The functional importance of the intra-tumoral heterogeneity for extracellular vesicle release and uptake in colorectal cancer

Doctoral Thesises

Andrea Kelemen

Semmelweis University
Doctoral School of Molecular Medicine

Supervisor:
Zoltán Wiener, Ph.D., associate professor

Opponents:
Ágnes Enyedi, Ph.D., senior research fellow
Krisztina Vellainé Takács, Ph.D., associate professor

Head of the Complex Examination Committee:
Dóra Szabó, MD, D.Sc, professor

Members of the Complex Examination Committee:
Mónika Lippai, Ph.D., senior lecturer
Balázs Mayer, Ph.D., senior research fellow

Budapest
2022.

1.Introduction

Colorectal cancer (CRC) is one of the most prevalent cancer types. In addition, its high mortality rate also highlights the importance of studying this major public health problem. According to the Vogelstein model, the first and the most common driver mutation in patients (over 80 % occurrence) is the inactivation of the adenomatous polyposis coli (*APC*) gene. This mutation in the *APC* gene leads to the constitutive and ligand independent activation of the Wnt pathway which causes an uncontrolled cell proliferation. Intratumoral heterogeneity (ITH) is a commonly overlooked hallmark of cancer that causes major difficulties in the treatment. It is a well-known fact that the tumor itself is not just a simple mass of uniform cells. It has several cell types beside the cancer cells, such as fibroblasts, immune cells, and endothelial cells. Interestingly, there are subpopulations with different functions among cancer cells, too. Understanding the critical role of this intra-tumoral cellular heterogeneity in drug resistance, relapse and metastasis has just started to emerge.

Extracellular vesicles (EV) are membrane-surrounded vesicles that are released by virtually all cell types and they participate at intercellular communication. EVs can be clustered according to their cellular origin: whereas the smallest exosomes are derived from the multivesicular bodies, microvesicles are shed from the plasma membrane. EVs are also frequently clustered based on their size and isolation methods: large, medium-sized (mEV) and small EVs (sEV). Since they carry biologically important molecules as cargo in a protected form, they are

a potential tool to deliver molecules to target cells. Moreover, the EV cargo may reflect the molecular composition of the releasing cells and thus, EVs may hold a great promise for tumor diagnostics. Despite their potential importance in clinical applications, heterogeneity for EV release and uptake in CRC has not yet been addressed.

Thus the major aim of my dissertation is the characterization of ITH for EV release and uptake, focusing on markers that are connected to the Wnt pathway and that markcell subpopulations of aggressive features. CD44 is a glycoprotein with a single transmembrane domain, localized in the plasma membrane. The expression of CD44 is upregulated in several types of cancers and it is a well described molecular marker for cancer stem-like cell subpopulations with aggressive features. Transcription of CD44 is partially activated by Wnt signaling, and its overexpression is an early event in the transition from colorectal adenoma to carcinoma. CD133, also called Prominin-1, is a five-transmembrane protein that is located in the plasmamembrane of cellular protrusions and microvilli. CD133 is one of the first documented cancer stem-like cell markers in a wide array of tumor types. In addition, CD133 plays an inductor role in the Wnt pathway as the suppression of this protein has been described to inhibit the localization of β-catenin into the nucleus and the activation of the Wnt pathway. Inactive tyrosine-protein kinase 7 (PTK7), also called colon carcinoma kinase 4 (CCK4), is a member of the receptor protein tyrosine kinase (RTK) family. Several studies

described that this protein is highly expressed in many cancers, in addition, PTK7+ CRC cells have a high Wnt and colony-forming activity in cell line models, suggesting that PTK7 marks an aggressive CRC cell population. IFITM1 is localized in the plasma membrane and interacts with other transmembrane proteins, such as tetraspanins (e.g. CD81). In a few studies, it has been reported that elevated IFITM1 expression correlates with worse outcomes and more aggressive phenotypes in CRC. Increased IFITM1 expression promoted, whereas decreased IFITM1 expression inhibited cell migration and tumorigenicity *in vitro*.

2. Objectives

1. Are there subpopulations in CRC which release EVs at different level?
2. Do the subpopulation derived EVs have different miRNA cargo?
3. How do they contribute to fibroblast activation and tumor progression?

4. Which marker could characterize CRC cells with differential EV uptake?
5. Do the subpopulations have different proliferation potential?
6. What is the effect of the differential EV uptake in tumor cell proliferation?
7. Does the marker has also a functional importance in EV uptake?

3.Methods

Organoid isolation and culturing

Tissue samples were isolated at the Uzsoki Hospital with an ethical permission and written informed consent was obtained from all patients. The samples were mechanically and enzymatically dissociated, and they were then embedded in 3D matrix (Matrigel). The tumor and adenoma organoids received no external Wnt ligands (R-spondin and Wnt-3a) to select for cells with *APC* mutation. Normal colon organoids required also R-spondin and Wnt-proteins for survival and proliferation.

Cell sorting

Organoids were removed from Matrigel and single cell suspension was prepared. They were labelled for the above mentioned markers and they were sorted into subpopulations with high and low level of the respective marker. The sorted cells were used for further analysis.

Whole-mount immunostaining

CRC organoids were cultured in 8-well chamber slides and fixed in 4% paraformaldehyde (PFA) for 20 min and washed with PBS. Blocking and permeabilization were carried out in whole-mount blocking buffer. Samples were incubated with primary antibodies at 4 °C overnight. After washing in PBS, labelled secondary antibodies were added overnight at 4 °C. The organoids were then mounted with mounting medium that contained DAPI and they were then analyzed with confocal microscopy. Images were evaluated by the ImageJ software.

EV isolation and characterization

EV isolation was performed by differential centrifugation from conditioned media. Both mEVs and sEVs were used. mEV and sEVs were isolated at 12 500g and 100 000g centrifugation steps, respectively. We analyzed EVs with the semi-quantitative bead-based method and flow cytometry, Nanoparticle tracking analysis (NTA), transmission electronmicroscopy (TEM) and capillary based Simple Western Blotting method (WES).

Real Time qPCR

Real-time quantitative PCR was used for the characterization of the gene expression profile of the sorted subpopulations. The primers were designed by our research group. We used GAPDH and HPRT1 genes as housekeeping controls.

Taqman Low Density Array (TLDA)

Conditioned media from CRC organoids were harvested, anti-CD63 and anti-CD81-coated beads were added to the supernatant after centrifugation, and the samples were incubated for 16 h at +4 °C. EVs bound to beads were then lysed. Total RNA was isolated with the miRNEasy Micro Kit (Qiagen) following the manufacturer's protocol.

The RNA was reverse transcribed with Megaplex RT primers, the samples were pre-amplified with Megaplex PreAmp Primers (Thermo Fisher) and samples were loaded onto TaqMan™ Array Human MicroRNA A Cards v2.0 (Thermo Fisher). Cards were then measured on an ABI 7900HT instrument according to the manufacturer's protocol.

CRISPR/Cas9 knockout system

Single cells produced from CRC organoids were suspended in the tranfection reagent according to the manufacturer's protocol (Amaxa SF Cell Line 4D-Nucleofector X Kit S, Lonza) in the presence of 1 µg IFITM1 CRISPR/Cas9 KO and 1 µg IFITM1 CRISPR/Cas9 HDR plasmids (Santa-Cruz Biotechnology, sc-416878 and sc-416878-HDR). Cells were electroporated with a 4D-Nucleofector (Lonza). Cells were then embedded in Matrigel in the presence of CRC medium. Successfully targeted cells were selected by puromycin. Organoid cells were used in our experiments >14 days after nucleofection.

Statistical analysis

Student's paired or unpaired t-tests, ANOVA, Mann–Whitney U-test or Kruskal–Wallis with Dunn post hoc test were applied with $*p < 0.05$, $**p < 0.01$ and $***p < 0.005$ significance levels. Microsoft Excel, and GraphPad softwares were used for statistical evaluation. For bioinformatical analysis, we used the NormFinder algorithm and custom Python scripts.

4.Results

When studying ITH for EV release, we found that $CD44^{high}$ cell-derived organoids released more EVs compared to the $CD44^{low}$ subpopulation. We observed this difference in different 3D matrices (Matrigel that is rich at laminin and collagen IV, collagen I, and the mixture of them at 1:1 ratio). In contrast, we found no difference in EV secretion in case of cell subpopulations with different CD133 and PTK7 levels. Since we normalized the EV

concentration for cell number, thus, our results did not reflect the different proliferation capacities of the cells. Collectively, our data suggest that high CD44 expression marks a CRC subpopulation with high EV release.

As the next step, we compared the cargo of EVs from the different sorted subpopulations. Based on our previous data we used anti-CD63 and anti-CD81-coated beads to capture EVs from the supernatant of the organoids. This method provided more pure EV samples with lower unspecific miRNA background compared to other methods. After the purification, we used TaqMan low-density miRNA arrays. As negative controls, we collected supernatants from cell-free Matrigel samples, the detected miRNAs were considered as background, and they were excluded from further analysis. We focused only on miRNAs that were present in all biological parallels at least in one of the different subpopulations. We analyzed the presence and expression of 377 miRNAs, and we detected 26, 26, and 19 miRNAs when examining the overlap between organoids with different CD44, CD133, or PTK7 levels, respectively. We found only one miRNA differentially present in $CD133^{high}$ and $CD133^{low}$ organoid-derived EVs and no miRNAs were found to be specific for $PTK7^{high}$ or $PTK7^{low}$ CRC cell-derived EVs. Interestingly, our analysis indicated that miR-95, miR-100, and miR-365 were specific for the $CD44^{high}$ and miR-345 for the $CD44^{low}$ organoid-derived EVs. Since we found a significant overlap in the miRNA cargos between the respective subpopulations, we normalized EVmiRNAs to miR-19b levels. We used these normalized fold change values for linear discriminant analysis (LDA) to find

miRNAs with differing levels between the experimental groups. Based on these results we concluded that EVs released by CRC subpopulations had only a marginal difference in their miRNA cargo.

To study the function importance of the differential miRNA cargo and secretion intensity of EVs from $CD44^{high}$ and $CD44^{low}$ CRC cells, we examined the effects of EVs on fibroblasts. Interestingly, when using EV amounts normalized to cell number, EVs secreted by $CD44^{high}$ organoids induced a higher proportion of KI67+ proliferating colon fibroblasts compared to $CD44^{low}$ cell-derived EVs. This proliferation capacity difference was not observed when testing EVs from identical numbers of $CD133^{high}$ and $CD133^{low}$ or $PTK7^{high}$ and $PTK7^{low}$ cells. To decide whether this effect was a purely dose-dependent phenomenon or based on the differential miRNA cargo, we repeated the experiments with an increasing amount of EVs isolated from $CD44^{high}$ and $CD44^{low}$ CRC cell subpopulations. Of note, we observed that the percentage of KI67+ fibroblasts depended on the EV concentration, but it was independent of whether they had been isolated from $CD44^{high}$ or $CD44^{low}$ organoids.

These results indicate that it is not the differential miRNA cargo, but the number of EVs that is the critical factor. Furthermore, the expression level of activation markers in colon fibroblasts depended on the number of EVs, and again, we could not find a difference between $CD44^{low}$ and $CD44^{high}$ cell-derived EVs. Interestingly, the increasing amounts of liposomes that were artificially produced and contained no miRNA cargo had a similar, dose-dependent

effect on both the proliferation rate and the expression level of activation markers in fibroblasts**,** suggesting that the common miRNA cargo of CD44 subpopulation cell-derived EVs is not critical either. Collectively, these results indicate that: (i) fibroblast activation is induced by EVs or liposomes in a dose-dependent manner; (ii) the higher EV secretion by $CD44^{high}$ CRC cells, and not the differential miRNA cargo is important in the differential effects of EVs derived from $CD44^{high}$ and $CD44^{low}$ cells.

As the next step, we aimed at identifying CRC cell subpopulations that take up EVs differently. Previously we had identified a set of genes that had been activated by the loss of p53 and the activation of the Wnt pathway (the p53-suppressed invasiveness signature, PSIS) and which may account for the induction of invasiveness. To find genes that may be involved in the malignant behavior of CRC cells and EV uptake by CRC cells, we focused on IFITM1, a member of the PSIS gene set and the interferon-induced gene family with cell surface localization. Our bioinformatical data analysis showed that IFITM1 is highly overexpressed in CRC samples compared to normal colon and rectum, suggesting that IFITM1 is regulated by the Wnt pathway. To test this hypothesis, previously our research group established organoid cultures from wild-type (WT) mouse small intestine and introduced an *Apc* mutation to constantly activate the Wnt pathway. We performed gene expression analysis in these organoids. As expected, highly elevated RNA levels of the known Wnt targets *Lgr5*, *Axin2*, and *Myc* were observed. In addition, *Apc* mutation resulted in a marked increase in the expression of *Prox1*, an intestine-specific Wnt target gene,

and, surprisingly, we detected a more than 2,500-fold increase in the expression of *Ifitm1*.

To further study the regulation of IFITM1, we isolated organoids from the normal colonic (NCO) and the tumor (CRCO) samples of CRC patients. In addition, we collected samples from patients diagnosed with colon adenoma (AO). AOs and CRCOs were cultured without the Wnt-agonist R-Spondin1 and Wnt3a, thus, samples were selected for organoids carrying *APC* mutation and harboring a constitutively active Wnt pathway. In our gene expression analysis, we detected higher RNA levels of not only *AXIN2*, *LGR5*, and *MYC*, but also of I*FITM1* in both AOs and CRCOs as compared to NCOs.

LGR5 marks a stem cell population in CRC and intestinal adenomas. To decide whether *Ifitm1* expression is specific for the Lgr5+ cells, we analyzed microarray data from sorted $Lgr5^{high}$ and $Lgr5^{low}$ mouse intestinal adenoma cells. Whereas the RNA levels of *Cd44* and *Cd133* were higher in the $Lgr5^{high}$ cell population, we found no difference in *Ifitm1*. Furthermore, whereas the RNA level of *CD133* was higher in $LGR5^{high}$ compared to $LGR5^{low}$ human CRC organoid cells in RNA expression datasets, we found no difference in *IFITM1* expression between the two cell populations. Collectively, these data suggested that *IFITM1* is under the regulation of the Wnt pathway, but it is not specific for the $LGR5^{high}$ CRC cells with stem cell features.

As the next step, we sorted CRC organoid cells based on their IFITM1 protein levels into $IFITM1^{high}$ and $IFITM1^{low}$ subpopulations. We counted the organoid

forming units in the subpopulations, however, surprisingly, we detected no difference in the numbers of organoids. The diameter of the organoids derived from IFITM1^{high} cells was significantly higher and they contained more KI67+ proliferating cells.

The IFITM1 cell surface protein is involved in inhibiting the uptake of membrane-surrounded viruses, raising the possibility that this molecule regulates EV uptake as well. Therefore, we studied whether cell populations with different Ifitm1 expression levels have different EV uptake abilities. To address this question, we cultured cells from wild type (WT) and *Apc* mutant mouse intestinal organoids in 2D conditions for short term and we added mEVs isolated from fibroblasts that had been pre-treated with a membrane labeling dye. Interestingly, *Apc* mutant organoids that express a higher level of *Ifitm*1, accumulated less fibroblast-derived mEVs compared to WT intestinal organoids. We next sorted IFITM1^{high} and IFITM1^{low} cells and we cultured them with labeled EVs. Of note, less IFITM1^{high} cells took up EVs both in the case of HT29 CRC cell-line-derived and human colon fibroblast-derived mEVs as compared to IFITM1^{low} CRC cells, detected by confocal microscopy. In addition, IFITM1^{high} cells that had taken up fibroblast-derived EVs showed a lower signal intensity for mEVs than IFITM^{low} cells. Thus, these results suggest that the difference in EV uptake is not restricted to one specific cell type-derived EVs.

We treated CRC organoid cells with fibroblast-derived labeled EVs in 2D cultures and then sorted cells with the

highest and lowest fluorescent signal, representing cell populations with high and low EV uptake ability, respectively. Whereas we found no difference in the expression of *AXIN2* and *MYC*, we measured a significantly higher RNA level of *IFITM1* in cells with low EV uptake. Since we found no increase in the expression of *IFITM1* in CRC cells after treatment with EVs, this confirms again that IFITM1^{high} cells take up less EVs.

Adding fibroblast-derived mEVs or sEVs to sorted IFITM1^{low} cells resulted in organoids with a markedly higher increase in the number of KI67+ cells as compared to IFITM1^{high} cell-derived organoids. In addition, the initial difference in the percentage of proliferating cells between the two CRC cell subpopulations disappeared after treatment with mEVs or sEVs. Thus, these results indicate that differential mEV or sEV uptake by CRC cell subpopulations critically modified the percentage of proliferating cells in an organoid model.

To test whether IFITM1 is functionally important for the enhanced proliferation rate and reduced EV uptake of cells with IFITM1^{high} expression, we inactivated this gene in CRC organoids by the CRISPR/Cas9 system. After 14 days selection for the genetically modified cells, both flow cytometry and immunocytochemistry proved the largely diminished level of IFITM1 in these cells. Of note, we observed a decreased diameter of organoids formed by IFITM1^{KO} cells. When using labeled human colon fibroblast EVs, we detected a markedly higher percentage of EV-positive cells derived from IFITM1^{KO} organoids

compared to IFITM1^{WT} cells, indicating that the lack of IFITM1 induced EV uptake. Similarly to the IFITM1^{low} and IFITM1^{high} populations, IFITM1^{KO} organoids showed a reduced percentage of KI67+ cells compared to IFITM1^{WT} organoids. However, fibroblast-derived mEVs or sEVs had a larger effect on the proliferation of IFITM1^{KO} organoids, indicating the functional effect of the increased EV uptake of CRC cells without IFITM1. Collectively, these data suggest that IFITM1 is not only a marker of a CRC cell population with reduced EV uptake ability, but this molecule is also functionally involved in inhibiting this process.

5.Conclusions

1. CD44^{high} CRC cells release more EV than CD44^{low} cells
2. CD44^{high} and CD44^{low} cell derived EV cargos differ only in a few miRNAs
3. CD44^{high} cell derived EVs induce the proliferation and the expression of activation markers in fibroblasts at a higher level compared to CD44^{low} cell EVs
4. However, this activation is dependent purely on the EV dose and independent from the miRNA cargo
5. IFITM1^{high} CRC cells form bigger organoids than IFITM^{low} CRC cells due to their higher proliferation capacity

6. IFITM1^{low} CRC cells take up more EVs than IFITM1^{high} CRC cells
7. Interestingly, IFITM1^{low} CRC cells are more responsive to the EV treatment than IFITM1^{high} CRC cells
8. IFITM1 plays a functional role in inhibiting EV uptake

Collectively, we identified CRC subpopulations with different EV releasing and uptake capabilities and we proved that this intratumoral heterogeneity has a functional role, too. Thus, we propose that intratumoral heterogeneity for EV uptake and release should be considered as a critical factor when designing targeted EV-based therapy in CRC.

6. Bibliography of the candidate's publications

Publications used for the dissertation:

A. Kelemen, I. Carmi, Á. Oszvald, P. Lőrincz, G. Petővári, T. Tölgyes, K. Dede, A. Bursics, E. I. Buzás, Z. Wiener, IFITM1 expression determines extracellular vesicle uptake in colorectal cancer. Cell. Mol. Life Sci. 78, 7009–7024 (2021).

A. Kelemen, I. Carmi, I. Seress, P. Lőrincz, T. Tölgyes, K. Dede, A. Bursics, E. I. Buzás, Z. Wiener, CD44 Expression Intensity Marks Colorectal Cancer Cell

Subpopulations with Different Extracellular Vesicle Release Capacity. Int. J. Mol. Sci. 23, 2180 (2022).

The cumulative impact factor of the publications used for the dissertation: 15.185

Other publications:

Á. Oszvald, Z. Szvicsek, M. Pápai, **A. Kelemen**, Z. Varga, T. Tölgyes, K. Dede, A. Bursics, E. I. Buzás, Z. Wiener, Fibroblast-Derived Extracellular Vesicles Induce Colorectal Cancer Progression by Transmitting Amphiregulin. Front. Cell Dev. Biol. 8 (2020), doi:10.3389/fcell.2020.00558.

Á. Oszvald, Z. Szvicsek, G. O. Sándor, **A. Kelemen**, A. Á. Soós, K. Pálóczi, A. Bursics, K. Dede, T. Tölgyes, E. I. Buzás, A. Zeöld, Z. Wiener, Extracellular vesicles transmit epithelial growth factor activity in the intestinal stem cell niche. Stem Cells. 38, 291–300 (2020).

Z. Szvicsek, Á. Oszvald, L. Szabó, G. O. Sándor, **A. Kelemen**, A. Á. Soós, K. Pálóczi, L. Harsányi, T. Tölgyes, K. Dede, A. Bursics, E. I. Buzás, A. Zeöld, Z. Wiener, Extracellular vesicle release from intestinal organoids is modulated by Apc mutation and other colorectal cancer progression factors. Cell. Mol. Life Sci. 76, 2463–2476 (2019).

J. C. Sági, B. Egyed, **A. Kelemen**, N. Kutszegi, M. Hegyi, A. Gézsi, M. A. Herlitschke, A. Rzepiel, L. E. Fodor, G. Ottóffy, G. T. Kovács, D. J. Erdélyi, C. Szalai, Á. F. Semsei, Possible roles of genetic variations in

chemotherapy related cardiotoxicity in pediatric acute lymphoblastic leukemia and osteosarcoma. BMC Cancer. 18, 704 (2018).

J. C. Sági, N. Kutszegi, **A. Kelemen**, L. E. Fodor, A. Gézsi, G. T. Kovács, D. J. Erdélyi, C. Szalai, Á. F. Semsei, Pharmacogenetics of anthracyclines. Pharmacogenomics. 17, 1075–1087 (2016).

A. Rzepiel, N. Kutszegi, J. Cs. Sági, **A. Kelemen**, K. Pálóczi, Á. F. Semsei, E. Buzás, D. J. Erdélyi, Extracelluláris vesiculák és hematológiai malignitásokban játszott szerepük. Orv. Hetil. 157, 1379–1384 (2016)

Cumulative impact factor: 40.274

Cumulative impact factor of the first author publications: 15.185