Methods in Pathology

Comparison of automated silver enhanced *in situ* hybridization and fluorescence *in situ* hybridization for evaluation of epidermal growth factor receptor status in human glioblastomas

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The epidermal growth factor receptor (EGFR) is amplified in approximately 40% of glioblastomas making it a compelling molecular target for therapy. Before starting a therapy targeting the EGFR pathway, accurate determining of EGFR status is a prerequisite. We evaluated the reliability of the novel automated silver enhanced *in situ* hybridization for the detection of EGFR gene amplification in human glioblastomas. EGFR-amplification status was assessed in 93 cases of glioblastoma by silver enhanced *in situ* hybridization and compared with results of fluorescence *in situ* hybridization and immunohistochemistry. In a second cohort, silver enhanced *in situ* hybridization status was correlated with EGFR gene expression data. The EGFR gene was amplified in 25/90 tumours (28%) by silver enhanced *in situ* hybridization, and in 28/93 tumours (30%) by fluorescence *in situ* hybridization. The concordance rate for silver enhanced *in situ* hybridization and fluorescence *in situ* hybridization was 98%. Two glioblastomas were scored as being amplified by fluorescence *in situ* hybridization but not by silver enhanced *in situ* hybridization. Polymerase chain reaction-based EGFR-amplification data were highly correlated with EGFR silver enhanced *in situ* hybridization. Altogether, 81 of 91 cases (89%) showed positivity for EGFR expression by immunohistochemistry. Although EGFR protein over expression was associated with gene amplification ($r = 0.40$, $P < 0.001$), there were 29 of 91 cases that showed a high EGFR protein level and no EGFR amplification by fluorescence *in situ* hybridization. The high concordance rate of silver enhanced *in situ* hybridization and fluorescence *in situ* hybridization for the detection of EGFR amplification in paraffin-embedded glioblastomas samples demonstrates that silver enhanced *in situ* hybridization is a valid and attractive alternative to fluorescence *in situ* hybridization. Silver enhanced *in situ* hybridization combines the advantages of bright field microscopy with fully automated analysis in a cost-effective way thereby emphasizing its use for routine application in surgical pathology.

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Introduction

Glioblastoma is the most aggressive form of adult primary astroglial tumour with median survival of <12 months.¹ One reason for the dismal prognosis is the lack of therapeutic agents to eliminate remaining diffusely infiltrating tumour cells after surgery. Molecular profiling of genetic alterations in glioblastoma enables classifying tumours into categories amenable to novel targeted treatment

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modalities. One of these therapeutic targets is the epidermal growth factor receptor (*EGFR*). *EGFR* is a transmembrane glycoprotein that belongs to the erb tyrosine kinase receptor family including *EGFR* (*HER1* or *erbB-1*), *erbB-2* (*HER2*), *erbB-3* (*HER3*) and *erbB-4* (*HER4*). *EGFR* overexpression has been found in approximately 40% of patients with glioblastoma.² It has been reported that *EGFR* amplification is a negative prognostic factor.³ In contrast, there are publications opposing a prognostic relevance.^{4,5} Currently, the possibility of *EGFR*-targeted therapies has initiated the development of a variety of agents directed either towards the extracellular ligand-binding domain or the intracellular tyrosine kinase domain including the monoclonal antibody Erbitux (IMC-C225, ImClone/Bristol Myers Squibb), and the *EGFR* tyrosine kinase inhibitors Gefitinib (ZD1839, Iressa, AstraZeneca) and Erlotinib (OSI-774, OSI/Genentech/Roche).⁶ Although these *EGFR*-targeted therapies are already in phase III clinical trials, it still remains to be determined, which subgroup of patients will most likely benefit.⁷ Several methods are available for testing *EGFR* status, including Southern blot analysis, polymerase chain reaction, fluorescence *in situ* hybridization and chromogenic *in situ* hybridization. Disadvantages of the first two methods are the lack of consideration of cell morphology. The advantage of both chromogenic *in situ* hybridization and fluorescence *in situ* hybridization is the ability to illustrate gene alterations on a tumour tissue section, which enables the comparison of tissue morphology with gene abnormality. Fluorescence *in situ* hybridization is globally accepted and used as a standard method to determine *HER2* status in breast cancer.^{8,9} Disadvantages of fluorescence *in situ* hybridization testing are an expensive fluorescent microscope, extensive user training and rapid loss of gene signals at room temperature, thereby hindering convenient slide storage. Currently, fluorescence *in situ* hybridization is the gold standard. However, it has been recently reported that chromogenic *in situ* hybridization is an attractive alternative to fluorescence *in situ* hybridization.¹⁰ Chromogenic *in situ* hybridization is based on the same principle as fluorescence *in situ* hybridization but uses a chromogen to detect gene signals. Even though chromogenic *in situ* hybridization can deal with some of the mentioned problems it has not yet found wide acceptance in routine laboratories. Chromogenic *in situ* hybridization requires manual processing, overnight hybridization, and two hybridizations, one for the gene of interest and one for detection of chromosomal copy number. Furthermore, due to signal weakness, it can be difficult to discriminate gene signals. An alternative may be a novel fully automated silver enhanced *in situ* hybridization protocol. This rapid method yields a stable and discrete chromogenic reaction product, which is analysed by standard light microscopy. Moreover, the manual steps that have to be performed for silver

enhanced *in situ* hybridization are reduced to minimum. However, technical and clinical validation is required before silver enhanced *in situ* hybridization might be accepted as a standard practice. This study compares the different methods and validates silver enhanced *in situ* hybridization as a method for assessing *EGFR* gene amplification in glioblastoma tumour samples.

Materials and methods

Tumour Materials

A series of 95 randomly selected glioblastomas, which had been diagnosed between 2006 and 2008, was collected from the tumour bank archive of the Department of Neuropathology, University Hospital Heidelberg. The study was approved by the local ethics committee. Before paraffin embedding, tumour tissues were fixed in 4% neutral buffered formalin for 5–36 h. The slides were cut to 3- μ m thick sections and then processed in parallel for fluorescence *in situ* hybridization (LSI *EGFR* SpectrumOrange/CEP 7 SpectrumGreen Probe, Vysis/Abbott), silver enhanced *in situ* hybridization (INFORM *EGFR* Probe and INFORM chromosome 7 probe, Ventana Medical Systems) and immunohistochemistry (CONFIRM anti-*EGFR* (3C6), Ventana). The mean patient age was 57 years and 60% (57/95) of the patients were male. From the 95 glioblastomas, 92 samples had been derived from gross total resections, two samples had been biopsied stereotactically, and one was frozen section tissue. All cases were diagnosed according to the current classification criteria of the World Health Organization.¹¹ In a second step, a group of patients ($n=26$), with known *EGFR*-amplification status determined by a semiquantitative polymerase chain reaction-based assay was screened for *EGFR* amplification by silver enhanced *in situ* hybridization. These samples were kindly provided by the Brain Tumour Reference Centre located at the Institute of Neuropathology, University of Bonn (Director Prof Dr Pietsch).

Immunohistochemistry

The sections were immunostained with CONFIRM anti-*EGFR* primary antibody (clone 3C6, Ventana Medical Systems, Tucson, AZ) on the BENCHMARK XT automated stainer (Ventana). Immunohistochemistry was performed following the manufacturer's recommendations. Immunohistochemical semiquantitative assessment of *EGFR* protein expression was based on the fraction of stained cells and the intensity of the membranous staining. Regardless of a possible cytoplasmic staining, the membranous staining for *EGFR* was specified on a scale of 0 to 3 as follows: 0 = negative (no staining of glioblastoma cells); 1 = weak staining; 2 = moderate staining;

3 = strong staining. *EGFR*-positive staining was defined as any immunohistochemical staining of tumour cell membranes above background level regardless of complete or incomplete circumferential staining. Cytoplasmic staining without membranous staining was scored as negative. Same slides of all tissue samples were scored by two primary independent observers according to the Dako *EGFR* pharmDx Interpretation Manual. Additionally, the scoring algorithm according to Hirsch *et al*¹² was used. This algorithm is based on multiplication of the percent fraction of stained cells with the staining intensity of tumour cells, thereby resulting in an immunohistochemistry score. Scores of 0–149 were considered negative/no expression of *EGFR*, and scores of 150–300 were considered positive/high expression of *EGFR*. Endothelial cells within the glioblastoma tumour samples served as an internal negative control for the *EGFR* staining procedures. Additional negative controls were obtained by the omission of the primary antibody. In each immunohistochemistry run one glioblastoma sample with known strong staining reaction was included as a positive control.

Silver Enhanced *In Situ* Hybridization

Automated silver enhanced *in situ* hybridization was performed according to the manufacturer's protocol for the INFORM *EGFR* probe and chromosome 7 probe. Both probes were labelled with dinitrophenol and optimally formulated for use with *ultraVIEW* SISH Detection Kit and accessory reagents on BENCHMARK series of automated slide stainers (Ventana). The *EGFR* DNA probe was denatured at 95°C for 12 min; hybridization was performed at 52°C for 3 h for *EGFR* and 52°C for 2 h for centromere 7 probe. After washing the *EGFR* and chromosome 7 dinitrophenol-labelled probes were visualized using the rabbit anti-dinitrophenol antibody and the *ultraVIEW* silver SISH Detection Kit. For details see Dietel *et al*.¹³ The silver precipitation was deposited in the nuclei and a single copy of the *EGFR* gene or the centromere 7 was visualized as a black dot. After counterstaining 60 tumour cells, nuclei were evaluated in three different areas (20 nuclei each).

Fluorescence *In Situ* Hybridization

For fluorescence *in situ* hybridization, consecutive sections from the same blocks used for silver enhanced *in situ* hybridization and immunohistochemistry were cut and mounted on SuperFrost +/+ slides. Deparaffinising, pre-treatment and protease digestion procedures were performed according to the DAKO Histology FISH Accessory Kit. Probe mixes were hybridized at 37°C between 14 and 24 h, washed and counterstained with DAPI. A minimum of 60 tumour cell nuclei in three different

areas (20 nuclei in each area) were scored using LEICA epi-fluorescence microscopes equipped with $\times 100$ oil immersion objective and DAPI/Spectrum Green/Orange single filters.

Fluorescence *In Situ* Hybridization and Silver Enhanced *In Situ* Hybridization Scoring Criteria and Algorithms

The scoring procedure for *EGFR* fluorescence *in situ* hybridization and silver enhanced *in situ* hybridization was performed similar to the INFORM *Her2* DNA probe (for details see <http://www.her2sish.com>). The slide was screened for a suitable tumour target area. For fluorescence *in situ* hybridization: *EGFR* gene and centromere 7 signals were counted in 60 cells in three different areas (20 nuclei in each area) and the sum was built for both probes. Then the ratio between *EGFR* gene signals and centromere 7 signals was calculated. For silver enhanced *in situ* hybridization: First, the *EGFR* gene signals were counted in 20 cells. Then the same area was located in the centromere 7 stained slide and again 20 cells were counted. This was repeated two times until 60 cells were examined. Finally the ratio between *EGFR* gene signals and centromere 7 signals was calculated in analogy to fluorescence *in situ* hybridization. To determine the cut-off value for *EGFR* amplification, the *EGFR* gene status of normal human brain tissue samples was examined. The normalized ratio (*EGFR* Gene number/centromere 7 number) varied between 0.7 and 1.8 (data not shown). As previously reported cut-off level of ≥ 2 has been proven to be useful for *EGFR* gene status assessment.^{3,14} We decided to perform the analyses in analogy to the *Her2* guidelines of the American Society of Clinical Oncology/College of American Pathologists. This scoring system includes an equivocal/borderline group. *EGFR* gene amplification status was classified applying the following criteria:

A *EGFR*/centromere 7 ratio less than 1.8 was defined as negative for *EGFR* gene amplification. A *EGFR*/centromere 7 ratio between 1.8 and 2.2 was defined as ambiguous (not determinable) for *EGFR* gene amplification. A *EGFR*/centromere 7 ratio greater than 2.2 was defined as positive for *EGFR* gene amplification.

Semiquantitative Polymerase Chain Reaction-Based Assay

Semiquantitative polymerase chain reaction-based assay was performed to determine the ratio between *EGFR* and a control gene, *Interferon- γ* (*IFN- γ*), as described earlier.¹⁵ Amplification was defined as a ratio of *EGFR* and *IFN- γ* higher than 4.16. The ratio of 2.09–4.16 was defined to be ambiguous for *EGFR* and a *EGFR*/*IFN- γ* ratio of < 2.09 was defined as normal/negative for *EGFR*. For comparison with

silver enhanced *in situ* hybridization, 10 semiquantitative polymerase chain reaction-based assay amplified, 4 ambiguous and 12 nonamplified samples were screened.

Statistics

Statistical analysis was done with SAS version 9.1WIN. Concordance between silver enhanced *in situ* hybridization and fluorescence *in situ* hybridization methods and between silver enhanced *in situ* hybridization and semiquantitative polymerase chain reaction-based assay were determined using the Pearson's correlation coefficient. The kappa statistics were calculated with Cohen's kappa.

Results

Failure Rate

The failure rate for both methods was very low. Fluorescence *in situ* hybridization failed in 2 of 95 cases (2%), silver enhanced *in situ* hybridization showed no signals in 3 of 95 cases (3%).

EGFR Gene Amplification Pattern

In total, 86 of 90 dual evaluable (for fluorescence *in situ* hybridization and silver enhanced *in situ* hybridization informative) cases showed a homogeneous *EGFR*-amplification pattern with rather constant gene signals in all tumour cells. Four cases illustrated a heterogeneous expression pattern with amplified gene signals in some interspersed tumour cells. This observation was independent from the

applied method (Figure 1a and b). In *EGFR* amplified cases, the gene ratio was rather high (mean value = 13.7). The *EGFR* gene signals in amplified cases reached numbers up to 60 in each cell.

EGFR-Amplification Status by Silver Enhanced *In Situ* Hybridization and Fluorescence *In Situ* Hybridization

From 95 cases entered in this study 90 cases could be evaluated for both, silver enhanced *in situ* hybridization and fluorescence *in situ* hybridization. Three cases were excluded due to the lack of signals (two with no fluorescence *in situ* hybridization/silver enhanced *in situ* hybridization signals and one with absent silver enhanced *in situ* hybridization signals). One of the excluded cases was the frozen tissue section. Two cases had to be excluded due to small sample size without any detectable tumour tissue on consecutive sections. In all, 93 of 95 samples were informative for fluorescence *in situ* hybridization, with an amplification rate of 30% (Figure 2a and b). No case showed an ambiguous *EGFR* gene status. In comparison, 90 of 95 samples were informative for silver enhanced *in situ* hybridization, with an amplification rate of 28% (Figure 2c and d). One case showed an *EGFR* gene ratio of 1.87 and was consequently termed 'ambiguous for *EGFR* gene amplification'.

Discrepancy between the fluorescence *in situ* hybridization and silver enhanced *in situ* hybridization occurred in only 2 of 90 cases, both positive for fluorescence *in situ* hybridization and negative for silver enhanced *in situ* hybridization. A case-by-case analysis revealed the following:

Figure 1 Amplified single cells by fluorescence *in situ* hybridization and silver enhanced *in situ* hybridization. **(a)** Fluorescence *in situ* hybridization: in this example, the cell in the centre has two green signals (centromere 7 probe) with clusters of red signals (*EGFR* gene locus), while all other cells have two green and two red signals. **(b)** Silver enhanced *in situ* hybridization: in this example, the cell in the centre has clusters of black signals (*EGFR* gene locus), while the surrounding tumour cells show only 1–3 signals. Magnification: **(a)** 1000 × oil objective; **(b)** 630 × air objective.

Figure 2 Representative images of fluorescence *in situ* hybridization and silver enhanced *in situ* hybridization *EGFR* in two glioblastoma tumour samples. In fluorescence *in situ* hybridization (a,b), green signals represent the centromere of chromosome 7; red signals represent the *EGFR* gene locus on chromosome 7p12. In silver enhanced *in situ* hybridization (c,d), black signals represent the *EGFR* gene locus. (a) and (c) are *EGFR* not amplified cases; (b) and (d) are *EGFR* amplified cases. In (d), endothelial cells serve as internal negative control (arrow). Magnification: (a,b) 1000 $\times$ oil objective; (c,d) 630 $\times$ air objective.

Case 1 (151/07b): The silver enhanced *in situ* hybridization ratio was negative for *EGFR* gene amplification (ratio of 1.54). The fluorescence *in situ* hybridization yielded a positive result (ratio of 2.39). In both methods, the *EGFR* gene signals were increased (fluorescence *in situ* hybridization = 4.90; silver enhanced *in situ* hybridization = 7.45) but the number of centromere signals was different (fluorescence *in situ* hybridization = 2.05; silver enhanced *in situ* hybridization = 4.83). It is therefore assumed that this discrepancy was caused by a polysomy of chromosome 7, which had not been recognized in the fluorescence *in situ* hybridization slide. This might have been due to weak green (centromere 7) fluorescence *in situ* hybridization signals, which were presumably overlooked by the observer.

Case 2 (285/08a): The silver enhanced *in situ* hybridization ratio was 1.78 (negative for *EGFR* gene amplification but very close to the ambiguous category). The fluorescence *in situ* hybridization

yielded a clear positive result with a ratio of 8.49. This discrepancy had been caused by heterogeneity of the signal distribution. Both methods identified amplified single cells, but the percentage of these cells differed in variable areas of the slide. This heterogeneity might have lead to a drifting result.

As a conclusion, the concordance rate for silver enhanced *in situ* hybridization and fluorescence *in situ* hybridization set at cut-off point 2.2 was 98% ($\kappa = 0.95$, 95% CI [0.87,1.0]). The Pearson correlation coefficient for the detailed data of silver enhanced *in situ* hybridization and fluorescence *in situ* hybridization was 0.85 ($P < 0.0001$).

Immunohistochemistry

Altogether, 10 of 91 glioblastomas (11%) were scored negatively (staining intensity 0) on *EGFR* immunohistochemistry. From 91 glioblastoma 81 cases (89%)

Table 1 Correlation of *EGFR*-gene amplification with silver enhanced *in situ* hybridization and protein expression with immunohistochemistry

IHC	Pos. <i>EGFR</i> -Gene amp.	Neg. <i>EGFR</i> -Gene amp.	Total
0	0	9	9
1+	0	8	8
2+	0	17	17
3+	26	29	55
	26	63	89

IHC, immunohistochemistry; pos., positive; neg., negative; amp., amplification.

revealed a weak, medium or strong (staining intensity 1–3) *EGFR* protein expression. Applying the immunohistochemistry score (0–149 negative, 150–300 positive) 39 of 91 glioblastomas (43%) were positive.

Correlation of Silver Enhanced *In Situ* Hybridization with Immunohistochemistry

In all, 89 cases were evaluable for both, immunohistochemistry and silver enhanced *in situ* hybridization. All cases (27/91) with *EGFR* gene amplification were also tested positive with immunohistochemistry. In all, 26 of the 27 glioblastomas with amplified *EGFR* showed staining intensity of three and one tumour demonstrated a weak-staining intensity (Table 1). In contrast, 29 of 64 cases without amplification exhibited high *EGFR* expression (staining intensity 3). *EGFR* protein expression was significantly associated with gene amplification. The correlation between staining intensity and fluorescence *in situ* hybridization was $r=0.402$ ($P<0.0001$). The correlation for immunohistochemistry score (staining intensity $\times$ percentage stained cells) and fluorescence *in situ* hybridization was $r=0.570$ ($P<0.0001$).

EGFR Expression by Polymerase Chain Reaction-Based Assay and Silver Enhanced *In Situ* Hybridization

EGFR DNA expression by a polymerase chain reaction-based assay was highly associated with *EGFR* silver enhanced *in situ* hybridization (Table 2). All semiquantitative polymerase chain reaction-based assay positive samples ($n=10$) were highly amplified with silver enhanced *in situ* hybridization (mean ratio = 10.7). All semiquantitative polymerase chain reaction-based assay negative cases ($n=12$) were not amplified with silver enhanced *in situ* hybridization (mean ratio = 1.03). The correlation for semiquantitative polymerase chain reaction-based assay and silver enhanced *in situ* hybridization was $r=0.79$ ($P<0.0001$). In the ambiguous semiquantitative polymerase chain reaction-based assay group ($n=4$) all silver en-

Table 2 Association of *EGFR* DNA amplification by polymerase chain reaction-based assay compared with silver enhanced *in situ* hybridization

PCR-A	SISH		Total
	Neg.	Pos.	
Amplified	0	10	10
Ambiguous	4	0	4
Normal	12	0	12
Total	16	10	26

(*EGFR/IFN- γ* ratio >4.16 amplified; *EGFR/IFN- γ* ratio <2.09 normal; *EGFR/IFN- γ* ratio from 2.09 to 4.16 ambiguous).

SISH, silver enhanced *in situ* hybridization; PCR-A, semiquantitative polymerase chain reaction-based assay; neg., negative; pos., positive.

Figure 3 Boxplot of *EGFR* silver enhanced *in situ* hybridization ratio in tumours determined by semiquantitative polymerase chain reaction-based assay to be normal, ambiguous and amplified.

hanced *in situ* hybridization results were negative. The mean value for *EGFR* gene ratio in this group was slightly higher than in the nonamplified group (2.95 signals per cell vs 2.68 signals per cell) but this was statistically not significant ($P=0.49$). The mean silver enhanced *in situ* hybridization ratio differences between the semiquantitative polymerase chain reaction-based assay positive and negative group were highly significant ($P<0.001$) but not between ambiguous cases and nonamplified cases ($P=0.57$) (Figure 3).

Discussion

The number of clinical studies using *EGFR*-based therapies in addition to the conventional radiochemotherapy is growing constantly. For treatment of glioblastoma, there are several clinical protocols

including Erlotinib, Gefitinib or Cetuximab in addition to the standard treatment regime.^{16–20} Accurate assessment of *EGFR* status is a prerequisite to identify subgroups of patients, which will benefit most likely from this treatment. Mellinghoff *et al*²¹ supported this concept by demonstrating that glioblastomas with coexpression of *EGFRvIII* (a mutant form of *EGFR*) and *PTEN* showed better clinical response when treated with erlotinib or gefitinib. Thus, need for rapid and reliable tests to determine *EGFR* status seems to be likely. The current genomic tests usually include fluorescence *in situ* hybridization. Although there are several articles demonstrating chromogenic *in situ* hybridization as a viable alternative, fluorescence *in situ* hybridization is still the gold standard and widely used.^{10,22–24}

In this study, we compared the novel method silver enhanced *in situ* hybridization with fluorescence *in situ* hybridization for the assessment of *EGFR* amplification in a large series of 90 human glioblastomas. Both methods showed low failure rates (fluorescence *in situ* hybridization 2%; silver enhanced *in situ* hybridization 3%). The incidence of *EGFR* amplification detected with silver enhanced *in situ* hybridization was 28% (fluorescence *in situ* hybridization 30%), according to previous literature.^{25,26} The concordance rate between fluorescence *in situ* hybridization and silver enhanced *in situ* hybridization was 98% ($\kappa=0.95$, 95% CI), demonstrating that silver enhanced *in situ* hybridization is a valid alternative testing method for the determination of *EGFR*-amplification status in glioblastoma. These results are consistent with previous studies reporting on concordance rates for *HER2* silver enhanced *in situ* hybridization vs. fluorescence *in situ* hybridization of 96% ($\kappa=0.75$, 95% CI) and 99%.^{13,27}

Additionally, we assessed the *EGFR* gene status with a semiquantitative polymerase chain reaction-based assay and compared the results with silver enhanced *in situ* hybridization. Semiquantitative polymerase chain reaction-based assay results concurred excellent with silver enhanced *in situ* hybridization findings ($P<0.001$) and showed high association with *EGFR* silver enhanced *in situ* hybridization. This result is consistent with a previous study illustrating a good correlation of polymerase chain reaction tested *EGFR* gene doses with fluorescence *in situ* hybridization.²⁸

All cases that yielded ambiguous results with semiquantitative polymerase chain reaction-based assay ($n=4$) showed negative *EGFR* amplification with silver enhanced *in situ* hybridization. The slightly higher *EGFR* gene ratio in this group might have been caused by trisomy 7 as often seen in glioblastoma.²⁹ However, this result was statistically not significant, due to the small number.

We demonstrated a significant association of *EGFR* protein overexpression with gene amplification according to previous literature.^{26,30,31} Although

EGFR protein overexpression was highly associated with gene amplification, there were 29 of 64 cases that showed a high *EGFR* protein level and no *EGFR* amplification by fluorescence *in situ* hybridization or silver enhanced *in situ* hybridization. All cases with gene amplification showed immunohistochemistry positivity. The observation that the frequency of elevated *EGFR* immunoreactivity far exceeded that of *EGFR* gene amplification is consistent with prior results in adult high-grade gliomas.^{32,33}

Although fluorescence *in situ* hybridization is globally accepted as a standard method for determining *EGFR* status, pathologists have been reluctant to adopt fluorescence *in situ* hybridization for routine clinical laboratory testing. The reasons for this include the technical challenges associated with fluorescence *in situ* hybridization assays,³⁴ the expenses, the required time for interpretation³⁵ and the relative lack of community-based experience with this technology. Although the material costs are slightly higher for silver enhanced *in situ* hybridization than for fluorescence *in situ* hybridization (approximately costs per each test for silver enhanced *in situ* hybridization: 200\$, for fluorescence *in situ* hybridization: 130\$), the technician working time for silver enhanced *in situ* hybridization is much shorter compared with fluorescence *in situ* hybridization (pure working time for silver enhanced *in situ*: 20 min, for fluorescence *in situ* hybridization 3 h). In addition, fluorescence *in situ* hybridization requires access to a cost-intensive fluorescent microscope. However, the fluorescence *in situ* hybridization method comprises one advantage: one hybridization is sufficient for diagnostic purpose compared with SISH, which needs to be performed on two slides per case (one for the *EGFR* probe and one for chromosome 7).

The excellent concordance rate of fluorescence *in situ* hybridization and silver enhanced *in situ* hybridization proves equivalent diagnostic value. Silver enhanced *in situ* hybridization offers several advantages. It is performed as a 6-h automated protocol and can be completed within one working day. Without the need of specialized microscope equipments, the pathologist can easily integrate the tests in the routine day work. It allows simultaneous demonstration of interesting results for teaching purposes by using routinely available multiheaded microscopes. Silver enhanced *in situ* hybridization generates a stable chromogenic reaction product, which allows the permanent storage of the slides. This point is particularly important when the tests are used in routine diagnostics when proper documentation and archival storage is required for certification and quality management. Furthermore, the possibility to work with low microscopic magnifications allows a much easier handling of difficulties due to tumour heterogeneity. Most of the silver enhanced *in situ* hybridization signals are already recognizable at 200 $\times$ magnification (for fluorescence *in situ* hybridization a magnification of

400 × up to 1000 × is often necessary) and the intensity and sharpness of signals in silver enhanced *in situ* hybridization is much better than in chromogenic *in situ* hybridization.

Many pathologists are experienced in evaluating silver enhanced *in situ* hybridization-like peroxidase-based immunostainings but not familiar with fluorescence microscopy. Therefore, the time and effort needed for acquisition of silver enhanced *in situ* hybridization is presumably shorter than for fluorescence *in situ* hybridization.

In conclusion, the present results convincingly demonstrate that silver enhanced *in situ* hybridization is a powerful tool for the detection of *EGFR* amplification in archival paraffin-embedded glioblastoma samples. The universal need to standardize, simplify and accelerate the diagnostic process renders silver enhanced *in situ* hybridization a cost-effective alternative to fluorescence *in situ* hybridization.

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Conflict of interest

All authors declare no conflict of interest. No conflict exists for drugs or devices used in this study. Authors thank Ventana for generously providing the silver enhanced *in situ* hybridization probe and reagents.

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