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Amplified *met* Gene Linked to Double Minutes in Human Glioblastoma

Bernd Wullich, Hans-Werner Müller, Ulrike Fischer, Klaus-Dieter Zang and Eckart Meese

The *met* proto-oncogene was found to be amplified in a human glioblastoma cell line (T3095) established from a glioblastoma multiform WHO grade IV. Amplification of epidermal growth factor receptor, transforming growth factor α and N-*myc* which have been described previously in glioblastoma were not observed in T3095. There was, however, an 8-fold *met* amplification. Giemsa-stained metaphases of T3095 cells revealed multiple (>5) double minutes (dmins) in the majority of cells. Following xenografting in nude mice there was a significant increase in the number and frequency of dmins. The increase in dmins correlates with the level of *met* amplification (50-fold), suggesting localisation of the amplified *met* on dmins. Here we report the first case of *met* amplification in glioblastoma. Correlation between *met* amplification and extrachromosomal elements (dmins) has not been reported previously.

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INTRODUCTION

GENE AMPLIFICATION, a process that increases gene copy numbers, has been demonstrated for a variety of different neoplasias [1]. Amplification of oncogenes is thought to contribute to a transformed phenotype via elevated expression of oncogenes. In some instances, detection of gene amplification within tumour cells has been shown to be of prognostic significance. Amplification and expression N-*myc* correlates with the stage of disease in patients with neuroblastoma [2–4]. In human breast cancer, amplification of *neu* gene is a significant predictor of both overall survival and relapse time, at least in a subset of patients [5]. In ovarian cancer, amplification of *erb*B2 is correlated with survival rates [6].

In glioblastoma the genes for epidermal growth factor receptor (EGFR), transforming growth factor α (TGFα), N-*myc*, *myc* and *gli* have been documented to be amplified [7–9]. Structural and numerical chromosome changes occur in approximately 75% of glioblastomas. Most striking is the gain of one or more entire

copies of chromosome 7 and the loss of chromosome 10 [10–12]. Furthermore, cytogenetic analyses indicate the presence of double minutes (dmins) in nearly 50% of gliomas with higher malignancy grades [8].

Dmins were first recognised as cytogenetic manifestations of somatic gene amplification [13]. Interestingly, amplification of EGFR coincides with the appearance of dmins in gliomas [8]. Here we report the results of a cytogenetic and molecular analysis of the T3095 cell line derived from a human glioblastoma multiform. The oncogene *met* was found to be amplified in T3095. The level of amplification correlates with the number of dmins, suggesting localisation of amplified *met* on dmins.

MATERIALS AND METHODS

Cell culture

The T3095 cell line was established from a biopsy of a left parieto-occipital brain tumour as described elsewhere [14]. Histologically, the tumour was characterised as glioblastoma multiform WHO grade 4 with cytoplasmic and nuclear atypia and pleomorphism, increased cellularity, mitoses, necrosis and endothelial proliferation. 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 μg/ml). The T3095 cell line has now been maintained in culture for 72 passages.

Xenografting

T3095 cells from passage 16 were injected subcutaneously into nude mice aged 2 to 3 months. Tumour growth was detected 6 weeks after inoculation. Following removal the tumour was divided, with one part used for cell culture and the other transplanted in nude mice. The tumour has now been maintained in nude mice for three serial passages. Following the first *in vivo* passage, cell line TX3095 was derived from tumour tissue. Cell culturing was performed as described for T3095. Cell line TX3095 has subsequently been propagated *in vitro* for 35 passages.

Cytogenetic studies

Exponentially growing cells were exposed to 0.02 μg/ml Colcemid for 2 h. Following trypsinisation and hypotonic treatment (0.0375 mol/l KCl for 7 min at room temperature) cells were fixed in methanol–acetic acid and spread on to slides. GTG-banding was performed according to standard protocols. The chromosomes were classified according to ISCN nomenclature [15].

RNA isolation and northern blot analysis

RNA isolation was performed on T3095 cells prior to xenografting according to a standard protocol [16]. Briefly, cells were lysed with the detergent NP40 and proteins were denatured with sodium dodecylsulphate and urea. Following final chloroform extraction, RNA was precipitated with sodium acetate and ethanol. Denatured RNA was fractionated by formaldehyde gel electrophoresis, transferred to nylon membranes and hybridised to random primer-labelled probes [17].

Gel electrophoresis and Southern blotting

High molecular weight DNA was extracted from cell cultures and peripheral blood according to standard protocols. Genomic DNA (10 μg) was completely digested with a specific restriction enzyme and the fragments were separated through 0.8% agarose gel electrophoresis. DNA was alkali denatured and transferred to Gene Screen nylon membranes. DNA probes were labelled with ^{32}P by the random primer method of Feinberg and Vogelstein [17]. Prehybridisation was done in 500 mmol/l phosphate buffer (pH 7.2), 1 mmol/l ethylenediaminetetraacetic acid (EDTA) and 7% sodium dodecylsulphate (SDS) at 65°C for 2 h. Following a 24-h hybridisation the filters were washed in 450 mmol/l phosphate buffer (pH 7.2), 1% SDS for 15 min and in 250 mmol/l phosphate buffer (pH 7.2), 1% SDS for 10 min.

Probes

Probes used were JAC.1 (*jun*) [18], p52 (N-*ras*) [19], pmetH (*met*) [20], p*Eco*RIB (*src*) [21], pHER-A64-1 (EGFR) [22], pbc-N1 (H-*ras*) [23], pHSR-1 (*myc*) [24], pNB1 (N-*myc*) [25], pAL1 (*wnt*1) [26], pGMB (*sec*) [27], KO3 (*ros*1) [28], λhTGF1-10 (TGF-α) [9], *gli* [29], PGP1 (*mdr*1) [30].

RESULTS

In the T3095 tumour cells direct cytogenetic preparation revealed a near-triploid to near-tetraploid chromosomal complement counting 55–83 chromosomes per cell. Structurally abnormal chromosomes including dmins have been identified. A detailed karyotype analysis has been performed on T3095 cells from passages 11 and 39 (Table 1, Fig. 1). Immunological characterisation of *in vitro* propagated T3095 cells showed expression of glial fibrillary acidic protein. The model chromosome numbers were found to be 69 and 66, respectively. Karyotyping revealed clonal chromosomal abnormalities typically observed in malignant glioma [14]. By passage 39 additional marker chromosomes appeared in T3095 cells with the original markers retained. The number of dmins found in both passages indicated a decline in dmins during cell culturing (Fig. 2). The overall decrease in dmins resulted both from a decrease in cells bearing dmins and a decrease in dmins per cell.

Following xenografting in nude mice, tumour cells termed TX3095 revealed a modal chromosomal number of 60 at passage 0 and of 58 at passage 13. As summarised in Table 1, karyotyping

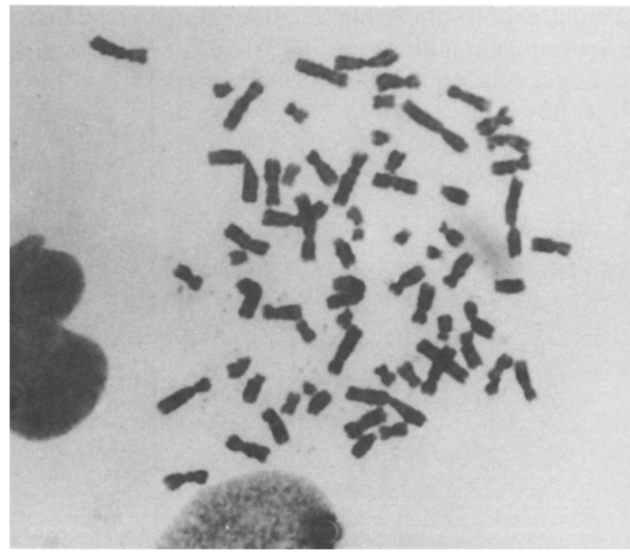

Fig. 1. Representative metaphase of TX3095 cells showing multiple dmins. Dmins were found in 95% of TX3095 cells (passage 0) ranging from 1 to 100 per cell. Note the presence of single minutes.

Correspondence to E. Meese.
The authors are at the Department of Human Genetics, Medical School, University of Saarland, 6650 Homburg/Saar, Germany.
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Table 1. *Cytogenetic data of the T3095 and TX3095 cell lines subsequent to serial* in vitro *cultivation. TX3095 was established by heterotransplanting the T3095 cell line in athymic nude mice*

Cell line	Passage no.	Composite karyotype
T3095	11	64-75 ⟨3n⟩, XXYY, +der(1)del(1)(q21), −2, +7, +8, +9, −10, −13, −14, −15, −16, −17, −18, +20, −22, +5mar, 3–50 dmin
	39	59-67 ⟨3n⟩, XXYY, +der(1)del(1)(q21), −2, −5, −6, +7, +9, −10, −13, −14, −15, −16, −17, −18, −19, −22, +7mar, 2–20 dmin
TX3095	0	59-68 ⟨3n⟩, XXYY, del(1)(q21), +add(1)(p36), −2, −5, +7, −10, −12, −14, −15, −16, −17, −18, −19, −20, −21, −22, +6mar, 1–100 dmin
	13	57-69 ⟨3n⟩, XXYY, del(1)(q21), +add(1)(p36), −2, −5, +7, −10, −12, −13, −15, −16, −17, −18, −19, −22, +8mar, 1–100 dmin

revealed additional marker chromosomes compared to the original T3095 cells. Chromosomal abnormalities characteristic of glioma were retained. There was also a significant increase in the overall number of dmins following xenografting (Fig. 2).

Since cytogenetic analysis indicated amplified DNA sequences, high molecular weight DNA was initially analysed for the amplification of the genes EGFR, TGF-α, N-*myc* and *gli* (Fig. 3). No amplification was detected for any of these genes that were previously found to be amplified in glioblastoma. To extend this analysis DNA from T3095 (passage 19) and TX3095 cells (passage 4) was hybridised with *wnt*1, *jun*, *mdr*1, *met*, *myc*, H-*ras*, N-*ras*, *sec*, *src* and *ros*1.

Met was the only gene found to be amplified. The degree of *met* amplification determined by densitometry of autoradiographs was approximately 8-fold in the T3095 cells (passage 19). Interestingly, northern blot analysis revealed expression of the oncogene *met* in T3095 cells (Fig. 4). The level of expression was, however, lower than expected from the level of amplification.

The *met* amplification cannot be accounted for by the gain of entire copies of chromosome 7. Figure 5 shows representative hybridisation results with N-*myc* and *met*. The hybridisation pattern did not indicate any structural alteration of the analysed genes.

The amplified *met* gene was also analysed after xenografting in nude mice. Following xenografting there was an 50-fold *met* amplification. This increase correlated with the higher number of dmins found following xenografting. These results are indicative of amplified *met* predominantly localised on dmins. This has been corroborated by *in situ* hybridisation experiments demonstrating chromosome 7-derived material on dmins.

DISCUSSION

Amplification of several proto-oncogenes and growth factor genes including EGFR, TGFα, N-*myc* and *myc* has previously been reported in glioblastoma [7, 8, 9]. This is, however, the first report on *met* amplification in glioblastoma. Furthermore, our preliminary studies indicate that amplification of the oncogene *met* is not an isolated incident in gliomas.

Met which maps to 7q was recently found to be amplified and overexpressed in human gastric adenocarcinoma [31]. Amplification of *met* in other tumour types has not been reported. *Met*

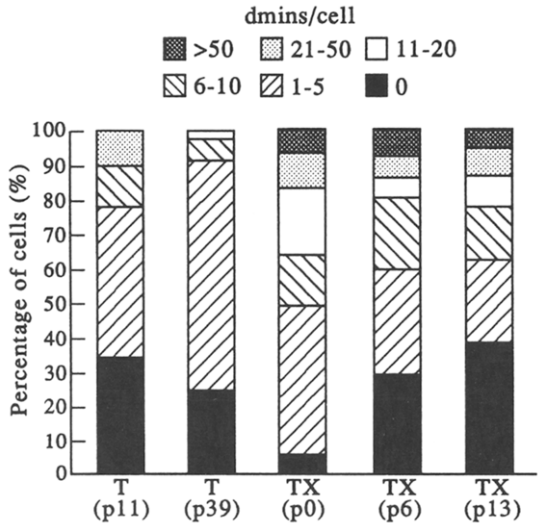

Fig. 2. Distribution of dmins in T3095 tumour cells before (T) and after xenografting (TX). *In vitro* culturing was accompanied by loss of dmins. Following xenografting the frequency of dmins increased to 95% of all tumour cells. Left ordinate, percentage of cells carrying dmins.

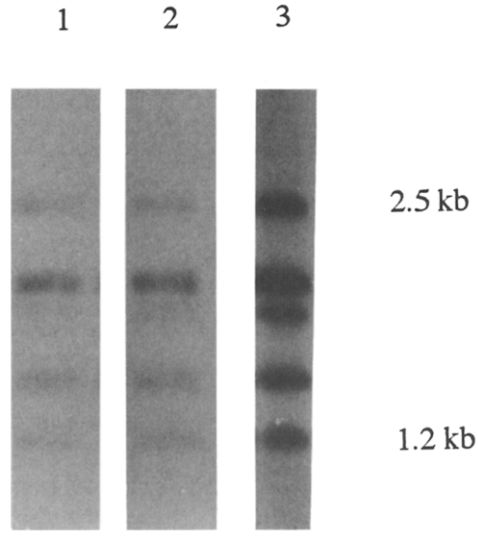

Fig. 3. Southern blot screening for EGFR amplification. DNA was digested by *Eco*RI, separated by gel electrophoresis, transferred to a nylon membrane and hybridised. Lane 1, lymphocyte DNA; lane 2, DNA from TX3095 tumour cells; lane 3, DNA from breast cancer cell line MDA-468 which carries a known EGFR amplification. Right ordinate of autoradiograms, size of the alleles in kilobase pairs (kb).

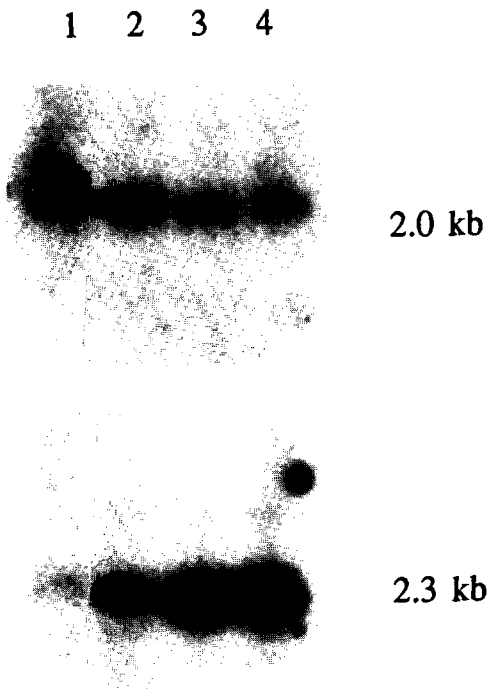

Fig. 4. Representative Southern blot demonstrating *met* amplification in T3095 tumour cells. DNA was digested by *Eco*RI, separated by gel electrophoresis and transferred to a nylon membrane. Following hybridisation with N-*myc* (upper panel) the membrane was stripped of the probe and rehybridised with *met* (lower panel). Lane 1, lymphocyte DNA; lane 2, T3095 DNA (passage 19); lane 3, TX3095 DNA (passage 4); lane 4, TX3095 DNA (passage 4).

encodes a 190 kD growth factor receptor with tyrosine kinase activity and with binding specificity for hepatocyte growth factor [32].

The *met* amplification found in T3095 and TX3095 is higher than can be explained by the polysomy of chromosome 7. Our study failed to identify amplification of EGFR which is also located on chromosome 7. Previous studies showed overexpression and gene amplification of EGFR in glioblastoma [33].

The level of *met* amplification correlates with the number of dmins in T3095 and TX3095. A similar correlation has been observed for the EGFR proto-oncogene in glioblastoma [8]. The number of dmins coincided with the overexpression of the EGFR gene in glioblastoma. It seems legitimate to speculate that the formation of extrachromosomal structures bearing *met* and EGFR, respectively, is more than a mere coincidence in glioblastoma development. Both genes stem from chromosome 7 of which numerical changes have been documented in glioblastoma. Numerical changes of chromosome 7 are the most frequent chromosomal deviation occurring in approximately 80% of cytogenetically aberrant glioblastomas [34].

However, structural alterations of chromosome 7 are infrequently seen in glioblastomas [8, 11]. Chromosomal rearrangements which are likely to result from the formation of dmins might be too small to be identified by cytogenetic analysis.

The increase in dmins that was observed after xenografting in nude mice is consistent with previously reported results [35]. It has been suggested that the *in vivo* environment favours extrachromosomal amplification whereas *in vitro* conditions favour intrachromosomal amplification.

In summary, amplified *met* gene was linked to dmins in glioblastoma T3095. Additional molecular and *in situ* hybridisation analyses (which are currently underway) are needed to further evaluate the biological significance of this finding. By analogy to other cancers, it is possible that the *met* amplification may indicate a role for the proto-oncogene *met* in the pathology of glioblastoma.

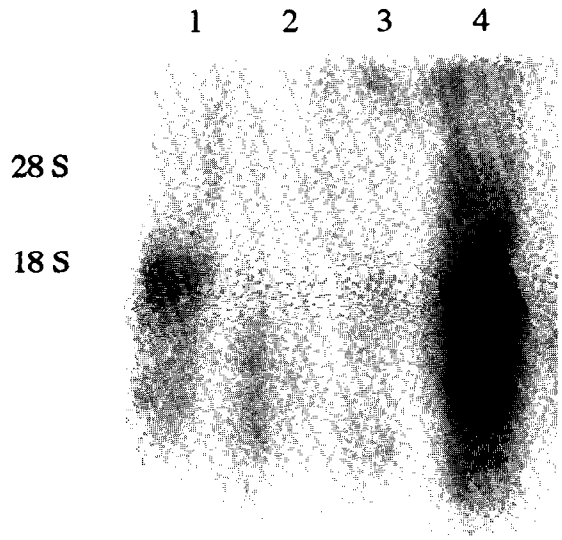

Fig. 5. Northern blot analysis of the proto-oncogene *met*. Lane 1, NIH/3T3 RNA; lane 2, HeLa RNA; lane 3, meningioma RNA; lane 4, TX3095 RNA. The strong signal in lane 4 is due to an increased amount of RNA as revealed by subsequent hybridisation to glyceraldehyde phosphate dehydrogenase (GAPDH).

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CD44 is Associated with Proliferation in Normal and Neoplastic Human Colorectal Epithelial Cells

A.M. Abbasi, K.A. Chester, I.C. Talbot, A.S. Macpherson, G. Boxer, A. Forbes, A.D.B. Malcolm and R.H.J. Begent

Flash-frozen biopsies obtained from surgical specimens of three adenomatous polyps and 22 colorectal adenocarcinomas (19 primary and three metastatic) were tested by immunohistochemistry for CD44 expression using F10-44-2 monoclonal antibody. CD44 positivity was correlated with proliferative status defined by Ki-67 monoclonal antibody reactivity. In normal colonic mucosa, CD44 was expressed in the proliferative zone of crypts. In tumours, CD44 expression was associated with proliferative areas irrespective of tumour stage or differentiation. Non-proliferating areas of the carcinomatous epithelium did not express CD44 although non-proliferating stromal lymphoid tissue did. There was no apparent association with tumour progression. F10-44-2-defined CD44 is consistently expressed during proliferation by normal colorectal epithelial cells and by both benign and malignant colorectal tumour cells.
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INTRODUCTION

CD44, AN ABUNDANT and ubiquitous cell adhesion molecule, has been linked with the development and spread of cancer. A distinct form of the CD44 molecule found on some epithelial cells appears to be increased by neoplastic transformation [1] and in nude mice CD44 has been associated with an increase in the number of lung metastases formed by a human melanoma cell line [2]. Furthermore, in rat pancreatic adenocarcinomas a CD44 splice variant has been indicated to confer metastatic potential when transfected into non-metastasising cells [3], and