Investigating tumor cell motility under hypoxia and therapeutic resistance in cancer models

Ph.D. thesis

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1. Introduction

To date, lung cancer is currently recognized as the most lethal form of malignant disease worldwide, often diagnosed at an advanced metastatic stage, resulting in a poor prognosis. Similarly, malignant melanoma of the skin is known for its highly metastatic nature and gives treatment challenges due to its resistance to therapies. Over the past two decades, it has become evident that the tumor microenvironment plays a crucial role in modulating tumor aggressiveness, motility, metastasis and the development of resistance to treatments. Metastasis is a process whereby malignant cells migrate from the primary tumor site, infiltrate neighboring tissues, enter the circulatory system and establish secondary tumors in distant organs. Therapy resistance can be defined as the ability of cancer cells to tolerate the effects of treatment and reduce or eliminate the therapy's success. Both metastasis and resistance mechanisms remain a significant challenge in cancer treatment, making it crucial to understand the underlying processes. In the first part of my thesis, I investigated the impact of chronic hypoxia on the expression of epithelial-mesenchymal transition markers, proliferation and motility in a panel of human lung adenocarcinoma cell lines. Hypoxia, an environmental condition that is commonly found in tumors due to the unmatured

vascularization is characterized by low oxygen levels and has been associated with tumor progression and the metastasis development. Therefore, investigation of the molecular mechanisms behind the development of lung cancer under hypoxia is crucial to establish more rational therapeutic approaches for the treatment of lung adenocarcinoma (LUAD) patients. In the second part of my thesis, I established a patient-derived tumor xenograft (PDTX) model from a melanoma patient harboring BRAF V600E mutation to investigate potential resistance-relevant mechanisms and markers against Vemurafenib treatment. Currently, the literature on the potential mechanisms of resistance to BRAF inhibitors has been overly focused on models based on cell lines. Vemurafenib is effective in BRAF V600E mutant cells by blocking ERK phosphorylation, however other pathways may contribute to tumor cell resistance to vemurafenib in melanoma. Therefore, investigating these resistance mechanisms and markers using PDTX models is crucial for developing more effective treatment strategies and improving patient outcomes.

2. Objectives

In this study, we aimed to investigate the followings:

1.) The effects of reduced oxygen level (hypoxia) or hypoxia mimicking ($CoCl_2$ treatment) on the expression of hypoxia-related proteins and genes in lung adenocarcinoma.

2.) The impact of hypoxia and $CoCl_2$ exposure on the single-cell and collective migration ability of lung adenocarcinoma cancer cells.

3.) Identifying resistance-associated changes using RNA sequencing in *in vivo* PDTX model of vemurafenib-resistant malignant melanoma.

4.) Validating the identified expression differences at RNA and protein level.

3. Methods

3.1. Cell culture

Human lung adenocarcinoma cell lines with different driver mutations (PF901, PF139, H838 H1975) were cultured in DMEM supplemented with 10% FBS and 1% P/S and incubated at 37°C in a humidified 5% CO_2 atmosphere. Cells were cultured in 1% O_2, 5% CO_2 and 94% N_2 for hypoxia measurements.

3.2. Motility studies

Videomicroscopy was performed with $4x10^2$ cells/well seeding concentration. One field of view per well was imaged every 10 min during the 48 hours treatment period. Distance from the origin and total length data were obtained from trajectories of the cells that remained in the field of view through the given time interval. The results were plotted and statistically evaluated using GraphPad Prism 5 software. For scratch assay cells were seeded at $4x10^4$ cells/well density and left for overnight to attach. The next day, scratches were made using the MuviCyte™ scratcher device. Wound closure was monitored in four parallel wells for each cell line for 72-hour period. $T_{1/2}$ wound closure data was calculated with GraphPad Prism 5 software using nonlinear regression.

3.3. Western blot

With immunoblot analysis we investigated the effects of $CoCl_2$ treatment or hypoxia on the protein expression of HIF-1α, p-Histone H3, FAK, p-FAK, p38, p-p38 and β-tubulin. For the PDTX samples protein expressions of CD27, IFI27, mTOR, p-mTOR, AKT, p-AKT, p44/42 (ERK1/2), p-p44/42 (p-ERK1/2), PDGFRB, p-MEK 1/2, p-c-RAF, p53 and GAPDH were analysed.

3.4. Real-time reverse transcription PCR

RNA was isolated from the lung tumor cell lines and from the PDTX samples. The RNA was reverse transcribed and quantitative real-time PCR reactions were performed. The following genes were analyzed: *vimentin, E-cadherin, N-cadherin, IFI27, CD27* and *RLPL0*.

3.5. PDTX Animal Model of Vemurafenib Treatment

KINETO Lab Ltd. provided PDTX samples from BRAF V600E mutant melanoma tissue. Under anesthesia, 5-10 mm^3 tumor tissue pieces were implanted subcutaneously under the right dorsal skin. When tumor sizes reached approximately 100 mm^3, *per os* treatment was carried out with 100 mg/kg vemurafenib five times a week. When either the tumor sizes reached 1500-2000 mm^3 or the animals reached the age of about 6 months

(treated groups), the tumor tissue was serially transplanted into new young animals.

3.6 Data processing and statistical analysis

Single-cell motility data were analyzed for normal distribution using the Shapiro-Wilk test, while in case of proliferation data, we employed the Kolmogorov-Smirnov test. Datasets with normal distribution were analyzed by ANOVA followed by Tukey's Multiple Comparison test, otherwise Kruskal-Wallis test was applied followed by Dunn's Multiple Comparison test. Data from scratch assays were analyzed using non-linear regression and compared with the sum-of-squares F test comparing Hillslope and logIC50 data. RNA and protein expression data were analyzed using the unpaired t-test with Welch correction.

4. Results

4.1. In vitro modeling of the migration ability and changes in EMT markers of LUAD cell lines under hypoxic conditions

4.1.1. The effect of hypoxia and $CoCl_2$ treatment on hypoxia-related protein expression

First, we conducted immunoblot analysis to follow the alterations in HIF-1α, NF-κB, and phosphorylated NF-κB levels, which serve as key regulators for cellular adaptation to hypoxia. Additionally, we sought to evaluate the occurrence of apoptosis by examining the presence of cleaved PARP in LUAD cells cultured *in vitro* and subjected to $CoCl_2$ treatment or hypoxia (1% O_2). HIF-1α was found to be upregulated across all cell lines upon hypoxia or $CoCl_2$ treatment. Although we did not detect any significant changes in total NF-κB levels, we noted a slight decrease in the levels of its phosphorylated form in all cell lines examined under hypoxic conditions.

4.1.2. The effect of hypoxia on cellular proliferation

We performed SRB viability assay and immunoblot analysis of the p-Histone H3 proliferation marker protein to examine the effect of hypoxia on cellular proliferation. Compared to normoxic conditions, we did not observe any significant changes in cell viability, however, we did note a reduction in p-Histone H3 protein expression under 1% O_2.

4.1.3. The effect of hypoxia on important signaling pathways

We aimed to examine the potential alterations in the activation of key signaling pathways in response to hypoxic conditions. Specifically, we focused on the activation levels of FAK, p38 and SRC. While the total level of P38 remained unchanged, the phosphorylated form showed a significant decrease in response to hypoxia across three cell lines examined. There were no changes observed in either the total FAK or phosphorylated forms in three out of four cell lines. However, in PF139, we noted a slight increase in the total FAK level, along with a visible rise in its phosphorylated form upon hypoxia.

4.1.4. Effect of hypoxia on motility

To investigate the effect of hypoxia on the migratory activity of the lung tumor cell lines we performed single-cell and collective migration assay. In the migratory activity of individual tumor cells, treatment with $CoCl_2$ did not significantly affect cellular motility in any of the cell lines. However, under hypoxic conditions, three out of four cell lines exhibited decreased motility. In contrast to the single-cell motility results, hypoxic conditions resulted in significantly faster wound closure for the H838, H1975, and PF901 cell lines compared to normoxia. The PF139 cell line was unable to completely close the wound under

hypoxia, whereas it successfully closed the scratch within 60 hours at normal oxygen levels.

4.1.5. Cell line-specific alterations in mRNA expression levels and evaluation of EMT markers in hypoxia

To investigate the molecular response to hypoxia on motility, we measured the mRNA levels of key epithelial-mesenchymal transition (EMT) markers, specifically vimentin, N-cadherin, and E-cadherin, in our panel of lung adenocarcinoma cell lines. PF139 and PF901 exhibited significantly higher baseline expression of N-cadherin compared to H838, which demonstrated the lowest baseline motility. Although none of the cell lines showed significant changes in the studied markers under hypoxic conditions, some variations were noted. In H838, levels of N-cadherin and vimentin increased under hypoxia, while E-cadherin expression remained unchanged. In H1975, hypoxia slightly increased vimentin expression alongside a strong decrease in E-cadherin levels. In PF139, none of the genes were affected by hypoxia. In PF901 cells, hypoxia resulted in a marked reduction of E-cadherin levels and a slight increase in vimentin expression. In addition, we examined the intracellular distribution of the vimentin protein. Under normoxic conditions, vimentin showed an uneven distribution, primarily localized around the nucleus. In contrast, low oxygen

concentrations led to a significant reorganization of vimentin filaments, resulting in a morphology characterized by a uniform distribution that aligned with the cell shape.

4.2. Analysis of resistance mechanisms in vemurafenib-resistant PDTX model

4.2.1. PDTX growth and vemurafenib resistance

To investigate the molecular changes associated with the development of vemurafenib resistance, we established a subcutaneous patient-derived tumor xenograft (PDTX) model of malignant melanoma and treated it with vemurafenib to monitor acquired drug resistance. Initially, the treatment effectively inhibited tumor growth during the first two generations. However, by the third to sixth generations, resistance had developed.

4.2.2. Identification and validation of resistance-associated changes

The first generation (G1), resistant groups from the third (G3) and fifth generations (G5) were sequenced at the mRNA level. With the bulk RNA sequencing we identified a total of 3,991 significant differences between the untreated and treated groups, with 434 of these differences being common to both treated generations. Two genes were selected for further validation by qPCR, CD27 and IFI27, as both have been identified as a prognostic marker in cancer patients. In line with the RNA-seq

data, CD27 was systematically upregulated, whereas IFI27 was consistently downregulated following vemurafenib treatment.

4.2.3. Immunoblot validation of CD27 and IFI27

To further validate our findings, we performed immunoblot analysis of these proteins. The results showed that CD27 and IFI27 were present in both treatment naïve (G1) and resistant (G5) samples. However, no significant changes could be detected due to high variance within the groups.

4.2.4. Examination of known resistance mechanisms in the present resistance model

Generally, upon analyzing various signaling pathways in protein level, including the amplification of BRAF and activation of CRAF, no significant accumulations or activation of these proteins were detected in the resistant groups. There were no changes in the total protein levels of AKT and mTOR, and none of these proteins showed systematic activation, as indicated by the absence of elevated phosphorylated forms. Additionally, p-MEK 1/2 levels remained unchanged at the protein level. While ERK 1/2 expression was elevated in one of the three resistant tumors, in-group variance was high, therefore no significant changes could be proved. Unexpectedly, PDGFRB, which showed increased mRNA expression during resistance development according to RNA sequencing data had lower

protein expression levels. Furthermore, there was no evidence of p53 accumulation.

5. Conclusions

In this study, we aimed to determine the role of hypoxia on the motility of lung adenocarcinoma cell lines and the acquired resistance mechanisms of a vemurafenib-resistant patient-derived tumor xenograft PDTX model. Our findings indicate that:

- (1) Hypoxic conditions decreased the activation level of SRC, P38 of FAK pathways in the LUAD cell lines as well as the level of proliferation marker phospho-Histone H3, indicating that it may act as a cellular stressor for this time exposure.
- (2) 48-hour exposure to hypoxic conditions suppressed single-cell motility in three of the four cell lines, however invasion under confluent conditions was enhanced upon hypoxia for most of the cell lines.
- (2) $CoCl_2$ failed to replicate the effects of true hypoxic conditions on single-cell motility, warranting caution in the interpretation of hypoxic studies solely based on the use of this hypoxia-mimicking agent.
- (2) Changes in the EMT markers are consistent with enhanced motility and metastasis-promoting effect of hypoxia.

- (3) The established PDTX model of melanoma developed resistance to vemurafenib.
- (4) We identified new potential markers of resistance at RNA level, however, failed to validate them on the protein level.
- (4) Resistant tumors exhibited different resistant mechanisms from the reactivation of RAF/MEK/ERK pathway reported in the literature.

6. Bibliography of the candidate's publications (∑ IF: 29.2)

Publications related to the thesis:

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