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DNA Amplifications on Chromosomes 7, 9 and 12 in Glioblastoma Detected by Reverse Chromosome Painting

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Biopsies and cell culture, respectively, of four human glioblastoma multiforme (WHO 4) have been evaluated for gene amplification using reverse chromosome painting. Three of the tumours showed amplified domains within chromosome bands 12q13-15. The exact localisation and extension of the amplified domains, however, varies within this region. Southern blot analysis revealed amplification of the *GLI* oncogene in two of the glioblastomas which were found to contain amplified domains within 12q13-15. Reverse chromosome painting also identified amplified domains within bands 7q21 and 9p23-24. Amplification within region 9p23-24 has previously not been reported in glioblastoma. The amplified domain encompassing 9p23-24 was detected in the same glioblastoma which contained an amplification unit within bands 12q13-14. These data, together with previous reports, indicate that amplifications are predominantly found on chromosomes 7, 9 and 12 in glioblastoma. In addition, this study provides further evidence that coamplification is not a rare event in glioblastoma.
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INTRODUCTION

AMPLIFIED GENES have been identified in a variety of different human tumours, most notably *N-MYC* in neuroblastoma and *ERB2* in breast cancer [1–4]. Although gene amplification has been found at a low frequency in non-tumorigenic cells, gene amplification in tumour cells is generally believed to contribute to the transformed phenotype [5, 6].

In glioblastoma, several genes have been reported to be amplified with the epidermal growth factor receptor (EGFR) gene amplified in approximately 30% of all glioblastoma [7]. Genes *GLI* and *N-MYC* account for approximately 4% of all amplifications reported in glioblastoma [8, 9]. Amplifications of other genes have only been found sporadically [7, 10]. The search for amplified genes in glioblastoma has, however, been largely limited to the analysis of known oncogenes.

Comparative genomic *in situ* hybridisation (CGH) allows detection and mapping of amplifications in tumour cells without prior information about the amplified locus [11, 12]. Tumour DNA and normal DNA are labelled with two different fluorochromes and used in a two-colour fluorescence *in situ* hybridisation on normal human metaphase chromosomes. The ratio of the fluorescence intensities reflects the level of amplified domains in the tumour DNA. Alternatively, labelled tumour DNA is mixed with unlabelled Cot-1 DNA and used as a probe in *in situ* experiments [13]. Amplified sequences in the tumour DNA are indicated by intense fluorescence signals. This approach, which is termed reverse chromosome painting, has been successfully used to detect coamplification in glioblastoma [13, 14].

The use of tumour DNA as a complex probe for *in situ* experiments has several advantages over alternative approaches previously employed for analysing gene amplification. While previous techniques frequently depend on tissue culture, CGH or reverse chromosome painting circumvent problems of *in vitro* artefacts. In addition, CGH and reverse chromosome painting give immediate information about the exact location of an amplification unit. Thus, these techniques are most suited to identifying novel amplification units, providing starting points for the isolation of target genes.

Here, we employed reverse chromosome painting for the identification of amplified domains in human glioblastoma. DNA was isolated from four glioblastoma biopsies and one cell culture started from a glioblastoma multiforme. Amplified domains which were previously reported to contain known oncogene amplifications were analysed by Southern blotting.

MATERIALS AND METHODS

Tumour samples and cell culture

Tumour specimens were stored in liquid nitrogen immediately after surgical removal. Histologically, the tumours were characterised as glioblastoma multiforme WHO grade 4. Cell culture was established from glioblastoma biopsy T3564, as described elsewhere [15]. Long-term cell culturing was performed in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, penicillin (100 U/ml) and streptomycin (100 mg/ml).

DNA isolation and probe labelling for reverse chromosome painting

Tumour DNA and peripheral blood DNA was isolated according to Sambrook *et al.* [16]. Tumour DNA was labelled by nick

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translation using biotin-16-dUTP according to the manufacturer's instruction (Boehringer Mannheim, Germany). Peripheral blood DNA was sonicated to an average size of 500 basepairs. Reverse chromosome painting was largely performed according to du Manoir and colleagues [12]. In brief, biotin-labelled tumour DNA and unlabelled sonicated blood DNA (250 ng) were mixed and hybridised in the presence of 50 μg Cot-1 DNA. The amount of tumour DNA varied between 170 and 250 ng. Prior to hybridisation the probe was denatured for 5 min at 75°C and pre-annealed for 20–30 min at 37°C. Metaphase spreads have been prepared from peripheral blood of normal individuals (46, XX) according to standard protocols [17]. Slides were prepared for hybridisation as described by Göttert and colleagues [18]. Hybridisation was carried out in 10% dextran sulfate, 50% formamide, 2 × SSC and 50 mM sodium phosphate (pH 7.0) for 15 h at 37°C. In a modified approach, biotin-labelled tumour DNA was solely hybridised in the presence of a 50-fold excess of Cot-1 DNA without competition through sonicated blood DNA.

Detection

Following hybridisation, slides were washed to a stringency of 0.1 × SSC at 60°C. Biotin-labelled probes were visualised by using avidin conjugated to fluorescein isothiocyanate (FITC) and amplified once by using goat anti-avidin antibodies. Chromosomes were counterstained with DAPI at a concentration of 1 mg/ml, sealed in fluorescence antifading buffer and evaluated with a Leitz Orthoplan microscope.

DNA preparation, gel electrophoresis and Southern blotting

High molecular weight DNA was extracted from cell cultures and frozen tissue samples according to standard protocols. Genomic DNA (5 μg) was completely digested with the *Eco*RI restriction enzyme and the fragments were separated through 0.8% agarose gel electrophoresis. DNA was alkali denatured and transferred to Gene Screen (Dupont) nylon membranes. DNA probes were labelled with ^{32}P by the random primer method of Feinberg and Vogelstein [19]. Prehybridisation was carried out in 500 mM phosphate buffer (pH 7.2), 1 mM EDTA and 7% SDS at 65°C for 1 h. Following a 24-h hybridisation the filters were washed in 450 mM phosphate buffer (pH 7.2), 1% SDS for 15 min and in 250 mM phosphate buffer (pH 7.2), 1% SDS for 15 min. Probes used were WNT1 (pal1) [20], EGFR (pHER-A64-1) [21] and GLI (pKK36) [9].

RESULTS

Reverse chromosome painting was employed to analyse four glioblastoma multiforme for DNA amplification. DNA used for chromosome painting was isolated from biopsies and cell culture. Tumour DNA was labelled by biotin-16-dUTP mixed with normal unlabelled human genomic DNA at a 1 : 1 ratio and hybridised to a normal metaphase (46, XX). To obtain an optimal signal-to-noise ratio, various amounts of tumour material were used while the amount of normal DNA was kept constant with 250 ng used in all experiments.

Using 170 ng tumour DNA an amplified domain was detected in chromosome region 7q21 in a biopsy of glioblastoma T3788 (Figure 1a). Standard Southern blot analysis revealed a 30-fold amplification of the EGFR gene. In three other glioblastomas amplification units were found on chromosome 12 using between 200 and 250 ng tumour DNA. The regional assignment of the amplified domain, however, varies between the three glioblastomas. Culture cells derived from glioblastoma T3564 were found to carry an amplified domain within bands 12q13-15.

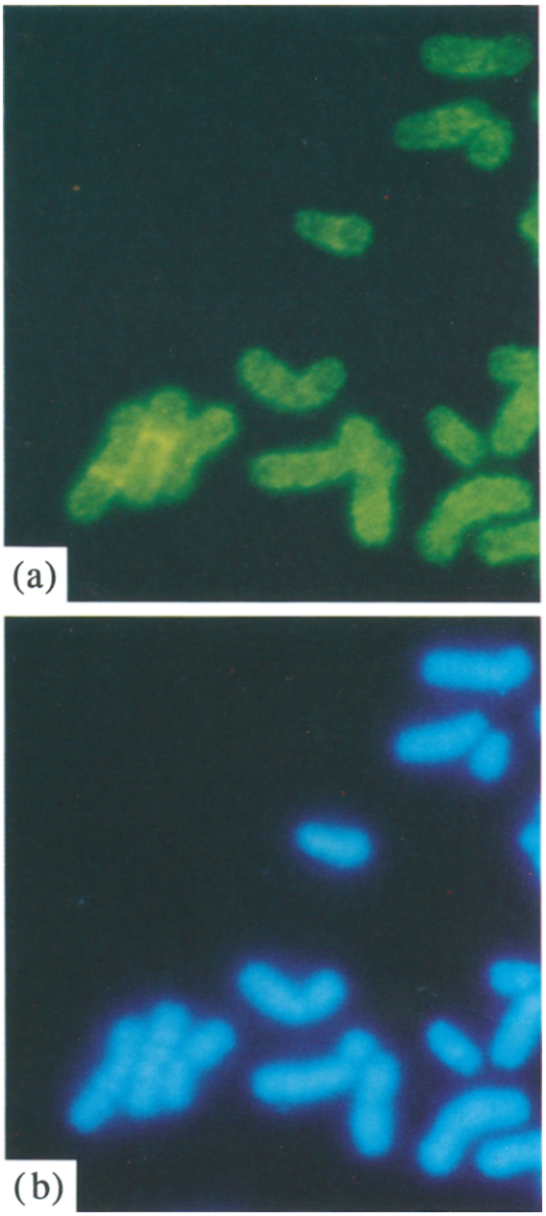

Figure 1. (a) Fluorescence signals of amplified DNA in cells of tumour T3788. Hybridisation signals were detected on 7q21 by reverse chromosome painting. DNA from glioblastoma biopsy T3788 was labelled by biotin-16-dUTP, mixed with 250 ng normal unlabelled genomic DNA at a 1 : 1 ratio and hybridised to a metaphase spread in the presence of 50 μg Cot-1 DNA. (b) Metaphase spreads counterstained with DAPI.

Biopsies of glioblastomas T3508 and T3868 contain an amplified domain within bands 12q13-14 and 12q13, respectively. In tumour T3508 the entire long arm of chromosome 12 was intensely stained with region 12q13-14 showing the strongest fluorescent signal (Figure 2a). Reverse chromosome painting revealed a second amplification unit within chromosome region 9p23-24 in glioblastoma T3508.

Interestingly, chromosome region 12q13-14 is commonly involved in tumour-related rearrangements and contains several oncogenes (e.g. *SAS*, *GLI*, *WNT*1). As demonstrated by standard Southern blot analysis *GLI* was found to be amplified in glioblastomas T3564 and T3868 (Figure 3). The level of amplification varies between 20-fold in tumour T3868 and 80-fold in tumour T3564. In addition, T3564 was found to contain an approximately 20-fold amplified *WNT*1 gene. Southern blot

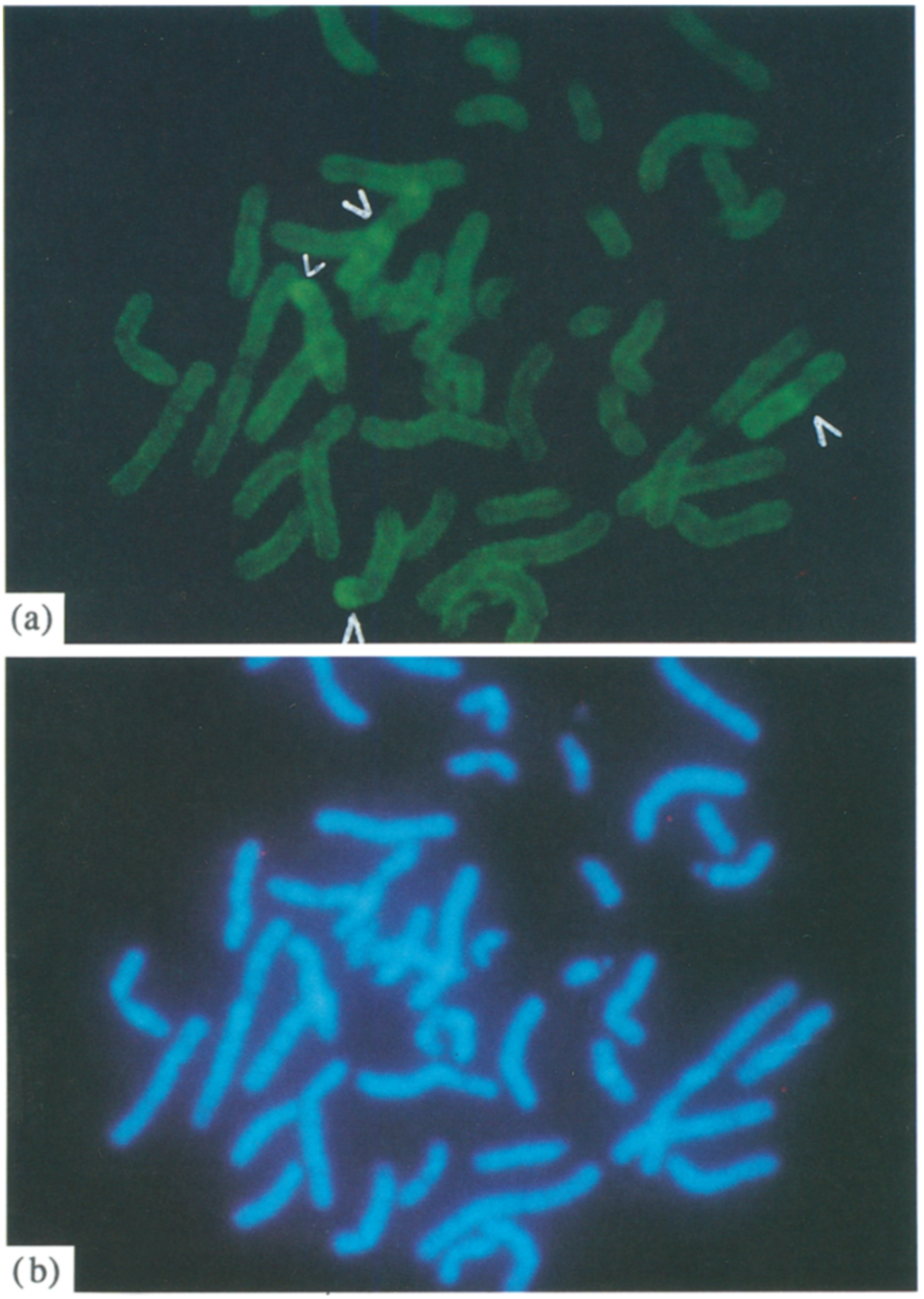

Figure 2. (a) Fluorescence signals of amplified DNA in cells of tumour T3508. Hybridisation signals were detected on 9p23-24 and 12q13-14 by reverse chromosome painting. The bands are indicated by arrows. DNA from glioblastoma biopsy T3508 was labelled by biotin-16-dUTP, mixed with 250 ng normal unlabelled genomic DNA at a 1 : 1 ratio and hybridised to a metaphase spread in the presence of 50 μg Cot-1 DNA. (b) Metaphase spreads counterstained with DAPI.

analysis of tumour T3508, however, failed to reveal an amplification of either *GLI* or *WNT*1 gene. The results of Southern blotting and reverse chromosome painting are summarised in Table 1.

DISCUSSION

Reverse chromosome painting allows the identification of novel amplification domains in human tumours. It is, however, necessary to bear in mind that this technique is fraught with several limitations. As previously reported, the amplification level must be greater than 20-fold [13]. In addition, our data indicate that the extension of the amplified domain impacts the intensity of the fluorescent signal. Small amplification units are less likely to be detected by reverse chromosome painting. This might explain why the amplified EGFR domain in tumour T3788 was not detected by reverse chromosome painting. Previous reports indicate amplifications of the EGFR gene (7p13) in approximate 30% of all glioblastoma. In addition, our recent studies indicate amplifications of the *MET* gene which is also localised on chromosome 7 [22]. Thus, standard Southern blot analysis and reverse chromosome painting should be applied complementarily for the analysis of DNA amplifications.

The identification of amplified DNA sequences on chromosome 12 is consistent with studies by Joos *et al.* [13] who also reported amplification within bands 12q13-15. *ERBB*3 localised within this region has been suggested as a possible target gene

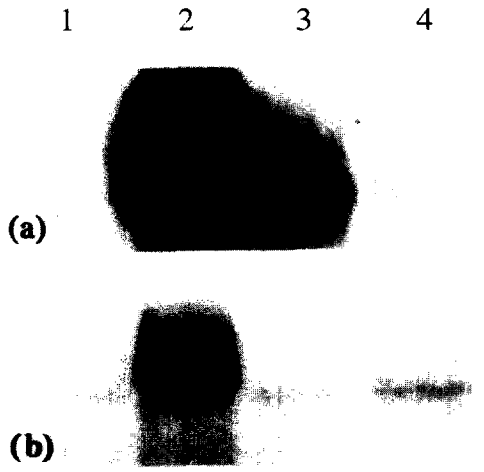

Figure 3. Representative Southern blot demonstrating *GLI* and *WNT1* amplification in glioblastoma T3564. DNA was digested by *Eco*RI, separated by gel electrophoresis and transferred to nylon membrane. Following hybridisation with probe GLI (a) the membrane was stripped of the probe and rehybridised with WNT1 (b). Lane 1, lymphocyte DNA; lane 2, DNA from the glioblastoma T3564; lane 3, DNA from glioblastoma T3868; lane 4, DNA from glioblastoma T3508.

Table 1. *Amplifications in four glioblastoma identified by Southern blot analysis and reverse chromosome painting*

	Southern blot analysis			Reverse chromosome painting
Glioblastomas	*GLI*	*WNT1*	*EGFR*	Chromosomal localisation
T 3508				12q13-14, 9p23-24
T 3564	X	X		12q13-15
T 3788			X	7q21
T 3868	X			12q13

Amplifications of the genes *GLI*, *WNT1* and *EGFR* are indicated by X.

[13]. Our studies, however, failed to reveal an amplification of EGFR-related genes on chromosome 12 (data not shown). While glioblastoma T3564 and T3868 carry amplified *GLI* or *WNT1* genes, these genes were not found to be amplified in tumour T3508. The results of reverse chromosome painting demonstrating an amplification within bands 12q13-14 are indicative of additional amplified genes on chromosome 12 in T3508. Further studies are needed to determine the exact location and extension of the amplified domains on chromosome 12 in glioblastoma.

This study provides additional evidence for a significant role of the oncogene *GLI* in glioblastoma. To the best of our knowledge the latest reports on *GLI* amplification in glioma were from Kinzler *et al.* [9] and Wong *et al.* [8]. The *GLI* gene has been found to be a member of the zinc finger genes. The exact role of the amplified *GLI* gene, however, remains elusive.

To better understand the role of *GLI* in the development of glioblastoma it might be necessary to further analyse the role of the other amplification units in glioblastoma. In previous reports several different oncogenes have been found to be amplified in gliomas. Our reverse chromosome painting experiments indicate additional amplification units on chromosomes 7, 9 and 12. Thus, the frequency and number of the amplification events in glioblastoma might be far more complex than previously suggested.

In summary, we identified several amplification domains in glioblastoma using reverse chromosome painting. In glioblastoma T3508, two independent amplified domains on chromosome 9 and 12 have been identified. In two glioblastomas, amplification events on chromosome 12 were identified with GLI as a possible target gene. The amplification units within bands 9p23-24 and 7q21 have as of yet not been reported.

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