Investigation of molecular factors determining targeted therapy sensitivity of BRAF mutant solid tumors

Synopsis of PhD thesis

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1. INTRODUCTION

Point mutation of the BRAF kinase occurs in number of malignancies. Among solid tumors BRAF mutation is common in melanoma (40-60%), thyroid (30-80%), colorectal (5-15%), ovarian cancer (10-60%) and in lung adenocarcinoma (2-3 %). The most frequent BRAF mutation is V600E alteration. However, the significance of non-V600 BRAF mutations in certain tumor types (melanoma, lung adenocarcinoma, colorectal cancer) is not negligible. Targeted therapy already exists against V600 BRAF mutant tumors, but due to the acquired resistance mechanisms, overall survival could and should be further improved. Furthermore, there are currently no approved targeted therapies for non-V600 BRAF mutant cancer patients. In my PhD thesis, molecular factors determining the targeted therapy sensitivity of BRAF mutant solid tumors was investigated in three studies. First, we studied a potential prenylation-inhibition-based therapeutic approach for melanoma with V600E BRAF mutation and PTEN loss. Second, we investigated the resistance mechanisms and phenotypical alterations on vemurafenib pre- and posttreatment isogeneic human melanoma cell line pairs. Finally, antitumor effects of panRAF and MEK combined treatment on preclinical models of non-V600 BRAF mutant tumor cells was explored. Altogether, our novel findings may contribute to the optimization of targeted therapy of BRAF mutant malignancies

2. AIMS

During our work, molecular factors determining the targeted therapy sensitivity of BRAF mutant solid tumors was investigated. Our aims were the following:

1. Comparison of antitumor effects of zoledronic acid on V600E BRAF melanoma cell lines with or without PTEN expression.

2. Investigation of resistance mechanisms and phenotypical changes on pre- and posttreatment isogeneic human melanoma cell line pairs, treated with vemurafenib.
Specifically, the evaluation of proliferative and migratory capacity of cell line pairs and the determination of the molecular background of emerging phenotypical alterations at the mRNA and protein level. Finally the assessment of inhibitors against the acquired resistance mechanisms.

3. Testing of pan-RAF and MEK combination inhibition treatment on preclinical models of non-V600 BRAF mutant tumors (melanoma, lung adenocarcinoma, breast cancer).

3. METHODS

3.1. Cell lines

Ten melanoma, two lung adenocarcinoma and one breast cancer cell line and also six pre- and posttreatment isogeneic human melanoma cell line pairs were used in our experiments.

3.2 Inhibitors

The prenylation-inhibitor zoledronic acid, the V600E BRAF mutant specific inhibitor vemurafenib, the MEK-inhibitor selumetinib, the pan-RAF-inhibitor sorafenib and AZ628, the EGFR-inhibitor erlotinib and the PI3K/mTOR dual inhibitor BEZ235 have been evaluated..

3.3 Viability assays

Total protein amount of the cells was determined via photometric measurement. Interactions between drugs were assessed using the combination index calculations in the CompuSyn software (ComboSyn Inc).

3.4 Cell migration analysis

Migrated distance was calculated by averaging displacement for each single cells in a defined time frame at least three independent experiments and three microscopic fields.

3.5 TUNEL assay

Labeling of terminal deoxynucleotidyl transferase—mediated dUTP nick end (TUNEL) was performed according to the manufacturer's instructions (Roche Diagnostics, Basel, Switzerland).

3.6 Immunblot

Activation of Erk1/2, S6, Akt, CRAF and expression of EGFR, PTEN PARP/cleaved PARP was determined via immunblot assay.

3.7 Cell cycle analyses

NucleoCounter NC-3000™ system (Chemometec) was used to define quantify cell cycle changes upon treatment.

3.8 Quantitative PCR

After RNA isolation reverse transcription was performed. mRNA levels of epithelial mesenchymal transition (EMT) and immune checkpoint proteins in pre- and posttreatment isogeneic human melanoma cell line pairs was evaluated via quantitative real time PCR.

3.9 In vivo orthotropic xenograft model

MDAMB231 cells were injected into the mammary fat pad of female NSG mice. Two weeks after injection, mice were randomly divided

into four groups and treated with vehicle, 25 mg/kg sorafenib and 10 mg/kg selumetinib or both intraperitoneally every day except weekends for 18 days. After treatment mice were euthanized and the tumor tissue was removed and weighed.

3.10 Statistical analyses

All statistical analyses were performed in GraphPad Prism 5 (GraphPad Software Inc, USA, San Diego, CA).

4. RESULTS

4.1 Antitumor effects of zoledronic acid on V600E BRAF mutant melanoma in the function of PTEN expression

PTEN expression of melanoma cell lines were evaluated by immunblot assay. Both short-term and long-term viability assays showed that melanoma cell lines with V600E BRAF mutation and wild type PTEN were more sensitive to vemurafenib, while melanoma cells with V600E BRAF mutation and lacking PTEN expression were more sensitive to zoledronic acid treatment. Additionally, TUNEL and cleaved PARP apoptosis assays were performed. We found that zoledronic acid could induce apoptosis in melanoma cells with V600E BRAF mutation and lacking PTEN expression. This finding was further confirmed on the V600E BRAF mutant isogeneic melanoma cell line pairs as well.

4.2 Exploring effects of long-term vemurafenib treatment on pre- and posttreatment isogeneic human melanoma cell line pairs

Our aim was to investigate the effects of long-term vemurafenib treatment on V600E BRAF mutant pre and posttreatment melanoma cell line pairs. First, we evaluated the vemurafenib IC50 values of the cells. Results showed that the pretreatment cells intrinsically had high IC50 value in two out of six cell line pairs (Mel JL and MM90906) while in the rest four cell line pairs (Mel KD, Mel JR, MM90911, MM040111) vemurafenib IC50 values significantly increased. Proliferation capacity of the cell lines was determined via SRB assay. In four out of six cell line pairs proliferation capacity decreased in posttreatment cells (Mel KD, Mel JL, MM90911, MM040111), while in the rest two cell pairs proliferation capacity increased (Mel JR, MM90906) in posttreatment cells. Migratory capacity of the cell lines was evaluated by videomicroscopy. Cell migration increased in all cell line pairs in the posttreatment cells. Next, we further investigated the molecular background of the increased migration. mRNA level of EMT markers (SNAIL, ZEB-1, vimentin, N-cadherin, E-cadherin MMP-3, MMP-1,) transcription factors (MITF, FRA-1), epidermal growth factor receptor (EGFR) and immun checkpoint proteins (PDL-1, PD-1, PDL-2, B7-1) was determined via qPCR. Since migratory capacity elevated in all cell line pairs, we expected increased mRNA level of the EMT markers

(except for E-cadherin) in all posttreatment cells. However, no consistent EMT marker patterns were seen in the cell line pairs. We found no E-cadherin expression in any of the cell lines investigated. Regarding the immune checkpoint proteins, PDL-1 and PDL-2 mRNA levels either decreased or increased in posttreatment cells. Next, we correlated (Spearman correlation) the mRNA expression levels of the investigated factors with the proliferation and migration of the cells. We found that migratory capacity was negatively correlated with MITF expression, while proliferation capacity negatively correlated with FRA-1 mRNA level. Also both transcription factors correlated with EGFR mRNA expression. Furthermore, we explored the correlation between Akt activation and PTEN expression as well as between Erk activation and EGFR expression. Cell lines with very low or not detectable PTEN expression demonstrated significantly higher Akt activation than PTEN wild type cells. Furthermore, we found that in cell lines with high EGFR expression Erk activation is significantly higher than in cells with low EGFR expression. Interestingly, vemurafenib sensitive cells (IC50<3 μM) express EGFR in lower levels when compared to vemurafenib resistant cells (IC50>3 μM). Next, we tested the EGFR inhibitor erlotinib and the PI3K/mTOR inhibitor BEZ235 in these cell lines, since Erk and Akt activation showed correlation with EGFR and PTEN expression, respectively. Interestingly, cells with low EGFR levels were more sensitive to erlotinib than cells with

high EGFR expression. Regarding PTEN expression we found no consistent BEZ235 sensitivity differences. Finally, we demonstrated that combination inhibition with erlotinib and BEZ235 could induce synergistic or additive growth inhibition.

4.3 Effects of panRAF and MEK combination inhibition on non-V600 mutant cell lines

Six BRAF mutated human tumor cell lines were treated with a pan-RAF inhibitor (sorafenib), a MEK inhibitor (selumetinib) or their combination. Since sorafenib also has inhibitory effect on several other kinases, we performed growth inhibition and PARP cleavage assay with a highly selective pan-RAF inhibitor (AZ628) on our panel of non-V600 BRAF mutant cells.. Combination indices (CI) were calculated from the data of viability assays of combination treatment. All cell lines - including the V600E BRAF mutant A375 at lower selumetinib concentration - showed lower CI value than 1 indicating synergistic interaction in combination treatment. Cell cycle analyses revealed that combination treatment reduced cell proliferation by arresting cell cycle in G0/G1. To investigate the effect of selumetinib and sorafenib or combined treatment on CRAF, Erk1/2, Akt and S6 activation, cell lines were treated with single agents or the combination and were analyzed by Western blot. We observed a decrease of Erk1/2 activation in all non-V600 BRAF mutant cell lines after both sorafenib and selumetinib treatment. In

comparison with the single agent treatment, combination of drugs resulted in further decrease in p-Erk1/2 levels in all cell lines, except A375, where selumetinib treatment alone caused complete Erk1/2 deactivation. Upon combination treatment we observed slightly increased p-Akt levels in WM3629 and CRL5922, however, in the other cell lines Akt activation decreased or not altered. Combination of drugs either decreased or did not impact the activation of S6 in these cell lines. Notably, in WM3629 and WM3670 melanoma cells single selumetinib treatment also caused increased activation of Akt. Furthermore, we found that in the WM3629 cell line selumetinib caused significantly elevated migration which could be diminished by adding sorafenib. However, combination treatment has no significantly stronger effect on migration than single agent treatment in the other cell lines. We observed that combination treatment significantly increased the level of apoptotic cells compared to single treatment. Finally, we examined the effect of selumetinib, sorafenib or combined treatment on *in vivo* growth of MDAMB231 cells transplanted into 14-weeks-old NSG mice. Both sorafenib or selumetinib treatments reduced tumor growth, however the combination treatment resulted in an enhanced growth inhibitory effect.

5. CONCLUSION

1. Treatment with zoledronic acid resulted in tumor growth inhibition and apoptosis induction in melanoma cells with V600E BRAF mutation and lacking PTEN expression. In contrast, in V600E BRAF mutant cells without PTEN mutation there was no significant antitumor effect upon treatment.

2. In isogeneic V600E BRAF mutant melanoma pre and posttreatment cell line pairs the later showed a more invasive phenotype. Our results suggest that EGFR expression in part determines the migratory and proliferative capacity of cells via MITF and FRA-1 transcription factors, respectively. Finally, we found that in BRAF and EGFR inhibitor resistant melanoma cells EGFR and PI3K/mTOR combination might be a promising approach.

3. In non-V600 BRAF mutant cell lines the combination of panRAF inhibitor (sorafenib/AZ628) and MEK-inhibitor (selumetinib) treatment synergistically enhanced therapeutic response.

6. LIST OF PUBLICATIONS

6.1. Publications related to the thesis:

Molnar E, Rittler D, Baranyi M, Grusch M, Berger W, Dome B, Tovari J, Aigner C, Timar J, Garay T, Hegedus B Pan-RAF and MEK vertical inhibition enhances therapeutic response in non-V600 BRAF mutant cells. BMC CANCER 18:(1) p. 542. (2018)

IF=3.288

Garay T, Kenessey I, **Molnar E**, Juhasz E, Reti A, Laszlo V, Rozsas A, Dobos J, Dome B, Berger W, Klepetko W, Tovari J, Timar J, Hegedus B Prenylation Inhibition-Induced Cell Death in Melanoma: Reduced Sensitivity in BRAF Mutant/PTEN Wild-Type Melanoma Cells. PLOS ONE 10:(2) Paper e0117021. 18 p. (2015)

IF=3.057

6.2. Publications not related to the thesis:

1. Spirek M, Mlcouskova J, Belan O, Gyimesi M, Harami GM, **Molnar E**, Novacek J, Kovacs M, Krejci L Human RAD51 rapidly forms intrinsically dynamic nucleoprotein filaments modulated by

nucleotide binding state. NUCLEIC ACIDS RESEARCH epub ahead of print Feb 22: p. 1. (2018)

IF=11.561

2. Hegedűs L, Garay T, **Molnár E**, Varga K, Bilecz A, Török S, Padányi R, Pászty K, Wolf M, Grusch M, Kállay E, Döme B, Berger W, Hegedűs B, Enyedi A The plasma membrane Ca2+ pump PMCA4b inhibits the migratory and metastatic activity of BRAF mutant melanoma cells INTERNATIONAL JOURNAL OF CANCER 140:(12) pp. 2758-2770. (2017)

IF=7.36

3. Tatrai E, Bartal A, Gacs A, Paku S, Kenessey I, Garay T, Hegedus B, **Molnar E**, Cserepes MT, Hegedus Z, Kucsma N, Szakacs G, Tovari J Cell type-dependent HIF1 alpha-mediated effects of hypoxia on proliferation, migration and metastatic potential of human tumor cells ONCOTARGET 8: pp. 44498-44510. (2017)

IF=0

4. Garay T*, **Molnar E***, Juhasz E, Laszlo V, Barbai T, Dobos J, Schelch K, Pirker C, Grusch M, Berger W, Timar J, Hegedus B

Sensitivity of Melanoma Cells to EGFR and FGFR Activation but Not Inhibition is Influenced by Oncogenic BRAF and NRAS Mutations PATHOLOGY AND ONCOLOGY RESEARCH 21:(4) pp. 957-968. (2015)

IF=1.94

5. Garay T, Juhasz E, **Molnar E**, Eisenbauer M, Czirok A, Dekan B, Laszlo V, Hoda MA, Dome B, Timar J, Klepetko W, Berger W, Hegedus B Cell migration or cytokinesis and proliferation? - revisiting the "go or grow" hypothesis in cancer cells in vitro. EXPERIMENTAL CELL RESEARCH 319:(20) pp. 3094-3103. (2013)

IF=3.378