Universitätsklinikum Hamburg-Eppendorf

Abschlussbericht

Thema

Functional characterization and clinical relevance of the new putative metastasis suppressor gene HERC5 in non-small cell lung cancer (NSCLC)

Funktionelle Charakterisierung und klinische Relevanz des neuen putativen Metastasensuppressorgens HERC5 in nicht-kleinzelligen Lungenkrebs

Antragstellerin

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Background

In our previous studies we found a significant association between loss of 4q12-q32 and early tumor cell dissemination and poor prognosis. Further analyses identified HERC5 as a potential target metastasis suppressor gene in this region. HERC5 methylation was significantly associated with both positive disseminated tumor cell (DTC) status as well as dismal prognosis. HERC5 has so far not been studied in association with cancer. Therefore, the focus of this project is to investigate the functional role of HERC5 in lung cancer metastases and its prognostic relevance in the context of early lung cancer dissemination.

M.Sc. Svenja Schneegans started her PhD project funded by the Hamburger Krebsgesellschaft e.V. 1st of July 2017. Below a short report of the results obtained during the funding period from Hamburger Krebsgesellschaft.

1. Establishment of functional cell line models.

The establishment of an HTB56 HERC5 overexpression cell line had already been previously completed in our lab. The *HERC5* mRNA overexpression in HTB56 O.E. cell line was shown by RT-qPCR and western blot to be elevated up to appr. 250 fold. In addition to that, an overexpression of HERC5 in H1395 cell line was established to serve as a second O.E. model. Western blot results showed a strong overexpression and RT-qPCR results showed an upregulation of appr. 280 fold. The cloning of the CRISPR–Cas9 plasmid, which should target the *HERC5* gene was successfully done and was used to transfect A549 cells. After FACS sorting and expanding the individual clones they were analyzed by sequencing and western blot. Four of the clones were chosen to generate the A549 *HERC5* K.O. pool cell line. The A549 K.O. pool cell line was analyzed by western blot which confirmed the loss of HERC5 protein.

2. Testing of different antibodies

For the detection of HERC5 protein different commercially available antibodies were tested. Eventually, we were able to establish a protocol using Abnova Anti-HERC5 (915-1024) pAb. The antibody, however, is not working for immunohistochemistry (IHC).

3. Proliferation analysis

In order to analyze a possible differential behavior in proliferative capacity an MTT assay was performed using HTB56 O.E./empty vector control, H1395 O.E./empty vector control and A549 K.O./parental cell lines. The proliferation was measured at day 0, 3 and 5 and the growth curves were compared. No significant differences were observed for either of the cell lines. The assay was also performed incubating the cells in conditioned media from either astrocytes or murine osteoblasts (MC3T3-E1) to examine a possible influence of the brain or bone microenvironment on the proliferative behavior. However, no significant differences in proliferation were observed in any of the assays.

4. Anchorage-independent growth

The ability of cells to grow under anchorage independent conditions was studied by performing a soft agar assay. 1000 cells were seeded and the amount of colonies exceeding a diameter of 100 µm were counted 14 days after seeding. The HTB56 HERC5 overexpression cell line grew significantly less colonies in comparison to the empty vector control cells ($p=0.021$; $n=5$, fig. 1A); indicating a less aggressive phenotype, whereas the A549 K.O. cell lines did not show any significant differences. No colonies were formed in soft agar by the H1395 cell line, making an analysis of differential growth impossible.

5. Clonogenic potential

The colony formation assay can be used to determine the ability of single cells to grow into a colony and therefore to undergo unlimited division. The assay was performed using standard culture media for either HTB56, H1395 or A549. Furthermore, the cells were also grown in conditioned astrocytes or MC3T3-E1 media and their respective media to serve as a control. The HTB56 OE cells show a significantly lower clonogenic growth ($p<0.0001$, n=5, fig. 1B). The difference in clonogenic growth is, however, not increased by the brain and bone microenvironment. Neither the modified cell lines H1395 nor A549 showed any significant differences in clonogenic potential.

6. Migratory potential

The influence of differential HERC5 expression on the migratory capability was analyzed in both cell lines by seeding HTB56, H1395 and A549 cells in boyden chambers. The migration assay was performed by seeding either astrocytes or MC3T3-E1 cells at the bottom of the wells or by simply using culture media containing FCS while the cells in the boyden chambers were FCS deprived. The amount of migrated cells was counted 16h after seeding. The HTB56 OE cells have a significantly lower migratory capability than the empty vector control cells ($p=0.012$, n=5, fig. 1C) but astrocytes and pre-osteoblasts do not influence migratory behavior. The A549 parental cells also have a significantly lower migratory capability than the K.O. cells ($p=0.042$, n=3, fig. 1D). The astrocyte or pre-osteoblast environment does not result in significant differences in migration. When using H1395 cells, no migrated cells were found in the FCS controls. Therefore, migratory capacity could not be assessed and was not performed using astrocytes or pre-osteoblasts.

7. Invasive potential

The invasive potential can be a linked to the metastatic capacity of cells. To study this capacity, an invasion assay was performed using the overexpression and K.O. cell lines. Due to the negative results of H1395 cells in the soft agar and migration assays, it was assumed that this rather non-aggressive cell line is not suitable for invasion assay. Both HTB56 O.E./empty vector control and A549 K.O./parental cell lines did not show any significant differences in invasive potential.

8. RNAseq

To study potential alterations in downstream pathways resulting from HERC5 expression changes gene expression was assessed by RNAseq. For this, total mRNA of the cell lines HTB56 O.E./empty vector control were used in triplicates. Results show a significant upregulation of *HERC5* (control), *FGF7* and a significant downregulation of *COL12A1*. Using DAVID database, functional annotation clustering was performed to look for enriched and functionally-related gene groups but no significant cluster enrichment was found.
A possible explanation for the low amount of significantly regulated genes could be that HERC5, which acts as the main E3 ligase for ubiquitin-like modifier ISG15 in the cell, might be predominantly regulated on the protein level rather than the mRNA level and is described to be only induced after viral infection or activation of the inflammasome.

9. HERC5 induction by cytokines

As HERC5 serves as a regulator of the innate immune system and plays a role in viral infections, it can be induced by cytokines such as interferons. To study whether this can be conferred on the inflammatory microenvironment of lung cancer patients or on different lung cancer cell lines, HERC5 levels were assessed by RT-qPCR and western blotting after treatment with pro-inflammatory cytokines IFNγ and TNFα. Both cytokines led to an induction of HERC5 in A549 cells, however, their combination gave a much strong induction of HERC5 expression, but not in the corresponding K.O. cell line. Furthermore, ISG15 protein levels were increased as well as the amount of ISGylated proteins in A549 parental cells, but hardly in the HERC5 K.O. cell line, indicating that HERC5 acts as the main ISG15 ligase also in A549 lung cancer cells.

10. Summary and future studies:

The project has proceeded according to the plans and functional assays showed that HERC5 is involved in features rendering the cells more aggressive. The bone and brain microenvironment does not seem to have a large influence on this behavior.

Ongoing studies include a SILAC-based analysis of immune-precipitated proteins after induction of inflammation with IFNγ and TNFα using ISG15 as bait. The A549 parental and K.O. cell lines will be used to analyze proteins that have been targeted by HERC5, hopefully further elucidating its role in lung cancer progression.

Furthermore, a cell line model of functionally inactive HERC5 is being established by using site-directed mutagenesis of the active site. By introducing a C994A mutation and repeating functional assays mentioned above, the role of the E3-ligase activity with regard to its metastatic potential will be further elucidated.

In the future, we are planning to analyze the effect of immune cells on the differential expression of HERC5. Both overexpression and knock-out cell lines will be used in the assays mentioned before in the same way as the astrocytes or pre-osteoblasts were used.

Finally, *in vivo*-studies will be performed to analyze if HERC5 depletion leads to a more aggressive phenotype. Writing allowances for animal experiments is already planned.

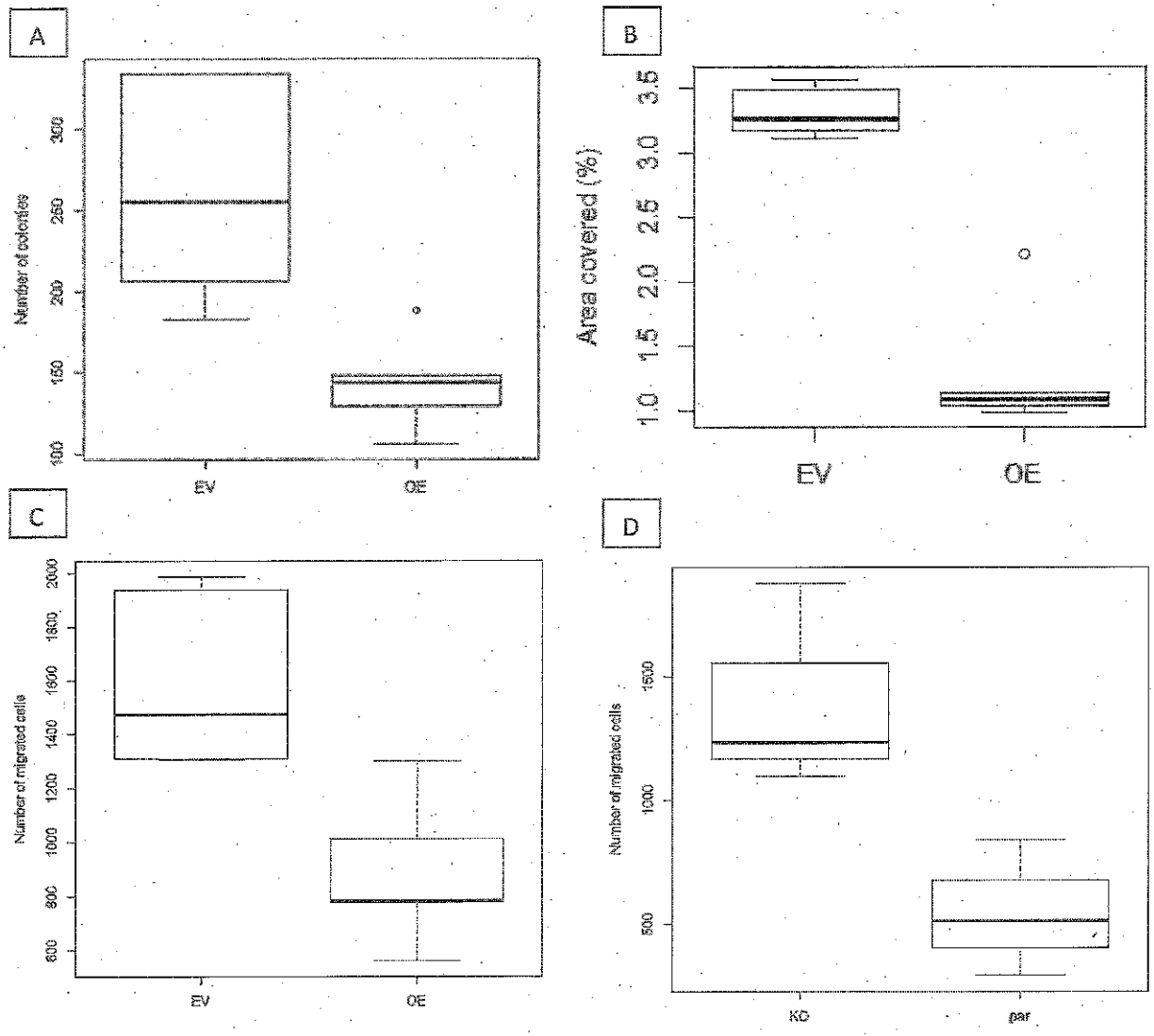

Figure 1: A) Anchorage-independent growth of HTB56 empty vector (EV) and HERC5 overexpressing (OE) cells (p=0.021) **B)** Clonogenic potential of HTB56 EV and OE cells (p<0.0001) **C)** Migratory potential of HTB56 EV and OE cells (p=0.012) **D)** Migratory potential of A549 HERC5 knock-out (KO) and parental (par) cells (p=0.042)