

SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D.)

Molecular interactions of ErbB receptor tyrosine kinases and integrin $\beta 1$:
implications for tumor therapy

by Miklós Petrás

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UNIVERSITY OF DEBRECEN
DOCTORAL SCHOOL OF MOLECULAR MEDICINE

DEBRECEN, 2013

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Head of the **Examination Committee**: Prof. László Csernoch, D.Sc.
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The Examination takes place at Library of the Department of Physiology, Medical and Health
Science Center, University of Debrecen
Debrecen
3 May, 2013

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The Ph.D. Defense takes place at the Lecture Hall of the Building "A" of Department of
Medicine, Institute for Internal Medicine, Medical and Health Science Center, University of
Debrecen
3 May, 2013

Introduction

Treatment of malignant tumors does still often meet insurmountable difficulties despite ever-broadening diagnostic and therapeutic modalities. In the year 2008, 12.7 million new cases of cancers were diagnosed worldwide, which is predicted to be increased by ~ 68 % - upto over 21 million - until 2030. Additionally, tumors of the central nervous system stand now as the second most frequent cause of cancer mortality under age 35, showing a dramatically increased incidence (up to 300%) over the past decades.

In the background of these distressful data, therapy resistance and even metastatic ability of the tumors evolving during treatment are the major factors causing the failure of treatment. In this line, the decreased chemo- or radiosensitivity and the developing uncontrollable metastasizing ability of the tumor cells frequently hamper therapeutic breakthrough, even though significant development in imaging and molecular marker detection (early diagnosis), in surgical technique and adjuvant therapy (tumor specific targeting) have been achieved over the recent years.

To face these therapeutic hindrances, numerous studies have been conducted on tumorigenesis that revealed the role of overexpression, gene amplifications or mutations of particular growth factor receptors - including ErbB proteins - in tumor proliferation and developing therapy resistance. After the discovery of their mitogenic potential, members of the ErbB family of type I receptor tyrosine kinases have undergone comprehensive investigations. They were found to form homo- or heterodimers with each other that - stimulated by the enhancing effect of ligand binding - results in transphosphorylation on their tyrosine residues. This latter activation process leads to the recruitment of intracellular signaling molecules and translates into diverse cell fates. Heterodimerization - compared to homodimerization - was shown to extend their transphosphorylating dynamism, yielding more potent mitogenic signals.

Recent findings have also provided evidence for the functional crosstalk between certain cell adhesion molecules and receptor tyrosine kinases suggesting that they may contribute to therapy resistance. Given that ectopic (e.g. brain) metastases of primarily invasive tumors are peculiarly isolated at their new localization - virtually retracting their invasive ability to infiltrate - a role for molecular interactions of unique, organ and tumor specific cell adhesion and extracellular matrix molecules is suggested, which may restate the theory of "cell adhesion mediated therapy resistance".

Molecular interactions of ErbB receptors and integrin cell adhesion molecules

Alterations of several tumor suppressors and oncogenes have been identified as being critical to the initial steps of transformation and progression to malignancy. In this respect, members of the ErbB superfamily (ErbB1-4) of transmembrane receptor tyrosine kinases have been studied extensively. It has been revealed that certain tumor cells may express multiple ErbB family members, which can homo- and heteroassociate during signal transduction. Thorough investigation disclosed that dimerization and ligand binding leads to receptor trans-activation and induction of versatile downstream signaling pathways.

In addition, the proliferation and survival of cells also depend on the interactions between cells and their environment, including the extracellular matrix (ECM). Among the specific cell adhesion proteins, integrins are the most dynamic and versatile group, from which downstream signals enable cancer cells to detach from neighbouring ones and promote survival even in a foreign environment. Integrins are evolutionarily ancient proteins, which consist of two subunits (α and β), and bind to other cell adhesion molecules, ECM components or specific blood proteins. To date, 18 distinct α subunits and 8 β subunits have been characterized in humans forming 24 distinct $\alpha\beta$ heterodimers.

The three key downstream signaling routes of the ErbB receptor family are the Ras/MAPK, PI-3K/Akt and PLC- γ /PKC signal routes. Of these, the PI-3K/Akt pathway is known to contribute to cell growth, invasion and anti-apoptotic processes. Furthermore, deregulation of PI-3K was also shown to be the most common loss-of-function alteration in glioblastomas, which may be involved in trastuzumab responsiveness, as well.

When tissue cells spread out on the extracellular matrix, integrins form multimeric clusters with structural and/or signaling roles in focal adhesion complexes composed of a number of receptors. Signals from these receptors then cause the activation and phosphorylation of focal adhesion kinase (FAK) and activate the PI-3K/Akt-PKB pathway, which promotes cell survival. Recent studies revealed that FAK activation also co-localized with ErbB1 and integrin $\alpha 5\beta 1$ expression on astrocytomas, which provides further evidence for their considerable cross-talk in human malignant gliomas.

Significance of ErbB interactions in tumor chemo- and radioresistance

Members of the ErbB family are influential representatives of growth factor receptors responsible for tumorous transformations since alterations of these cell membrane proteins are common and quite frequent events that occur in the first steps of tumor development.

ErbB2 alterations in breast cancers

ErbB2 amplification was reported first in human breast and ovarian cancer, but its overexpression has been also often accused in the background of other malignancies. Upon revealing the pathogenic role of ErbB2, trastuzumab (a recombinant, humanized anti-ErbB2 antibody) is now included not only in the management of metastatic breast cancer but also in (neo)adjuvant regimens. Several inter-molecular and downstream signaling events (down-modulation, internalization or mono-ubiquitination of ErbB2, activation of PTEN, increase in p27^{kip1} and decrease in cyclin D1/cyclin-dependent kinase 2 activity or inactivation of MAPK/PI-3K pathways) substantiated the anti-proliferative effect of trastuzumab *in vitro*, while onward research proved that the majority of the inhibitory effect of trastuzumab *in vivo* is attributable to antibody dependent cellular cytotoxicity.

Although clinical application of trastuzumab has drastically improved the therapy outcome for patients with ErbB2 positive breast tumors, success is far from complete, as resistance to trastuzumab is quite frequent; initial efficacy is 30-50%, and many of the initial responders have a relapse after 6-9 months. Various mechanisms have been suggested to explain trastuzumab resistance including autocrine production of EGF-related ligands, activation of the IGF-I receptor pathway, and masking of the target epitope for trastuzumab by MUC4 sialomucins or CD44/hyaluronic acid complexes. The background of this common resistance to trastuzumab thus seems to be multitudinous and may vary case by case, which can significantly limit the potential of this new treatment modality.

ErbB1 alterations in astrocytoma

Since the discovery of the ErbB receptor family, substantial evidence demonstrated that alterations in the ErbB1 signaling route can be frequently accounted for gliomagenesis. In addition to adjuvant systemic chemotherapy, which means almost exclusively temozolomide combined with radiotherapy, newly proposed anti-tumor therapies tend to include anti-ErbB1 agents, as well. Inhibition of integrin $\alpha V\beta 3$ signaling with the RGD mimetic peptide cilengitide has also reached the clinical phase, however, the positive outcomes were more likely attributable to direct effects on endothelial cells and tumor vascularization. Furthermore, ongoing preclinical studies now address PI-3K-related pathway inhibitors to sensitize chemotherapy-induced apoptosis via disrupting the PI-3K / Akt / mTOR / GSK-3 β signaling routes. However, overall survival of patients suffering from astrocytic tumors is still devastatingly low, and recurrence generally occurs within a year after diagnosis.

Several mechanisms to radioresistance have been proposed so far, but the underlying molecular interactions still remain elusive. In addition to more efficient repair of DNA damage, ErbB proteins appeared to participate in the response of tumor cells to ionizing radiation, and increased expression of ErbB1 correlated with decreased radiosensitivity.

Based on emerging evidence on ErbB-integrin interactions, treatment strategies are growingly focusing on cell adhesion mediated therapy resistance, because the challenge of treating malignant gliomas arises from their infiltrative extension rather than from mass expansion. Several studies demonstrated that PI-3K / Akt signaling has a crucial role in enhanced DNA repair, in the development of radioresistance through ErbB1 activation and in prolonged cell survival via modulation by integrin β 1. Moreover, most recent findings highlighted integrin β 1 inhibitors as potentially promising radiosensitizers *in vitro*.

Accordingly, interactions of ErbB1 and integrin β 1 molecules could play an important role in the development of increased resistance to irradiation and may present a novel diagnostic and therapeutic target.

Analysis of multi-faceted molecular interactions of membrane proteins

One of the best approaches for studying molecular rearrangements is fluorescence resonance energy transfer (FRET), which is a sensitive method for measuring intra- or intermolecular distances in the 1-10 nm range. For mapping physical associations of membrane proteins, flow cytometric and microscopic applications of the FRET phenomenon have been established. The flow cytometric FRET (FCET) technique can provide statistically accurate information on the distribution of FRET efficiency on thousands of cells. In contrast, microscopic FRET methods are statistically less accurate, but can reveal heterogeneity in cell surface distributions and interactions of membrane receptors within even a single cell.

Nevertheless, the role of molecular interactions involved in oncogenesis could be more reliably analyzed after characterizing their mutual relationship. Thus, a direct demonstration of the (anti)correlation of two distinct interactions would vastly enhance our understanding.

However, molecular associations of ErbB and integrin molecules may differ on individual tumors *in situ*, which may prejudice treatment and patient outcome. Steadily improving FRET techniques have now evolved to the potential of characterizing molecular interactions in tissue sections. Indeed, precisely quantitated molecular interactions on tumor samples *in situ* could be conjoined with clinical data that may allow for discernment of prognostically diverse patient subgroups and addressing optimal combined therapy.

Objectives

Based on recent emerging findings on the importance of integrins in ErbB2-mediated transmembrane signaling, we first set out to demonstrate the interaction of ErbB2 and integrin β 1 proteins and to compare the functional significance of this interaction in trastuzumab sensitive and resistant cell lines. We aimed:

- to determine molecular interactions of ErbB2 and integrin β 1 on ErbB2 positive tumor cell lines and
- to compare expression patterns and the degree of homo- and heteroassociation of ErbB2 and integrin β 1 molecules on trastuzumab sensitive and resistant cell lines.

As molecular interactions can be influenced by other neighbouring proteins - especially at signaling membrane platforms (lipid rafts) harbouring multimolecular complexes - we proposed to establish a new method, two-sided FRET, to characterize multimolecular interactions

- to measure the relationship between the association states of two molecule-pairs of three arbitrarily chosen molecular species and
- to untangle the correlation of ErbB2 homoassociation and ErbB2 - integrin β 1 heteroassociation on trastuzumab sensitive and resistant cell lines.

Given the critical contribution of integrins to growth factor downstream signaling in glioma cell survival and adhesion, we set out to characterize the interaction of these proteins in clinical samples *in situ* and cellular models of glioma *in vitro*. We wished

- to determine the extent and molecular background of chromosome 7 or erbB1 gene gain-related radiation resistance of U251-derived astrocytoma subclones,
- to assess the contribution of ErbB1 overexpression and ErbB1 interaction with integrins to radioresistance on erbB1 gene transfected astrocytoma cell lines and
- to quantitate *in situ* the molecular interactions of ErbB1 and integrin β 1 molecules on fresh frozen intraoperative astrocytoma sections and correlate findings with clinical data as well as with results from the *in vitro* model system.

Material and Methods

Antibodies

Monoclonal antibodies 528 against ErbB1, TS2 against integrin β 1, L368 against β 2-microglobulin, W6/32 against HLA-A,B,C and Hermes3 against CD44 were purified from supernatants produced by the hybridoma cell lines 528, TS2/16.2.1, L368, W6/32 and Hermes3 (ATCC), respectively. Against the ErbB2 molecule, 2C4, 7C2 (a gift from Genentech Inc.), trastuzumab (Hoffman-La Roche AG) and their Fab' fragments were used. ErbB3 and ErbB4 were targeted with the H3.90.6 (against ErbB3), and 72.8 or 77.16 (against ErbB4) (NeoMarkers, Lab Vision Corporation). The monoclonal antibodies anti-CD29/integrin β 1, anti-CD104/integrin β 4, anti-ITGB5/integrin β 5, anti-CD61/integrin β 3, anti-CD49a/integrin α 1, anti-CD49b/integrin α 2, anti-CD49d/integrin α 4, anti-CD49f/integrin α 6, anti-CD51/integrin α V (Sigma, Dako-Cytomation, Research Diagnostics Inc.) were used for labeling members of the integrin family. Cy3- and Cy5-conjugated goat anti-mouse antibodies (GaMIg) and Fab' fragments (Jackson ImmunoResearch) were used for indirect secondary labeling of unconjugated primary antibodies. Labeling of antibodies with Cy3 and Cy5 (Amersham Biosciences Europe), Alexa Fluor[®] 546, Alexa Fluor[®] 555, Alexa Fluor[®] 647 and XFITC (Molecular Probes) was carried out according to the manufacturers' specifications. The dye to protein labeling ratio determined by spectrophotometry was in the range of 2:1 - 3:1 and 1:1 in the case of whole IgGs and Fabs, respectively.

Labeling of cells with antibodies

For flow cytometric measurements, cells were trypsinized and then resuspended at 1×10^6 cells in 50 μ l PBS supplemented with 0.1 % BSA and labeled with a saturating concentration (10–20 μ g/ml) of Alexa546/647 antibodies on ice (4°C) for 30 min. After labeling, cells were washed 3x in PBS and fixed in 500 μ l of 1% formaldehyde-PBS. For microscopic measurements, adherent cells were washed 3x in PBS, labeled with saturating concentrations of X-FITC, Cy3, Alexa555 or Cy5 antibodies (singly or in combination) on ice for 30 min, then washed 3x in PBS and fixed in 500 μ l of 4% formaldehyde-PBS for 20 min. Lipid rafts were labeled with 8 μ g/ml Alexa488- or Cy5-labeled subunit B of cholera toxin (CTX-B) for 30 minutes on ice (Sigma, Molecular Probes). For cross-linking, CTX-B labeled lipid rafts or secondary labeled integrins β 1 were incubated at 37°C for 30 minutes. After cross-linking, samples were placed on ice for 15 minutes before continuing the labeling protocol.

Cell cultures

The gastric adenocarcinoma cell lines MKN-7 (Immuno-Biological Laboratories Cell Bank) and NCI-N87 (ATCC), and the breast cancer cell lines SK-BR-3 (ATCC) and JIMT-1 (from Jorma Isola, University of Tampere) were grown to subconfluence.

The parental U251 NCI cell line (National Cancer Institute - hereafter U251) was established from a grade IV astrocytoma (glioblastoma multiforme) and the parental cell line was shown to be considerably sensitive to X-ray radiation. It was grown to subconfluence in MEM EBSS + 10% FCS + Non Essential Amino Acids at 37°C, in 5% CO₂. Previously, subclones of the U251 parental line were established by transferring into them extra chromosome 7 (chr7) pieces (carrying the erbB1 gene) through fusion with microcells made from mouse A9 hybrids of human chr7.

Cellular Model Systems

Chr7 transferred U251 NCI subclones:

Earlier it has been shown that gain of chr7 material in glioblastoma is associated with radiation resistance, which coincides with independent findings that ErbB1 overexpression is associated with radiation resistance, and with the fact that erbB1 gene is located on chr7p.

To reveal the extent, and molecular mechanisms of chr7 gain-related radiation resistance attributable to signaling by ErbB1, we have used a set of U251 subclones – c5, c9, and c55 – with increasing amounts of chr7, which also showed parallel increasing radioresistance. Clones that acquired pieces of 7p together with proximal 7q were radiation resistant, whereas those that acquired smaller pieces of distal 7q remained more sensitive. The radioresistance of this set of U251 NCI fusion subclones (U251 parental, c5, c9 and c55) was characterized previously in agar colony forming assay (LD₅₀ values of 4, 4.7, 5, and 5.7 Gy, respectively) at our collaborating institute (Barrow Neurological Institute/St. Joseph's Hospital and Medical Center, Phoenix).

The U251 Hyg⁺ subclone was used as a control line to the clones armed with extra chromosome 7 material, carrying no chromosome 7 pieces but also expressing the hph gene product that confers resistance to hygromycin B used at 0.4 mg/ml to maintain selection pressure on the subclones. Thus, all compared lines could be grown under equivalent conditions. Upon reaching subconfluence, cells were harvested by trypsinization before labeling for flow cytometric measurements, or subcultured on 12 mm diameter coverslips or on Lab-Tek II chambered coverglass for microscopic experiments (Nalge Nunc).

Stable transfected U251 NCI sublines

To confine the results to the clear effect of ErbB1 overexpression, in order to differentiate from the regulatory effect of other genes coded on chr7p, U251 transfectant sublines that express various amounts of extra ErbB1 were generated. The erbB1 gene in the plasmid pCDNA3 carrying geneticin resistance - a kind gift from Yosef Yarden, Weizmann Inst. of Science - was introduced by electroporation using the nucleofector device of Amaxa (Cologne) with solution V and protocol T-20. Previously, the functionality of the expression vector was verified using a GFP reporter fused C-terminally to the ErbB1, and transfecting this eGFP-ErbB1 into CHO cells, which normally do not express ErbB1.

U251 cells transfected with erbB1 were selected with 0.4 mg/ml geneticin, and sorted in a FACSVantage SE with DiVa option (Becton Dickinson) into subclones of low, medium and high ErbB1 expressors. Further cultivation under selection pressure resulted in the splitting of the medium expressor population, yielding subpopulations similar to the low and high expressors. Furthermore, in spite of selection pressure, a subset of the high ErbB1 expressors gradually drifted to low expression, and thus flow cytometric sorting had to be repeated several times. Eventually, a low ErbB1 (~150 thousand copies per cell, U251 E1L) and a high ErbB1 (~1 million copies per cell, U251 E1H) subclone was created, which could be reliably used as a stable expression system under selection pressure for 3-4 passages.

Fresh frozen sections

Intraoperative tumor samples were stored at -80 °C in the Brain Tumor and Tissue Bank of the Department of Neurosurgery, Medical Health Science Center, University of Debrecen with permission of the Regional Ethical Committee and the National Research Ethical Committee. Each patient signed an informed consent form prior to surgery. Histological classification of the tumors was done as part of the routine clinical pathology process. Samples immediately adjacent to the region used for the histopathological diagnosis were snap-frozen in isopentane (Sigma-Aldrich) cooled in liquid N₂. After embedding in Tissue-Tek O.C.T. (Sakura Finetek), 15 µm thick sections were cut by a cryostat-microtome system (Shandon Cryotome, AS-0620E, Thermo Fisher Scientific Inc.) at -20 °C. Sections on silane-coated slides were dried, fixed in 4% PFA-PBS for 30 min, blocked in 1% BSA-PBS for 30 min, and labeled with 20 µg/ml Cy3-conjugated anti-ErbB1 antibody (clone 528) and 20 µg/ml Cy5-conjugated anti-integrin β1 antibody (clone TS2) overnight in a wet chamber at 4°C, washed 3x and mounted in glycerol.

Determination of radiosensitivity

Radiosensitivity of transfectants against the parental U251 cell line was tested both in conventional proliferation assay using EZ4U (Biomedica) as a colorimetric indicator of mitochondrial activity and determined from colony forming after exposure to 0, 2, 4, 6 and 8 Grays of ^{60}Co γ irradiation. Aliquots exposed to 2 Gy were also treated with 20 nM wortmannin (Sigma-Aldrich) starting 1 hour before irradiation. Six replicates of 200 single cells of each subline were placed and incubated until attachment in a flat bottom 96 well plate, and cultured in and irradiated through indicator-free DMEM medium. To minimize radiation scattering, plates were submerged into water bath and irradiating doses were corrected for the absorbance of the thickness of the liquid layer. Seven days after irradiation methylene-blue (0.2% w/v in 70% ethanol) stained colonies were counted in a Nikon Eclipse TS100 microscope (Nikon Instr). Alternatively, colonies were imaged in an imaging cytometer (CompuCyte Corp) using 633nm transmission images for counting and contouring of clusters.

Surviving fractions were calculated and normalized to plating efficiency of cells receiving no radiation. Survival curves were fitted and plotted using the Linear Quadratic Model by the software Sigmaplot v10.0 (Systat Software).

Activation of cells with EGF, heregulin and trastuzumab

Cells were treated with 50 ng/mL EGF, 50 ng/mL β 1-heregulin (R&D Systems) or 50 $\mu\text{g/mL}$ trastuzumab (Herceptin®) on coverslips or in suspension for 30 minutes in a CO_2 incubator in serum-free medium followed by washing in PBS and labeling.

Western blot analysis

To assess the activity of the PI-3K/Akt-PKB survival pathway, we performed Western blot analysis on Akt and p-Akt proteins. As activation of signaling pathways can vastly depend on their initial equilibrium, both serum-starved and serum-fed cells were tested. Cells were serum-starved overnight in a serum-free medium (SFM) and then treated with 50 ng/ml EGF for 30 min at 37°C. PBS-washed cell pellets were solubilized in 5x SDS-sample buffer, sonicated, centrifuged at 16,000x g for 5 min, and supernatants were subjected to standard SDS-PAGE (7% gel) followed by ECL-visualized, peroxidase-based immunoblotting. Akt and pAkt was detected with specific antibodies (Upstate/Millipore) and as loading control, β -actin (AC40, Sigma-Aldrich) was used. For each lane of the blot, the signal intensity of Akt/pAkt specific bands was quantified, corrected for background, and normalized to β -actin.

Flow cytometric determination of cell surface receptor expressions

Quantitation of receptor expression was carried out on a FACSVantage SE with DiVa option (Becton Dickinson) using the FL-6 channel (excitation at 633 nm, detection through a 650 nm long pass filter) in linear mode. The background corrected mean of histograms from 20,000 events was converted to antigen number using QIFIKIT (DakoCytomation). Unconjugated 528 or TS2 primary antibodies were detected with AlexaFluor 647 conjugated goat-anti-mouse (H+L) secondary antibodies (Molecular Probes).

Flow cytometric fluorescence resonance energy transfer measurements (FCET)

To determine the homo-, and heteroassociation states of the labeled receptors, flow cytometric fluorescence resonance energy transfer measurements were performed either on the FACSVantage SE instrument with DiVa option equipped with 488, 532 and 633 nm lasers or on a FACSArray bioanalyzer (Becton Dickinson) equipped with 532 and 635 nm lasers. In experiments on the FACSVantage SE instrument, the FRET efficiencies were calculated with cell-by-cell correction for autofluorescence.

The distribution of cells in forward and side scattering channels was used for discarding debris and dead cells. The cellular autofluorescence was measured in the FL1 channel (excited by the 488 nm laser) through a 530/30 nm band pass filter. Donor intensities were recorded in the FL4 channel through a 585/42 nm bandpass filter, while acceptor and sensitized acceptor intensities were detected in FL6 and FL5 channels, respectively, through 650 nm long pass filters. The donor (Alexa546) and acceptor (Alexa647) molecules were excited by the 532 and 633 nm laser lines, respectively.

In measurements on the FACSArray bioanalyzer the donor intensities (excited by the 532 nm laser) were recorded in the Yellow channel through a 585/42 nm bandpass filter, while the acceptor and sensitized acceptor intensities were excited by the 633 nm and the 532 nm lasers, respectively, and were detected in the Red and Far-Red channels through a 661/16 nm bandpass and a 685 nm long pass filter, respectively. List mode data were stored in FCS 3.0 file format. Calculation of cell-by-cell FRET efficiency was carried out using the software REFLEX with correction for autofluorescence. FRET efficiencies are presented as mean values - indicating the standard error of the mean - of approximately normally distributed, unimodal FRET histograms of 20,000 cells.

Confocal microscopy

A Zeiss LSM 510 confocal laser-scanning microscope (CLSM, Carl Zeiss AG) with a Plan-Apochromat 63×/1.40 NA oil immersion objective and an UV/488/543/633 beam splitter was used to image cells. X-FITC, Cy3 and Cy5 fluorophores were excited at 488, 543 and 633 nm (argon ion and He-Ne lasers) and detected through 505-535, 560–605 nm bandpass and 650 nm longpass filters, respectively. Horizontal, 1-3 μm thick, 4x averaged optical sections (512 x 512 pixels at 12 bit) were imaged, taken either the top or bottom flat layer of the cell membrane using 2.51 μs pixel dwell time and 3× zoom (100 nm per pixel, 2 times oversampling) in frame mode. Before abFRET measurements, the optimal bleaching time for reducing the Cy5 fluorescence to less than 20 percent of the prebleach value was determined. For assessing expression levels of ErbB1 and integrin β 1 on fresh frozen sections, images were taken in tile mode allowing the surveillance of larger tissue areas, while maintaining high resolution and confocality. Images were acquired in multi-track mode to avoid crosstalk between channels. The alignment of detection channels was regularly checked with samples of FocalCheck fluorescent microspheres (Molecular Probes).

Calculation of image cross-correlation

Colocalization between two different fluorescent labels was calculated according to Pearson's formula. Low intensity pixels were excluded from the analysis. The value of the cross-correlation coefficient, C ranges from +1 to -1. Values close to 1, 0 and -1 indicate high and low degree of colocalization and anticorrelation, respectively. C values were computed using Zeiss LSM v3.2 or a home-made software written in LabView (National Instruments).

Donor photobleaching method (dbFRET)

In donor photobleaching, the energy transfer is calculated by comparing the photobleaching kinetics of the donor in the presence and absence of the acceptor. This kinetics can be described by a time constant (τ) which is inversely related to the donor quantum yield.

In these experiments the specimens were bleached by acquiring 40-50 images as time series using the 488 nm Ar-ion laser at high (20-40%) power. Images were median or low-pass filtered when necessary (3x3 kernel, LSM v3.2 software), then exported and fit after thresholding by the dbFRET software, yielding images of the photobleaching time constants.

Acceptor photobleaching method (abFRET)

In the case of tissue samples a microscopic method needs to be applied to measure FRET in adherent cells and tissues. A quick and simple procedure is based on irreversibly destroying the acceptor fluorophore (Cy5) by photobleaching it and thereby de-quenching the donor molecule (Alexa555). FRET efficiency (E) in each pixel then can be measured from the change of the donor fluorescence value before and after photobleaching the acceptor with correcting for shifts, backgrounds, photobleaching of the donor and crosstalk of the acceptor label into the donor channel. Correction for incomplete acceptor bleaching was not necessary, because samples with less than 80% depletion of acceptor were not evaluated.

The measurement protocol was implemented on an LSM 510 CLSM (Carl Zeiss AG). Adherent cells were washed 3x in PBS, labeled with saturating concentrations of respective donor – and/or acceptor-conjugated antibodies on ice for 30 min, then washed 3x in PBS and fixed in 500 μ l of 4% formaldehyde-PBS for 20 min. Coverslips were mounted in glycerol onto precleaned slides. Image acquisition was performed as described above. To evaluate image sequences and calculate mean FRET efficiency, a custom C algorithm in SCIL Image (TNO, Institute of Applied Physics) and also custom written ImageJ plugin AccPbFRET was used.

Statistical analysis

Data analysis was done with SigmaStat version 3.5.0.54 (Systat). Parameters of Grade II and IV astrocytic tumor groups were compared with Student's t-test or upon lack of normality (Kolmogorov-Smirnov with Lilliefors' correction) with Mann-Whitney rank sum tests. Normally distributed variables of more than two classes were compared using ANOVA, followed by Tukey's post hoc test. Predicting power of ErbB1 and integrin β 1 expression, and ErbB1 – integrin β 1 heteroassociation on recurrence time was assessed by stepwise forward multiple regression, and on the grade of tumor by stepwise multiple binary logistic regression. Overall and relapse-free survival were analyzed by the Kaplan-Meier method comparing Grade II against grade IV tumors, as well as pairs of groups created by splitting the whole cohort into below and above average values of ErbB1 or integrin β 1 expression or ErbB1 – integrin β 1 heteroassociation. Patients enrolled later than the start of the observation were censored from further analysis at their survival time point at the end of the study. Those undergone incomplete tumor resection were also censored. Statistical comparison were done using log-rank test with *post-hoc* Holm-Sidak methods.

Results

Interactions of ErbBs and integrin β 1 on breast and gastric cancer cell lines

Lower ErbB2 expression is accompanied by higher integrin β 1 levels

We first characterized the expression levels of ErbB1-4 proteins, and integrins β 1-, β 3- and α 6 in trastuzumab resistant (JIMT-1, MKN-7) and sensitive (SK-BR-3, N87) cell lines by flow cytometry. Trastuzumab resistant cell lines showed lower ErbB2 (112.000 and 500.000 per cell) and higher integrin β 1 (2.460.000 and 566.000 per cell) expression levels (JIMT-1 and MKN-7, respectively). The trastuzumab resistant JIMT-1 line displayed an extremely high integrin β 1 level (2.460.000 per cell), whereas its trastuzumab sensitive counterpart, the SK-BR-3 cell line had the lowest expression level (81.000 per cell). Expression levels of other ErbB and integrin molecules revealed no characteristic differences among the cell lines.

Strong concurrent associations of ErbB2 and integrin β 1 to lipid rafts are not influenced by ErbB-mediated signaling

Significant colocalization was found between ErbB2 and integrin β 1 on all the examined tumor cell lines (0.61 ± 0.08 - 0.76 ± 0.02), which was independent of trastuzumab resistance. Additionally, both ErbB2 and integrin β 1 were found in lipid rafts. The previously documented, spatially non-overlapping transferrin-ErbB2 receptor pair was chosen as a negative control yielding a C value of 0.13 ± 0.02 . Positive control samples labeled with two non-competing monoclonal antibodies against ErbB2 (Cy5-2C4, Cy3-trastuzumab) yielded a C value of 0.75 ± 0.06 .

None of the treatments with trastuzumab, EGF and heregulin changed the ErbB2 - integrin β 1 colocalization significantly; however, trastuzumab-treated breast cancer cells showed a significantly decreased integrin β 1-lipid raft and ErbB2-lipid raft colocalization.

Crosslinking of ErbB2, integrin β 1 or lipid rafts disrupts their colocalizations with the other two regardless of cytoskeleton anchoring

CTX-B-induced crosslinking of lipid rafts significantly decreased their colocalization with both ErbB2 and integrin β 1 on all cell lines (C values of 0.60-0.75, 0.10-0.20 and 0.15-0.30 for ErbB2-integrin β 1, integrin β 1-lipid rafts, and ErbB2-lipid raft pairs, respectively).

Cross-linking of integrin $\beta 1$ caused a decrease in colocalization values involving integrin $\beta 1$ (C values 0.10-0.20 for both integrin $\beta 1$ -ErbB2 and integrin $\beta 1$ -lipid rafts) in all cell lines implying that cross-linking liberated integrin $\beta 1$ from its complex with ErbB2 and lipid rafts.

Cytochalasin D treatment caused no significant differences in any of the colocalization values indicating that binding to the actin cytoskeleton does not affect the interactions of ErbB2, integrin $\beta 1$ and lipid rafts with each other.

Interaction between ErbB2 and integrin $\beta 1$ is independent of integrin $\beta 1$ expression level

Colocalization measurements proved that ErbB2 and integrin $\beta 1$ are located in the same membrane domains, but FRET measurements are needed to estimate their molecular interactions. The heteroassociation of ErbB2 and integrin $\beta 1$ was always measured using the more abundant of the two proteins as the acceptor. Significant interaction between ErbB2 and integrin $\beta 1$ was detected on JIMT-1 ($9.7 \pm 1.8\%$) and N87 ($5.8 \pm 2.2\%$), whereas on SK-BR-3 and MKN-7 cells FRET for the association was lower ($3.3 \pm 2.0\%$, $7.1 \pm 1.3\%$, respectively).

High level of homoassociation of ErbB2 was detected on the trastuzumab sensitive SK-BR-3 ($16.1 \pm 1.2\%$) and N87 ($11.5 \pm 0.1\%$) cells using 2C4 antibody, whereas the resistant JIMT-1 and MKN-7 cells showed FRET below 5%. In addition, none of the cell lines showed significant changes in FRET after treatment with trastuzumab, EGF or heregulin.

Molecular interactions between ErbB2 and integrin $\beta 1$ do not alter trastuzumab-mediated ErbB2 tyrosine phosphorylation

Because integrins have been shown to modulate ErbB-mediated signal transduction, it seemed possible that the high expression level of integrin $\beta 1$ on the trastuzumab resistant cell lines affects trastuzumab-mediated ErbB2 tyrosine phosphorylation to a greater extent. We stimulated serum-starved SK-BR-3 and JIMT-1 cells with trastuzumab with or without prior crosslinking of cell surface integrins $\beta 1$. ErbB2 tyrosine phosphorylation was lower on non-stimulated JIMT-1 than SK-BR-3 cells in accordance with previous results.

Trastuzumab induced the tyrosine phosphorylation of ErbB2 both in the trastuzumab sensitive and resistant cell lines. Cross-linking of integrin $\beta 1$ did not alter the level of trastuzumab-induced ErbB2 tyrosine phosphorylation even in the trastuzumab resistant JIMT-1 cell line expressing a high amount of integrin $\beta 1$. We conclude that in spite of the high cell surface density of integrin $\beta 1$ on JIMT-1 cells, the first signaling steps initiated by trastuzumab are not affected in this aspect.

Associations of ErbBs are dynamically modulated by integrin β 1 molecules - application of the newly established two-sided FRET method

Excess integrin β 1 inversely correlates with ErbB2 level of trastuzumab resistant lines

First, we recharacterized the expression levels of ErbB1, ErbB2 and integrin β 1 extended for CD44 and MHC-I on trastuzumab resistant (JIMT-1, MKN-7) and sensitive (SK-BR-3, N87) cell lines by flow cytometry. Trastuzumab resistant cell lines showed lower ErbB2, but higher integrin β 1 (see before) and CD44 (1.1 - 2.3 million per cell on resistant and 100 - 200 thousand on sensitive lines) expression levels. The relative numbers of ErbB1 and MHC-I did not reveal characteristic differences between the examined resistant and sensitive cell lines.

ErbB2 interactions are shifted towards heteroassociations with integrin β 1

As we observed differences in the expression levels of ErbB2, integrin β 1 and CD44, we tended to assess the molecular interactions using FCET. Homoassociation of ErbB2 correlated with its expression level giving rise to high ErbB2 homoassociations in trastuzumab sensitive lines. Heteroassociations were measured using the more abundant protein as acceptor. Significant interaction between ErbB2 and integrin β 1 was detected on JIMT-1 cells ($13.1 \pm 1.1\%$), whereas MKN-7 and N87 cells displayed moderate ($9.1 \pm 1.2\%$ and $7.1 \pm 1.3\%$) and SK-BR-3 cells low ($<5\%$) FRET in this respect. Heteroassociation of CD44 with ErbB2 or integrin β 1 was high on the resistant JIMT-1 ($19.9 \pm 1.5\%$ and $10.5 \pm 1.0\%$) but weak on the similarly resistant MKN-7 cells ($4.2 \pm 0.7\%$ and $3.7 \pm 0.5\%$).

Model system for two-sided FRET (tsFRET)

As proof-of-concept for the tsFRET approach, we devised a triple labeled model system applicable in CLSM. In this, the first fluorophore (XFITC) is a donor dye of the first FRET pair (D1), and its acceptor (A1) is the second fluorophore (Cy3 or Alexa555). This dye also acts as a donor (D2) for the third fluorophore (Cy5), which is the acceptor in this second FRET pair (A2). Consequently, the second fluorophore is both an acceptor and a donor ($A1 \equiv D2$), although in two different measurement protocols.

Accordingly, the energy transfer efficiency can be measured sequentially using the abFRET method (between $A1 \equiv D2$ and A2) followed by the dbFRET method (between D1 and $A1 \equiv D2$). As a model system, we used the ErbB2 molecule labeled with three non-competing monoclonal antibodies - XFITC-7C2 (D1), Cy3/Alexa555-2C4 ($A1 \equiv D2$) and

Cy5-4D5 (A2). Contour plot from the corresponding pixel-by-pixel dbFRET and abFRET efficiencies and the pixel-by-pixel 2D matrix of the contour plot was calculated and plotted on 5 - 10 % FRET ranges. The dependence of the FRET efficiencies on each other was derived by computing a trendline showing the mean abFRET values plotted against the binned dbFRET values. In our model system, the contour plot and the trendline (slope = 0.003) showed no correlation between the FRET values, since both dbFRET and abFRET values represent intramolecular distances in this case.

Application of two-sided FRET: ErbB2 homoassociations are disintegrated by integrin $\beta 1$

We characterized the effect of integrin $\beta 1$ -ErbB2 heteroassociation (dbFRET) on ErbB2 homoassociation (abFRET) on trastuzumab sensitive lines (SK-BR-3, N87). JIMT-1 and MKN-7 were not examined in this respect, because the homoassociation state of ErbB2 was low on both cell lines as determined by FCET and abFRET. On the contour plot for N87 cells, two different peaks could be identified at 13% (abFRET) – 20% (dbFRET) and at 7% (abFRET) – 30% (dbFRET), and trendline slope (m) occurred -0.042 implying anti-correlation between the associations. Similar anti-correlation could be observed on SK-BR-3 cells ($m = -0.080$); however, contour plot had a single peak at 12% (abFRET) – 26% (dbFRET).

Analysing the correlation of ErbB2–integrin $\beta 1$ and integrin $\beta 1$ –CD44 heteroassociations, modest positive correlation was observed on the contour plot for MKN-7 cells (two discrete peaks at 10% (abFRET) – 22% (dbFRET) and at 20% (abFRET) – 32% (dbFRET), and $m = 0.077$). On the contrary, there was only one peak at 12% (abFRET) – 21% (dbFRET) on the contour plot for JIMT-1 cells with $m = -0.002$, indicating no correlation between FRET efficiency values. Assessing the correlation of ErbB1–ErbB2 heteroassociation and ErbB2 homoassociation, and the interactions of ErbB1, integrin $\beta 1$ and MHC-I molecules, neither the contour plots nor the trendlines showed clear correlations.

By comparing data, FCET values were notably lower than corresponding microscopic FRET values. To exclude the possible overestimation of microscopic FRET, we compared dbFRET values for ErbB2–integrin $\beta 1$ in SK-BR-3 and MKN-7 cells after trypsinization and cyto-centrifugation of cells ($1.1\pm 0.3\%$, $3.8\pm 0.5\%$) with those measured on adherent, non-trypsinized cells ($26.3\pm 1.3\%$, $15.2\pm 2.4\%$). The results substantiated that it is trypsinization and not the FCET method, which lowers the FRET efficiency. Measurements through various optical slices over the "z" axis proved integrin $\beta 1$ and CD44 to distribute inhomogenously in the membrane, and increasingly heteroassociated at the plane of adherence.

Molecular interactions of ErbB1 and integrin β 1 molecules reliably predict clinical outcome and correlate with Akt-mediated *in vitro* radioresistance of astrocytic tumors

Higher ErbB1 and integrin β 1 levels correlate with pronounced radioresistance

We screened expressions of integrins α V, α 1, α 2, α 4, α 6, β 1, β 3, β 4 and β 5 on Hyg+, c5, c9, c55 clones. In line with increasing extra chr7 and radioresistance (LD50 of 4.0, 4.7, 5.0, 5.7 Gy), clones expressed not only increasing numbers of ErbB1 (65, 77, 83, 270 thousand) but also increasing numbers of integrin β 1 (360, 400, 420, 540 thousand), respectively.

Ectopic overexpression of ErbB1 alone also induces higher integrin β 1 levels

Initially, both E1L and E1H sublines expressed increased numbers of ErbB1 (230 and 940 thousand) accompanied by increased expression of integrin β 1 (360 and 380 thousand), over parental U251 (70 and 240 thousand for ErbB1 and integrin β 1). In parallel with stably sustained ErbB1 and integrin β 1 expression, ratio of the E1H/H subpopulation decreased to 5% gradually over the passages. Notably, E1H/H subpopulation did not show significant further increase in integrin β 1 expression over E1L, despite the extreme increase in ErbB1.

Excess ErbB1 and integrin β 1 contribute to post irradiation survival, whereas additional surplus ErbB1 contributes primarily to colony forming ability

After irradiation, surviving fraction of transfectants increased parallel with increasing ErbB1 and integrin β 1 expression, but additional surplus ErbB1 of the E1H subline did not contribute to higher survival rate. However, presence of surplus ErbB1 promoted colony forming capability of E1H compared with parental U251 and even E1L. Both parameters α and β differed between parental U251 and transfectants, but not between the two transfectants (α : 0.33, 0.10 and 0.10; β : 0.09, 0.06 and 0.06 for U251, E1L and E1H, respectively)

Additional integrin β 1 is crucial for increasing Akt-dependent radiation resistance, which can be overcome by the inhibition of PI-3K

Western blot of Akt and pAkt proteins disclosed significant, 85% increase in pAkt levels of both U251 E1L and E1H. Also, the pAkt/Akt ratio in both U251 E1L and E1H increased (1.6 ± 0.2 , 1.8 ± 0.2) over parental U251 (1.1 ± 0.1) after EGF treatment. Increased radioresistance of E1L could be reverted by inhibiting PI-3K with wortmannin; colony growth after irradiation dropped back from 80% to 35% in the presence of PI-3K inhibitor.

ErbB1 interactions are shifted towards heteroassociations with integrin β 1

FCET measurements - shift of balance towards ErbB1-integrin β 1 heteroassociations

The results revealed anticorrelation between decreasing ErbB1 homoassociation (11 \pm 1%, 6 \pm 1%, 4 \pm 1%, 1 \pm 0.5%) and increasing ErbB1-integrin β 1 heteroassociation rates (6 \pm 1%, 7 \pm 1%, 8 \pm 1%, 9 \pm 2%) on Hyg+, c5, c9, c55 U251 clones, as well as on parental U251, E1L, E1H/L and E1H/H (13 \pm 1%, 10 \pm 0.1%, 10 \pm 0.2%, 7 \pm 0.5% and 5 \pm 1%, 7 \pm 0.5%, 7 \pm 0.5%, 10 \pm 1%, respectively). FRET was also measured in the integrin β 1-ErbB1 direction by reversing donor-acceptor labeling, and a similar tendency was observed (2%, 3%, 4%, 18%).

tsFRET experiments - integrin β 1 recruits ErbB1 from ErbB1 homoclusters

Both E1H/L and E1H/H showed anticorrelation between the ErbB1 homoassociation and ErbB1 - integrin β 1 heteroassociation, indicating a competition by excess integrin β 1 for ErbB1. Contour plot peaks showed that this tendency was stronger in E1H/H.

Expressions and interaction of ErbB1 and integrin β 1 are increased in grade IV astrocytoma

Ten grade II (age 18 to 59 years, mean 37.8 years, 5 males and 5 females) and 10 grade IV (age 42 to 73 years, mean 61.4 years, 3 males and 7 females) astrocytoma patients were studied. Background corrected mean fluorescence intensities of tissue sections were higher in grade IV than in grade II tumors both for ErbB1 and integrin β 1. Overall, abFRET *in situ* on grade IV tumors showed stronger ErbB1-integrin β 1 heteroassociation than on grade II tumors. The clear separation of the two disease groups was verified by binary logistic regression.

Association of ErbB1 and integrin β 1 alone is a potential predictor of therapy outcome

In addition to a significant difference in ErbB1-integrin β 1 association on grade IV and II tumors ($p < 0.001$), this interaction was identified as a single determinant of tumor grade by stepwise binary logistic regression (OR=8716). Multiple stepwise linear regression also highlighted FRET efficiency as a predictor of time-to-relapse both for pooled grade II and IV and for grade IV cases alone ($p = 0.094$ and 0.085 , respectively). Overall and progression-free survival, estimated using Kaplan-Meier analysis, was determined best and equally well by histopathological grading and ErbB1-integrin β 1 heteroassociation ($p < 0.001$), whereas ErbB1 and integrin β 1 levels showed less, but still significant differences ($p = 0.014$, $p = 0.004$) in comparison of grade II and IV groups, and groups formed by splitting into below versus above overall mean expression levels of ErbB1, integrin β 1 and heteroassociation state.

Abstract

We set out to investigate the molecular interactions of ErbB receptor tyrosine kinase proteins and integrin molecules to shed light on their possible interfering, correlative roles behind trastuzumab resistance of breast cancers and radioresistance of astrocytomas. To study the contingent mutual interplay of neighbouring receptors, we have implemented a method, two-sided FRET (tsFRET), for the investigation of the relationship of associations of two molecule pairs of three arbitrarily chosen molecular species. For the evaluation of individual differences of clinical samples, we adapted the abFRET method for quantitatively assessing *in situ* molecular interactions on intraoperative fresh frozen tissue sections.

According to our results, it is tempting to speculate that the homoassociation state of ErbB2 is dynamically modulated by the interaction with integrin $\beta 1$ on breast cancer cells *in vitro*, although a strong correlation toward trastuzumab resistance could not be affirmed on the basis of our study. It may, however, be assumed that higher integrin $\beta 1$ expression alters the signal transduction of breast cancer cells, thereby presenting an alternative pathway for the activation of the MAPK and PI-3K/Akt routes and giving rise to the metastasizing ability.

As our preliminary experiments confirmed dynamically modifiable ErbB-integrin $\beta 1$ interactions, and a protein level screen on astrocytoma cells proved integrin $\beta 1$ overexpressed along with ErbB1, we aimed to assess their possible interaction behind gliomagenesis.

Two cellular model systems showing expression profiles similar to low and high grade astrocytoma tumors have been generated and we showed that increased ErbB1 expression was consequently followed by the presence of excess integrin $\beta 1$ in the membrane. Decreasing radiosensitivity correlated with increasing ErbB1 and integrin $\beta 1$ expression levels, and also with decreasing ErbB1 homoassociation and increasing ErbB1 – integrin $\beta 1$ heteroassociation rates. Radioresistance could be explained by the increased basal Akt phosphorylation, and augmented output from the PI-3K/Akt pathway. After EGF stimulation in the sublines with higher ErbB1 and integrin $\beta 1$ expression this increased radioresistance could be reverted by inhibition of PI-3K. The enhanced ErbB1 - integrin $\beta 1$ interaction was also detectable in grade IV versus grade II astrocytoma fresh frozen sections and appeared to be an efficient predictor of patient survival and disease relaps.

The abFRET procedure proved to be a useful, cell function-related diagnostic method in assessing *in situ* ErbB1–integrin $\beta 1$ heteroassociation, which implies potential exploitation of similar investigations that may allow for discernment of prognostically diverse patient subgroups warranting the application of certain single or combined therapeutic modalities.

Acknowledgments

Foremost, I would like to thank my advisors *Professor János Szöllősi* and *Álmos Klekner* for supporting me over the years and providing me the opportunity to finish my thesis.

I will always remember with grateful respect the late *Professor György Csécei*, who conducted my first "footsteps" in the medical science, but unfortunately could not live to be here with us at the finish of our work.

I wish to express the most sincere gratitude to *Professor György Vereb* for the insightful discussions that have driven me along the way and helping me in every aspect of my work.

I pay great honour to *Professor Kálmán Nagy*, who always gave me enough time to do my scientific work besides my clinical duty, whose advisement led me on the way and helped to get over difficulties, and without whose selfless support this work could have not been done.

I am also very grateful to the *members of our research group*: Zsolt Fazekas, Zsolt Sebestyén, Péter Nagy, Tamás Lajtos, Maria-Magdalena Mocanu, Elza Friedlander, Barbara Zsebik, Gábor Horváth, Ákos Fábián, Márk Barok, László Ujlaky-Nagy, Zsuzsanna Pályiné Kreck, to Andrea Dóczy-Bodnár and Gergely Szentesi. I acknowledge the help of Mária Kern and Ms. Gabriella Óri, Ms. Tünde Terdik and Ms. Hajnalka Toldi in the technical assistance, and the help of the staff of the Departments of Biophysics and Cell Biology and Neurosurgery at the University of Debrecen, and also that of the Children Health Care Center at the Borsod County and University Hospital.

I owe a lot to *my wife* for providing this loving atmosphere, patiently encouraging me, and enduring till the end over assurgent "time tunnels and traps".

I am grateful to *my family* for giving me the opportunity to accomplish my plans.

The experimental work was performed by the support of the TÁMOP-4.2.2.A-11/1/KONV-2012-0025 project and the Baross Gábor Program (REG-EA-09-1-2009-0010). The doctoral training program was supported by the TÁMOP-4.2.2/B-10/1-2010-0024 project. The projects are co-financed by the European Union and the European Social Fund.



Register Number: DEENKÉTK/104/2013.

Item Number:

Subject: Ph.D. List of Publications

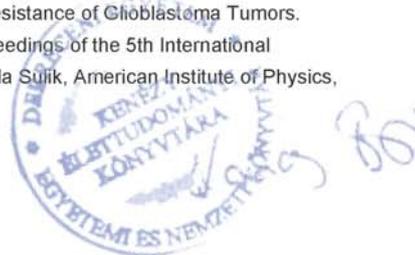
Candidate: Miklós Petrás

Neptun ID: ELUGCO

Doctoral School: Doctoral School of Molecular Medicine

List of publications related to the dissertation

1. **Petrás, M.**, Lajtos, T., Friedländer, E., Klekner, Á., Pintye, É., Feuerstein, B.G., Szöllösi, J., jr. Vereb, G.: Molecular interactions of ErbB1 (EGFR) and integrin-beta1 in astrocytoma frozen sections predict clinical outcome and correlate with Akt mediated in vitro radioresistance. *Neuro-Oncology*. "accepted by publisher", 2013.
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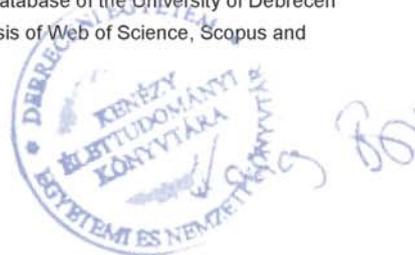
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IF:2.115

Total IF: 14.618

Total IF (publications related to the dissertation): 12.031

The Candidate's publication data submitted to the Publication Database of the University of Debrecen have been validated by Kenezly Life Sciences Library on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

14 March, 2013



Citable abstracts:

1. *Magda Tufeanu, Zsolt Fazekas, Miklós Petrás, Jorma Isola, György Vereb, János Szöllősi*: Association of ErbB2, β 1 integrin and lipid rafts on tumor cells. *Cytometry A*, 2004. May, 59A: 79
2. *Zsolt Fazekas, Miklós Petrás, György Vereb, János Szöllősi*: Double-sided fluorescence energy transfer: A new method for the in situ examination of the associations of ErbB receptors on the surface of tumor cells. *Cytometry A*, 2004. May, 59A: 86
3. *Barbara Zsebik, Miklós Petrás, Jorma Isola, János Szöllősi, György Vereb*: ErbB2 homodimerization and activation in Herceptin resistant and sensitive cell lines. *Cytometry A*, 2004. May, 59A: 113

List of presentations related to the dissertation:

- ErbB1 – Integrin interakció szerepe gliatumorok terápia rezisztenciájában.
Petrás Miklós, Lajtos Tamás, Friedländer Elza, Klekner Álmos, Pintye Éva, Burt G. Feuerstein, Szöllősi János, Vereb György
(Miskolci Akadémiai Bizottság tudományos ülése, 2011)
- The ErbB1/EGFR - Integrin Beta 1 Axis: Evidence for Increased Molecular Association in Higher Grade Clinical Glioma Samples Leading to Enhanced Akt Activation and Improved Radiation Resistance.
György Vereb, Miklós Petrás, Tamás Lajtos, Álmos Klekner, Éva Pintye, Burt G. Feuerstein, János Szöllősi (ISAC XXV. International Congress, Seattle, 2010.)
- Az EGFR (ErbB1) - integrin kölcsönhatás szerepe gliatumorok sugárrezisztenciájában. *Vereb György, Petrás Miklós, Lajtos Tamás, Klekner Álmos, Pintye Éva, Szöllősi János* (Magyar Neuronkológia Társaság X. Kongresszusa, Debrecen, 2009.)
- Role of extra chromosome 7p material and epidermal growth factor receptor overexpression in the radiation resistance of glioblastoma tumors.
György Vereb, Miklós Petrás, Tamás Lajtos, Álmos Klekner, Éva Pintye, János Szöllősi (Radiation Damage in Biomolecular Systems, RADAM, Debrecen, 2008.)
- ErbB2, β 1-integrin és lipid raft asszociációja Herceptin (Trastuzumab) szenzitív és rezisztens tumorsejteken. *Petrás Miklós* (TDK - PhD Konferencia, Debrecen, 2005.)
- Association of ErbB2, β 1 integrin and lipid rafts on tumor cells.
Magda Tufeanu, Zsolt Fazekas, Miklós Petrás, Jorma Isola, György Vereb, János Szöllősi (ISAC XXII. International Congress, Montpellier, 2004.)
- Double-sided fluorescence energy transfer: A new method for the in situ examination of the associations of ErbB receptors on the surface of tumor cells.
Zsolt Fazekas, Miklós Petrás, György Vereb, János Szöllősi
(ISAC XXII. International Congress, Montpellier, 2004.)
- ErbB2, β 1-integrin és lipid raft asszociáció tumor sejteken.
Petrás Miklós (TDK - PhD konferencia, Debrecen, 2004.)
- ErbB1 és β 1-integrin molekuláris közelségének hatása az ErbB2 homoasszociációra.
Petrás Miklós (TDK - PhD konferencia, Debrecen, 2003.)

List of posters related to the dissertation:

- ErbB receptorok asszociációjának in situ vizsgálata tumorsejtek felszínén kétoldali fluoreszcencia energiáttranszfer segítségével.
Petrás Miklós, Fazekas Zsolt, Vereb György, Szöllősi János (XXXIII. Membrán Transzport Konferencia, Sümeg, 2003.)
- ErbB2 receptorok homoasszociációja és tirozin foszforilációja humán emlőtumor sejtekben. *Zsebik Barbara, Fazekas Zsolt, Petrás Miklós, Szöllősi János, Vereb György* (XXXIII. Membrán Transzport Konferencia, Sümeg, 2003.)
- Kétoldali fluoreszcencia energiáttranszfer: egy új módszer ErbB receptorok asszociációjának in situ vizsgálatára tumorsejtek felszínén.
Fazekas Zsolt, Petrás Miklós, Vereb György, Szöllősi János (Magyar Biofizikai Társaság XXI. Kongresszusa, Szeged, 2003.)
- ErbB2 receptorok homoasszociációja és tirozin foszforilációja humán emlőtumor sejtekben. *Zsebik Barbara, Fazekas Zsolt, Petrás Miklós, Szöllősi János, Vereb György* (Magyar Biofizikai Társaság XXI. Kongresszusa, Szeged, 2003.)
- Examination of associations of ErbB2, β 1-integrin and lipid rafts on Trastuzumab (Herceptin®) resistant and sensitive tumor cell lines.
Magda Tufeanu, Zsolt Fazekas, Miklós Petrás, Jorma Isola, György Vereb, János Szöllősi (IV. Magyar Sejtanalitikai Konferencia, Budapest, 2004.)
- Mapping multimolecular complexes on cell surfaces by dual fluorescence energy transfer imaging. *Zsolt Fazekas, Miklós Petrás, György Vereb, János Szöllősi* (IV. Magyar Sejtanalitikai Konferencia, Budapest, 2004.)
- ErbB2 Homodimerization and Activation in Herceptin Resistant and Sensitive Cell Lines. *Barbara Zsebik, Miklós Petrás, Jorma Isola, János Szöllősi, György Vereb* (Outstanding Poster Award, ISAC International Congress, Montpellier 2004.)
- ErbB1, β 1-integrin, CD44 és lipid tutajok asszociációjának szerepe U251 NCI glioblastoma klónok eltérő sugárrezisztenciájában.
Petrás Miklós, Horváth Gábor, Burt G. Feuerstein, Klekner Álmos, Szöllősi János, Vereb György (Magyar Biofizikai Társaság XXII. Kongresszusa, Debrecen, 2005.)
- Molecular interactions of ErbB1 (EGFR) and β 1-integrins in the membrane of astrocytoma cell lines, and intraoperative samples, correlate with malignance and radiation resistance. *Miklós Petrás, Álmos Klekner, Burt G. Feuerstein, János Szöllősi, György Vereb* (9th European Congress of Neuropathology, Athens, 2008.)
- Expression pattern of invasion-related extracellular matrix molecules in astrocytomas. *Klekner Álmos, Varga Imre, Bognár László, Hutóczki Gábor, Petrás Miklós, Kenyeres Annamária, Tóth Judit, Hanzély Zoltán, Scholtz Beáta* (EORTC-EANO Congress Budapest, 27-28 March, 2009.)
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