Tumor hypoxia: Impact on gene amplification in glioblastoma

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Abstract. Gene amplification is frequently found in human glioblastoma but the mechanisms driving amplifications remain to be elucidated. Hypoxia as hallmark of glioblastoma is known to be involved in the induction of fragile sites that are central to gene amplification. We analyzed the potential of hypoxia (pO_2 0%) and mini hypoxia (pO_2 5%) to induce fragile sites within a homogeneously staining region (HSR) at 12q14-15 in a glioblastoma cell line (TX3868). Treatment of cells by hypoxia or by mini hypoxia induced double minutes (DMs) and caused breakage of the HSR structure at 12q14-15, suggesting a novel hypoxia inducible fragile site on 12q. Treatment with aphidicolin, a known fragile site inducer, indicates that the hypoxia inducible fragile site is a common fragile site. Reintegration of amplified sequences and occurrence of anaphase-bridge-like structures shows that mini hypoxia and hypoxia are able to initiate amplification processes in human glioblastoma cells. Hypoxia as known tumor microenvironment factor is crucial for the development of amplifications in glioblastoma. The identification and characterization of novel common fragile sites induced by hypoxia will improve the understanding of mechanisms underlying amplifications in glioblastoma.

Introduction

DNA amplifications are frequently found in numerous human tumors including glioblastoma (1). Among the most frequently found amplifications in glioblastoma are chromosome regions 7p11-12 and 12q14-15 (2-4). Cytogenetically, gene amplifications manifest as double minutes (DMs) and homogeneously staining regions (HSRs). DMs develop through extra-chromosomal mechanisms and are found in approximately 50% of glioblastoma (5,6). HSRs expand by breakage-fusion bridge (BFB) cycles and are less frequent in glioblastoma (7,8). A study demonstrated initiation of BFB cycles by activation of a common fragile-site and thus provided first evidence that amplifications in human tumors are inducible *in vivo* by fragile-site activation (9). Common fragile sites are chromosome regions that tend to form gaps and breaks after partial inhibition of DNA synthesis. Those fragile sites are normally stable in cultured human cells, but are induced when cells are grown under conditions of folate or thymidylate stress or with low doses of aphidicolin that only partially inhibit DNA synthesis (10). Fragile site expression is also induced by chemotherapeutic drugs including methotrexate (MTX) and N-(phosphonacetyl)-L-aspartate (PALA) (11). However, most glioblastoma with amplified genes were derived from patients that underwent surgery before chemotherapy and/or radiation. Thus, the cause of the frequent gene amplifications in glioblastoma remains an open question.

The tumor microenvironment is likely to be crucial for DNA-amplification in human tumors. Perfusion in malignant glioma is decreased and dynamic studies of glioma microcirculation have shown low functional efficacy of tumor angiogenesis (12). The resulting hypoxic microenvironment promotes genomic instability and specifically induces fragile site expression (13). Recent studies on the influence of hypoxia on gene amplifications mainly investigated drug-selected cells. We investigated in our study glioblastoma cells without prior drug selection and addressed the following questions. Does hypoxia cause the induction of DNA-amplification? Does hypoxia impact the expression of fragile sites associated with the DNA-amplification? Are the effects of hypoxia on DNA-amplification comparable to the effects caused by drugs like aphidicolin? The basis for our investigations is the glioblastoma derived cell line TX3868. This cell line contains two HSRs with amplified sequences from chromosome 12q14-15 (14).

Materials and methods

Cell culture. Cell line TX3868 was established from glioblastoma multiforme and xenografted to mice (15). Cultured cells were maintained in DMEM (Invitrogen, Germany) supplemented with 10% fetal calf serum (FCS) (PAA, Germany) and 1 μg/l penicillin/streptomycin (PAA). Exponentially growing TX3868 cells were seeded at a density of $5x10^5$ cells per 10-cm diameter Petri-dish. For aphidicolin treatment cell culture medium was supplemented with 1.0 μg/ml aphidicolin, dissolved in DMSO. Lower concentrations of aphidicolin (0.4 and 0.6 μg/ml) did not induce detectable double minutes in TX3868 cells. Cells were incubated for 18 h and then either harvested or allowed to recover in

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normal growth medium for 6 h. Control cells for aphidicolin treatment were treated with DMSO only.

For mini hypoxia and hypoxia, culture dishes were incubated 24 h (mini hypoxia) and 24 or 30 h (hypoxia) in hypoxia or oxygen reducing (mini hypoxia) incubation bags (Merck Biosciences, Germany) (16). Hypoxia bags completely removed oxygen and the partial pressure of CO_2 is increased to a maximum of nearly 19%. In mini hypoxia bags, the oxygen level is adjusted at ≥5% O_2, whereas the level of CO_2 is adjusted at ≤9%. Control cells of mini hypoxia and hypoxia were grown at 5% pCO_2 and 37˚C.

After treatment, cells were incubated either with nocodazole for 2 h (aphidicolin) or with colcemid for 45 min (mini hypoxia, hypoxia) for subsequent metaphase chromosome preparation and FISH studies.

In situ hybridization. A single BAC clone was selected as probe for the *KUB3* locus (clone RP11-58A17, Roswell Park Cancer Institute Human BAC Library). A BAC and a cosmid clone were selected as probes for the *GAS41* locus (BAC: RP11-159A18; cosmid clone LLNLc132K1140Q2, RZPD, Germany) locus. BAC and cosmid probes were directly labeled using High Prime Labeling System (Roche Molecular Biochemicals, Germany). One μg of BAC-/cosmid-DNA each were labeled with Cyanine-3-dCTP (Cy3) or Cyanine-5-dCTP (Cy5) (Perkin-Elmer, Germany), according to the manufacturer's instructions. Sixty ng of Cy3-labeled DNA (*KUB3* or *GAS41*) and 100 ng of Cy5-labeled DNA (*GAS41* or *KUB3*) and 1 μg human Cot-1 DNA were precipitated. Samples were resuspended in hybridization mix (50% formamide, 2X SSC, 0.05 M sodium phosphate buffer, 10% dextrane sulphate). Probe DNA was denatured at 75˚C for 5 min and preannealed for 30-45 min at 37˚C.

Pretreatment of metaphase chromosomes. Chromosome preparations were dropped on glass slides. Slides were RNase treated (100 μg/ml RNaseA in 2X SSC) for 1 h at 37˚C and pepsin-treated (0.005% in 0.01 M HCl at 37˚C) for 10 min. Postfixation was done by 1% formaldehyde/1C PBS for 10 min at room temperature. Finally, slides were dehydrated by an ascending ethanol series (70%/80%/96%) and air-dried.

Hybridization. Metaphase chromosomes were denatured in 70% formamide/2X SSC/50 mM sodium phosphate buffer for 2.5 min at 80˚C and subsequently dehydrated by an ascending ice cold ethanol series. The denatured probe mixture was applied onto the slide on a 37˚C heating plate, covered with a cover slip, sealed with rubber cement and allowed to hybridize in a humid chamber at 37˚C for 16-48 h.

Post hybridization washes were performed in 50% formamide/2X SSC (4x5 min; 45˚C) followed by 0.1X SSC (3x5 min) at 60˚C. DNA was counterstained with DAPI (4',6'-diamidino-2-phenylindole) (1 μg/ml in PBS) for 5 min and mounted with VectaShield mounting medium (Vector Laboratories, Orton Southgate, UK) for microscopic analysis.

Microscope imaging and analysis. FISH results were evaluated using an Olympus AX70 fluorescence microscope. Digital images were taken at 630-fold magnification using the ISIS software (MetaSystems, Altlussheim, Germany). To analyze changes in fluorescence intensities of the hybridization signals within HSRs, recorded pictures were magnified four times. The HSRs were then framed manually and absolute (abs. FI) and relative (rel. FI) fluorescence intensities as well as sizes of the HSRs were measured. Because of variable chromosome length, results were normalized by dividing the abs. FI of the regarded HSR by the total area of the same HSR. Results are thus given as: abs. FI/μm^2.

Sequence analysis. Five hundred kb flanking DNA sequences of the BAC RP11-58A17 (AC084033) sequence were analyzed for content of different types of repetitive elements using RepeatMasker program (http://www.repeatmasker.org/). Each sequence consists of 98 kb and starts at the following bp positions on human chromosome 12: sequence 1 at 56,080,284; sequence 2 at 56,178,284; sequence 3 at 56,276,284; sequence 4 at 56,374,284; sequence 5 at 56,472,284; sequence 6 at 56,813,301; sequence 7 at 56,911,301; sequence 8 at 57,009,301; sequence 9 at 57,107,301 and sequence 10 at 57,205,301.

Results

The effects of hypoxia and mini hypoxia on amplified DNA-domains. We first analyzed whether hypoxia and mini hypoxia were capable of inducing fragile sites within a homogeneously staining region (HSR) at 12q14-15 in the glioblastoma cell line TX3868. An overview of the amplicon and localization of known fragile sites on 12q is shown in Fig. 1.

We found double minutes (DMs) both in cells treated with hypoxia or mini hypoxia. Untreated TX3868 cells revealed 1-2 double minute chromosomes in <5% of cells. Hypoxia treated cells revealed 3-6 DMs in 50% of mitoses and 8-13 DMs in 50% of mitoses. Mini-hypoxia treated cells revealed 3-6 DMs in 50% of mitoses. In general, mini hypoxia induced slightly less DMs than hypoxia. Newly generated double minutes contained amplified genes as indicated by fluorescence *in situ* hybridization with BAC clones for *GAS41* (*YEATS4*) and *KUB3* (*XRCC6BP1*) gene regions that both map within 12q14-15. Most DMs showed signals for *KUB3* and as well *GAS41* (Fig. 2A and B). Mini hypoxia for 24 h and hypoxia treatment for 30 h also caused breakage of the HSR structure at 12q14-15, suggesting a novel hypoxia inducible fragile site on 12q (Fig. 2C).

Breakage of the HSR structure was accompanied by a split fluorescence signal for the *KUB3* BAC suggesting a novel fragile site (FRA12F) that maps close to the *KUB3* gene (Fig. 1). In several cases mini hypoxia or hypoxia treatment induced double minutes and broken HSRs both in the same cell.

Following hypoxia treatment we also detected signals for both *GAS41* and *KUB3* within chromosome Xq indicating reintegration of amplified sequences. There are four fragile sites on the long arm of chromosome X including FRAXA, FRAXC, FRAXD. In addition after hypoxia treatment cells displayed large extra-chromosomal structures that hybridized with both *KUB3* and *GAS41* probes (Fig. 3).

Treated TX3868 cells also displayed anaphase-bridge-like structures that hybridized with both the *KUB3* and *GAS41* probes (Fig. 4A and B). Those structures were clearly

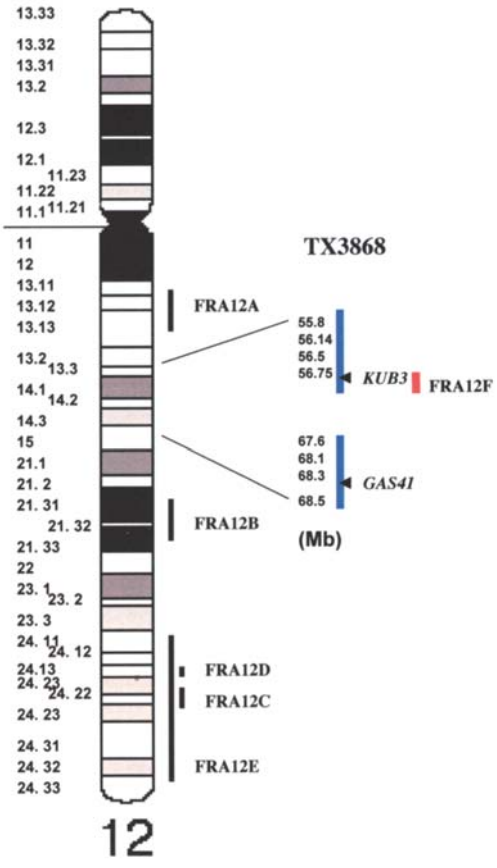

Figure 1. Chromosomal localization of fragile sites on 12q and the analyzed amplicon of TX3868. Amplicon size in TX3868 cells is indicated by blue bars, localization of the novel fragile site FRA12F is indicated by a red bar and localization of genes *GAS41* and *KUB3* by arrows.

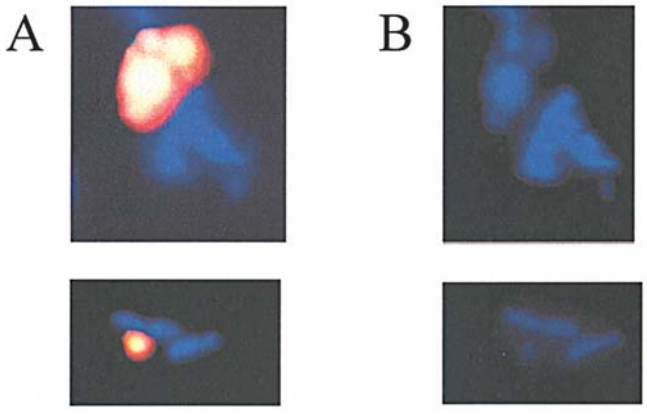

Figure 3. *KUB3* and *GAS41* 'overreplication'. (A) Large extra-chromosomal structures (overreplication) after hypoxia for 30 h are identified by *KUB3* (red) and *GAS41* (green) probes. (B) Extra-chromosomal structures are counterstained with DAPI.

different from structures resulting from micronuclei formation (Fig. 4C and D).

In summary, induction of DMs, breakage of HSRs, and occurrence of anaphase-bridge-like structures strongly indicate that mini hypoxia and hypoxia initiate amplification processes in human glioblastoma cells.

The effect of aphidicolin on amplified DNA-domains. Aphidicolin treatment induced DMs in TX3868 cells. Similar to the results detected after hypoxia treatment, newly generated double minutes harbored amplified *GAS41* and *KUB3* sequences. We also found hybridization signals for *GAS41* within chromosome 1q indicating reintegration of

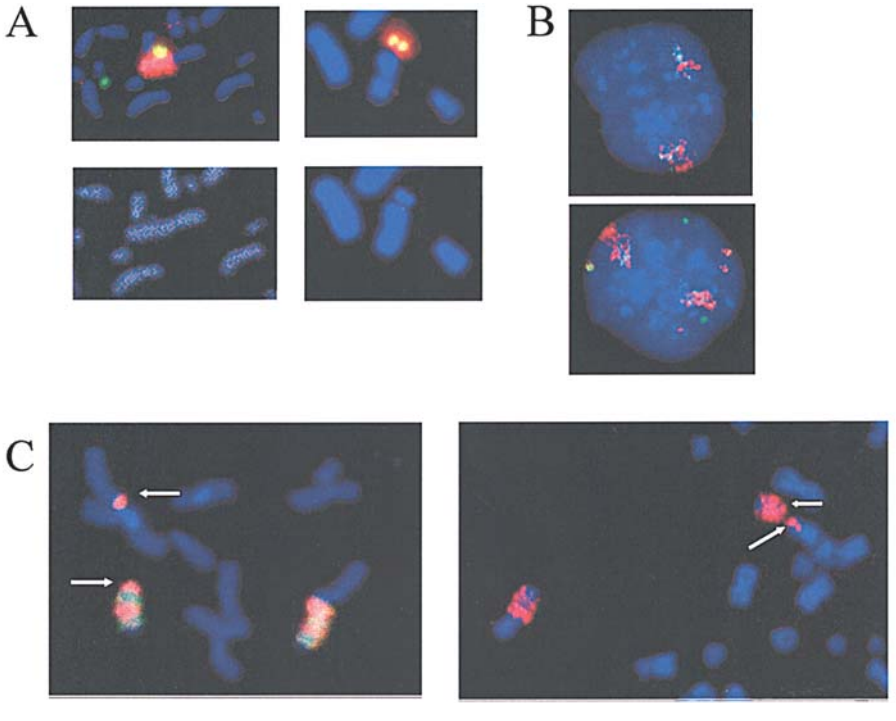

Figure 2. Double minute induction and HSR disruption after hypoxia or mini hypoxia. Fluorescence *in situ* hybridizations using probes specific for *KUB3* (red) and *GAS41* (green). Hypoxia treatment induced DMs hybridizing with both *KUB3* and *GAS41* indicated by a yellow signal or with *GAS41* only indicated by a green signal. (A) Examples of DMs hybridizing with *KUB3* and *GAS41* probes and DAPI-counterstain; (B) Interphase nucleus with *KUB3* and *GAS41* hybridization signals within the two HSRs (upper panel) and interphase nucleus with additional *KUB3* and *GAS41* hybridization signals (lower panel); (C) Disrupted HSRs after hypoxia and after mini hypoxia treatment. Arrows indicate the split parts of the HSR.

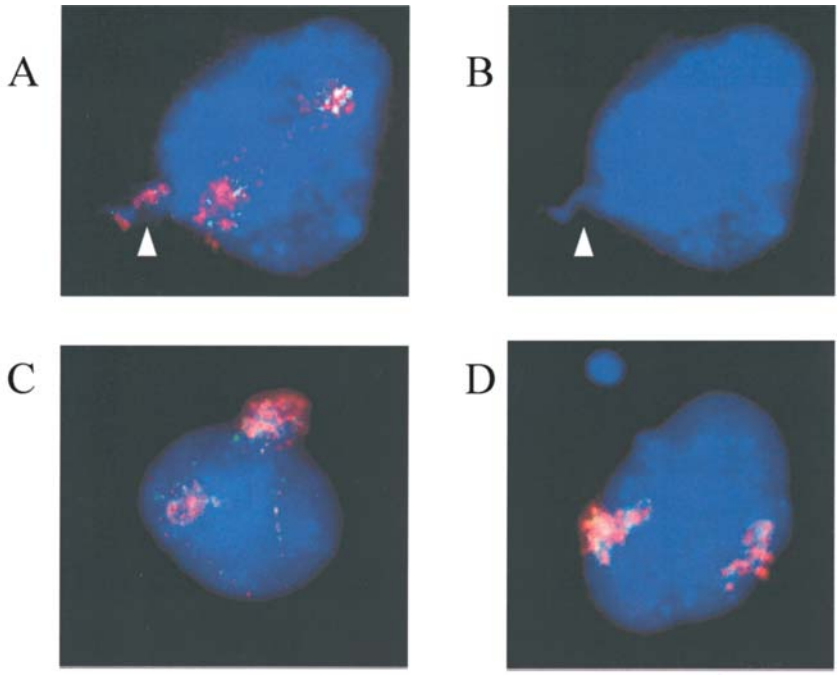

Figure 4. Formation of micronuclei and anaphase bridges. (A) Hybridization with *KUB3* (red) and *GAS41* (green) revealed a structure similar to an anaphase-bridge. (B) Anaphase-bridge like structure counterstained with DAPI. Arrows indicate the disrupted bridge-like structure. (C) *KUB3* and *GAS41* hybridization signals on a micronucleus released from the nucleus. (D) Micronucleus formation without hybridization to *KUB3* or *GAS41*.

Table I. Loss of *KUB3* and *GAS41* sequences on one homologous HSR after aphidicolin or mini hypoxia treatment.

	Area (μm^2)	Cy3 abs. FI	Cy3 FI/μm^2	Cy5 abs. FI	Cy5 FI/μm^2	*GAS41* labeling	Loss of *KUB3* or *GAS41*
HSR A	5.1	37,192	7,292.5	105	20.6	Cy3	
HSR B	5.1	36,365	7,130.4	39	7.6	Cy3	*KUB3*
HSR A	3.4	13,002	3.824,1	53	15.6	Cy3	
HSR B	3.4	13,512	3,974.1	3	0.9	Cy3	*KUB3*
HSR A	1.8	58,023	32,235.0	336	186.7	Cy5	*KUB3*, *GAS41*
HSR B	2.0	79,690	39,845.0	643	321.5	Cy5	
HSR A	1.5	52,402	34,934.7	36	24.0	Cy5	*KUB3*
HSR B	2.3	84,395	36,693.5	18	7.8	Cy5	*GAS41*
HSR A	3.3	150,684	45,661.8	6,897	2,090.0	Cy5	
HSR B	3.8	114,530	30,139.5	5,020	1,321.1	Cy5	*GAS41*, *KUB3*
HSR A	3.4	116,084	34,142.4	22,962	6,753.5	Cy5	
HSR B	3.7	91,653	24,771.1	22,526	6,088.1	Cy5	*KUB3*, *GAS41*

Fluorescence intensities (FI) for *KUB3* and *GAS41* were displayed as ratio of fluorescence intensity/area for Cy3 and Cy5. HSR A and B are examples of homologous HSRs per cell.

GAS41 sequences into 1q. Aphidicolin treatment and mini-hypoxia led to significant differences of fluorescent intensities between homologous HSRs (summarized in Table I and shown in Fig. 5). These results show that aphidicolin induces double minutes, alters the HSR structures, and induces a new common fragile site at 12q14.1.

Sequence analysis of KUB3 BAC. The *KUB3*-gene region represented in BAC RP11-58A17 (AC084033) contains three interrupted AT-dinucleotide islands localized between nt 64668-64821, nt 121823-122004, and nt 122053-122124 bp (Fig. 6). AT-dinucleotide islands were previously reported to lead to a high DNA torsional flexibility and were found in several of the characterized common fragile sites (17).

We analyzed a 500 kb region telomeric to FRA12F and 500 kb centromeric to this fragile site for content of different types of repetitive elements. As shown in Fig. 7 sequence of BAC RP11-58A17 revealed a higher content of LINE1

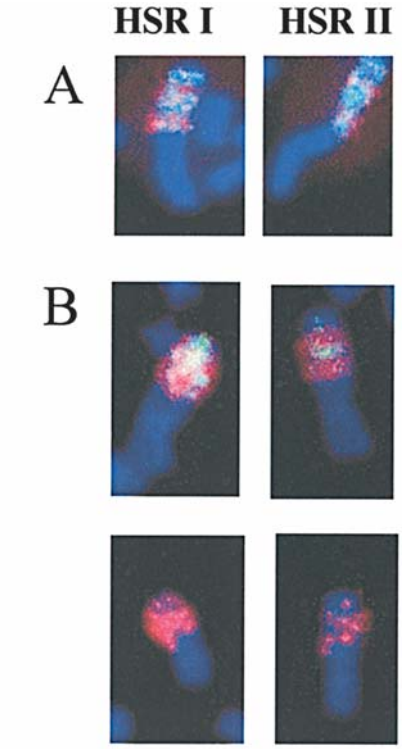

Figure 5. HSR alterations after mini hypoxia. (A) Hybridization of *KUB3* (red) and *GAS41* (green) against two homologous HSRs (I and II) revealed a ladder-like hybridization pattern. (B) Hybridization of *KUB3* and *GAS41* after mini hypoxia treatment revealed loss of signal intensity for *GAS41* and *KUB3* on one of the HSRs (II).

A
ATATATATAC ACATATATAT
ATGTGTGTGT ATATATATGT
ATATATGTAT ATATGTATGT
ATGTATATAT GTATATGTAT
ATATGTATAT GTATATGTGT
ATATGTATAT ATAGATATGT
AAAAATATAT ATAGAGACAC
ACATATACAT ATAT

B
TATATATATA TATATATATA
TATATATATA TATGGCACAC
ACACACACAC ATATATACAC
ACCTACACAT ATATATATAC
ACACACACAT ATATATACAC
ACATATATAT ACACACACAC
ACATATATAT ACACACACAC
ACATATATAT ACACACACAC
ATATATATAT ACACATATAT
AT $(N)_{49}$
ATATATATAT ATACACATAC
ATATATACAT ATATATATAT
ATATATATAT ATACACACAT
ATATACACAT AT

Figure 6. Sequence of two AT-dinucleotide rich flexibility-islands from BAC RP11-58A17. (A) AT-dinucleotide island at BAC position 64668-64821 bp; and (B) AT-dinucleotide island at BAC position 121823-122124 bp. AT-dinucleotide runs in bold letters.

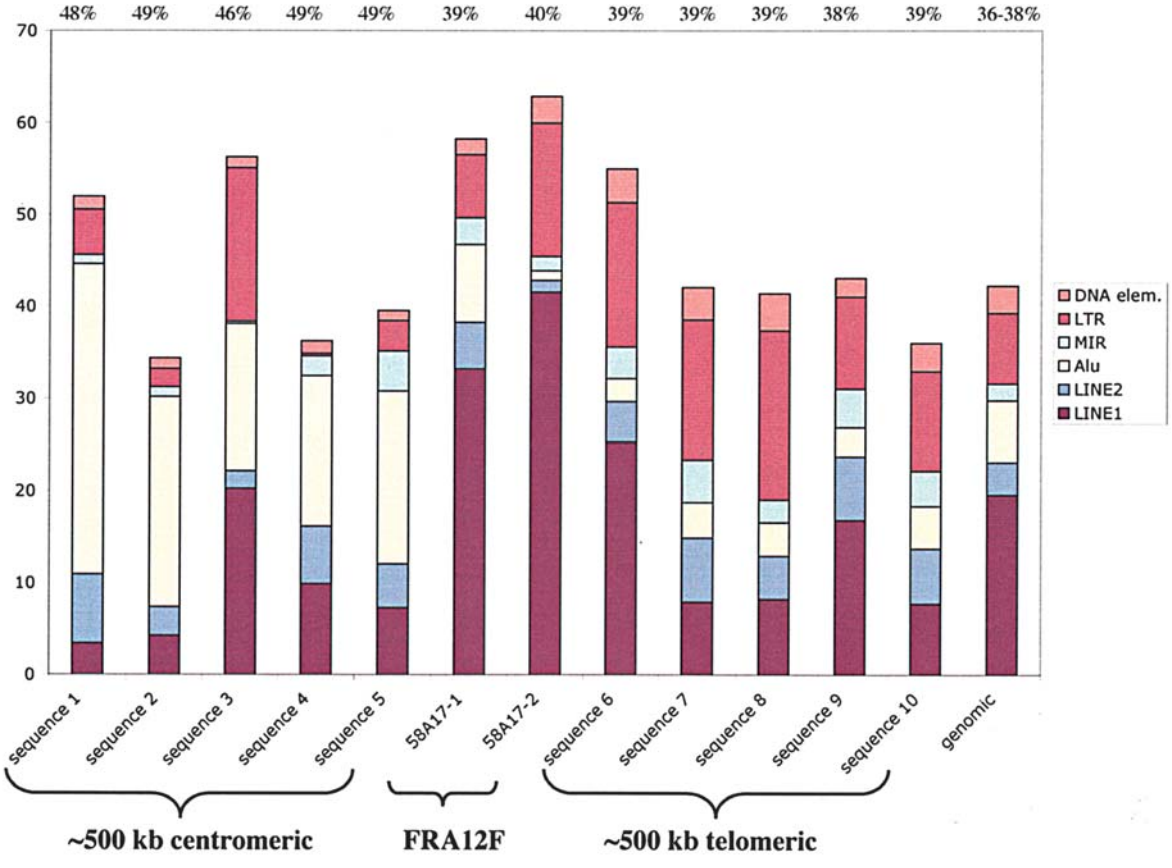

Figure 7. DNA repeat composition of FRA12F. Sequence of FRA12F and its flanking region was analyzed for content of repetitive elements. Content of repetitive elements is displayed in %. Data for the genomic content were from Smit (23).

sequences (41%) when compared to the 500 kb flanking sequences and to genomic DNA. An elevated content of LINE1 sequences was recently associated with characterized fragile sites (18). Our findings of AT-dinucleotide runs and an elevated LINE1 content provide further evidence that the *KUB3* region at 12q14.1 contains a novel common fragile site.

Discussion

Glioblastoma develop a large tumor mass with remarkable necrotic regions. Although glioblastoma is strongly vascularized, novel blood vessels result in an inefficient supply (19) finally producing a hypoxic microenvironment. The aim of our study was to analyze whether the process of gene amplification can be activated through fragile-site induction by hypoxia in glioblastoma. Gene amplification by the induction of fragile sites is frequently accompanied by several typical features, including generation of DMs of varying sizes, breakage at fragile sites, HSR breakdown and anaphase-bridges indicating BFB-cycles. Aphidicolin was used for its known ability to induce fragile-sites and thus allowed us to compare our results with former studies that also used drugs for fragile-site induction, such as MTX, PALA or aphidicolin (11).

We detected most of the above-mentioned features including DMs of varying sizes, breakage of the HSR and structures consistent with anaphase-bridges. Frequently we found loss of one HSR or a partial breakdown of an HSR into DMs. HSR breakdown became evident by loss of fluorescence intensities or by a reduced size of the HSR. We did not detect a complete breakdown of the HSRs into DMs as reported in other studies. Since the cell line TX3868 used carries two HSRs, breakdown of one HSR always leaves one HSR unaltered that still confers possible advantage for cell survival. Many of the newly induced DMs showed highly increased hybridization signal intensities indicating subsequent amplification within DMs.

Reintegration of DMs was frequently found during fragile site-induction by hypoxia or aphidicolin (11,20). Our study identified reintegrations of the genes *KUB3* and *GAS41* into the chromosomal region der (Xq) and reintegration of the *GAS41* gene into the chromosomal region 1q. Such reintegrations may result from induction of fragile sites on Xq and 1q.

Although we found 3 anaphase-bridge like-structures in chromosome preparations after hypoxia only one of them hybridized with both *KUB3* and *GAS41* probes. These results indicate that hypoxia induces additional fragile sites other than the ones on chromosome 12. Similar bridge-like structures were described and linked to breakage-fusion-bridge type mitotic disturbances in several tumor cell cultures (21).

In addition to the known features of gene amplification we frequently found large DNA containing structures that strongly hybridized with both *KUB3* and *GAS41* probes. Such structures always displayed a light bulb-like shape and were detected only in cells treated for 30 h with hypoxia. Since identical structures were never found during cell propagation, they likely have a very short live span. The very strong hybridization signals indicate extreme amplification within those structures.

Analysis of chromosomal region 12q in the Genome Data Bank indicated that the amplification process at this locus could be initialized at FRA12B (12q21.3). The size of the amplification unit is likely determined by FRA12A (12q13.1) (22). As shown in Fig. 1 the 12q amplicon in the TX3868 cells does not contain a known fragile site. However, our analysis demonstrated breakages within the HSR. Most breakages were detectable by a split fluorescence signal for the *KUB3* specific BAC probe. This suggests a new aphidicolin- and hypoxia-sensitive fragile site close to the *KUB3* gene. This idea is further supported by several AT-dinucleotide runs localized within the *KUB3*-containing BAC RP11-58A17. AT-dinucleotide runs have been reported for several characterized common fragile sites (17). Further sequence analysis revealed a higher content of LINE1 sequences in the BAC RP11-58A17 when compared to 500 kb of flanking DNA sequences. The average content of LINE1 sequences in the human genome was reported to be 19.6% (23). An elevated content of LINE1 sequences was recently shown for several human fragile sites (18). At FRA3B, LINE1 elements have been proposed to contribute to fragility (24). It remains to be seen whether LINE1 elements also contribute to fragility at FRA12F.

Hypoxic conditions as a prominent factor in glioblastoma microenvironment were used to investigate possible triggers of gene amplifications. Our study demonstrates that gene amplification processes are initiated by hypoxia in glioblastoma cells. In contrast to previous studies the amplification process on 12q14-15 was analyzed without drug selection. The identification of a novel common fragile site at 12q14.1 is important to understand the development of the 12q14-15 amplicon. Our results support the idea that hypoxia and the tumor microenvironment are crucial for the development of amplifications in glioblastoma.

Acknowledgements

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