genetic studies to reveal

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*Abbreviations:* CGH, comparative genomic hybridization; DAPI, 4,6-diamidino-2-phenylindole; EGFR, epidermal growth factor receptor; GBM, glioblastoma multiforme; HR-CGH, high-resolution comparative genomic hybridization; I-FISH, interphase-fluorescence *in situ* hybridization; TSG, tumour suppressor gene

*Key words:* comparative genomic hybridization, high-resolution comparative genomic hybridization, glioblastoma multiforme, molecular cytogenetics

subtle chromosomal rearrangements in dysmorphic and mentally retarded individuals with normal karyotypes (16,17) as well as for the diagnostic and prognostic classification of haematological malignancies (18).

As with previously published methods (14), we developed our own HR-CGH modifications using specialized software from Laboratory Imaging, Ltd. (Prague, Czech Republic). Subsequently, HR-CGH together with conventional CGH was employed in the analysis of samples from 18 patients with GBM in order to compare the sensitivity of these two methods. HR-CGH was very effective in the detection of chromosomal changes. Compared with CGH, HR-CGH was able to reveal nearly 50% more aberrations. Because GBM is characterized not only by intertumoral but also by intratumoral heterogeneity in both histomorphology and genetic changes (19-21), we also compared the incidence of various chromosomal changes in both the peripheral and central parts of tumours. Nevertheless, our results showed that these two sections were not genetically different.

## Materials and methods

**Tumour material.** The samples of GBM from 18 adult patients (12 male/6 female; median age, 65.5 years; range, 28-76 years) were obtained from the Department of Neurosurgery, University Hospital in Brno. All the samples were histologically characterized according to the WHO classification (1) as astrocytoma grade III and IV. The central part of the tumour came from the geometric hub of the necrotic and cystic tumour tissue and the peripheral part was taken from the line dividing the pathological and healthy tissue. The tumour samples were frozen in liquid nitrogen immediately after resection and were stored at  $-70^{\circ}\text{C}$ . Portions of both sections of each tumour were used for tumour touch imprints. Briefly, a slice of the tumour was removed, and the surface created by the cutting was printed on a microscopic slide. The slides were then dried and fixed in Carnoy's fixative. These tumour touch imprints were utilized for the interphase-fluorescence *in situ* hybridization (I-FISH) examination. The DNA was extracted from the remaining frozen tissue using standard chloroform extraction procedures and examined by CGH/HR-CGH.

**CGH and HR-CGH protocols.** Phytohemagglutinin-stimulated normal lymphocytes from karyotypically normal males were prepared as the metaphase targets for the CGH/HR-CGH experiments using standard protocols. CGH was performed according to the manufacturer's protocol (Abbott-Vysis, Inc., Downers Grove, IL, USA). Briefly, 2 ng tumour DNA, extracted from native frozen tissue using chloroform extraction, was labelled with Spectrum Green-dUTP (Abbott-Vysis) by nick translation. Simultaneously, normal human genomic DNA from peripheral blood lymphocytes was labelled with Spectrum Red-dUTP (Abbott-Vysis). The test and reference DNA were then ethanol-precipitated in the presence of 15  $\mu\text{g}$  human Cot-1 DNA (Abbott-Vysis) and dissolved in 10  $\mu\text{l}$  hybridization buffer (50% formamide/10% dextran sulfate/2x SSC, pH 7.0). The probe mixture was denatured and hybridized to denatured normal metaphase preparations at  $37^{\circ}\text{C}$  in a humidified chamber for 48-72 h.

After hybridization, the slides were washed in 0.5x SSC at  $74^{\circ}\text{C}$  and 2x SSC at room temperature and then counterstained with 4,6-diamidino-2-phenylindole (DAPI) solution.

Representative images of at least 10 metaphases per case were captured using an Olympus BX61 microscope and a CCD Camera COHU 4910. The chromosomes were karyotyped and ratio profiles were calculated using the software program, LUCIA G 4.82 - KARYO/FISH/CGH/CGH-Advanced Statistics (Laboratory Imaging).

For the CGH analysis, the losses and gains of the chromosomal regions were detected when the green:red ratio profiles deviated 20% from a ratio of 1 ( $<0.80$ , losses;  $>1.20$ , gains). Amplification was implied in the profiles with sharp rising peaks relative to the rest of the chromosomal profiles. The centromeres and acrocentric p-arms were excluded from the CGH analysis.

As mentioned above, HR-CGH was developed according to the published methods (14). In our study, the 99.5% dynamic standard reference interval was based on an average of 17 CGH analyses from healthy donors with normal karyotypes. The interval for each chromosome was then analyzed by a special software program, LUCIA G 4.82 Advanced Statistics (Laboratory Imaging). Chromosomal abnormalities were detected by comparing this 99.5% dynamic standard reference interval to the 99.5% confidence interval of the mean ratio profile of the test samples. Regions where the two sets of intervals did not overlap were considered aberrant. The centromeres, telomeres and acrocentric p-arms were excluded from the HR-CGH analysis.

For system sensitivity and specificity tests, patients with microdeletion syndromes (Prader-Willi/Angelman and DiGeorge) were analyzed and the sensitivity of the system was established at an interval of 4-5 Mb. To determine clone prevalence that can be identified by HR-CGH, particular chromosome aberrations detected by HR-CGH were verified by I-FISH with specific probes on the tumour touch imprints as described below.

**(I-FISH).** To verify the HR-CGH results, I-FISH was performed on the respective tumour touch imprints. Slides with tumour imprints were fixed in Carnoy's fixative, co-denatured and allowed to hybridize with commercially available centromeric probes from Aquarius<sup>®</sup>, Cytocell, UK [Enumeration Probe Chromosome 10 (Red)], and [Enumeration Probe Chromosome 7 (Green)], locus-specific probes from Q-BIOgene, France [17p13 specific probe (Green)/17  $\alpha$ Satellite Probe (Red)], and centromeric or locus-specific probes from Abbott-Vysis [LSI 1p36 SO/1q25 SG Probe, LSI ALK (2p23) Dual Color Probe, LSI IgH SG/ MYC SO/CEP 8 SA Probe, Her2/Neu17q11.2-12 SO/CEP 17 SG, LSI 19q13 SO/19p13 SG Probe, and LSI abl SO/bcr SG] according to the manufacturer's instructions. After post-hybridization washes, the slides were counterstained with DAPI and evaluated using an Olympus BX61 fluorescence microscope. At least 100 nuclei were captured and evaluated using a CCD camera and LUCIA software as described above.

**Statistical analysis.** The spectrum and frequency of the chromosomal aberrations were compared between CGH and HR-CGH as well as between the central and peripheral tumour

Table I. The number of DNA copy number changes in the tumour samples from 18 patients with GBM detected by CGH and HR-CGH.

Patient	Sex	Age	CGH			HR-CGH		
			G	L	T	G	L	T
1	M	65	0	0	0	0	4	4
2	M	66	2	2	4	2	6	8
3	M	44	8	8	16	10	11	21
4	M	59	2	0	2	4	5	9
5	M	71	0	0	0	4	4	8
6	M	74	3	2	5	3	5	8
7	F	65	2	7	9	4	10	14
8	M	28	0	0	0	0	0	0
9	F	76	0	0	0	0	0	0
10	M	70	4	8	12	5	11	16
11	F	57	2	5	7	2	6	8
12	F	69	2	2	4	3	2	5
13	F	63	2	0	2	2	5	7
14	F	47	1	2	3	4	3	7
15	M	61	5	2	7	6	3	9
16	M	67	0	0	0	5	8	13
17	M	69	2	1	3	2	6	8
18	F	67	2	2	4	7	2	9
Total			37	41	78 <sup>a</sup>	63 <sup>b</sup>	91	154
Median			2	2	3.5	3.5	5	8
Range			0-8	0-8	0-16	0-10	0-11	0-21

Patients: M, male; F, female. The number of DNA copy number changes: G, gain; L, loss; T, total. <sup>a</sup>p<0.05 CGH vs HR-CGH, <sup>b</sup>p<0.05 gains vs losses by HR-CGH.

parts. The nonparametric Wilcoxon rank-sum test was used for the variables with non-normal distributions, and the paired t-test was used for the variables with normal distributions. Differences of p<0.05 were considered statistically significant. Each of the two chromosomal arms was counted individually in the total chromosomal aberrations summary. An aberration had to be identified in at least 15% of the cases in the central or peripheral tumour parts in order to be considered for the comparison between these two sections.

## Results

*Chromosomal aberrations detected by CGH and HR-CGH.* In order to make a comparison of CGH and HR-CGH sensitivity, samples from the central part of GBMs were chosen. A total of 78 different changes were observed using conventional CGH (0-16 per tumour, median 3.5) and 154 using HR-CGH (0-21 per tumour, median 8) (p<0.05) (Table I). The frequency of the losses and gains of chromosomal material was equivalent when using CGH (median 2 vs median 2), while the losses were more frequent than the gains when using HR-CGH (median 5 vs median 3.5) (p<0.05). There were five patients with no evidence of genetic changes using CGH, while HR-CGH was able to disclose changes for three of them (Table I).

The representation of the most frequent chromosomal changes revealed by both methods was similar: The loss of 10 and 9p, the gain of 7, 3q and 19, and the amplification of 7p12 and 12q13-q15. However, HR-CGH was always more effective in chromosomal change detection and it was also able to detect new aberrations that were only rarely detectable by CGH: The loss of 6, 14q, 15q and 18q, and the gain of 19 (Fig. 1A and B).

*Re-evaluation of HR-CGH.* In order to re-evaluate the HR-CGH results, I-FISH was performed in select cases, including when the CGH profiles did not exceed the given thresholds while HR-CGH showed a gain or loss of chromosomal sections or of the whole chromosome. In each case, the chromosome that showed a normal HR-CGH profile was chosen as the reference chromosome and was co-hybridized with centromeric or locus-specific probes for the re-evaluation of chromosomes or chromosomal loci (Table II). Using this approach, the clonal proportion of chromosomal changes detectable by HR-CGH was established to be ~20%.

*Chromosomal aberrations in central and peripheral parts of GBM.* The spectrum and frequency of chromosomal changes detected by HR-CGH in the central and peripheral sections of

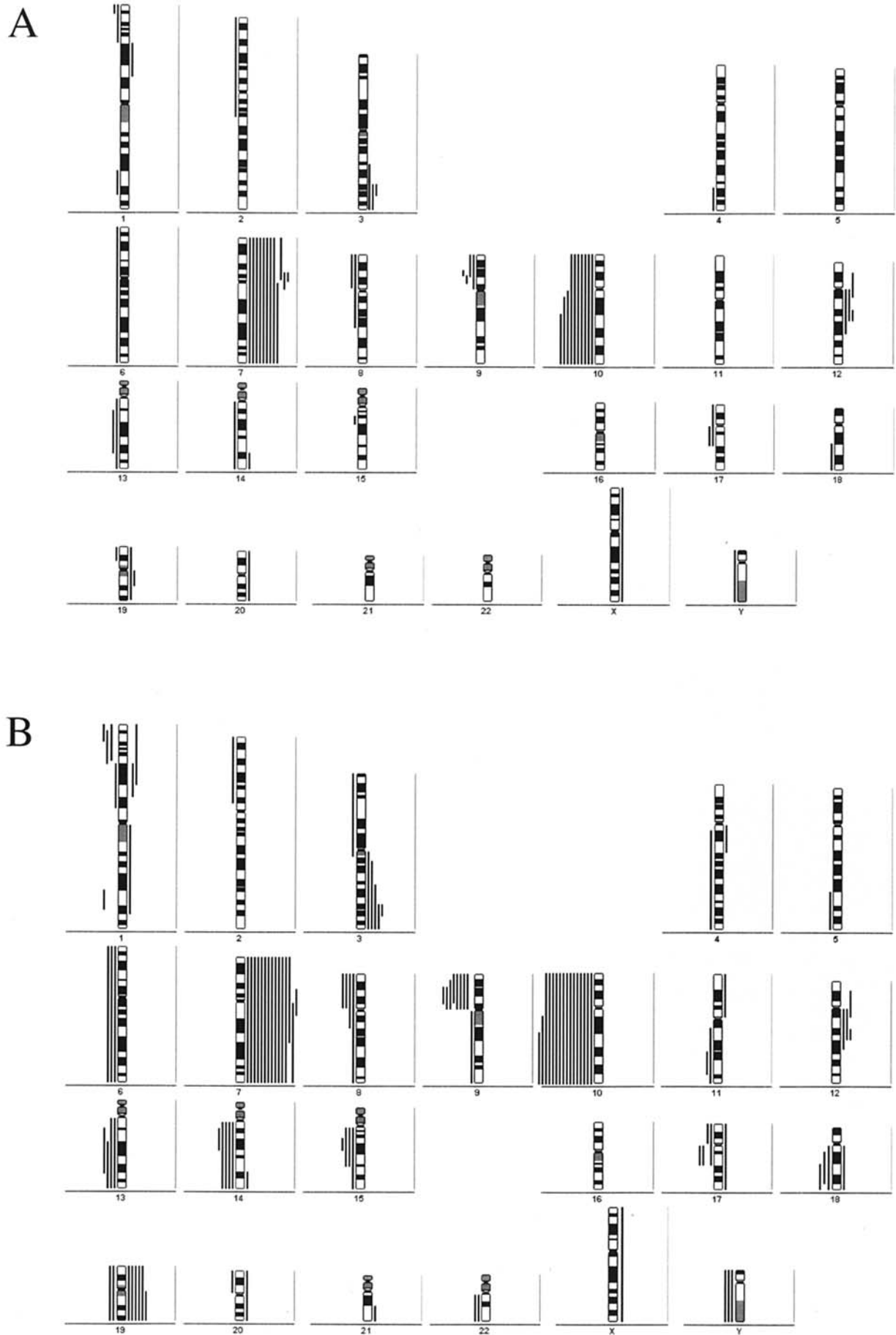


Figure 1. A summary of the copy number alterations in the tumour samples from 18 patients with GBM analysed by CGH (A) or HR-CGH (B). The vertical lines on the left of each chromosome ideogram represent losses, and on the right correspond to gains.

Table II. HR-CGH re-evaluations performed on fixed tumour touch imprints by the I-FISH technique.

HR-CGH change	Chromosome probe	Locus of interest	I-FISH % (number of signals)
-1p36	Vysis LSI 1p36 SO/1q25 SG Probe	1p36	18 (2x SG, 1x SO)
-2p	Vysis LSI ALK Dual Color Probe	2p23	36 (1x)
+7	Aquarius® Enumeration Probe Chromosome 7 (Green)	cep 7	27 (3x)
+7	Aquarius® Enumeration Probe Chromosome 7 (Green)	cep 7	33 (3x)
-9q	Vysis LSI abl SO/bcr SG	9q34	53 (2x SG, 1x SO)
-10	Aquarius® Enumeration Probe Chromosome 10 (Red)	cep 10	30 (1x)
-10	Aquarius® Enumeration Probe Chromosome 10 (Red)	cep 10	31 (1x)
-14q	Vysis LSI IgH SG/MYC SO/CEP 8 SA Probe	14q32.3	17 (2x SA, 1x SG)
-17q11.2-12	Vysis Her2/Neu SO/CEP 17 SG	17q11.2-12	50 (2x SG, 1x SO)
+17	Q-BIOgene 17p13 specific probe (Green)/17 αSatellite Probe (Red)	cep 17	26 (3x SG, 3x SO)
-19q	Vysis LSI 19q13 SO/19p13 SG Probe	19q13	53 (2x SG, 1x SO)
+19	Vysis LSI 19q13 SO/19p13 SG Probe	19p13, 19q13	52 (3x SG, 3x SO)

HR-CGH was able to detect clones with a prevalence of 17-50%, that were not detectable by CGH. Fluorophores: SO, Spectrum Orange™; SG, Spectrum Green™; SA, Spectrum Aqua™.

Table III. A summary of the DNA copy number changes detected by HR-CGH in the central and peripheral tumour parts in the samples taken from 18 patients with GBM.

	Central part			Peripheral part		
	G	L	T	G	L	T
Total	63 <sup>a</sup>	91	154	71 <sup>a</sup>	88	159
Median	3.5	5	8	3.5	5	9
Range	0-10	0-11	0-21	0-11	0-10	0-21

The number of DNA copy number changes: G, gain; L, loss; T, total. <sup>a</sup>p<0.05 gains vs losses.

GBM were almost equivalent. A total of 154 different changes were observed in the central parts (0-21 per tumour, median 8) and 159 in the peripheral parts (0-21 per tumour, median 9) (Table III). The losses of chromosomal material were more frequent than the gains in both tumour parts (p<0.05). The most frequent losses were detected at chromosomes 10, 9p and 14q, while the most frequent gains occurred at chromosomes 7, 19 and 3q (Table IV). Amplifications were frequent at loci 7p12 and 12q13-q15 in both tumour parts.

In general, 22 aberrations were present in the central tumour parts in at least 15% of the tumours, whereas three of these aberrations (the loss of 8p, 13q14, and 18q12-q21) occurred at a lower frequency in the peripheral parts (Table IV). However, these differences were not statistically significant. Therefore, no genetic change was predominantly associated with either one of the tumour parts.

Table IV. A comparison of the common aberrations revealed by HR-CGH in the central and peripheral parts of the tumour samples from 18 patients with GBM.

Abnormality (minimal region)	Total number (%) of samples with the respective change	
	Central part	Peripheral part
<b>Losses</b>		
1p (1p36.1)	3/18 (17)	4/18 (22)
6p	3/18 (17)	3/18 (17)
6q	3/18 (17)	3/18 (17)
8p	4/18 (22)	2/18 (11)
9p (9p21)	8/18 (44)	10/18 (56)
10p	13/18 (72)	12/18 (67)
10q (10q22-qter)	15/18 (83)	14/18 (78)
13q(13q21-q31)	4/18 (22)	5/18 (28)
14q (14q12-q21)	5/18 (28)	4/18 (22)
15q (15q15-q21)	4/18 (22)	4/18 (22)
17q (17q11-q21)	3/18 (17)	3/18 (17)
18q (18q12-21)	3/18 (17)	2/18 (11)
Y	3/18 (17)	4/18 (22)
<b>Gains</b>		
3q(3q26.1-26.3)	5/18 (28)	4/18 (22)
7p	14/18 (78)	14/18 (78)
7q (7q11.1-q22)	15/18 (83)	14/18 (78)
19p	5/18 (28)	6/18 (33)
19q	6/18 (33)	6/18 (33)
<b>Amplifications</b>		
7p12	4/18 (22)	4/18 (22)
12q13-q15	3/18 (17)	3/18 (17)

## Discussion

This study was designed to compare conventional CGH with the newly developed HR-CGH technique in order to ascertain the potential of HR-CGH in aiding the routine classification of GBM tumours. A series of 18 histologically characterized GBM tumours was examined using these two methods to compare their detection sensitivity, as well the spectrum of chromosomal changes in the central and peripheral parts of these tumours.

The detection limit of HR-CGH was established at 4-5 Mb, with an aberrant clone prevalence of at least 20% (Table II). HR-CGH detected clones with a prevalence of 20-50% which were not detectable by CGH, and thus was able to reveal nearly 50% more aberrations than CGH. This increment was especially prominent in the number of losses. Similar results, i.e. more frequent losses than gains, were also observed in another CGH study (22). One possible explanation is that duplications are more difficult to detect than deletions. The green:red ratio for duplications is 3:2, while the one for deletions is 1:2 (23). This is a very important fact to realize especially in the case of small aberrations and/or aberrations with lower prevalence detection. Even three apparently negative patients were shown to possess some genetic alterations by using HR-CGH instead of CGH.

Our study supported the trend of prominent chromosomal abnormalities in GBM described in other reports. (3,5,6,9,13,19,22,24-28). Chromosomal changes most often involved chromosomes 7, 10, 9p, 19, 3q and 13q, which are important indicators of GBM progression. As documented in Fig. 1A and B, these prominent changes were identified using both CGH and HR-CGH methods, although HR-CGH demonstrated a higher prevalence. The HR-CGH representation of the chromosomal abnormalities was comparable not only with previous CGH studies including a similar number of patients examined but also with studies that involved a higher number of patients (3,13,25).

HR-CGH revealed two times more alterations of 9p loci than conventional CGH (Fig. 1A and B). Tumour suppressor genes (TSGs) that play important roles in cell cycle regulation were shown to be located in this region (29).

Losses in chromosome 13q have been reported previously in studies of high-grade gliomas (25,26,28), possibly implicating the inactivation of the retinoblastoma gene at 13q14. A commonly deleted region in our study was 13q21-q31, which was also found to be lost in the study of Mohapatra *et al* (3). Possible TSGs localized in this region could also be associated with tumour progression.

There are certain TSGs on 19q that are frequently involved in the progression from low-grade astrocytoma to secondary glioblastoma (30). We detected the loss of chromosome 19 in only a few cases, whereas there were frequent gains in both arms of chromosome 19 (Fig. 1B). Similar results were observed by another team in 72 primary GBMs (3). Gains of chromosome 19 were detected mostly by HR-CGH, which is possibly due to the fact that these abnormalities occurred at a lower frequency. However, the presence of this chromosome could have a significant impact on patient treatment. Huhn *et al* (9) has shown that the simultaneous gain of chromosomes 7 and 19 was found in

30% of the radiation-resistant cases but was absent in the radiation-sensitive group.

Nevertheless, there were some changes that were observed predominantly or only with HR-CGH, and their impact on the survival of patients with GBM has also been documented (7). One of these changes is the invariable finding of a partial or complete loss of 14q. This loss has been described in oligodendrogliomas. However, alterations of this region are not often described in GBM studies. Kros *et al* (21) reported the loss of the entire chromosome 14q in the high-grade tumour regions of a large tumour resection specimen. Using high-resolution genome-wide allelotyping analysis, two common regions of deletion on chromosome 14 were mapped to 14q22.3-q32.1 and 14q32.1-qter (31). However, we found 14q12-q21 as a commonly deleted region, so there could be more regions whose deletion plays an important role in the pathogenesis of GBM.

The loss of the whole of chromosome 6 is another alteration detected primarily by HR-CGH. Several studies have described the potential role of the 6q deletion in malignant gliomas (32,33). Weber *et al* (34) found that the 6q loss was more common in the shorter progression-free interval group. Ichimura *et al* (35) confirmed the high frequency of chromosome 6 deletions in anaplastic astrocytomas and GBM using array-CGH, and identified two novel commonly deleted regions that could harbour TSGs. Previous studies have also shown that the loss of chromosomes 6 and 14 occurs more frequently in recurrent than in primary tumours. This suggests that these changes could play a role in GBM recurrence and/or the development of resistance to therapy (24).

Other affected chromosomal regions were 8p, 15q and 18q (Fig. 1B). Some studies, in accordance with our results, revealed the loss of the whole or part of the long arm of chromosome 15 (3,21), while others report gains and even the amplification of some regions of this chromosome (36,37). The prognostic effects of these alterations remain unreported in literature.

The frequent loss of 18q in the context of gliomas was described in 22 glioma-derived cell lines using a custom-made CGH array (38). Wooten *et al* (32) described the loss of the long arm of chromosome 18 and the short arm of chromosome 8 as the most frequently deleted loci specific to GBM, which can either lead to *de novo* GBM or permit existing astrocytomas to progress to GBM. The frequent loss of chromosome 8p, observed in our study by HR-CGH, has not yet been described in CGH studies of gliomas.

The lesser-known chromosome alterations we report here can be useful for higher-resolution mapping studies to precisely identify novel genes which could serve as targets for the future treatment of this malignancy.

The term 'multiforme' in GBM indicates the histological heterogeneity, and within a single tumour, areas with low- and high-grade histological features can be visible. Intratumoural genetic heterogeneity has been detected using CGH on microdissected glioblastoma tumour cells (19,21), and a large number of novel genes with distinct expression patterns in high- and low-grade gliomas have been identified (39). In contrast, Walker *et al* (40) found identical genetic losses in all areas of histological differentiation in 13 glioma tumours with intratumoural heterogeneity. We identified certain

differences in the numbers and representation of chromosomal aberrations between the central and peripheral parts of single tumours using newly developed HR-CGH. In our study, the comparison of the genetic change distribution in both areas showed that there are minimal differences in the representation of common genetic changes (Table III). Although aberrations of chromosomes 11 and 16 were observed in the peripheral but not the central tumour parts, this fact had no statistical significance. This contrast with other CGH studies could be due to the fact that our examined tumour parts were not histologically graded. They differed only in topological localization and therefore both parts could conceivably contain both low- and high-grade areas.

We have confirmed that GBM harbours multiple genetic abnormalities and that DNA isolated from single tumours varies widely in the number and location of chromosomal changes observed. However, genetic heterogeneity did not exist within the same tumour if compared localities differed only topologically, and not histologically. The molecular classification of gliomas is becoming increasingly important clinically as an adjunct to histopathological diagnosis. Our results have shown that HR-CGH is a comprehensive and rapid methodological approach to the analysis of global and locus-specific genomic alterations in glial tumours. Therefore, it could serve as a molecular tool for improved diagnostics and therapeutic decision-making in these tumours.

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