

Identification of Postoperative Margins of Glioblastoma Multiforme Using Gold Nanoparticles Conjugated to Epidermal Growth Factor Antibodies

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Abstract

Background: Glioblastoma multiforme (GBM) is the most common primary malignant brain tumor in adults. Complete resection is impossible. Overexpression of epidermal growth factor receptor (EGFR) by the tumor has been associated with the level of malignancy and possibly with prognosis. Monoclonal antibodies may be used to identify tumor borders. The aim of the present study was to investigate the value of immunostaining with anti-EGFR antibodies conjugated to gold nanoparticles (GNPs) for detecting tumor infiltration into adjacent tissue.

Methods: Fresh GBM samples were collected intraoperatively from 14 patients treated at a tertiary medical center in 2014-2015, in accordance with the guidelines of the local and national ethic committees. Demographic, clinical, and outcome data of the patients were recorded. Analysis for *EGFR* overexpression by polysomy or amplification was determined by calculating the ratio of expression between pairs of the following genes: *EGFR*, *GPER*, and *RNaseP*. Tumor sample with known overexpression of *EGFR* underwent immunostaining with GNP-conjugated anti-EGFR antibodies followed by light-scattering spectroscopy and electron microscopy. The findings were compared with routine hematoxylin and eosin staining.

Results: On molecular analysis, *EGFR* overexpression was identified in 10/14 GBM samples (71%). Amplification was detected in 50% and polysomy in 27%, and one sample showed both. Patients whose samples were characterized by an overexpression of *EGFR* died within 3-10 months, whereas 2 of the patients with normal *EGFR* expression were still alive 9 and <15 months after surgery. Tumor borders identified by anti-EGFR-GNP immunostaining exceeded the area considered "healthy" on routine pathologic study.

Conclusion: The high level of expression of *EGFR* in the majority of GBM tumors makes it possible to selectively distinguish tumor infiltration using anti-EGFR antibodies conjugated to GNPs. As staining with GNPs is tumor-functional-dependent, the findings may have important prognostic value. Preoperative mapping of tumor extent may facilitate complete resection of these difficult-to-resect tumors.

Keywords: GBM; Glioblastoma multiforme; Gold nanoparticles; GNPs; Tumor margins; EGFR; Epidermal growth factor; Overexpression

Abbreviations: GBM: Glioblastoma Multiforme; GNP: Gold Nanoparticles; H&E: Hematoxylin and Eosin; EGFR: Epidermal Growth Factor Receptor

Received: October 23, 2016; **Accepted:** November 25, 2016; **Published:** November 28, 2016

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Background

The epidermal growth factor (*EGFR*) gene (7p12.1) codes a cell membrane protein receptor with intrinsic tyrosine kinase activity. It has been the subject of rigorous investigation owing to its involvement in several human cancers and as a potential target of therapy [1]. The EGFR protein is activated by ligand binding which leads to tightly regulated stimulation of proliferative and survival cell-signaling pathways, most notably the RAS/RAF/MEK/MAPK and PI3K/Akt [2-4].

Glioblastoma Multiforme (GBM) is the most common primary brain tumor in adults. Despite current therapies, it has a rapidly progressive and fatal course [5]. A recent study of the molecular genetics of GBM reported overexpression of the *EGFR* gene in the majority of tumors evaluated, either by amplification, which results in multiple copies of the gene in chromosomal regions (amplicons) [2] or by polysomy of chromosome 7 which contains the *EGFR* gene [6]. In very rare cases, *EGFR* overexpression may be due to a *de novo* mutation [4]. As a result of these findings, attention has been addressed to overexpression of the EGFR protein as a potential prognostic indicator in GBM [5]. However, its accuracy remains unclear [3,5,7,8].

Complete resection of GBM with clear margins is nearly impossible owing to its aggressive nature and diffuse structure [9,10]. GBM has a high recurrence rate after surgery even when combined with chemotherapy or radiotherapy [9,11]. The estimated one-year mortality rate is as high as 65% [10]. The diffuse structure of the tumor can also hinder intraoperative and post-resection mapping of the tumor margins by standard imaging techniques. Therefore, a better method for mapping tumor margins would be invaluable to neurosurgeons and could contribute to patient survival.

The application of biocompatible gold nanoparticles (GNPs) for *in vivo* molecular imaging has attracted much interest in various scientific disciplines, such as biomedicine, engineering, and chemistry [12]. Recently, our group suggested a new method for cancer detection based on optical measurements of GNPs [13]. The GNPs were bioconjugated to EGFR-expressing head and neck cancer cells. The accumulation of the GNPs in the cancer cells caused an increase in their absorption properties, resulting in a specific reflection signal of the tumor site [14,15].

Prompted by these findings, in the present study, we sought to determine if GNP-conjugated anti-EGFR antibodies can be used to mark tumor borders and to identify tumor infiltration into surrounding tissue.

Methods

Ethics, consent and permissions

The study was approved by the national and institutional ethics committees.

Glioblastoma tissue samples

The study was conducted on fresh GBM tumor samples from 14 consecutive patients treated at Rabin Medical Center in 2014-

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Citation: Hazon D, Marianayagam NJ, Barinfeld O, et al. Identification of Postoperative Margins of Glioblastoma Multiforme Using Gold Nanoparticles Conjugated to Epidermal Growth Factor Antibodies. *Nano Res Appl.* 2016, 2:2.

2015. The diagnosis of GBM was made clinically, radiologically, and pathologically. All participants gave written informed consent prior to surgery, and the samples were collected intraoperatively in accordance with the guidelines of the ethics committees. The tissue diagnosis was confirmed by a pathologist (S.H.F.) using the glioma grading scale of the World Health Organization [16].

Patient data

The demographic and clinical data of the patients were recorded, and the clinical course was compared with the molecular data and outcome.

Molecular analysis

DNA extraction from tumor tissue. The fresh tumor samples were immediately placed in Proteinase K for 12 hours at 56°C for digestion of all cellular components except DNA. DNA was extracted using the Blood and Tissue DNAeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. All DNA obtained from the tumor samples was stored at -20°C for further analysis.

Testing EGFR expression levels in GBM samples

To determine *EGFR* expression in the GBM samples, we measured the expression of the *GPER* and *RNaseP* genes, as recently described [6]. The primer sequence was kindly provided by Dr. Bieńkowski and his group. The following sense and antisense primers were used:

EGFR: 5'-CACACCCCTGACTCTCCACT-3' and 5'-GAGACAATCCTGT-GAGCTTGG-3';

GPER: 5'-CATCTGGACGGCAGGTAAGT-3' and 5'-CCCTCAGCCGG-TAGTTTTC-3';

RNaseP: 5'-GGGAGATGCGGAAGAATGT-3' and 5'-CCTCCAGT-CAGCCACAGAA-3'.

GPER lies in close proximity to *EGFR* on chromosome 7; *RNaseP* is a housekeeping gene on chromosome 15. A ratio of *GPER* expression to *RNaseP* expression above 1.5 is considered indicative of chromosome 7 polysomy. A ratio of *EGFR* expression to *RNaseP* expression above 1.5 is considered indicative of *EGFR* amplification [6]. Cut off was calculated according to a previous publication [6].

Real-time-polymerase chain reaction (RT-PCR)

RT-PCR was performed with the SYBR® Fast PCR system (Applied Biosystems). DNA samples were loaded with the appropriate primers and reagents to a final volume of 10 µl, with a final concentration of 10 ng DNA. The following materials were added to the reaction mixture: Sybr Green (5 µl), 10 µM reverse primer (0.5 µl), 10 µM forward primer (0.5 µl), DNA (10 µl) and ddH₂O (3

μl). The thermal cycling conditions for RT-PCR were as follows: enzyme activation, 19 minutes at 95°C; denaturation, 40 cycles of 15 seconds at 95°C; annealing and extension, 1 minute at 60°C. Melting curves were generated to analyze the specificity of gene amplification. The expression levels of each gene of interest were calculated from the generated data [6,9,16].

Immunostaining

Preparation of GNPs and their conjugation to anti-EGFR antibody. Rod-shaped GNPs were used, with a spectral peak of 660 nm [17,18]. The GNPs were conjugated to two different anti-EGFR antibodies: a recombinant human/mouse chimeric EGFR monoclonal antibody [Cetuximab (Erbix), Merck KGaA, Germany] and an affinity purified rabbit polyclonal antibody (PDGFR-α, Santa Cruz Biotechnology, Santa Cruz, CA, USA). The GNPs were bioconjugated to the anti-EGFR antibodies using polystyrene sulfonate, according to the method described by Lvov [19].

Immunostaining with ant-EGFR conjugated to GNPs

Paraffin-embedded block from each patient containing tumor tissue were collected from the pathology department. Two 4 μm sections were cut on glass slides. One slide was stained with hematoxylin and eosin (H&E) and evaluated by a pathologist for tumor borders. The other was de-paraffinized, marked with the tumor borders identified on the H&E-stained slide, and stained with anti-EGFR antibody conjugated to GNPs, as previously described [13,14].

Hyperspectral imaging of immunostained slides

The reflectance of GNPs within the tissue samples was measured with the hyperspectral imaging system (Nuance, CRI, Woburn, MA, USA). For imaging in RGB mode, we used a 40x objective (0.75 NA) 32-bit ultrasensitive charge-coupled device camera

detector (N-MSI-EX) under halogen illumination (UN2-PSE100, Nikon, Tokyo, Japan) Microscopy was performed with a Nikon 80i microscope. Images were acquired using Nuance 2.1 software (Burlington, MA, USA). The reflectance is presented as arbitrary intensity units (IU).

Each slide was scanned before (negative control) and after GNPs where added. Under both conditions, the reflectance spectra were measured in 3 areas on the tumor and 3 areas on the area considered healthy by routine pathology. For image processing analysis, the results without GNPs were subtracted from the results with GNPs. We subtracted the spectra of the pure glass slide in order to reduce noise.

Electron microscopy of immunostained slides

To directly visualize GNPs in the tissue, we scanned the GNP-stained slides with the airSEM™ (B-Nano, Krapina, Croatia), an innovative high- resolution scanning electron microscope that sidesteps the main obstacles stemming from the need for vacuum as a prerequisite for capture of high-resolution images. The technology overcomes fundamental electron beam imaging-related challenges, such as scattering of electrons by gas molecules and efficient signal collection, leading to a resolution better than 5 nm under ambient conditions.

Results

The patient data are summarized in **Table 1**. A sample of GBM neuroimaging is shown in **Figure 1**.

EGFR overexpression in GBM

EGFR overexpression was observed in 10 of the 14 (71%) GBM samples examined (**Table 1**). Amplification was found in 5 tumor samples, polysomy in 4 samples, and the both amplification and polysomy in one sample. The remaining 4 samples showed normal *EGFR* expression patterns.

Table 1 Characteristics of patients with GBM: Demographics and EGFR expression status and suggested mechanism.

Pt. no.	Sex	Age (yr)	Date of diagnosis	Date of last f-u or death	Length of survival (mos)	EGFR over-expression	Mechanism	GPER/RNase P	EGFR/GPER	EGFR/RNase P
1	F	71	04.2014	2.2015	10	Yes	Amplification	1.28	39.49	50.65
2	F	59	03.2013	Alive	>30	Yes	Amplification	1	3.4	3.4
3	F	70	5.2014	Alive	>18	No	Normal	1.47	1.15	1.7
4	M	35	2015	No data	No data	No	Normal	1.06	0.97	1.03
5	M	86	07.2014	08.2015	11	Yes	Polysomy	1.74	1.37	2.39
6	F	76	07.2014	04.2015	9	No	Normal	0.76	1.62	1.24
7	M	84	07.2014	10.2014	3	Yes	Amplification	1	10.06	10.14
8	M	60	07.2014	04.2015	9	Yes	Amplification	0.9	2.43	2.2
9	M	62	08.2014	Alive	< 15	No	Normal	0.81	1.27	1.04
10	M	47	10.2014	03.2015	5	Yes	Polysomy	1.78	1.4	2.61
11	N/A	70	10.2014	Alive	N/A	Yes	Amplification	0.43	13.74	32.22
12	N/A	71	10.2014	Alive	N/A	Yes	Amplification and polysomy	2.67	17.27	46.10
13	M	64	09.2014	Alive	< 2	Yes	Polysomy	1.74	1.00	1.75
14	F	49	09.2014	No data	No data	Yes	Polysomy	7.48	1.37	10.27

EGFR-epithelial growth factor receptor, f-u: follow-up, N/A: not available

Demographics and EGFR expression status and suggested mechanism with the comparison to the clinical parameters and outcome are shown on **Table 1**.

Immunostaining with GNP-conjugated anti-EGFR antibodies and comparison to routine pathologic staining

EGFR-conjugated GNPs were detected following immunostaining of EGFR-overexpressing tumor samples. Hyperspectral imaging was performed on the tumor and surrounding neural tissue under different magnifications [20]. Representative results are presented in **Figure 2**. High reflectance at the peak GNPs wavelength (550 nm to 660 nm) was found in areas identified histologically as GBM, and also in areas considered histologically to be free of tumor.

Electron microscopy for direct visualization of GNPs in the tissue

On scanning of the GNP-stained slides with a high-resolution scanning electron microscope (airSEM™) [21], the anti-EGFR-conjugated GNPs could be visualized in the tissue (**Figure 3**). As shown in **Figure 3**, a higher concentration was detected in cells in the tumor area than in the infiltrating cells in the adjacent area. Some areas did not stain. The GNP-stained slides showed infiltrating cells that were not detected on the H&E-stained slides.

Discussion

On DNA analysis of the expression of the *EGFR* gene in fresh GBM samples from 14 patients, we found that 71% of the samples had increased levels of *EGFR* expression via either polysomy of chromosome 7 or amplification of the *EGFR* gene. Patients whose samples were characterized by an overexpression of *EGFR* died within 3-10 months, whereas 2 of the patients with normal *EGFR* expression were still alive 9 and <15 months after surgery. These findings suggest a possible prognostic value of *EGFR* overexpression in GBM. There is currently no known link

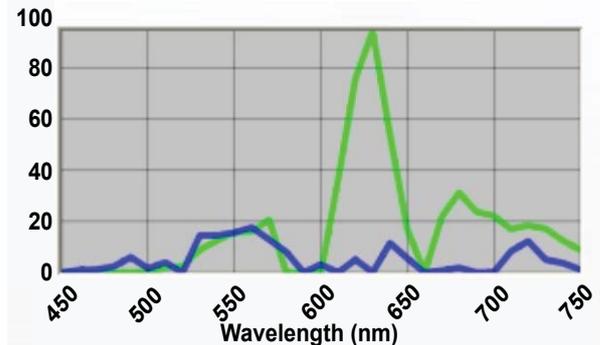


Figure 2 Hyperspectral spectra of tumor (green line) and surrounding neural tissue (blue line). The Y axis is the reflectance intensity in arbitrary intensity units (IU).

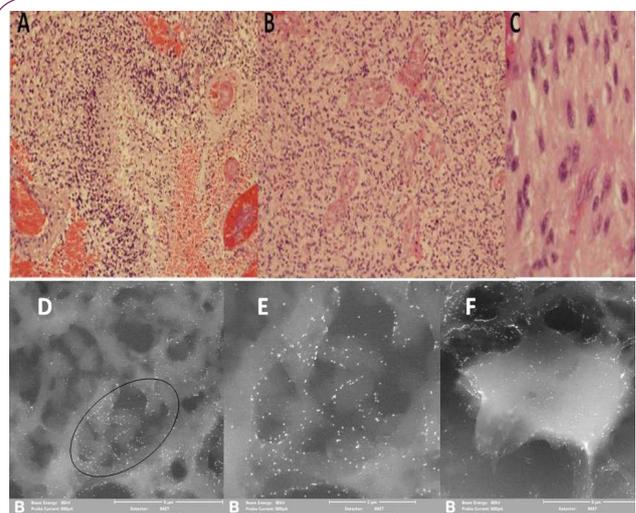


Figure 3 Histopathology of GBM and airSEM. Upper line: (A) pseudo palisading in necrotic area (X5), (B) microvascular proliferation (X10) and (C) mitosis (X20). Lower line: airSEM measurements of tumor and healthy regions of GBM in micrometric (D) and nanometric (E) resolutions. (D). Tumor (up to the dark line) and healthy (down to the dark line) sites of GBM tissue in brain. (E) and (F). airSEM pictures of healthy and tumor sites. GNPs appear as bright dots. Note: An evident difference in the GNPs concentration is clearly seen.

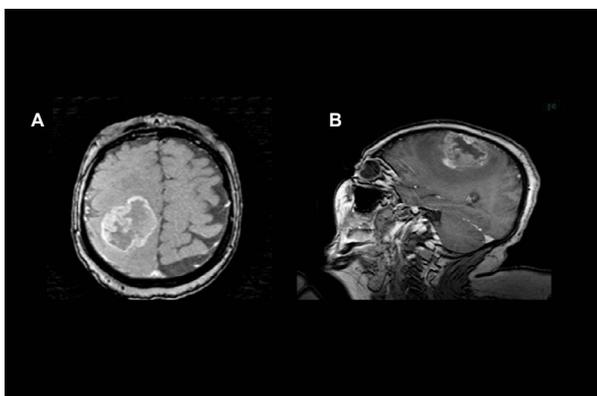


Figure 1 MRI brain imaging. T1 axial (A) and sagittal (B) post contrast images showing a large intraxial heterogeneous enhancing mass with local infiltration and mass effect probably consistent with high grade tumor.

between chromosome 7 polysomy and GBM prognosis, and findings in the literature for the prognostic significance of *EGFR* overexpression are contradictory. One study suggested that it is indicative of poorer outcome [19] but others failed to support this conclusion [5,17,18,22], except in pediatric GBM, in which overexpression of *EGFR* was linked to a worse prognosis [23]. A decreased expression of *EGFR* is clearly not associated with tumor recurrence [5].

Whether or not *EGFR* overexpression has predictive value for outcome, it apparently has importance as a marker of tumor borders and tumor-cell infiltration. On the basis of studies showing that monoclonal antibodies can be utilized to identify tumor borders [24], we immunostained samples of GBM with anti-*EGFR* antibodies conjugated to GNP. In this manner, we

were able to differentiate the tumor tissue even from brain tissue considered healthy by standard histological methods. On both reflectance spectroscopy and electron microscopy, cells infiltrating surrounding areas could be clearly identified.

The high levels of *EGFR* present in GBM makes them a potentially valuable tool for targeted imaging and selective detection of tumor cells within normal brain parenchyma using monoclonal anti-EGFR antibodies conjugated to GNPs. Other studies have used anti-EGFR mutant antibody conjugated to iron oxide nanoparticles [25]. The iron oxide nanoparticles effectively reached the brain tumor by convection-enhanced delivery [25,26] which bypasses the blood-brain barrier for more efficient tumor penetration [26].

Additionally, given the difficulty of complete resection of GBM tumors because of their diffuse structure and highly aggressive behavior, by using GNPs conjugated to anti-EGFR antibodies, researchers may potentially be able to preoperatively map the entire extent of tumor infiltration in order to guide the surgeon. GNP technology can also be used intraoperatively as a contrast agent on magnetic resonance imaging to highlight areas of unresected tissue that may not be visible with conventional intraoperative imaging [27].

Several groups have employed conjugated anti-EGFR antibodies in the treatment of cancer, either as a drug delivery vehicle [28] or as an aid to kill tumor cells by photothermal or radiofrequency ablation [29-32]. In one study, GNPs were used for radiofrequency ablation of pancreatic and colorectal adenocarcinomas in animal models [33]. Although the high levels of EGFR in some forms of GBM also make it an accurate and desirable therapeutic target, current therapies targeting *EGFR*, including tyrosine kinase inhibitors and RNA interference, have proved to be ineffective [7,8,34]. Therefore, attention is being addressed to other means: the delivery of novel antitumor agents across the blood-brain barrier, gene therapy, and brain tumor vaccines [35-37]. One study found that the use of metallic nanoparticles conjugated to anti-EGFR antibody combined with cisplatin could eradicate tumor cells when subjected to radiation *in vitro* [38]. It may therefore be possible to conjugate chemotherapeutic agents to GNP along with anti-EGFR antibodies to facilitate the accurate *in vivo* delivery of these agents to the tumor bed.

There are some drawbacks to conjugation of monoclonal antibodies to nanoparticles, particularly the large size of the conjugate and difficulty in achieving the necessary chemical reaction for conjugation [27]. Furthermore, the use of GNP in particular poses some risk. Although gold is inert, it has a tendency to aggregate in blood vessels, which may lead to toxicity and renal failure. Therefore, the GNP dosage must be carefully titrated with renal function [39].

GNP conjugated to EGFR may enable detection of tumor margins when EGFR is overexpressed. This may limit the use of this technique to approximately 60% of the tumors, in comparison to the existing 5 ALA methods, which has a similar 40% suboptimal detection ability [40]. Therefore, the GNP-EGFR new method suggested herein may be as good as 5 ALA, if not

better. When comparing coloring methods, two techniques have been investigated *in vivo* to date. The nano-encapsulated visible dye was tested in animal model (rats) for intraoperative visual delineation of tumor margins [41] and the intravenous fluorescein sodium detected by specific camera [42]. Liposomal nanocarriers containing Evans blue dye (nano-EB) clearly aids in distinguishing tumor tissue from healthy tissue in rat model. The injection of unencapsulated EB failed to distinguish tumor from healthy brain tissue due to diffusion of dye from the tumor into healthy tissue [41]. Using high doses of fluorescein sodium under white light somewhat improved total resection of GBM. The new fluorescein-specific camera that allows for high-quality intraoperative imaging while using very low dose fluorescein has improved this method. However, fluorescein sodium does not appear to selectively accumulate in astrocytoma cells but in extracellular tumor cell-rich locations. It shows leakage areas of compromised blood-brain barrier, in correlation with enhanced areas on magnetic resonance imaging [42].

There are some limitations to this study. We describe a small cohort that cannot be analyzed statistically. Although this study has shown that GNPs conjugate with anti-EGFR antibodies preferentially home to the GBM tissue expressing EGFR, this concept still needs to be tested *in vivo*. As not all tumors show EGFR overexpression, and healthy neuronal tissue may also express this receptor, the efficiency of the method is yet to be examined. The added value of detecting GNPs intraoperatively depends on the structures and resectability of the tumor. This study has not yet intervened with GNP injection to patients, and therefore no measurement of toxicity is available. It is possible that in an *in vivo* system the conjugated GNPs could behave differently than in an *in vitro* system. To date, the use of GNP did not reveal any toxicity. We have set the foundation for a possibly useful tool to help achieve full resection of GBM. The next phase of this work will be to test this methodology *in vivo*. This study could be useful for future pre- and peri-operative mapping of tumor margins and for delivery of direct chemotherapy to the tumor cells. Although 5 ALA has been investigated for the last decade, and proven to have a good safety profile [40,43,44] with relative efficacy [43,44], the benefits of its use are less than expected. The differentiation between tumor and normal cerebral tissue was suboptimal. Herein, we suggest a new method, that may overcome some of the obstacles. However, further investigation is needed.

Conclusions

In summary, we have shown that GBM overexpresses *EGFR* mechanisms by polysomy of chromosome 7 and amplification. In addition, we show that GNPs conjugated to anti-EGFR antibody preferentially home to areas of tumor within normal brain parenchyma and are therefore useful for the detection of tumor cells that infiltrate surrounding neuronal tissue. They may also serve as a tool for preoperative and intraoperative mapping of resection margins and possibly for the delivery of chemotherapeutic agents directly to the tumor bed and *in vivo* killing of tumor cells using focused radiation.

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