Environmental Effects on Molecular Biomarkers Expression
In Pancreatic and Brain Cancer

Lawrence B. Mensah1*, Srivalleesha Mallidi1*, Iqbal Massodi, Sriram Anbil1, Zhiming Mai1,
Tayyaba Hasan1

*Authors have equal contribution
1Wellman Center for Photomedicine, Harvard Medical School,
Massachusetts General Hospital, 40 Blossom St, Boston, MA, USA 02114

ABSTRACT

A complete understanding of the biological mechanisms regulating devastating disease such as cancer remains elusive. Pancreatic and brain cancers are primary among the cancer types with poor prognosis. Molecular biomarkers have emerged as group of proteins that are preferentially overexpressed in cancers and with a key role in driving disease progression and resistance to chemotherapy. The epidermal growth factor receptor (EGFR), a cell proliferative biomarker is particularly highly expressed in most cancers including brain and pancreatic cancers. The ability of EGFR to sustain prolong cell proliferation is augmented by biomarkers such as Bax, Bcl-XL and Bcl-2, proteins regulating the apoptotic process. To better understand the role and effect of the microenvironment on these biomarkers in pancreatic cancer (PaCa); we analysed two pancreatic tumor lines (AsPc-1 and MiaPaCa-2) in 2D, 3D in-vitro cultures and in orthotopic tumors at different growth stages. We also investigated in patient derived glioblastoma (GBM) tumor cultures, the ability to utilize the EGFR expression to specifically deliver photosensitizer to the cells for photodynamic therapy. Overall, our results suggest that (1) microenvironment changes affect biomarker expression; thereby it is critical to understand these effects prior to designing combination therapies and (2) EGFR expression in tumor cells indeed could serve as a reliable and a robust biomarker that could be used to design targeted and image-guided photodynamic therapy.

Keywords: Three-dimensional tumor models, (3D models), glioblastoma multiforme (GBM), pancreatic cancer, biomarkers, photodynamic therapy.

1. INTRODUCTION

Over the years the conventional chemotherapy has remained only moderately successful in treatment of many cancers including pancreatic and brain cancers [1, 2]. Pancreatic cancer (PaCa) is very aggressive and resistant to most traditional chemotherapeutic agents including radiotherapy [3-5]. In about 80% of the patients, the disease is diagnosed often at late stage as an advanced or metastatic adenocarcinoma [3, 6]. The late stage diagnosis drastically reduces the median survival time to about 6 months. Surgical resection and chemotherapy extended survival time only by a few years in less than 20% of the patients [7-9]. Glioblastoma multiforme (GBM) also remains one of the most common and very aggressive primary brain tumor in adults [10-12]. It is fatal in almost all cases [13]. Surgical debulking and radiation therapy remains the mainstay of treatment. Though these radiation and chemotherapeutic treatments are aggressive, most of the patients still experience tumor recurrence in regions within the treatment area or in close proximity to the treatment area. This resilient nature of the cancer has reduced the mean survival time to typically 6-18 months post-therapy [1, 14, 15].

Recent advances in identification of proteins associated with specific cancers have opened a new frontier for targeted therapy [16, 17]. These classes of proteins are often referred to as molecular biomarkers and are known to play critical role in cancer progression and resistance to therapeutic agents [18, 19]. The application of these molecular biomarkers in detection, treatment and therapy monitoring is depicted in Figure 1[20, 21]. The clinical application of molecular biomarkers has made it feasible to identify (diagnose) specific cancer types and their level of progression (staging) to give valuable information on treatment type [22-25]. Understanding the expression pattern of these biomarkers in a particular cancer provides critical information on the type and frequency of the treatment to be administered. Periodic
testing for the presence of these key molecules also makes it possible to monitor disease response to treatment (surveillance) as summarized in Figure 1 [20, 26, 27].

Most of these molecular biomarkers are members of critical growth factors such as the epidermal growth factor receptor (EGFR), vascular endothelial growth factor receptor (VEGFR) and members of the apoptotic machinery that drives cell proliferation and survival. Interestingly, a vast majority of PaCa (30-85%) and GBM (60-65%) carry EGFR mutations which result in high express level of EGFR due to posttranslational amplification [28-31]. The EGFR signaling drives cell proliferation and tumor metastasis and also cross-talk with the Bcl-2 family proteins that regulate cell survival and reduce apoptosis [28, 32, 33]. Hence, shutting down EGFR signaling pathway in PaCa and GBM as a means of therapy remains an intense focus of research [34, 35].

![Figure 1: Schematic representation of the areas where molecular biomarkers play an important role in cancer treatment.](image)

Several novel approaches have been initiated to enable therapeutic agents achieve optimum treatment response [32, 36]. Among these novel treatments, include specific delivery of photodynamic therapy (PDT) agents in combination with inhibitors of key biomarkers such as EGFR has been tested [37]. To better understand the expression profile of these clinically relevant biomarkers in PaCa, we examined the expression of EGFR, Bax, Bel-XL and Bcl-2 by western blot in 2D, 3D and in vivo orthotopic tumor in two transformed pancreatic cancer lines; AsPC-1 and MiaPaCa-2. In brief, the data show increased expression of EGFR and pro-survival molecule, Bcl-XL in in vivo system compared to in vitro cultures. This indicates that high levels of EGFR and Bcl-XL expression could drive cancer cells to resist apoptosis during treatment resulting in tumor resistance to therapy [33, 38] This study is particularly unique since it carefully characterized the key proliferative marker expression in two important pancreatic cell lines. We also investigated whether the expression pattern of EGFR biomarker would be useful for designing and guiding of therapy in GBM. We used a photoimmunoconjugate (PIC) that is Cetuximab, EGFR antibody conjugated to photosensitizer benzoporphyrin derivative (BPD) to specifically deliver BPD to GBM cells that overexpress EGFR. Taken together, these results indicates that EGFR may will be suitable candidate target for treatment and disease monitoring in both GBM and pancreatic cancer.

**MATERIALS AND METHODS**

2.1. 2D Cell Culture

Two pancreatic cell lines; AsPC-1 (American Type Culture Collection [ATCC] CRL1682) and MiaPaCa-2 (ATCC, CRL-1420) monolayer (2D) were cultured in RPMI and DMEM medium (Mediatech Inc., Herndon, Virginia, USA) supplemented with 10% heat inactivated fetal calf serum (GIBCO Life Technologies, New York, USA), 100 U/mL penicillin, and 100 µg/mL streptomycin respectively. Cells were cultured at the density of 7,500/ml per well in a 24 well
plates at 37°C in 5% CO2 incubator and media changed every 48-72hrs. Cells were harvested at either day 5 or 12 post initial plating and lysed with chilled 200µl MPER lysis buffer (Thermo Scientific, Rockford, IL, product # 89900) containing cocktail of phosphatase inhibitor (Sigma, St. Louis, MO, product #P5726) and protease inhibitor (Sigma, P8340) for 30 minutes on ice with repeated pipetting followed by centrifugation at 14,000 rpm for 15 minutes. The clear supernatant protein lysate were carefully removed and preserved.

2.2. 3D Cell Culture
To prepare protein lysates from three-dimension (3D) micronodule samples, AsPc-1 and MiaPaCa-2 cells of 7,500/ml per well in a 24 well plates were cultured on 250µl growth factor reduced (GFR, Matrigel) media for 5 and 12 day with 48-72hr media change [37, 39]. The culture media was carefully aspirated and 200 µl of chilled MPER mammalian lysis buffer containing a cocktail of phosphatase and protease inhibitor was added to each well and incubated for 30 minutes with repeated pipetting followed by centrifugation at 14,000 rpm for 15 minutes. The clear supernatant protein lysate were preserved.

2.3. Orthotopic pancreatic tumor implantation:
Male athymic Swiss Nu/Nu mice 6-8 weeks old, weighing ~25 g, were used. Briefly, animals were anesthetized with a cocktail of 100 mg/kg ketamine and 10 mg/kg xylazine. For tumor implantation, a small left abdominal flank incision was made to exteriorize the pancreas. Subsequently, a suspension of 1 x 10^6 of either AsPc-1 or MiaPaCa-2 tumor cells in 25-50µl of chilled media mixed with an equal volume of chilled matrigel were injected into the body of the pancreas using a 1ml syringe and a 30 gauge needle and the incision carefully sutured.

Animals were monitored daily and AsPc-1 orthotopic tumor bearing mice were euthanized on day 10 and 15 while MiaPaCa-2 pancreatic tumor mice were euthanized on day 40. Tumors were carefully removed, transferred into a re-labeled tube and snap frozen on dry ice and stored at -80°C. Tumor tissue were carefully weighed and homogenized in chilled MPER lysis buffer at 0.5 mg of tumor per 1ml of lysis buffer and incubated on ice for 30 minutes for complete lysis of cells. Cell lysate were spun at 14,000 rpm for 15 minutes at 4°C and clear supernatant lysate was preserved.

2.4. Serial passage of glioblastoma tumors
GBM6 (EGFR-positive) and GBM 22 (EGFR-negative) tumor tissues were obtained from Dr. Sarkaria group at Mayo Clinic, Rochester. The tissues were implanted subcutaneously on mice flank and serially passaged in vivo using procedure as previously established [40, 41]. Briefly, the tumor was separated from the mouse subcutaneous wall using sterile procedure, minced into smaller pieces and mixed with matrigel and implanted into the flank of subsequent mouse. To obtain short-term culture explants, the subcutaneous tumor tissue was extracted from the mouse after euthanasia, minced and cultured on matrigel coated flasks with DMEM (2.5% FBS, 1% penicillin/streptomycin) media. The media was changed to DMEM (10% FBS, 1% penicillin/streptomycin) 24 hours post initial plating as has been previously described[40, 41]. All animal experiments were conducted according to guidelines established by the Massachusetts General Hospital Institutional Animal Care and Use Committee (IACUC).

2.5. Tumor tissue Preparation
Mice were euthanized and tumor samples were removed. Scalpel blade was used to carefully remove as much blood as possible from tissue and placed in pre-labeled 1.5 ml eppendorf tube and snap frozen on dry ice. A scalpel blade was used to cut 0.1g of frozen tissue and weighed without thawing the sample. Tissue was then homogenized using a pestle in chilled 3 ml MPER mammalian tissue lysis buffer containing cocktail of protease and phosphatase inhibitor. Homogenized sample was incubated on ice for 30 minutes with repeat pipetting to ensure complete lysis of cells. Protein sample was spun at 14,000 rpm and the clear supernatant protein lysate was preserved. The protein concentration was quantified using BCA assay kit.

2.6. Western Blot
The 2D cell culture, 3D cell culture and in vivo clear supernatant protein lysates were quantified using Pierce Bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, Rockford, IL, product # 23225). Protein samples were then separated on 4-15% SDS-PAGE gel (Bio-Rad, Hercules, CA) and transferred onto immune-blot PVDF membrane (BioRad, Hercules, CA). Membranes were blocked with 5% non-fat milk in 0.01% Tween 20 for 1 hour followed by overnight incubation at 4°C with rabbit anti-EGFR mAb (#4405), Bax(#5023), Bcl-XL (#2762), Bcl-2 (#2870) and beta-actin (#4970) primary antibodies.
Anti-rabbit IgG HRP-conjugated secondary antibody (#7074) was used to blot for all primary antibodies. All antibodies were obtained from (Cell Signaling Technology, Denver, MA) and used at 1:1000 dilutions. All proteins were detected under non-reducing condition. HRP signal from protein bands were detected using Immuno-Star WesternC substrate chemiluminescence (ECL) kit (BioRad, Hercules, CA) on Kodak image station 4000R. HRP signals from protein of interest were normalized to their respective β-actin loading controls. Densitometry was performed using Image J analysis software (NIH).

2.7. EGFR targeted delivery of PDT photosensitizer

Binding of 250 nM PS equivalent of BPD–Cetuximab photoimmunoconjugate (PIC) in patient derived short-term cultures was measured as previously described [27]. Briefly, cells were incubated for 15 hours with PIC and, after washing, were incubated with Solvable (Perkin Elmer, Waltham, MA) for 2 h at 37 °C. Fluorescence was measured from the cell lysate and plotted as relative fluorescence units (RFU) per µg cell protein.

3. RESULTS AND DISCUSSION

In order to delineate how the molecular blueprint of key biomarkers such as EGFR, a proliferative biomarker and Bax, a pro-cell death protein and Bcl-2 and Bcl-XL pro-survival could be used to better understand the heterogeneity of each cancer type and as well as understand the effect of the tumor microenvironment on the cancer on the biomarker expression; we cultured two different transformed pancreatic cancer cell lines; AsPc-1 and MiaPaCa-2 in monolayer (2D), 3D micronodules on matrigel gel, and subcutaneous in vivo tissues. We also investigated the effect of increased cell proliferation on the expression pattern of these biomarkers by culturing the 2D and 3D cells at two time point, day 5 and 12 and the in vivo AsPc-1 at day 10 and 15, except for MiaPaCa-2 in vivo sample where only one time point was obtained at day 40 for the preliminary experiment.

![Figure 2](http://spiedigitallibrary.org/)

**Figure 2**: EGFR and Bcl-2 family protein biomarkers expression profile in pancreatic cancer.

A. Western blot was used to detect the expression of EGFR, pro-apoptotic marker, Bax and pro-survival proteins Bcl-2 and Bcl-XL in 2D, 3D and in vivo AsPc-1 and MiaPaCa-2 pancreatic cell lines at day 5 and 12. The expression patterns of these biomarkers were also detected in day 10 and 15 AsPc-1 and day 40 MiaPaCa-2 xenografts tissues. B. Shows summary of protein densitometry in various cell types. Protein quantification data reveals slight increased expression of EGFR and Bax in in vitro 2D and 3D cells compared to in vivo tissues in both cell types. However, dramatic upregulation of pro-survival molecule, Bcl-XL was observed in both AsPc-1 and MiaPaCa-2 in vivo compared to in vitro cells.
Immunoblotting was used to analyze expression profile of the biomarker in all the samples (Figure 1A) and protein expression densitometry was done using Image J (NIH) expression (Figure 1B). Increased EGFR expression was observed in in vitro AsPc-1 cultures compared to in vivo AsPc-1. Conversely, the pro-survival molecule, Bcl-2 is significantly upregulated in all MiaPaCa-2 cells along with Bax, while downregulated in AsPc-1 cells. Similarly, Bcl-XL is moderately upregulated in AsPc-1 compared to MiaPaCa-2 in vitro system. Remarkably, Bcl-XL is significantly upregulated in both AsPc-1 and MiaPaCa-2 in vivo samples followed by in vitro system, with least the expression in 2D. These data suggests that the 3D system best recapitulates in vivo system compared to 2D.

The increased expression of EGFR in in vitro system was accompanied by slightly increased Bax levels, with no corresponding elevation in pro-survival in Bcl-XL and Bcl-2 which indicates that the 2D system capable of only short-term maintenance of cell proliferation. On the contrary, though EGFR expression in in vivo tissue was relative low compared to in vitro system, however it correlates with high expression of pro-survival protein Bcl-XL expression and decreased Bax levels (Bcl-XL/Bax basal ratio, Figure 1B) indicating the ability of the in vitro system to sustain long-term cell growth and proliferation. Interestingly, increased expression of Bcl-2 was observed in MiaPaCa-2 while Bcl-XL expression in AsPc-1 cells (Figure 1A) implying that; pancreatic cancers arising from these different sites might use slightly different molecular switches within the Bcl-2 family proteins to drive cell survival as indicated in other literature [42-46]. These data suggests that effective treatment could be attained in AsPc-1 and MiaPaCa-2 by targeting survival biomarkers such as EGFR, Bcl-XL and Bcl-2 in cell type dependent manner.

![Figure 3: (A) Maintenance of EGFR expression in murine GBM orthotopic cancer model. Several serial passages of organotypic primary cell line GBM22, a non-expresser of EGFR and GBM6, a positive expresser of EGFR were used to study the maintenance of EGFR expression in GBM over three to five generations in mouse subcutaneous flanks. Immunoblotting was to analyze levels of EGFR protein expression. Data from alternate passages or generations (1, 3 and 5) is shown here. (B) Short-term explant cultures obtained from the subcutaneous tumors were incubated with photoimmunoconjugate (PIC, EGFR targeted benzoporphyrin derivative) show greater uptake of BPD in the EGFR positive GBM 6 cell line compared to the EGFR negative GBM 22 cell line.](http://spiedigitallibrary.org/)

The understanding of the molecular biology of most cancers still remains elusive; hence the desperate need to model most malignant tumors in in vitro in order to allow for easy manipulation, testing of drug response and extraction of data in a way that is not feasible in in vivo systems. The robustness and reliability of these in vitro systems is dependent on the ability of the platform to maintain similar expression profile of clinically relevant biomarkers that drives tumor growth in vivo. To this the use of transformed cancer cells has not proven to be always reliable in disease remodeling due to tendency to misregulate biomarker expression due to sporadic mutations often acquired in culture.

In order to develop a more robust system, we use patient-derived glioblastoma GBM6 and GBM22 cell lines and...
investigated the duration for which the EGFR expression is sustained in GBM6 EGFR positive cells or be misregulated in GBM22 negative line. Figure 3A shows the western blots data for EGFR expression in GBM6 and GMB22 tumors for serial short-term passages 1, 3 and 5. Both GBM22 and GBM6 show no change in EGFR expression status over several serial in vivo passages as has been previously reported [40, 41] indicating the use of GBM primary cell lines provides a robust system to better model GBM and test efficacy of new drugs and treatment response.

We have previously shown in established ovarian cancer cell line that when cetuximab is conjugated to BPD (PIC), its anti-EGFR activity and phototoxic abilities are retained [47]. We tested the efficacy of PIC to deliver BPD to patient derived glioblastoma short-term explant cultures. Figure 3B shows the control group (no PIC) is in blue and fluorescence obtained from cells incubated with PIC is shown in red bar. Clearly the uptake of PIC is higher in GBM6 line that has higher EGFR expression than GBM 22. The competition assay performed by adding 10 fold excess C225 showed a decrease in BPD uptake in the cell lines. This study also indicates that mechanistic-based treatment targeting EGFR could be used to guide treatment in GBMs.

4. CONCLUSIONS

In conclusion, better understanding of clinically relevant molecular biomarker expression profile in cancer will undoubtedly provide sound rationale for designing and guiding treatment [23, 48, 49]. We have used immunoblotting to explore the expression blueprint of key proliferative and survival biomarkers EGFR, Bcl-XL, Bcl-2 and Bax in two transformed pancreatic cancer cell line AsPc-1 and MiaPaCa-2 in 2D, 3D and in vivo at two different proliferative time points. Interestingly, our data show that AsPc-1 is more likely to use Bcl-XL for survive while MiaPaCa-2 is dependent upon Bcl-2 expression in vivo system. We also show that EGFR expression of GBM cells can be utilized to specifically target photosensitizer for custom-designed PDT treatment. Overall, these conclusions suggest that effective mechanistic-based drug treatment design must be cancer cell-type specific dependent as suggested by the environment dependent changes in biomarker expression. This concept needs further detailed verification.

ACKNOWLEDGMENTS

This work was supported by the NIH grants R01CA156177 and PO1CA084203. Dr. Mallidi is supported by NIH grant F32CA165881. The authors would like to thank Dr. Sarkaria for providing the GBM tissues. We would also like to thank Dr. Huang-Chiao and Dr. Goldschmidt for providing MiaPaCa-2 in vivo tumor sample.

REFERENCES


42. Fiebig, A.A., et al., Bcl-XL is qualitatively different from and ten times more effective than Bcl-2 when expressed in a breast cancer cell line. BMC Cancer, 2006. 6: p. 213.


