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Analysis of tumor-draining vein secretome: A direct access to tumor-derived extracellular vesicles in surgical lung cancer patients

YANGYI HE[1,2]; DAVID SANCHEZ-LORENTE[3,4,5]; MELISSA ACOSTA-PLASENCIA[1]; MARC BOADA[3,4,5]; ANGELA GUIRAO[3,4,5]; RAMON M. MARRADES[4,5,6,7]; LAUREANO MOLINS[3,4,5]; ALFONS NAVARRO[1,4,5,*]

[1] Molecular Oncology and Embryology Laboratory, Department of Surgery and Surgical Specializations, Human Anatomy Unit, Faculty of Medicine and Health Sciences, Universitat de Barcelona (UB), Barcelona, 08036, Spain
[2] School of Basic Medical Sciences, Chengdu University, Chengdu, 610106, China
[3] Department of Thoracic Surgery, Hospital Clínic de Barcelona, Universitat de Barcelona (UB), Barcelona, 08036, Spain
[4] Thoracic Oncology Unit, Hospital Clinic, Barcelona, 08036, Spain
[5] Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Barcelona, 08036, Spain
[6] Department of Pneumology, Institut Clínic Respiratori (ICR), Hospital Clínic de Barcelona, Universitat de Barcelona (UB), Barcelona, 08036, Spain
[7] Centro de Investigación Biomédica en Red de Enfermedades Respiratorias (CIBERES), Instituto de Salud Carlos III, Madrid, 28029, Spain

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Abstract: Tumor-secreted extracellular vesicles (EVs) participate in the metastasis process through different mechanisms, including the preparation of the pre-metastatic niche to grant circulating tumor cells (CTCs) implantation and growth. The study of the metastasis process through the analysis of CTCs and tumor-derived EVs is difficult because of the dilution grade of these elements in peripheral blood. In early-stage lung cancer patients, the tumor-secreted products are even more diluted. An attractive strategy in surgical lung cancer patients is to purify them from a pulmonary tumor-draining vein where they are enriched. The information obtained from the analysis of EVs and CTCs purified from this source could give more accurate information about tumor biology and could be an important source of biomarkers to identify patients at high risk of relapse after curative surgery.

Introduction

Lung cancer remains one of the top leading causes of cancer-related deaths in 2022. According to GLOBOCAN statistics, lung cancer caused 18% of cancer-related deaths worldwide in 2020 (Sung *et al.*, 2021). Most of the patients are diagnosed with non-small cell lung cancer (NSCLC), the most common type of lung cancer (Jemal *et al.*, 2011). The 5-year survival of patients diagnosed with NSCLC cancer is still poor with only a median of 26% of survival (Allemani *et al.*, 2018). Treatment strategy depends mainly on the disease stage and in localized tumors, which can be treated with surgery, and the 5-year survival can increase up to 64%. Surgical resection is possibly the most curative therapeutic option for early and even locally-advanced stages (Vansteenkiste *et al.*, 2013). Nevertheless, around 35% of resected NSCLC patients develop recurrence and die of their disease (Hofman *et al.*, 2011; Vokes, 2000). So surgical treatment is less than perfect, even if a complete macroscopic resection can be performed (Hashimoto *et al.*, 2014). Numerous studies have tried to determine the elements involved in post-surgical relapse and recently the focus is on circulating tumor cells (CTCs) and tumor-secreted small extracellular vesicles (EVs). Small EVs term is used to define a group of vesicles ranging from 30 to 150 nm purified only according to physical characteristics like size (f.i. by ultracentrifugation) and includes two different populations: exosomes and microvesicles (Théry *et al.*, 2018). Small EVs are a heterogeneous group of cell-derived membranous structures, which originate from the endosomal system in the case of exosomes or are shed from the plasma membrane in the case of microvesicles (Théry *et al.*, 2009; van Niel *et al.*, 2018). Now, it has been proved that EVs, especially exosomes, play a crucial role in both local and distant intercellular communication.

Circulating tumor cells, extracellular vesicles, and metastasis

CTCs and tumor-derived EVs are the main actors in the metastasis process (Jerabkova-Roda *et al.*, 2022). During cancer progression, some tumoral cells detach from the

*Address correspondence to: Alfons Navarro, anavarroponz@ub.edu
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main tumor and can attach to endothelial cells to enter the circulation. Likewise, the primary tumor can communicate with CTCs and "control" them by releasing EVs to the circulation that can target the CTCs (Fu *et al.*, 2018; Ghoroghi *et al.*, 2021a). Moreover, EVs themselves participate in the development of the premetastatic niche (Ghoroghi *et al.*, 2021a; Peinado *et al.*, 2017). In fact, it is widely accepted that priming with EVs precedes secondary tumor growth and is an important step in the cancer metastasis process (Peinado *et al.*, 2017).

Recently, it has been shown that tumors exploit transmembrane cell adhesion molecules (CAMs), which participate in cell-to-cell interactions (Berrier and Yamada, 2007; Geiger *et al.*, 2009), to direct CTCs and EVs migration toward a specific organ (organotropism). EVs and CTCs share similar CAMs that allow their own interaction and at the same time direct both to the same metastatic site. Moreover, these CAMs participate in the adhesion to the endothelium during the extravasation process (Ghoroghi *et al.*, 2021b; Osmani *et al.*, 2019).

Taking into account all this information, it is clear that characterizing the CTCs and tumor-secreted EVs can allow a better comprehension of the metastasis process and they can act as a source of relapse biomarkers to identify patients at high risk of relapse, and even identify the potential metastatic site before it is clinically detectable (Gold *et al.*, 2015). But the question is, can this be properly performed in peripheral blood?

Tumor-draining vein as an enriched source of circulating tumor cells and extracellular vesicles

Despite great efforts in the development of more sensitive and efficient CTC isolation platforms, due to their ambiguity, rarity, and heterogeneity, isolation of CTCs remains a challenge due to their low abundance in peripheral blood (Bu *et al.*, 2016). Despite great efforts in the development of more sensitive and efficient CTC isolation platforms, due to their ambiguity, rarity, and heterogeneity, isolation of CTCs remains a challenge due to their low abundance in peripheral blood (Bu *et al.*, 2016). This is even more problematic in early-stage patients with no disseminated disease (Murlidhar *et al.*, 2017). Similarly, EVs, are released by virtually all cell types and not only by tumor cells (Ludwig and Giebel, 2012) producing an important dilution effect on peripheral blood (Sharma *et al.*, 2018) and requiring the identification of appropriate surface markers to allow the correct purification of the tumoral ones (Beltraminelli *et al.*, 2021; Hoshino *et al.*, 2020). So far, between the pool of identified surface exosomal proteins, the integrin family has emerged as one of the important elements involved in direct organ-specific colonization and pre-metastatic niche development (Hoshino *et al.*, 2015). However, their role in the specific purification of tumor-derived EVs has not been properly explored. Several authors, including our group, have explored the effectiveness of using tumor-draining vein (TDV) as an enriched source of tumor-secreted products for refining the detection of CTCs and tumor-derived EVs. To understand the importance of TDV, we need to review how pulmonary circulation works. Anatomically, the lung can be divided into lobules and subdivided into segments. The venous blood from each segment is collected by a segmental vein. Segmental veins converge towards pulmonary veins. There are four pulmonary veins: right superior pulmonary vein (collects blood from the upper and middle lobes of the right lung), right inferior pulmonary vein (collects blood from the lower lobe of the right lung), left superior pulmonary vein (collects blood from the upper lobe and lingula of left lung), and the left inferior pulmonary vein (collects blood from the lower lobe of the left lung). Finally, pulmonary veins drain blood to the left atrium of the heart from where it is widely distributed peripherally (Porres *et al.*, 2013). When a tumor is found in a specific lung lobule/segment, most cancer cells will disseminate through its specific pulmonary vein (Dudek and Louis, 2013). Therefore, the collection of blood from the pulmonary vein draining from the lobe where the tumor is located would allow the collection of tumor-secreted products (Buscail *et al.*, 2019) (Fig. 1). Confirming this hypothesis, numerous studies have shown that blood obtained from TVD is enriched in tumor-secreted products, including proteins (Geary *et al.*, 2019), CTCs (Crosbie *et al.*, 2016; Hashimoto *et al.*, 2014; Hattori *et al.*, 2019; Murlidhar *et al.*, 2017; Reddy *et al.*, 2016) and even small EVs (Castellano *et al.*, 2020; Han *et al.*, 2022; Navarro *et al.*, 2019) (Fig. 2). Geary *et al.* (2019) used blood from pulmonary TDV to profile the tumor secreted proteins and observed that the identified proteins correlated with features of the natural history of the tumor *in situ*. Many proteins were observed enriched in the TVD in comparison to a pulmonary vein from a non-cancerous lobe. They used the identified proteins to generate a panel biomarker of utility in the early diagnosis of lung cancer (Geary *et al.*, 2019). Several groups have quantified CTCs in TDV to evaluate their potential as a prognostic biomarker, especially to identify patients at high risk of relapse after a curative surgery. Sienel *et al.* (2003) analyzed CTCs in TDV in a

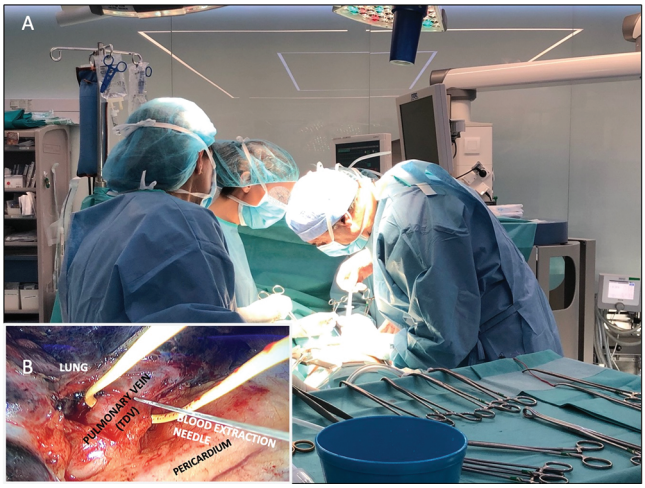

FIGURE 1. Intraoperative picture of the surgical procedure. (A) The image shows the moment when the surgeon is performing the extraction of blood from the pulmonary tumor-draining vein at the time of operation on the lung cancer to perform tumor resection. (B) Detail of the surgical field showing the pulmonary vein, the extraction needle, and the lung lobule where the tumor to be resected is located.

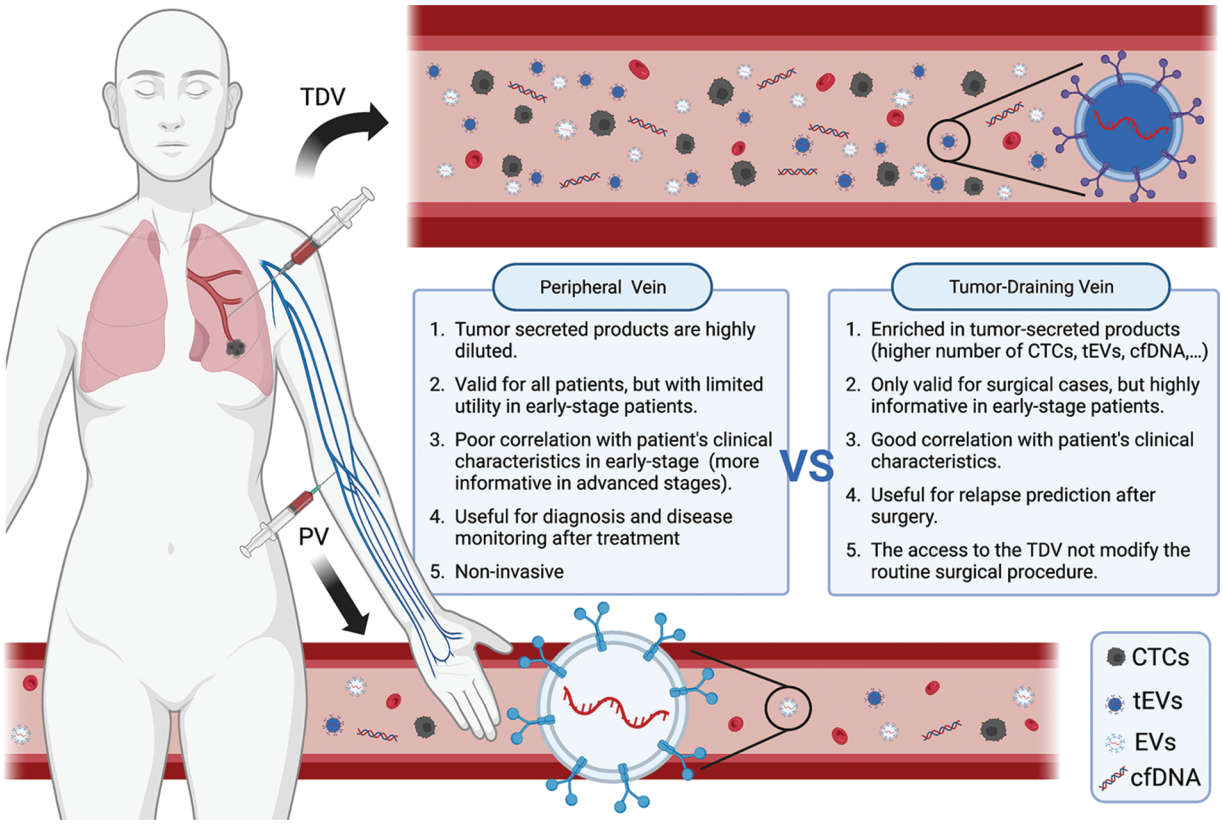

FIGURE 2. Scheme representing the main differences between peripheral and tumor-draining vein blood, especially in relation to circulating tumor cells (CTCs) and tumor-derived extracellular vesicles (EVs) content. Created with BioRender.com.

cohort of 62 patients; Funaki *et al.* (2011) studied 92 patients; Franco *et al.* (2012) analyzed 45 patients; and Crosbie *et al.* (2016) 30 patients. All these studies validate the superiority of the TDV in front of the peripheral vein at both the detection of CTCs and the capacity of relapse prediction.

Analysis of extracellular vesicles in the tumor-draining vein

Although the analysis of CTCs in TDV has been extensively analyzed, few groups have studied this source EVs. Our group (Navarro *et al.*, 2019), first quantified and analyzed the EVs size distribution in TDV in comparison with the paired peripheral vein. While we expected to find higher levels of EVs in TDV than in PV, we did not observe significant differences in quantity, but otherwise, differences in the size distribution of EVs were observed. A higher proportion of small-size EVs (30–50 nm) was found in TDV. Assessment of the relationship of the specific levels in each patient and the main clinical characteristics requires to be performed in the operating room and it is shown that the level of EVs in TDV were highly correlated with the disease stage, while no correlation was observed in PV. Additionally, the size of EVs from the pulmonary vein was significantly smaller in patients with relapsed compared to patients with non-relapsed lung cancer, while those differences were not detected in the peripheral vein (Navarro *et al.*, 2019). Choi *et al.* (2020) confirmed that quantification of EVs in pulmonary TDV showed a higher correlation with the disease stage compared to those in the peripheral vein. In their study, Choi *et al.* (2020) performed both *in vivo* (rabbits with lung cancer) and *ex-vivo* studies to validate the properties of TDV in relation to EVs quantification. Their study showed that EVs levels from TDV in both animals and patients having lung cancer were significantly higher than those in preoperative peripheral blood (Caivano *et al.*, 2015; Choi *et al.*, 2020). In both studies, Navarro and Choi showed that EV levels in the pulmonary TDV blood reflect more strictly the tumor stage and could be used as a superior prognostic tool for lung cancer patients who had undergone surgery (Choi *et al.*, 2020; Navarro *et al.*, 2019).

The study of the pulmonary TDV-derived EVs cargo is of great interest. EVs cargo contains assorted molecules like DNA, mRNAs, microRNA (miRNA), circRNAs, proteins, enzymes, and many more molecules, that can potentially cause epigenetic manipulations in the target cells (Kanada *et al.*, 2016; Vahabi *et al.*, 2022). One of the most enriched products in EVs are miRNAs (representing around 40% of RNA content) (Yuan *et al.*, 2016), which have recently gained significant attention as they were found to cause epigenetic regulation through translational silencing and mRNA instability (Ekstrom *et al.*, 2012; Qing *et al.*, 2018). Our group recently analyzed in a study by Han *et al.* the miRNA cargo of EVs purified from pulmonary TDV by small RNAseq and identified an miRNA signature composed of 17 EV-miRNAs able to predict relapse in NSCLC patients who had undergone surgery. The study of the miRNA with the highest levels, EV-miR-203a-3p, allowed its validation in an independent cohort of 70 patients the prognostic role of this EV-miRNA in TDV. When this EV miRNA was quantified in the paired

TABLE 1

Summary of tumor-draining vein EVs biomarkers

Biomarker	Method of detection	Outcome	Major findings	Reference
EVs quantification and measurement	Nanoparticle tracking analysis	Relapse, TTR, OS	The analysis of EVs size using the Mode value (cutoff <112 nm) was correlated with relapse and patient outcome. Patients with a higher quantity of smaller EVs had a worse outcome.	(Navarro *et al.*, 2019)
EV-miR-203a-3p	Small RNAseq and qRT-PCR	TTR	Patients with high TDV EV-miR-203a-3p had a shorter TTR than patients with low levels.	(Han *et al.*, 2022)
EV-lincRNA-p21	qRT-PCR	TTR, OS	High EV lincRNA-p21 levels were associated with shorter TTR and shorter OS.	(Castellano *et al.*, 2020)

Note: EVs: extracellular vesicles; TTR: time to relapse; OS: overall survival; qRT-PCR: quantitative real-time-polymerase chain reactio.

peripheral blood, we observed that in almost one-third of the patients, EV-miR-203a-3p was even not detected in peripheral blood and when detected, the levels in TDV were significantly higher (Han *et al.*, 2022). Before this screening analysis, our group in Castellano *et al.* (2020) analyzed the presence of a long non-coding RNA (lincRNA-p21) in TDV and also demonstrated its utility as a post-surgical relapse biomarker. We examined the role of lincRNA-p21 since previously we observed its utility as a biomarker when quantified in tumor tissues by regulating microvessel formation in the context of hypoxia (Castellano *et al.*, 2016). Unlike miRNAs, lncRNAs are RNA molecules longer than 200 nt with little or no protein-coding capacity (Wilusz *et al.*, 2009). Usually, lncRNA genes comprise fewer exons than mRNAs, since they are less constrained by selection during evolution; their expression is highly cell type/tissue-dependent showing a high tissue specificity (Cabili *et al.*, 2011) and a high cancer type specificity (Yan *et al.*, 2015). On analyzing lincRNA-p21 in EVs from lung cancer cell lines, we observed an overexpression under hypoxic conditions (Castellano *et al.*, 2016; Huarte and Rinn, 2010). Analysis of EVs purified from pulmonary TDV showed that patients with high EV-lincRNA-p21 had a worse outcome after surgery. Our *in vitro* studies demonstrated that EV-lincRNA-p21 promoted angiogenesis and modulated the EVs cargo, enriching EVs with pro-angiogenin miRNAs that might be transferred from tumor to endothelial cells through EVs (Castellano *et al.*, 2020; Castellano *et al.*, 2016). The main biomarkers described in tumor-draining vein EVs are summarized in Table 1.

Conclusions

The tumor-derived EVs cargo is enhanced in TDV because it is enriched in tumor-secreted products. However, much remains to be done in this promising area of research (Castellano *et al.*, 2020; Han *et al.*, 2022; Navarro *et al.*, 2019), which could be of special interest in early-stage tumors with low tumor dissemination grade. Nevertheless, some limitations need to be considered, especially when comparing its use with peripheral vein blood (Fig. 2). Compared to peripheral vein analysis, obtaining blood from the TDV can be considered invasive because requires to be performed in the operating room and it is limited to patients treated with surgery as a first treatment option. Yet, it is important to clarify that obtaining the TDV blood sample does not modify the routine surgical procedure. To obtain the blood, a gauge needle is used to puncture the visible pulmonary vein draining from the lobule/segment where the tumor is located. The surgeons need to locate and ligate the pulmonary vein prior to the process of the tumor resection anyway, irrespective of whether is going to extract pulmonary blood or not. Additionally, we must consider that this analysis is limited to NSCLC patients who undergo surgery and cannot be easily extended to advanced and even less to non-surgical NSCLC patients. However, as we have commented previously, TDV adds important information, especially in early-stage tumors, most of which are surgically treated, while in advanced stages the peripheral blood is already a valuable tool because of the higher dissemination grade of the disease in advanced stages.

Focusing on EVs, another limitation that is not exclusively associated with TDV, is that we need to consider the lack of a standard and reliable method for purifying EVs (Caivano *et al.*, 2015; Gardiner *et al.*, 2016). The EVs purification methodology needs to be standardized to improve its clinical application. The stability of EVs under different conditions in terms of maintaining viability and sample processing, allows them to be used in various fields without limitations (Kang *et al.*, 2017) being a good source for biomarker identification, although clinical standardization is required. Additionally, the increasing interest in the study of EVs isolated directly from tumors (Beltraminelli *et al.*, 2021; Gardiner *et al.*, 2016; Shi *et al.*, 2020) makes their study in TDV an excellent source, at least until the identification of appropriate surface markers that will allow specific purification of tumor-derived EVs. The clinical utility of quantification and characterization of EVs in TDV need further validation since most of the studies performed using TDV are retrospective studies that need to be confirmed in a prospective study, such as the ongoing clinical trial (NCT04939324) by the French group from the University Hospital of Limoges, that are recruiting early-stage NSCLC patients—who have undergone surgery—with the primary objective of evaluating size distribution, concentration and the molecular profiling of pulmonary vein exosomes and correlating them with patient characteristics and outcome. We will need to wait for the

results of this and other clinical trials to establish the clinical role of EVs on NSCLC patients having undergone surgery.

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