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# Relationships Between Ki-67 Labelling Index, Amplification of the Epidermal Growth Factor Receptor Gene, and Prognosis in Human Glioblastomas

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## Summary

The aim of this study was to determine possible relationships between Ki-67 labelling index (Ki-67 LI), amplification of the epidermal growth factor receptor (EGFR) gene, and prognosis in human glioblastomas. Ki-67 LI was determined on cryosections of biopsy specimens of 20 human glioblastomas with a mouse anti-human Ki-67 monoclonal antibody. Amplification of the EGFR gene was determined by slot blot and Southern blot analyses of DNA extracted from the tumour biopsies. The Ki-67 LI was higher in the glioblastoma group with EGFR gene amplification (8 tumours, median value of Ki-67 LI 4.2, range 0.4–24.6) than in those without EGFR gene amplification (12 tumours, median value of Ki-67 LI 0.8, range 0.2–11.8) ($0.05 > p < 0.1$). The glioblastoma patients with Ki-67 LI > 1.5 (10 tumours) had a statistically significant shorter survival than those with Ki-67 LI < 1.5 (10 tumours) ($p < 0.05$). The glioblastoma patients with EGFR gene amplification lived shorter time than those without EGFR gene amplification ($p > 0.05$).

*Keywords:* Glioblastoma; epidermal growth factor receptor; Ki-67; survival.

## Introduction

In human gliomas the proto-oncogene c-erb-B1 [homologous to the epidermal growth factor receptor (EGFR) gene] is commonly amplified and overexpressed[2, 8, 12, 15, 16, 24]. The EGFR is a transmembrane receptor protein with binding specificities for epidermal growth factor (EGF), transforming growth factor α, and amphiregulin[25]. Since EGFR gene amplification occurs mainly in glioblastomas, it is likely that this genetic event is related to the progression of gliomas. So far, EGFR gene amplification has shown no statistically significant correlation to survival of glioma patients[2].

The monoclonal antibody Ki-67 has been used to assess the growth fraction in tumour tissue[3]. Ki-67 detects a nuclear antigen expressed during the G1, S, G2, and M phases of the cell cycles, but absent in the G0 phase[9, 10]. In human gliomas the Ki-67 Labelling Index (Ki-67 LI) has shown a good correlation with histological grade[4, 6, 11, 17, 18, 20, 21, 27] and is comparable to other proliferative markers such as mitoses and bromodeoxyuridine labelling index (BrdU LI)[14, 18, 21]. BrdU LI has been shown to correlate with survival in glioblastoma patients[14]. For Ki-67 LI no overall correlation with survival has been shown[22], although five patients with an index below 2.5 lived longer than 40 weeks[27].

The aim of this study was to determine possible relationships between Ki-67 LI, EGFR gene amplification, and prognosis in glioblastoma patients.

## Materials and Methods

Twenty human patients with glioblastomas operated upon at the Department of Neurosurgery, University Hospital of Trondheim, Trondheim, Norway, in the time period 1986–89 were included in the study. No chemotherapy or radiotherapy was given before surgery. Immediately after surgical removal one portion of the tumour was fixed in 3.9% formalin for routine histopathology (the pathological diagnoses were based on the criteria of WHO[28]), and the rest was shock frozen in liquid nitrogen and stored at − 70 °C.

Acetone fixed frozen sections were incubated 1 h with the IgG1 murine monoclonal antibody Ki-67 (Dakopatts, Glostrup, Denmark) at a dilution of 1 : 50 in PBS. The staining procedure was performed by an avidin-biotin-peroxidase technique (Vectastain ABC kit, Vector Laboratories, Burlingame, CA, U.S.A.). The sections were lightly counterstained with haematoxylin. In each experiment frozen sections of human tonsils (rich in Ki-67 positive centrocytes)[9] were included as positive controls whereas in the negative controls Ki-67 was omitted. The negative control included regularly an irrelevant mouse monoclonal antibody instead of the primary antibody.

The Ki-67 LI was defined as the percentage of Ki-67 positive cells divided by the total number of cells[3]. Ten representative areas were evaluated at a magnification of × 400 with an eyepiece grid covering an area of 0.055 $mm^2$. 400–6000 cells were counted per section.

The mitotic index (MI) was determined as the percentage of mitotic cells divided by the number of cells counted[23]. Ten representative areas were examined on the formalin-fixed paraffin-embedded tumour tissue stained with haematoxylin/eosin at a magnification of × 400 using an eyepiece grid as described above.

Southern blot analyses of glioblastomas 1, 4, 9–15, and 18 have been published previously[12]. The remaining glioblastomas have been examined by Southern blot (tumours 2, 3, 6, and 17) and slot blot analyses (tumours 2, 3, 5–8, 16, 17, 19, and 20) (Torp *et al.*, subm.). Briefly, the concentration of the extracted tumour DNA was determined fluorometrically using the DNA binding dye Hoechst 33258 (Sigma, St. Louis, U.S.A.)[7]. For slot blot analyses serial dilution of each denatured DNA sample was applied to a nylon membrane filter using a slot blot manifold. For Southern blot analyses the DNA was digested with Eco RI or Hind III, electrophoresed on a 0.8% agarose gel, denatured, and transferred to a nylon membrane in alkaline solution. The hybridization and washing procedures were performed according to Church and Gilbert[5]. Before hybridization the membranes were washed in 0.5 M Na-phosphate buffer with 7% SDS (Sigma) and 1 mM EDTA (Merck, Darmstadt, Germany) for 15 min at 65 °C. The membranes were hybridized in a similar solution with a $^{32}$P-labelled 2.4 kb Cla I fragment of the pE7 plasmid with the cDNA of the human EGFR[26] at 65 °C for 1–3 days. Then the membranes were washed several times at 65 °C in 0.04–0.01 M Na-phosphate buffer with 1–0.5% SDS and autoradiographed at − 70 °C for 1–3 days. The blots were stripped and then rehybridized with the 1.7 kb Eco RI fragment of the pUNG-40 plasmid with the cDNA of the human uracil-DNA glycosylase residing on chromosome 12 (kindly provided by Dr. H. E. Krokan, UNIGEN, Trondheim, Norway)[1, 19] to control for loading differences between the samples. The autoradiographs were scanned by a laser densitometer to quantify the intensity of the hybridization signals, and the degree of EGFR gene amplification was estimated by comparing the signal intensity of serially diluted DNA blots of a given specimen with that of the control probe. A more than two fold increase in gene dosage was regarded as gene amplification. DNA from the tumour cell lines T-CAR1 with a ten fold amplification of the EGFR gene[13], from human full term placenta, and from normal human lymphocytes served as controls.

Kaplan-Meier estimates of survival data were calculated. The log rank test, the Mann-Whitney test, and correlation coefficient served to check for significant differences.

## Results

The results are presented in Table 1. The DNA analyses have been published previously[12] (Torp *et al.*, subm.). There were 8 glioblastoma patients with EGFR gene amplification with an age range of 34–76 (mean 55.5) and 12 glioblastoma patients with normal EGFR gene dosage with an age range of 30–79 (mean 60).

Ki-67 positive cells were heterogeneously dispersed throughout the tumour, and the positive cells were usually the small anaplastic ones whereas the giant bizarre cells were more often negative (Fig. 1). The Ki-67 LI for all the 20 glioblastomas showed a wide range of values (median 1.9, range 0.2–24.6). The 8 glioblastomas with EGFR gene amplification had a higher Ki-67 LI (median 4.2, range 0.4–24.6) than the 12 glio-

Table 1. *Summary of Results in 20 Human Glioblastomas*

| Tumour | Age at operation (yrs) | Sex[a] | Localization | EGFR gene amplification (yes/no) | Ki-67 labelling index (%) | Mitotic index (%) | Survival (months after operation) |
|---|---|---|---|---|---|---|---|
| 1 | 68 | F | fronto-parietal | yes | 3.2 | 0.55 | 6 |
| 2 | 46 | F | frontal | yes | 24.6 | 0.13 | 13.5 |
| 3 | 34 | M | parietal | yes | 18.4 | 0.10 | 1.5 |
| 4 | 76 | F | frontal | yes | 2.2 | 0.40 | 4 |
| 5 | 54 | M | frontal | yes | 0.4 | 0.12 | 9 |
| 6 | 57 | M | temporal | yes | 13.9 | 0.22 | 7 |
| 7 | 41 | F | temporal | yes | 1.1 | 0.06 | 17 |
| 8 | 68 | M | frontal | yes | 5.2 | 0.27 | 9 |
| 9 | 78 | M | frontotemporal | no | 0.4 | 0.13 | 32.5 |
| 10 | 46 | F | frontal | no | 1.5 | 0.15 | alive |
| 11 | 69 | M | parieto-occipital | no | 0.2 | 0.07 | 3 |
| 12 | 57 | M | temporal | no | 0.6 | 0.05 | 13 |
| 13 | 30 | M | temporal | no | 0.3 | 0.03 | 20 |
| 14 | 69 | F | parietal | no | 0.5 | 0.04 | 6 |
| 15 | 79 | M | temporal | no | 0.7 | 0.06 | 11 |
| 16 | 66 | M | temporal | no | 0.9 | 0.22 | 10 |
| 17 | 43 | M | occipital | no | 7.7 | 0.06 | 12 |
| 18 | 43 | F | temporal | no | 7.6 | 0.16 | 7.5 |
| 19 | 74 | F | temporo-parietal | no | 2.7 | 0.00 | 13.5 |
| 20 | 70 | F | occipital | no | 11.8 | 0.04 | 1.5 |

[a] F = female, M = male.

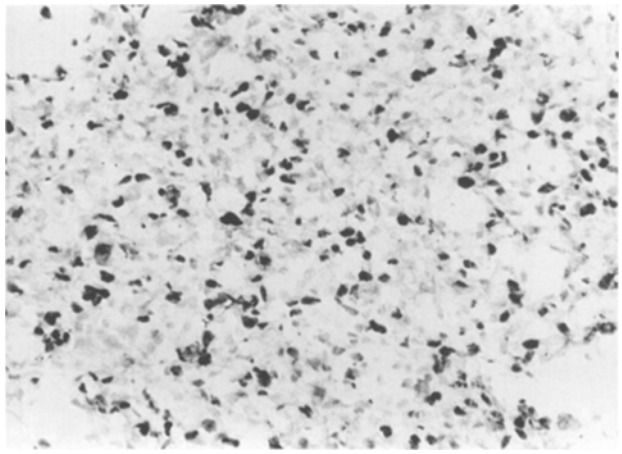

Fig. 1. Glioblastoma (tumour 2) with multiple Ki-67 positive cells (dark nuclei) × 200

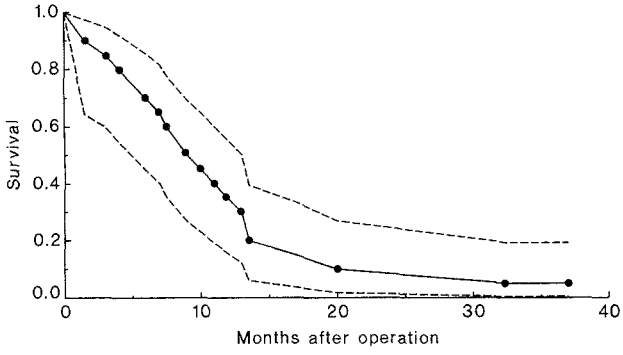


Fig. 2. Kaplan-Meier survival curves for all 20 glioblastoma patients (median survival 9 months) (●—●)/(----) with 95% confidence intervals

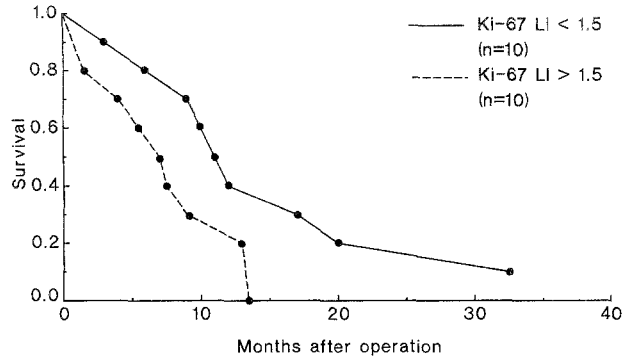


Fig. 3. Kaplan-Meier survival curves for glioblastoma patients with Ki-67 LI > 1.5 (n = 10) (median survival 7 months) and Ki-67 LI < 1.5 (n = 10) (median survival 11 months) ($p < 0.05$, log rank test)

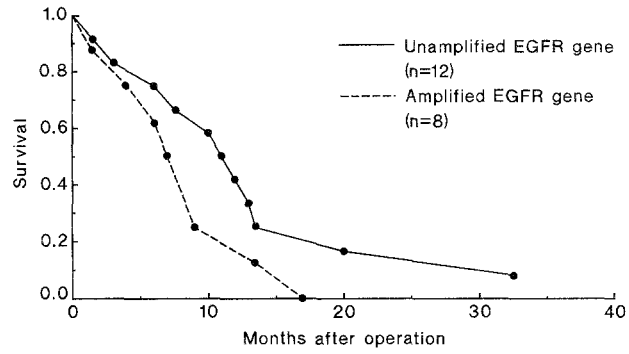


Fig. 4. Kaplan-Meier survival curves for glioblastoma patients with amplified EGFR gene (n = 8) (median survival 7 months) and unamplified EGFR gene (n = 12) (median survival 11 months) ($p > 0.05$, log rank)

blastomas with unamplified EGFR gene (median 0.8, range 0.2–11.8) (not statistically significant, $0.05 < p < 0.1$, Mann-Whitney test).

For MI there was also a wide range of values (median 0.11, range 0.00–0.55). The MI was higher in the glioblastomas with EGFR gene amplification (median 0.18, range 0.06–0.55) than in the glioblastoma group without EGFR amplification (median 0.06, range 0.00–0.22) (not statistically significant, $0.05 < p < 0.1$, Mann-Whitney test). There was no degree of relationship between Ki-67 LI and MI (correlation coefficient $r = 0.0076$).

The median survival for all the 20 glioblastoma patients was 9 months (Fig. 2). The median survival of the 10 glioblastoma patients with Ki-67 LI up to 1.5 was 11 months and 7 months for the 10 glioblastoma patients with Ki-67 LI higher than 1.5 (statistically significant, $p < 0.05$, log rank test) (Fig. 3). The 8 glioblastoma patients with EGFR gene amplification had a median survival of 7 months while the 12 glioblastoma patients with normal EGFR gene dosage had a median survival of 11 months (not statistically significant, $p > 0.05$, log rank test) (Fig. 4).

## Discussion

The high Ki-67 LI and MI in human glioblastomas found in this study are in agreement with other reports[4, 6, 11, 17, 18, 20, 21, 27]. The Ki-67 LI varied much intra- and intertumourally noted by others as well[4, 6, 11, 17, 18, 20, 21, 27] showing the heterogeneous distribution of proliferative cells in the glioblastomas.

The higher Ki-67 LI and MI in glioblastomas with EGFR gene amplification suggested a higher growth

fraction in these tumours than in those glioblastomas with normal EGFR gene dosage. These observations were also supported by the shorter survival of the glioblastoma patients with amplified EGFR gene.

The correlation between low Ki-67 LI (< 1.5) and longer survival is in accordance with the observation of Zuber *et al.*[27]. However, Pigott *et al.*[22] found no correlation between KI-67 LI and survival.

We could not confirm the positive correlation between MI and Ki-67 LI as reported by others[4, 18, 21]. This discrepancy might be due to tumour heterogeneity. Further, the tumour tissues examined for Ki-67 staining and mitosis counts were prepared by different techniques that could result in different assessment of proliferative activity[4, 23].

Our results should be interpreted with care because of the small number of patients and the pronounced heterogeneity of glioblastoma tumour tissue. Further, determination of Ki-67 LI and EGFR gene dosage are time consuming and their predictive values in clinical medicine seem still questionable.

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