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Genomic Markers for Glioblastoma Multiforme, Revealed by Array CGH Analysis

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Authors' contributions

This work was carried out in collaboration between all authors. Author ID designed the study, performed the analysis, wrote the protocol and wrote the draft of the manuscript. Author ND managed the analyses of the study. Author HR managed the literature searches, author NE collected the material and author TD provided the general support in all steps of the study. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: Despite of intensive studies of glioblastoma multiforme, still no unified concept for the most important specific molecular alterations exists for this tumor type. The method of array CGH has great potential for molecular characterization of glioblastoma. The aim of our study was to determine the type, frequency and fine mapping of unbalanced genomic changes and to suggest candidate genes for the emergence and development of brain tumors.
Study Design: Ten tumor samples were collected from patients with glioblastoma multiforme after taking informed consent. Histological examination was done to confirm the presence of tumor cells in more than 75% of the samples. DNA was isolated from each tumor sample.
Place and Duration of Study: The material was collected in Department of Neurosurgery (Medical University Sofia) and processed for analysis in Department of

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Medical genetics (Medical University Sofia) between June 2010 and December 2011.
Methodology: We used the method of array CGH with BAC clones, covering the entire genome, for investigation of copy number changes in tumors. We applied specific software microarray analysis (Blue Fuse, Blue Gnome, Cambridge, UK). For fine mapping of the most significant aberrations and to identify possible candidate genes: 1) we identified BAC clones with aberrations of high amplitude (ratio T / H> 0.5 for gains and <-0.5 for losses); 2) we select only those clones that have a frequency of aberrations more than 30% and have at least one adjacent clone with the same aberration. Doing this we also determined the smallest regions of overlap (SRO) of aberrations that occur in at least 50% of tumors.
Results: Trisomy 7 (70%) and monosomy 10 (80%) were the most common big aberrations in tumors. Regional aberrations across all chromosomes were characterized in details. The most frequent were: amplification of 1q43-1q44 (50-70%), deletion 1p36 (60%), gains 9p11-9p13 (50%), deletion 18q22 (50%), gains of 20q11-q13 (50%), loss of 22q12 (50%), deletion Xp21 (50%). By determination of smallest regions of overlap and based on their function, we suggested potential candidate-oncogenes (*RGS7*, *CDK5*, *OPN3*, *CDK5RAP1*, *PTPN1*) and tumor-suppressor genes (*NF2* and *OSM*).
Conclusion: Our study provide with basis for further studies in which the role of identified candidate-genes will be validated by other molecular genetics methods and at other levels - transcriptional and protein. This will lead to significant advances in knowledge of glioblastoma multiforme and suggestion of new more effective molecular-based prognostic and therapeutic indicators.

Keywords: Glioblastoma multiforme; array CGH; oncogenes; tumor-suppressor genes.

1. INTRODUCTION

Glioblastoma multiforme (GBM) is the most common and most aggressive of all primary brain tumors. In most cases patients survive one year after diagnosis and the effect of treatment is often unsatisfactory. Understanding the pathogenesis and molecular changes in this type of cancer is the key to its effective diagnosis, prognosis and therapy.

The occurrence of cancer is a consequence of changes in regulatory mechanisms playing central role in the main cell-specific functions - proliferation, differentiation and apoptosis, which underlie the transformation of a normal cell into malignant. Despite of intensive studies of glioblastoma, still no unified concept for the most important specific molecular alterations exist for this tumor type. Fine mapping of genomic aberrations in these tumors and summarizing the data from different labs will lead to the complete and accurate characterization.

Microarray-based comparative genomic hybridization (array CGH) is a modern molecular method, doing scan of the cellular DNA for quantitative changes [1-3]. It is able to demonstrate genomic losses and/or gains in tumor tissue, as it is characterized by high sensitivity and reliability of results. So far, microarray techniques have been widely used in profiling different types of cancer. Studies of brain tumors using microarrays, however are relatively limited. The clinical significance of microstructural changes established by this method has been described by several authors. According Korshunov and coworkers [4] significant aberrations with adverse prognostic effects on survival of patients with glioblastoma multiforme are monosomy 10, trisomy 7, trisomy 19, 7p12 amplification, deletions of 10q and 9p. On the other hand the authors found that the presence of trisomy 9

and/or 19q deletion have good predictive value. According to Nigro et al. [5] simultaneous presence of monosomy 10 and trisomy 7 has a negative impact towards the survival of patients. In 2007, Korshunov and coworkers [6] describe the presence of 1p36 amplification as a risk factor for leptomeningial dissemination of tumors. Glioblastoma multiforme cell lines have been analyzed by array CGH, showing high levels of amplification of cyclin-dependent kinase 4 (CDK4), GLI, MYCN, MYC, MDM2, PDGFRA, EGFR and some other genes, suggesting their role in the tumorogenesis of GBM [7].

Obviously, the method of array CGH has high potential for molecular characterization of glioblastoma. The aim of our study was to determine the type, frequency and fine mapping of unbalanced genomic changes in glioblastoma multiforme by array CGH, covering the entire genome and to suggest candidate genes for the emergence and development of brain tumors.

2. MATERIALS AND METHODS

2.1 Subjects and Samples

We have used 10 tumor samples from patients with GBM aged between 38 and 65 (median age of 51 years) for analysis by comparative genomic hybridization on DNA microarrays, covering the entire genome with an average density of 1 BAC clone/0.8 Mb. The study was approved by the local Ethical Committee of Medical University of Sofia.

In all patients the diagnosis of glioblastoma multiforme was histologically confirmed–based on presence of pleiomorphy, nuclear atypism, high mitotic activity, microvascular proliferation, vessel microthrombosis with large necrotic fields and/or small necroses with pseudopalisadal order of tumor cells around necrosis. All patients were treated by surgery, usually with subtotal resection, followed by radio- and chemotherapy in standard doses and regimen.

2.2 DNA Extraction

Tumor DNA was isolated by standard phenol-chlorophorm extraction. DNA concentration was measured by Nanodrop, as well as the purity of DNA was estimated. The ratio 260/280 for the last parameter was in the range of 1.8 - 2.0 for each sample. As an additional quality control, DNA was checked on 1% agarose gel: DNA of high molecular weight (> 50 kbp) indicated it suitable for use.

2.3 Genomic Aarrays

Array CGH was applied for analysis of the samples. The method allows detection of quantitative abnormalities in the genetic material of tumor cells compared to normal. In this work each tumor DNA was hybridized against DNA from normal individuals of the same sex. Genomic DNA was labeled by random priming. After successful incorporation of Cy3 and Cy5 the two labeled DNAs (tumor and control) were combined in the presence of Cot 1-DNA. The samples were precipitated, solved in hybridization buffer and hybridized on microarray for 16-22 hours at 47ºC. After three washings at 60ºC (2x SSC + 0,05% Tween 20; 1x SSC and 0,1x SSC) and one at RT (0,1x SSC) the slides were scanned on GenePix 4100A. The images were analyzed by software program GenePix Pro 6.0. Fluorescent intensities of all spots on the slide were calculated after elimination of the local background.

Intensities of Cy3 and Cy5 were normalized to the whole set of spots on the chip. The ratio Cy3/Cy5 (when tumor DNA was labeled with Cy3) was calculates taking the average of the ratio for the replicates for each clone (only clones with less than 15% deviation from the average of replicates were analyzed). All data were processed by the program BlueFuse, whereby the profile is generated for each tumor with log2 ratio for all clones of microarray on the ordinate and Mb positions of these clones on the abscissa. Ratio values above 0.3 are reported as a gain, while values below -0.3 are reported as a loss.

3. RESULTS

Using the method of comparative genomic hybridization (CGH) on DNA microarrays (covering the entire genome) we have performed whole-genome analysis of unbalanced genomic alterations in 10 patients with glioblastoma multiforme. Microarrays were commercial and contained clones of Bacterial Artificial Chromosomes (BAC) with an average density of 1 clone/0.8 Mb. Fig. 1 represents a single profile with log2 T/N ratios for all clones across all chromosomes in tumor No 1. The average number of aberrations found in our study was 32.2 aberrations per tumor, among them deletions dominated.

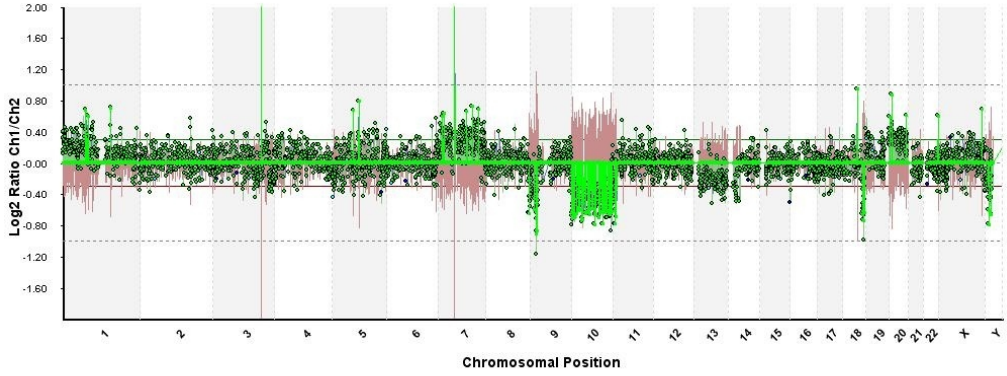

Fig. 1. Genomic profile for copy numbers across all chromosomes in patient No1, showing trisomy 7 and monosomy 10 along with other genomic aberrations.

The copy number alterations in each tumor were determined and Average Copy Number Alterations (ACNA) was summarized (Table 1). Tumors were divided in groups of genomic instability according to ACNA – group A with aberrations below ACNA and group B with aberrations above ACNA. The largest percentage of the analyzed tumors (60%) belonged to group A of genomic instability with prevalence of genetic losses. Four of the tumors showed higher degree of genomic instability (group B). These results could be a starting point for tracking the effects of therapy and the course of disease development in each patient to assess the impact of genomic instability on these parameters.

The incidence of large chromosome aberrations (gains and losses of whole chromosomes and chromosome arms) is presented in Table 2. The most commonly affected by gains of the entire chromosome or chromosome arm (Table 2) was chromosome 7 (80%). In 40% of tumors we observed duplications of chromosome 20. Such changes were detected in 30% of cases for chromosomes 1, 5, 9, 19 and 21. The highest frequency of large losses (Table 2) had chromosome 10 (monosomy in 80% of tumors), followed by chromosomes 13, 14 and 22 (40%), chromosome 16 (30%) and chromosomes 8, 18, X and Y (20%).

Table 1. Copy number alterations in each tumor and average copy number alterations (ACNA) determining two groups of genomic instability (GI)

Patient	Gain, N	Loss, N	Total aberrations, N	Group GI
1	11	14	25	A
2	14	20	34	B
3	11	30	41	B
4	9	9	18	A
5	12	14	26	A
6	20	29	49	B
7	19	31	50	B
8	20	11	31	A
9	7	11	18	A
10	11	19	30	A
Average aberrations	13,4	18,8	32,2	

Table 2. Frequency of genetic gain and loss for whole chromosomes and chromosome arms

Chr	Trisomy	Gain (p)	Gain (q)	Total	Monosomy	Loss (p)	Loss (q)	Total
1	20%	10%	0%	30%	0%	0%	0%	0%
2	10%	0%	0%	10%	0%	0%	0%	0%
3	0%	0%	0%	0%	0%	10%	0%	10%
4	0%	0%	0%	0%	0%	0%	0%	0%
5	10%	0%	20%	30%	0%	0%	0%	0%
6	0%	0%	0%	0%	0%	0%	10%	10%
7	70%	0%	10%	80%	0%	10%	0%	10%
8	0%	0%	0%	0%	20%	0%	0%	20%
9	20%	0%	10%	30%	0%	10%	0%	10%
10	0%	0%	0%	0%	80%	0%	0%	80%
11	10%	0%	0%	10%	0%	0%	10%	10%
12	0%	0%	0%	0%	0%	0%	10%	10%
13	0%	0%	0%	0%	10%	0%	30%	40%
14	0%	0%	0%	0%	20%	0%	20%	40%
15	0%	0%	10%	10%	0%	0%	10%	10%
16	0%	0%	0%	0%	0%	0%	30%	30%
17	0%	0%	0%	0%	0%	0%	10%	10%
18	0%	0%	0%	0%	0%	0%	20%	20%
19	0%	20%	10%	30%	0%	0%	10%	10%
20	30%	0%	10%	40%	0%	0%	0%	0%
21	20%	0%	10%	30%	0%	0%	0%	0%
22	0%	0%	0%	0%	10%	0%	30%	40%
X	0%	0%	0%	0%	20%	0%	0%	20%
Y	0%	0%	10%	10%	0%	0%	20%	20%

Fig. 2 represents the regional aberrations (comprising chromosome band) for the chromosomes 1-4. Genetic gains (green bars) dominated losses (red bars) in chromosome 1. The highest frequency of gain/amplification was detected in 1q43-1q44 (50-70%). The most frequent deletion was in 1p36, which occurs in 60% of cases. The highest frequency in chromosome 2 was detected for gain of 2p16 and 2q31 - 40%. In chromosome 3 genetic losses were prevalent. The highest frequency was in 3q23 -50%; 40% were deletions in

3p26 and 3p14. Regarding chromosome 4 the highest was the frequency for genetic gain in 4q34 (40%).

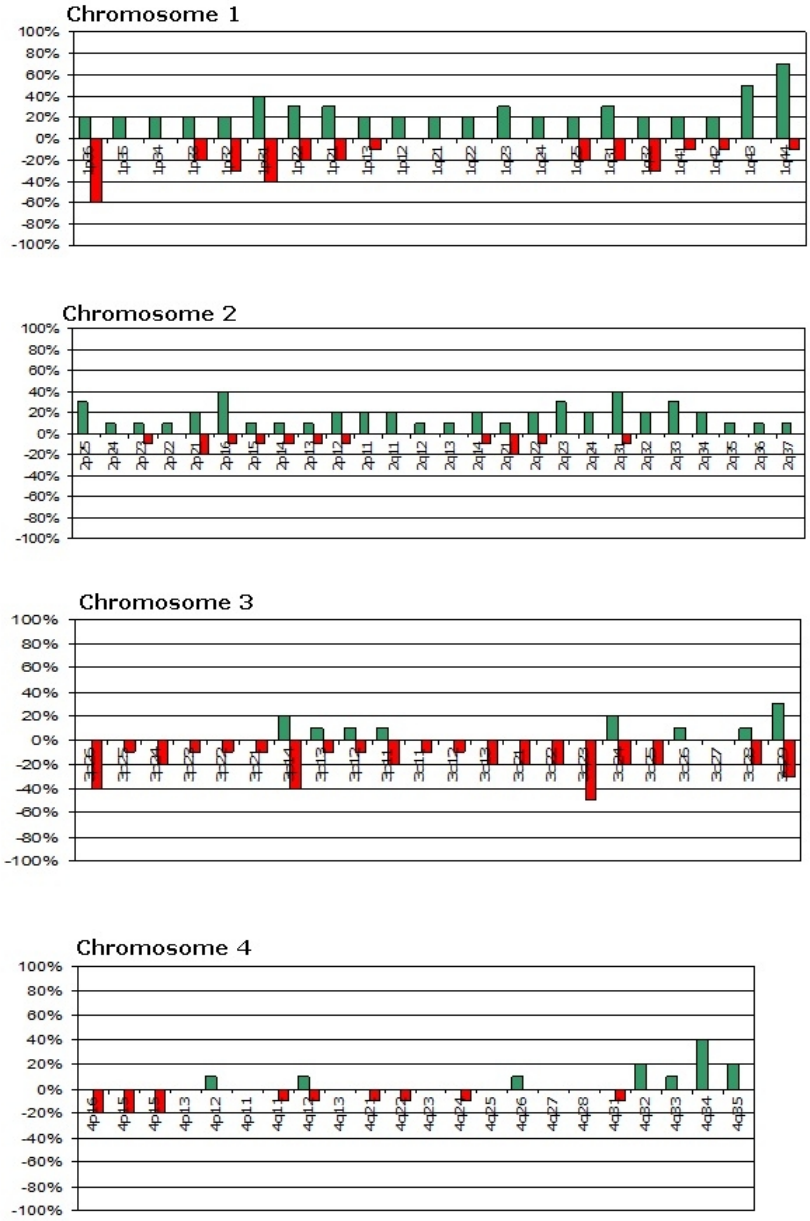

Fig. 2. Graphic representation of frequency of genetic gains and losses for each band of chromosomes 1-4 in studied GBM tumors.

Fig. 3 represents the regional aberrations for the chromosomes 5-8. The most common in chromosome 5 were genetic gains at 5q14 (40%) and genetic gains in regions 5q15-5q33 (30%). Genetic losses prevailed in chromosome 6, the most frequent were at 6p21 (50%).

Most of the tumors (70%) were with trisomy 7. In chromosome 8 deletions were most common – at 8q24 (50%) and at 8p12 (30%).

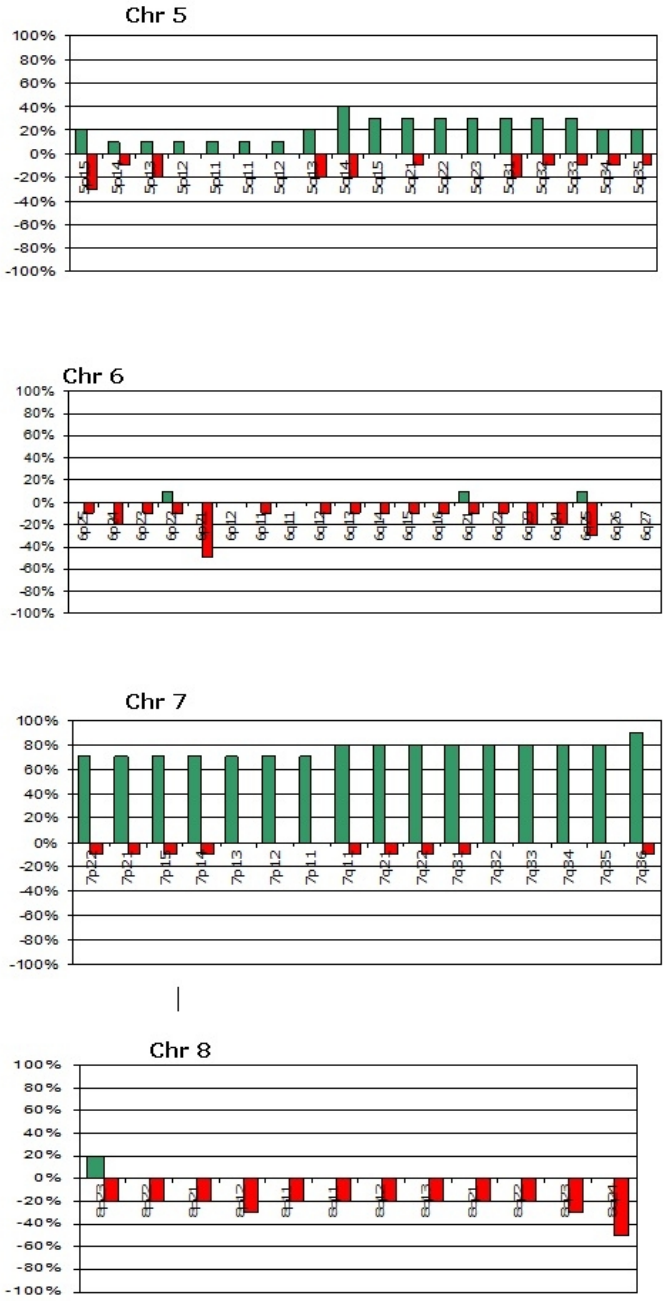

Fig. 3. Graphic representation of frequency of genetic gains and losses for each band of chromosomes 5-8 in studied GBM tumors.

Fig. 4 represents the regional aberrations for the chromosomes 9-16. Regarding chromosome 9 in 50% of tumors we observed gains at 9p13, 9p12 and 9p11. Monosomy 10

was detected in 80% of cases. In chromosome 11 for 30% of tumors gains were found in 11p14, 11p13 and 11q24. For chromosome 12 the most common was deletion in 12q24 (50%). For chromosome 13 deletions were detected also in 50% of cases for 13q31 and 13q32. The whole chromosome 14 was affected by gain in 50% of tumors. Deletions of 15q22, 15q24 and 15q25 were detected in 30% of cases. Half of the tumors were affected by deletions in 16q12.

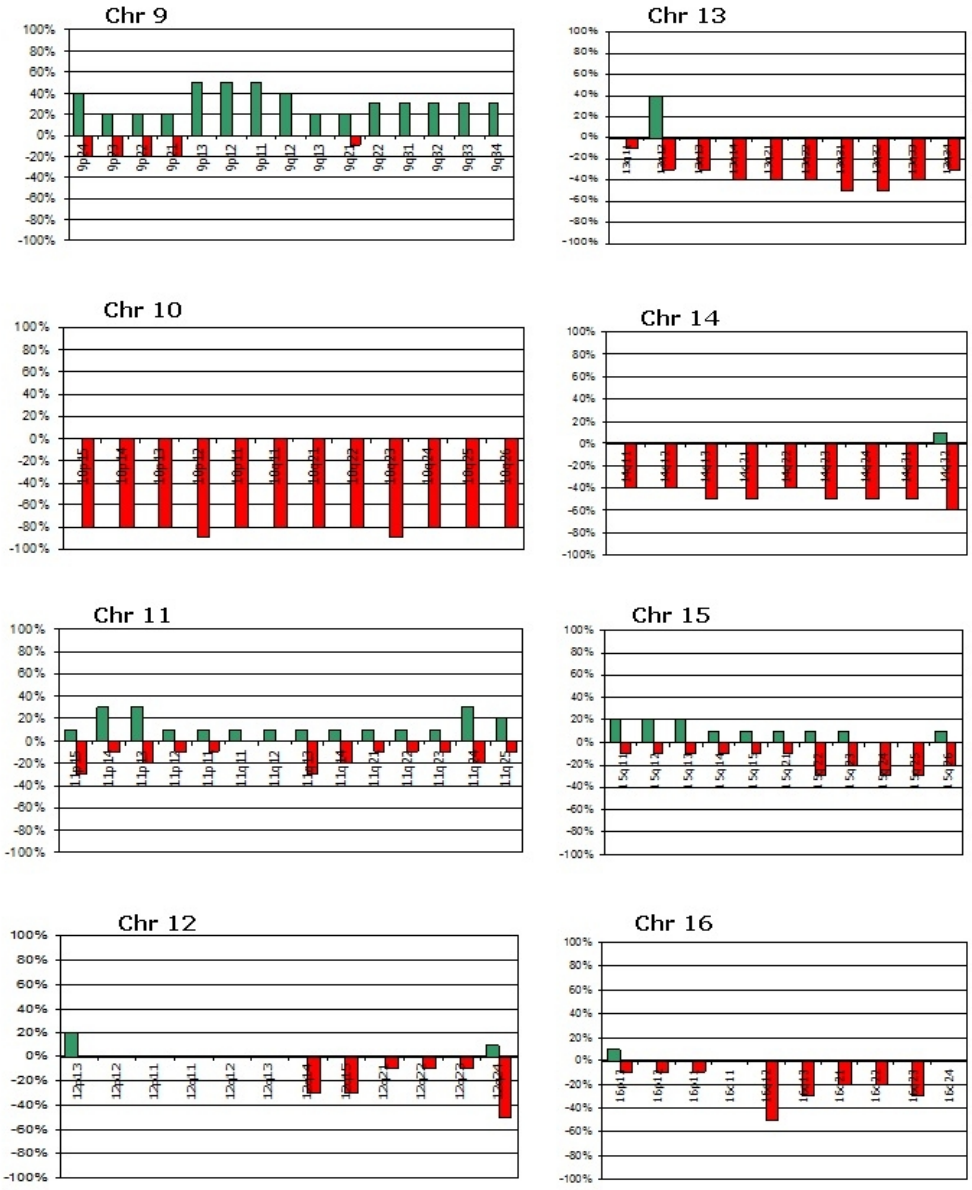

Fig. 4. Graphic representation of frequency of genetic gains and losses for each band of chromosomes 9-16 in studied GBM tumors.

Fig. 5 represents the regional aberrations for the chromosomes 17-22, X and Y. No genetic gains were discovered for chromosome 17, only low frequency's losses (20%) in 17q21 - 17q24. Deletions of 18q22 were found in 50% of tumors. Genetic gains along whole chromosome 19 were established in 20-40% of tumors. The frequency of gains in 20q11 and 20q13 was 50%. Frequency of genetic gains along chromosome 21 varied in 30-40%. The most frequent for chromosome 22 was loss in 22q12 (50%). For X chromosome deletions prevailed, especially Xp21 (50%). In male patients deletion of Yq11 was observed in all cases.

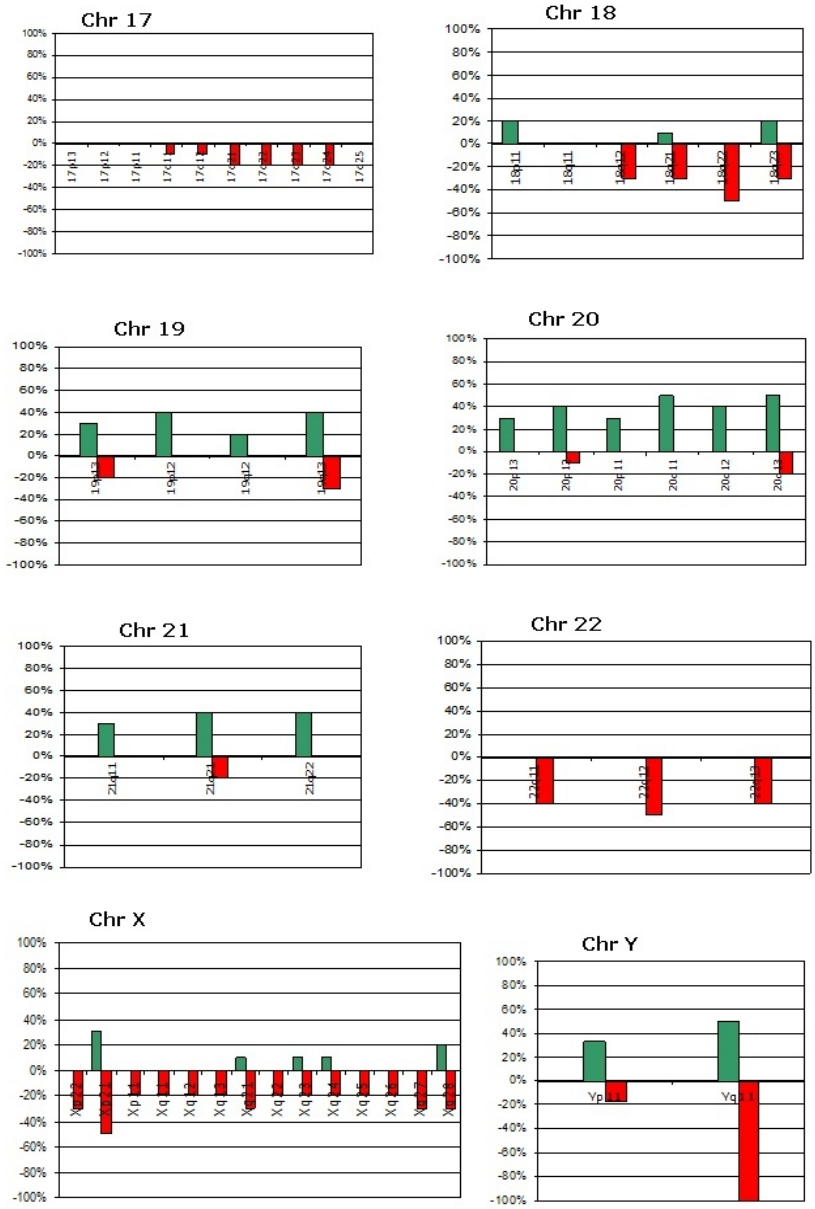

Fig. 5. Graphic representation of frequency of genetic gains and losses for each band of chromosomes 17-Y in studied GBM tumors

For fine mapping of the most significant aberrations and to identify possible candidate genes, we applied the following approach: 1) we identified BAC clones with aberrations of high amplitude (ratio T/H> 0.5 for gains and <-0.5 for losses); 2) select only those clones that have a frequency of aberrations more than 30% and have at least one adjacent clone with the same aberration. Doing this we also determined the smallest regions of overlap (SRO) of aberrations that occur in at least 50% of tumors. These regions and respective genes are shown in Table 3 for gains and Table 4 for losses. By this approach we detected in 90% of tumors genetic gain of 7q36.1-q36.3, containing several genes. Among the genes the best candidate for tumor development is CDK5. Other possible candidate GBM cancer-emerging genes, revealed from our study and based on their function are RGS7 in 1q44 (gained in 60% of tumors), as well as OPN3 in 1q44, CDK5RAP1 in 20q11 and PTPN1 in 20q13, all gained in 50% of tumors. Looking for potential tumor-suppressor genes, as they are located in highly deleted regions, in our study we highlighted the tumor-suppressor role of NF2 and OSM in 22q12, deleted in 50% of tumors.

Table 3. Smallest regions of overlap for gains and genes, located in them; potential candidat-oncogenes are highlighted

SRO	Tumors N	Start Mb	End Mb	Genes in the region
1q44	6	241417645	241525342	RGS7
1q44	5	241692178	242341646	KMO/OPN3/CHML/WDR64/ EXO1/MAP1LC3C/PLD5
7q36.1-7q36.3	9	150232192,5	157410964	GIMAP4/NOS3/ABCB8/SMARCD3/ CDK5/GBX1/MLL3/DNAJB6/SHH
9p13.1-9p11.1	5	41568845	46494788	ZNF658B/FOXD4L4/ DKFZp686I15204
20q11.21-20q11.23	5	29580150	34257717	BCL2L1/ID1/ASXL1/DNMT3B/ CDK5RAP1/ZNF341/NCOA6/BLP
20q13.13-20q13.2	5	48148645	49538625	PTPN1/BCAS4/CEBPB
20q13.32	5	55597837	56187108	BMP7/SPO11/BORIS/CTCFL

Table 4. Smallest regions of overlap for losses and genes, located in them; potential candidat-tumor supressor genes are highlighted

SRO	Tumors N	Start Mb	End Mb	Genes in the region
1p36.31	5	5954484.5	6499984.5	CHD5/GPR153/BACH
3q23	5	140627104	141352404.5	ZBTB38/RASA2/
6p21.1	5	45619656	45658506.5	------
10p12.31	9	21955728.5	21968744.5	MLLT10/AF10(CALM)/ DKFZp779J0967
10q23.32-10q23.33	9	93825200.5	94820919	CPEB3/IDE/KIF11/
13q22.2	5	74442733.5	75364760.5	KLF12
14q32.13-4q32.2	6	95282639.5	99065155.5	DICER1/TCL1A/TCL1B/VRK1
18q22.3	5	70293654	70710369.5	NETO1
22q12.1	5	25656648	26610024.5	MYO18B/ADRBK2/ SEZ6L
22q12.2-22q12.3	5	29965321.5	33318398.5	NF2/LIF/OSM/PES1/PATZ1/ SYN3

4. DISCUSSION

It is important GBM to be studied at the molecular and molecular-cytogenetic level. Thus we can extract as much information as possible about the genetic alterations and consequently arising biochemical and cytological changes, which may facilitate the diagnosis, prediction of disease progression and response of patients to various therapeutic agents.

Regions of gains and losses of genomic DNA are found in many types of cancer. Using whole-genome screening by DNA microarrays these aberrations can be detected at a very high resolution and investigated in order to identify candidate-genes responsible for disease [8]. This type of analysis was applied in patients with glioblastoma multiforme for the first time in Bulgaria. Our expectation was that the results would confirm the current data and will contribute to a better understanding of the mechanisms of the disease by revealing new information on potential oncogenes and tumor suppressor genes, aberrant in tumors of our patients.

The average number of aberrations found in our study was 32.2 aberrations per tumor, among them deletions dominated. Other studies have reported lower [9], larger [10] and a very similar rate of 31 aberrations per patient [11]. Expected number of aberrations correlated with the degree of histo-pathological malignancy. Also, in most studies genetic losses prevail over genetic gains. The high number of genomic aberrations shows that glioblastoma is genetically instable tumor. Moreover, it is genetically heterogenous in different intratumor areas [12].

Our results showed that the number of aberrations involving whole chromosomes (+7, -10, -14, +20) or chromosome arms (5q +, 13q-, 22q-), are common in patients with GM. Trisomy of chromosome 7 (in 70% of cases) and monosomy of chromosome 10 (80% of cases) were the most common aberrations in large series of tumors - a result fully confirming previous studies [13]. In these chromosomes are mapped some of the most important genes associated with the emergence and growth of malignant gliomas. These include EGFR-gene on chromosome 7 and PTEN and DMBT genes on chromosome 10. According to pooling data from array CGH analyses of 456 cases, EGFR gains/amplifications is one of the most common aberrations in glioblastoma, occurring in 35.7% of cases [14]. Deletion of 22q12, containing *NF2* gene, was detected in half of analyzed tumors in our study. This aberration was found to be associated with meningiomas [15], suggesting its early appearance in tumor development. *NF2* is a critical regulator of contact-dependent inhibition of proliferation and is the site of mutations causing neurofibromatosis II, characterizing by CNS tumors in addition to vestibular schwannoma before 30 years [16]. We delineated two putative tumor-suppressor loci in 22q chromosome, similarly to other investigators [17].

Gain in the long arm of chromosome 1 was first observed by other research groups. Takahashi and coworkers [18] associated it with a very good response to chemotherapy similar to that in patients with loss of 1p. These chromosomal aberrations are characterized by an imbalance between the copies 1p and 1q (1p <1q), which should be investigated to determine the treatment response of malignant gliomas. Loss of 1p36 was found in half of the analyzed tumors in our study and the comprising genes, in the smallest repeated region, were extracted. Among them *CHD5* was highlighted as down-regulated gene in one of the most comprehensive genetic studies of glioblastoma multiforme [19], which integrates data from array CGH, breakpoints and expression analyses.

Additional common aberration was gain of 20q13 in our study. This alteration was specifically related to tumor samples with higher number of CD133+ cells and without treatment response, in another study [20].

Identifying narrow regions with altered DNA copy number is an important part of the analysis of tumor genome, as genes mapped in these regions with amplifications/homozygous deletions represent potential candidate oncogenes or tumor suppressor genes. Hoelzinger and co-authors [21] did test 21 genes related to invasiveness of glioma. Much of the studied genes with increased expression more than twice were found in invasive cells. Among these genes was *RGS7*, which was found as gained in our study. Team of Ren Liu [22] examined five tumor samples and observed increased expression of *CDK5* (a gene, suggested as oncogene by our study) in comparison with normal astrocytes. There are other publications related this gene to glioblastoma multiforme development [23]. Other genes, located in 7q36 region (with very high frequency of gain in our study) are *GIMAP4*, *NOS3, ABCB8, SMARCD3, GBX1, MLL3, DNAJB6* and *SHH.* It is therefore necessary to analyze these genes for expression changes to relate them with malignant phenotype, since it is obvious that chromosome 7 harbors several amplicons, significant for GBM [24].

The other candidate genomic markers, found in our study are still not well investigated for their role in brain tumor development. OPN3, originally called encephalopsin, is highly expressed in brain tissue and regulates cAMP-related G protein-coupled receptor signaling [25]. CDK5RAP1 regulates the function of CDK5 and is found to suppress the differentiation of rat neuroblastoma cells [26]. PTPN1 is protein-thyrosin phosphatase, which is shown to play role in mammary tumorigenesis, especially in the process of lung metastases [27]. OSM gene, deleted in our study, encodes oncostatin M, which is shown to inhibit proliferation and changes in cellular morphology of a number of tumor cell lines derived from a wide variety of tissue types [28].

4. CONCLUSION

The limited number of tumors used in our study didn't allow us to make statistical analysis for correlation of genomic aberrations with prognosis – survival and therapy response. The study provide with basis for further studies in which the role of identified candidate-genes will be validated by other molecular genetics methods and at other levels - transcriptional and protein. This will lead to significant advances in knowledge of glioblastoma multiforme and suggestion of new prognostic and therapeutic indicators.

CONSENT

All authors declare that 'written informed consent was obtained from the patient (or other approved parties) for publication of this case report and accompanying images.

ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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