New prognostic markers in lung adenocarcinoma

Doctoral theses

Anita Horváth-Rózsás

Semmelweis University

Clinical Medicine PhD School

Consultants:

Dr. Balázs Döme, MD, Ph.D, Head of Department

Dr. József Tóvári, Ph.D, Head of Department

Official reviewers:

Dr. Marcell A. Szász, MD, Ph.D, Assistant Lecturer

Dr. Nóra Bittner, MD, Ph.D, Head of Department

Head of the Final Examination Committee:

Dr. Béla Szende, DSc, professor emeritus

Members of the Final Examination Committee:

Dr. Zoltán Süttő, MD, Ph.D, Senior Lecturer

Dr. Krisztina Czebe, MD, Ph.D, Head of Department

Budapest
2016

1 INTRODUCTION

Lung cancer has the highest mortality rate in cancers thus representing a major healthcare problem worldwide. The determination of some driver oncogene mutations can be prognostic or predictive marker in the molecular pathology of lung adenocarcinoma (ADC). The three generally known components are epidermal growth factor receptor (EGFR), mutation of Kirsten rat sarcoma viral oncogene homolog gene (KRAS), and anaplastic lymphoma kinase (ALK) gene arrangement. Since there are significant differences in the prognosis of same stage ADC patients, further biomarkers are urgently needed in this malignancy that facilitate early detection and predict therapeutic efficacy.

The aim of our study was to investigate whether one or more from the followings are of any significance in lung adenocarcinoma: erythropoietin receptor (EPOR) expression, level of circulating activin A (ActA) and follistatin (FST).

The erythropoietin (EPO) is a glycoprotein hormone, produced mainly by the peritubular cells of the kidney and also in smaller amounts by the liver. EPO controls erythropoiesis in the bone marrow, stimulating the proliferation, differentiation and it has also an anti-apoptotic effect. The human recombinant form of EPO is widely used in the clinical practice to treat tumor induced anaemia and kidney disease.

Its receptor is a 59 kDa glycoprotein transmembrane receptor, member of the cytokine receptor superfamily. Detection of EPOR on non-hematopoietic tissues indicates its pleiotrop effect. The expression of EPOR are detected in various human malignancies (also in NSCLC) and on the vascular components in the tumor stroma by human and experimental studies. This can also suggest that the EPO/EPOR signaling plays a significant role in cancer cell proliferation, migration, invasivity, and also regulates the angiogenesis.

In our study we also investigated the molecule ActA, a member of the TGF-beta superfamily. Multifunctional cytokines with various cell functions, which is feasible by their complex intracellular signaling pathways are included in this group. It was discovered as a cytokine that enhances the biosynthesis and secretion of follicle-stimulating hormone (FSH). It is formed v*ia* the covalent intracellular dimerization of two inhibin ßA (IHNBA) subunits. Its receptors have serine/threonine transmembrane domain and in addition of SMAD intracellular signaling pathway the activation of several alternative mechanisms are observed in different cell types. Furthermore, there are numerous regulating mechanisms of ActA signaling pathways. In our experiments we focused on the FST, the well-known circulating antagonist of ActA. However, ActA is also involved in a variety of biological functions including control of cellular differentiation, homeostasis of cell number and tissue architecture in multiple organs. It can effect on the tumor cells and the stroma as well and in tumoral environment it can regulate: inflammation, fibrosis, and angiogenesis, attracting macrophages, nitric oxide releasing, extracellular matrix protein and MMPs. In breast-, liver- and colon cancer, activin signals were found to inhibit tumor cell growth. The decreased levels of ActA were demonstrated in the tumor tissue, just like the increased levels of activin antagonists or the loss of functional activin receptors or SMAD proteins. In contrast, the high ActA expression increased the tumor cell aggressiveness in oral squamous cell carcinoma, esophageal ADC and malignant pleural mesothelioma. The expressions of ActA in clinical ADC samples were investigated in two studies resulting conflicting data.

2 OBJECTIVES

Our aim was to evaluate the magnitude of EPO/EPOR and ActA/FST system and also investigate as prognostic and diagnostic markers in lung adenocarcinoma.

1. Determine the EPOR expression and the *in vitro* effect of rHuEPOα and gemcitabine treatment on the proliferation of human lung adenocarcinoma cells.

2. Investigate the effect of rHuEPOα and gemcitabine treatment on xenograft tumors.

3. Analyse the EPOR mRNS expression levels of human bronchoscopy samples and assess the correlation with clinicopathological data. Investigate the EPOR level as prognostic marker.

4. Study the ActA and FST secretion and activin receptor expression and also analyse the effect of recombinant human (rh) ActA and rhFST *in vitro* treatment on the proliferation and migration of human lung adenocarcinoma cells.

5. Assess the circulating ActA, FST levels and the correlation with clinicopathological data. Investigate the circulating ActA and FST levels as diagnostic and prognostic markers.

6. Investigate the correlation of ActA level and KRAS mutation status in lung adenocarcinoma.

3 METHODS

Patients

In our clinical experiments the EPOR expression was determined in a total of 43 patients' bronchoscopy samples with stage III-IV ADC. Circulating ActA and FST levels were analysed in 64 blood samples of patients with ADC and in a sex-, age- matched control kohort (n=46) without tumor. To determine the ActA - KRAS status correlation, ActA level of plasma samples of patients with ADC and known KRAS status (n=34) were analysed.

Cell lines

For the *in vitro* and *in vivo* experiments A549, H1975, H358, H1650 and HCC827 human adenocarcinoma cell lines, K562 and HUVEC as control cell lines, and the HepG2 cell line for the activin A bioactivity test were used.

Drugs

In our *in vitro* and *in vivo* treatments the following drugs were used: recombinant human erythropoietin-alfa, gemcitabine, recombinant human activin A and recombinant human follistatin.

In vitro experiments

The expressions of EPOR and activin receptors were measured by real-time PCR in lung adenocarcinoma cell lines. The secretions of ActA and FST in the cell lines' supernatants were analysed by ELISA kit, according to the guidelines of the manufacturer. ActA and FST ELISAs were tested for interference with rhFST or rhActA, respectively. For the test of ActA ELISA, freshly prepared human plasma samples were treated with different doses of rhFST (2 ng/ml, 50 ng/ml, 100 ng/ml and control without treatment) and incubated for 2h at

37°C. In case of FST ELISA, the same procedure was performed with rhActA treatment.
In case of rHuEPOα and gemcitabine treatments after 48 hours incubation, cell growth was assessed by sulforhodamine B (SRB) colorimetric assay. The *in vitro* effect of rhActA and rhFST on the cell proliferation, after 72 hours incubation was determined with EZ4U kit, according to the guidelines of the manufacturer. The effects of rhActA and rhFST on the motility of the cells were investigated with Scratch assay.
To test biologically activity of the ActA, secreted by ADC cell lines, HepG2 cells were treated with the ADC cell lines' supernatant. After 30 min incubation at 37°C, cells were harvested in lysis buffer and the p-SMAD2, total SMAD and beta-actin was visualized by Western blot analysis.

***In vivo* experiments**

Female SCID mice were subcutaneously inoculated with H1975 tumor cells and treated with rHuEPOα, gemcitabine and the combination of rHuEPOα and gemcitabine. Control group was treated with saline. Tumor volumes were measured with a caliper. Endothelial and tumor cell proliferation were measured by 5-bromo-2'-deoxyuridine (BrdU), blood vessels and nuclei were visualized with anti-mouse CD31 and TOTO-3. The BrdU labeling index was determined by counting non-labeled and labeled H1975 and mouse endothelial cell nuclei in independent intratumoral areas. The labeling index was calculated by dividing the number of labeled nuclei by the total number of counted nuclei.

Statistical analysis

For the statistical analysis PASW Statistics 18.0, GraphPad Prism 5.0 and Statistic 9.0 programs were used.

4 RESULTS

Expression of EPOR and the *in vitro* effect of rHuEPOα and gemcitabine treatment on the proliferation of human lung adenocarcinoma cells

EPOR mRNA levels of the three human ADCs were determined by using quantitative real-time PCR analysis. H1650 and H358 cell lines expressed EPOR at low level. However, H1975 cells expressed EPOR mRNA at higher level than K562 cells. Importantly, rHuEPOα did not stimulate the *in vitro* proliferation rate of any of the three cell lines when compared to untreated cells but as expected treatment with gemcitabine significantly decreased cell proliferation in all examined human ADC cell lines but the anti-proliferative effect of gemcitabine was not affected by rHuEPOα at any concentrations.

In vivo effect of rHuEPOα and gemcitabine treatment

Next, we sought to study the effects of rHuEPOα and gemcitabine on the *in vivo* growth of the H1975 cell line that showed the highest EPOR expression. Tumor growth was significantly decreased in mice treated with gemcitabine alone. Surprisingly, a less robust but still significant growth-inhibitory effect of rHuEPOα was observed when administered alone. However, no additional synergistic effects could be achieved when the two drugs were given in combination. In mice treated with rHuEPOα alone, the proliferation index of H1975 cells and mouse blood vessel endothelial cells were determined by BrdU labeling. rHuEPOα not only resulted in accelerated endothelial cell proliferation *in vivo* but surprisingly it also significantly decreased the *in vivo* growth rate of the high EPOR receptor expressing H1975 ADC cells.

Association between the EPOR mRNA expressions of bronchoscopy brushes and the clinicopathological parameters of the patients

To determine the clinical relevance of tumor tissue EPOR expression, we performed comparative statistical analysis of bronchial brush EPOR mRNA expression and clinicopathological variables. No significant association with age, smoking history, gender, stage or treatment was detected.

EPOR expression level as a prognostic marker

Next we used Kaplan-Meier analysis to calculate the overall survival rates for advanced stage ADC patients with low and high EPOR levels. We elucidated that ADC patients with high EPOR levels had significantly longer overall survival than those with low EPOR expression. Multivariate analysis also indicated that pretreatment EPOR levels predicted outcome is independent of other variables.

Secretion of ActA/FST and expression of activin receptors

We analysed the *in vitro* ActA and FST secretions of five different ADC cell lines by ELISA. ActA was detectable in the supernatants of 3 cell lines (H1650, HCC827 and H358). In two of these cell lines, we found relatively low ActA concentrations, while H358 cells produced high amount of the protein. In the case of FST, all five cell lines secreted the protein. We also measured the mRNA levels of activin receptors real-time PCR. Each cell line expressed the type II receptors ActR-IIA and ActR-II, as well as the type I receptor ActR-IB.

Biological activity of the ActA, secreted by ADC cell lines

It has been demonstrated previously that HepG2 hepatoma cells are responsive to ActA. In these cells, treatment with exogenous ActA leads to the phosphorylation of SMAD2, so

this assay is suitable to measure the activity of ActA. When HepG2 cells were treated with conditioned supernatants of the five different ADC cell lines, phosphorylation of SMAD2 was induced in the cell models H358, in case of the other two cell lines, H1650 induced a lower level of SMAD2 phosphorylation, while HCC827 could not induce any difference.

***In vitro* effect of rhActA and rhFST treatment on the proliferation and migratory capacity of lung ADC cell lines**

Furthermore, the effects of rhActA and rhFST treatments on the proliferation of the activin receptor expressing tumor cells were also analysed. After 72h, any concentrations of the rhActA and rhFST treatments had no effect on the *in vitro* cell proliferation. As our clinical data showed a correlation between circulating ActA and distant organ- and lymph node metastasis (see below) we also investigated the effect of rhActA and rhFST treatments on the *in vitro* migratory capacity of ADC cell lines. Treatments had no significant effect on the ADC cell lines' motility.

Testing of activin A and follistatin ELISA assays

To test whether ActA/FST complexes interfere with the ELISA detection of ActA and/or FST alone, FST and ActA levels of plasma samples were determined after treatment either with rhActA or rhFST, respectively. No significant differences could be detected between ActA concentrations of the untreated plasma and the samples incubated with rhFST at different concentrations, demonstrating that the ActA ELISA detects both free (active) and FST-bound (inactive) ActA. In contrast, treatment of plasma with 50 and 100 ng/ml rhActA decreased the levels of measurable FST, indicating that only free (and not the ActA-bound form of) FST can be detected by the ELISA kit.

Correlation between circulating activin A levels and the clinicopathological parameters of patients with lung ADC

ActA levels were measured in serum samples of 64 ADC patients and 46 age- and gender-matched controls. In patients with ADC the concentrations of ActA were significantly higher compared to controls. We also observed a stage- and T and N status-dependent increase of circulating ActA concentrations.

Level of ActA as a diagnostic and prognostic marker

Serum ActA levels were significantly increased in patients with metastatic disease as compared to M0 patients. ROC curve analysis revealed that serum ActA had a sensitivity of 82.6% (95% CI: 61.2-95.1%) and a specificity of 63.4 %, (95 % CI: 46.9-77.9 %) to differentiate metastatic patients from M0 cases and the AUC was 0.806 (95% CI: 0.693- 0.919).

Because lymph node and organ metastatic ADCs were characterized by a significant increase in circulating ActA levels, next we used Kaplan-Meier analysis to calculate the overall survival rate for patients with low and high serum ActA levels. These classifications were based on the median values of ActA concentrations in our patient population. We found that ADC patients with high serum ActA levels had significantly shorter overall survivals than those with low circulating ActA concentrations. Multivariate analysis (including standard prognostic parameters such as patient age, gender and tumor stage) also showed that serum ActA concentration predicted outcome was independent of other variables.

Correlation between Activin A level and KRAS mutation in lung adenocarcinoma

Plasma ActA levels were determined in case of 34 ADC patients and 66 control subjects and the metastasis and overall

survival of ADC patients were investigated concerning the ActA concentration and KRAS mutation status. The level of ActA was significantly higher in the ADC group compare to control. When the ADC cohort was divided as patients with KRAS mutation (mut) or wild type (wt) we found no difference in the ActA level. Furthermore, KRAS status did not influenced the metastasis and the overall survival of the ADC patients. In the kohort of ADC patients with wt KRAS we could not detect the same correlations between the ActA level and M status or ActA level and overall survival what we found in the full ADC cohort. In the cohort of ADC patients with mutated KRAS status the same tendency was observed what we could detect in the full ADC cohort. Patients with high ActA level had a higher number of metastasis and also had a significantly shorter overall survival compare to the patients with low ActA level in the plasma.

Relevance of FST level in human lung adenocarcinoma

Since the activity of circulating ActA is regulated by FST, serum samples of 64 ADC patients and 46 age- and sex-matched controls were also analysed for FST concentrations. There was no difference in the FST serum levels between controls and ADC patients. When a separate analysis of males and females was conducted, we found similar serum FST levels in the male and female controls as well as in the male ADC patients. However, we detected significantly increased serum FST concentrations in female ADC patients. There was no association between FST levels and ActA concentrations, TNM stage or OS. Serum FST had no diagnostic value in the full cohort or in the female cohort.

5 CONCLUSIONS

1. EPOR mRNA levels of the human ADC cell lines were determined by using real-time PCR analysis. H1975 cells expressed EPOR at the highest level but importantly, rHuEPOα alone or in combination with gemcitabine did not stimulate the *in vitro* proliferation rate of these cells.

2. Tumor growth was decreased in mice treated with rHuEPOα and not only resulted in accelerated endothelial cell proliferation *in vivo* but surprisingly it also significantly decreased the *in vivo* growth rate of the high EPOR receptor-expressing H1975 ADC cells.

3. Advanced stage ADC patients with low EPOR levels had significantly shorter overall survival and the pretreatment EPOR levels predicted outcome was independent of other variables.

4. In case of ActA, three cell lines and in the case of FST, all five cell lines secreted the protein and the activin receptors were also expressed in each ADC cell lines. Nevertheless, the exogen rhActA and rhFST did not influence the proliferation and migration of the ADC cell lines.

5. Serum ActA concentration had significant correlation with the TNM status and overall survival of the ADC patients. The FST level was elevated only in the ADC female cohort. Level of ActA was a novel prognostic marker in the whole cohort and a potential diagnostic marker for the ADC patients with metastasis.

6. ADC patients with KRAS mutation and high ActA levels had a higher number of metastases and the overall survival was

significantly shorter compared to the ADC patients with low ActA level. However, in ADC patients with wild type KRAS, the level of ActA had no correlation with the number of metastases and overall survival.

6 LIST OF PUBLICATIONS

Publications related to the theses:

Hoda MA[*], **Rozsas A**[*], Lang E, Klikovits T, Lohinai Z, Torok S, Berta J, Bendek M, Berger W, Hegedus B, Klepetko W, Renyi-Vamos F, Grusch M, Dome B[**], Laszlo V[**]. (2016) High circulating activin A level is associated with tumor progression and predicts poor prognosis in lung adenocarcinoma. Oncotarget, 7(12):13388-99.
[*]These authors share the first authorship, [**]These authors are co-senior authors of this study

Rózsás A, Berta J, Rojkó L, Horváth LZ, Keszthelyi M, Kenessey I, László V, Berger W, Grusch M, Hoda MA, Török S, Klepetko W, Rényi-Vámos F, Hegedűs B, Döme B, Tóvári J. (2013) Erythropoietin receptor expression is a potential prognostic factor in human lung adenocarcinoma. PLoS One, 8(10):e77459.

Rózsás A, Ostoros Gy, Hegedűs B, Döme B, Tóvári J. (2013) Az erythropoietin jelátvitel szerepe humán nem-kissejtes tüdőrákban. Medicina Thoracalis, 66(1):21-30.

Publications not related to the theses:

Hoda MA, Pirker C, Dong Y, Schelch K, Heffeter P, Kryeziu K, van Schoonhoven S, Klikovits T, Laszlo V, **Rozsas A**, Ozsvar J, Klepetko W, Doeme B, Grusch M, Hegedues B,

Berger W. (2016) Trabectedin is active against malignant pleural mesothelioma cell and xenograft models and synergizes with chemotherapy and bcl-2 inhibition in vitro. Mol Cancer Ther., pii: molcanther.0846.2015.

Hoda MA, Dong Y, **Rozsas A**, Klikovits T, Laszlo V, Ghanim B, Stockhammer P, Ozsvar J, Jakopovic M, Samarzija M, Brcic L, Bendek M, Szirtes I, Reid G, Kirschner MB, Kao SC, Opitz I, Weder W, Frauenfelder T, Nguyen-Kim TD, Aigner C, Klepetko W, van Zandwijk N, Berger W, Dome B, Grusch M, Hegedus B. (2016) Circulating activin A is a novel prognostic biomarker in malignant pleural mesothelioma - A multi-institutional study. Eur J Cancer, 63:64-73.

Hegedűs B, Moldvay J, Berta J, Lohinai Z, **Rózsás A**, Cserepes MT, Fábián K, Ostoros G, Tóvári J, Rényi-Vámos F, Tímár J, Döme B. (2015) Excerpts from the collaborative lung cancer research program of Semmelweis University, the National Institute of Oncology and Korányi Institute of TB and Pulmonology (2010-2015)]. Magy Onkol, 59(4):282-5.

Kirschner MB, Pulford E, Hoda MA, **Rozsas A**, Griggs K, Cheng YY, Edelman JJ, Kao SC, Hyland R, Dong Y, László V, Klikovits T, Vallely MP, Grusch M, Hegedus B, Dome B, Klepetko W, van Zandwijk N, Klebe S, Reid G. (2015) Fibulin-3 levels in malignant pleural mesothelioma are associated with prognosis but not diagnosis. Br J Cancer, 113(6):963-9.

Laszlo V, Hoda MA, Garay T, Pirker C, Ghanim B, Klikovits T, Dong YW, **Rozsas A**, Kenessey I, Szirtes I, Grusch M, Jakopovic M, Samarzija M, Brcic L, Kern I, Rozman A, Popper H, Zöchbauer-Müller S, Heller G, Altenberger C, Ziegler B, Klepetko W, Berger W, Dome B, Hegedus B.

(2015) Epigenetic down-regulation of integrin α7 increases migratory potential and confers poor prognosis in malignant pleural mesothelioma. J Pathol, 237(2):203-14.

Lohinai Z, Hoda MA, Fabian K, Ostoros G, Raso E, Barbai T, Timar J, Kovalszky I, Cserepes M, **Rozsas A**, Laszlo V, Grusch M, Berger W, Klepetko W, Moldvay J, Dome B, Hegedus B. (2015) Distinct Epidemiology and Clinical Consequence of Classic Versus Rare EGFR Mutations in Lung Adenocarcinoma. J Thorac Oncol, 10(5):738-46.

Garay T, Kenessey I, Molnár E, Juhász É, Réti A, László V, **Rózsás A**, Dobos J, Döme B, Berger W, Klepetko W, Tóvári J, Tímár J, Hegedűs B. (2015) Prenylation inhibition-induced cell death in melanoma: reduced sensitivity in BRAF mutant/PTEN wild-type melanoma cells. PLoS One, 10(2):e0117021.

Ghanim B, Klikovits T, Hoda MA, Lang G, Szirtes I, Setinek U, **Rozsas A**, Renyi-Vamos F, Laszlo V, Grusch M, Filipits M, Scheed A, Jakopovic M, Samarzija M, Brcic L, Stancic-Rokotov D, Kern I, Rozman A, Dekan G, Klepetko W, Berger W, Glasz T, Dome B, Hegedus B. (2015) Ki67 index is an independent prognostic factor in epithelioid but not in non-epithelioid malignant pleural mesothelioma: a multicenter study. Br J Cancer, 112(5):783-92.

Torok S, Vegvari A, Rezeli M, Fehniger TE, Tovari J, Paku S, Laszlo V, Hegedus B, **Rozsas A**, Dome B, Marko-Varga G. (2015) Localization of sunitinib, its metabolites and its target receptors in tumour-bearing mice: a MALDI-MS imaging study. Br J Pharmacol, 172(4):1148-63.

Berta J, Hoda MA, Laszlo V, **Rozsas A**, Garay T, Torok S, Grusch M, Berger W, Paku S, Renyi-Vamos F, Masri B,

Tovari J, Groger M, Klepetko W, Hegedus B, Dome B. (2014) Apelin promotes lymphangiogenesis and lymph node metastasis. Oncotarget, 5(12):4426-37.

Cserepes M, Ostoros G, Lohinai Z, Raso E, Barbai T, Timar J, **Rozsas A**, Moldvay J, Kovalszky I, Fabian K, Gyulai M, Ghanim B, Laszlo V, Klikovits T, Hoda MA, Grusch M, Berger W, Klepetko W, Hegedus B, Dome B. (2014) Subtype-specific KRAS mutations in advanced lung adenocarcinoma: a retrospective study of patients treated with platinum-based chemotherapy. Eur J Cancer, 50(10):1819-28.

Höftberger R, Titulaer MJ, Sabater L, Dome B, **Rózsás A**, Hegedus B, Hoda MA, Laszlo V, Ankersmit HJ, Harms L, Boyero S, de Felipe A, Saiz A, Dalmau J, Graus F. (2013) Encephalitis and GABAB receptor antibodies: novel findings in a new case series of 20 patients. Neurology, 81(17):1500-6.

Zimmermann M, Nickl S, Lambers C, Hacker S, Mitterbauer A, Hoetzenecker K, **Rozsas A**, Ostoros G, Laszlo V, Hofbauer H, Renyi-Vamos F, Klepetko W, Dome B, Ankersmit HJ. (2012) Discrimination of clinical stages in non-small cell lung cancer patients by serum HSP27 and HSP70: a multi-institutional case-control study. Clin Chim Acta, 413(13-14):1115-20.

Berta J, Kenessey I, Dobos J, Tovari J, Klepetko W, Jan Ankersmit H, Hegedus B, Renyi-Vamos F, Varga J, Lorincz Z, Paku S, Ostoros G, **Rozsas A**, Timar J, Dome B. (2010) Apelin expression in human non-small cell lung cancer: role in angiogenesis and prognosis. J Thorac Oncol, 5(8):1120-9.

7 ACKNOWLEDGEMENTS

Hereby I would like to thank my supervisors Balázs Döme and József Tóvári for their long term professional help and support. I am also grateful to the heads of National Korányi Institute of TB and Pulmonology, Department of Thoracic Surgary and Translational Thoracic Oncology Laboratory at the Medical University of Vienna, Department of Experimental Pharmacology at the National Institute of Oncology and the Department of Thoracic Surgery at the Semmelweis University for their support and the opportunity to perform my research work.
Special thank for the help of the coworkers of the Department of Tumor Biology and the Translational Thoracic Oncology Laboratory.
I am grateful to the Hungarian Pulmonology Foundation, Austrian-Hungarian Action Foundation and EACR for their support.
Finally my special thanks are extended to my husband, my family and friends for their endurance, encouragement and the secure background.