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THEME | *Extracellular Vesicles in Cell Physiology*

Extracellular vesicles as biomarkers and therapeutic targets for cancer

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Urabe F, Kosaka N, Ito K, Kimura T, Egawa S, Ochiya T. Extracellular vesicles as biomarkers and therapeutic targets for cancer. *Am J Physiol Cell Physiol* 318: C29–C39, 2020. First published November 6, 2019; doi:10.1152/ajpcell.00280.2019.—Extracellular vesicles (EVs) are small lipid membrane vesicles that are secreted from almost all kinds of cells into the extracellular space. EVs are widely accepted to be involved in various cellular processes; in particular, EVs derived from cancer cells have been reported to play important roles in modifying the tumor microenvironment and promoting tumor progression. In addition, EVs derived from cancer cells encapsulate various kinds of tumor-specific molecules, such as proteins and RNAs, which contribute to cancer malignancy. Therefore, the unveiling of the precise mechanism of intercellular communication via EVs in cancer patients will provide a novel strategy for cancer treatment. Furthermore, a focus on the contents of EVs could promote the use of EVs in body fluids as clinically useful diagnostic and prognostic biomarkers. In this review, we summarize the current research knowledge on EVs as biomarkers and therapeutic targets and discuss their potential clinical applications.

biomarker; cancer treatment; exosome; extracellular vesicles; therapeutic target

INTRODUCTION

Intercellular communication is essential for maintaining cellular functions and tissue homeostasis in multicellular organisms. This intercellular communication involves direct cell–cell contact or the transfer of secreted molecules, such as cytokines, chemokines, and growth factors (85, 115). Recently, extracellular vesicles (EVs) have gained considerable attention as a novel mechanism of intercellular communication. Especially in the cancer field, accumulating evidence shows that EVs play important roles in tumor dissemination (74), e.g., establishment of a premetastatic niche (82), promotion of angiogenesis (51), destruction of the peritoneum (117) or the blood-brain barrier (99), induction of drug resistance (112), and formation of the heterogeneity of cancer-associated fibroblasts (73). Consequently, intercellular communication via EVs in the tumor microenvironment is currently expected to be a novel therapeutic target (55). Furthermore, EVs have been reported to carry a variety of molecules, such as proteins and nucleic acids, that reflect the phenotype of their parental cells. In addition, as EVs are stably circulated in almost all kinds of body fluids, they also have great potential as useful disease biomarkers (104). Conventional blood-based tumor markers, such as carcinoembryonic antigen (CEA), cancer antigen 125 (CA125), and squamous cell carcinoma antigen (SCC-Ag), are not ideal for general use, especially for the early detection of these cancers (25,110, 118). CEA is the most frequently accepted tumor marker when gastrointestinal tract tumors are suspected. However, an increase in CEA concentration rarely occurs in the early stages of the disease; on the other hand, it is usually observed in the presence of late-stage tumors (62). In addition, CA125 is a widely examined serum marker for ovarian cancer (68); however, it is a poor indicator of early-stage ovarian cancer, only appearing in ~40% of patients at the early stage (37, 38). Furthermore, SCC-Ag levels have been associated with the advanced stage of non-small cell lung cancer (NSCLC); however, SCC-Ag is not a useful tumor marker for the early diagnosis of NSCLC because of its low sensitivity (45, 75). Therefore, additional biomarker resources are clinically required. To date, a number of studies have reported that EVs collected from cancer patients are associated with disease status and are detectable even in the early stage of cancer (54). In this review, we summarize the current research knowledge on EVs as biomarkers and therapeutic targets and discuss their potential for clinical applications in cancer treatment.

THE DEFINITION OF EXTRACELLULAR VESICLES

EVs can be generally classified according to their size and origin: exosomes (50–200 nm) (17), microvesicles (100–1,000 nm) (18), apoptotic bodies (50–4,000 nm) (9), and prostasomes (40–500 nm) (95) (Fig. 1). Exosomes originate from the inward budding of the endosomal membrane and are secreted

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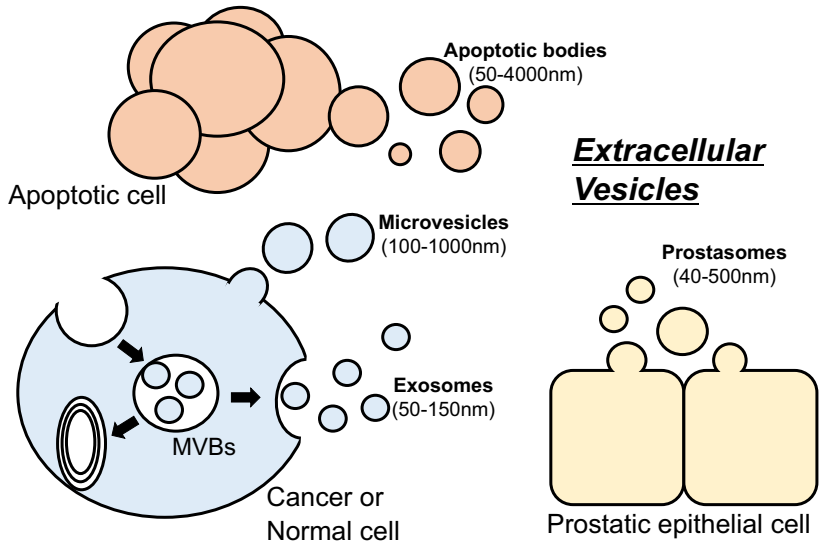

Fig. 1. Various kinds of extracellular vesicles (EVs). Several EVs are secreted from cells. Currently available technologies cannot completely distinguish between these types of small vesicles. MVBs, multivesicular bodies.

when multivesicular bodies (MVBs) fuse with the plasma membrane (116). Exosomes have been reported to contain several types of specific surface markers, such as tetraspanins (CD9, CD63, and CD81), heat shock proteins (Hsp70 and Hsp90), MVB synthesis proteins [ALG-2-interacting protein X (Alix) and tumor susceptibility gene 101 (*Tsg101*)], and membrane transporters and fusion proteins (annexins and flotillin) (27, 119). Microvesicles, which are larger than exosomes, are directly secreted from the plasma membrane via shedding or budding under normal circumstances or in response to stimuli (116). Several proteins have been identified as microvesicle specific, including CD40, ADP-ribosylation factor 6 (ARF6), selectins, phosphatidylserine, and Rho family members (3, 11, 70). Apoptotic bodies are released from the plasma membrane via indiscriminate blebbing when programmed cell death is induced and contain DNA fragments, noncoding RNAs, and cell organelles (4, 47). Annexin V and histone are reported as specific proteins of apoptotic bodies (19, 103, 114). Prostasomes, which are well-known EVs in reproduction and urology, are released from prostate epithelial cells in human seminal fluid and are reported to play decisive roles in the process of fertilization (107). To date, several papers have specified the kinds of proteins contained in prostasomes. Notably, the presence of a ubiquitous EV marker, CD9, and prostate cancer candidate markers, such as prostate-specific antigen (PSA), type 2 transmembrane serine protease (TMPRSS2), prostate stem cell antigen (PSCA), and prostate-specific membrane antigen (PSMA), has been repeatedly reported (1, 79, 87, 107). Although the origins of each kind of vesicle and their respective markers have been defined, currently available technologies cannot completely distinguish among those types of small vesicles. Indeed, the International Society for Extracellular Vesicles has recommended that researchers clearly state the process by which they collect vesicles and carefully use the proper terms for the different small vesicles (31). Therefore, in this review, we use "EVs" as an umbrella term for all kinds of small vesicles secreted in the extracellular space: exosomes, microvesicles, apoptotic bodies, and prostasomes.

THE BEGINNING OF EXTRACELLULAR VESICLE RESEARCH

Approximately 35 years ago, EVs were first reported as small vesicles secreted when multivesicular bodies fused with the plasma membrane in reticulocytes (33, 41). However, these vesicles did not attract much attention because they had long been thought of only as a "garbage can" for cells to discard unnecessary molecules (56). In 1996, EV research drastically changed when Raposo et al. (89) revealed the function of immune cell-derived EVs as activators of the immune system; since that discovery, the value of EVs has been increasingly recognized. In addition, in 2007, Valadi et al. (108) showed that microRNAs (miRNAs) as well as messenger RNAs (mRNAs) are contained in EVs and proposed that these molecules may function in recipient cells. Subsequently, in 2010, three independent groups, including our group, reported that miRNAs inside EVs are delivered to recipient cells and function within these cells (52, 81, 123). These studies have prompted many researchers to begin to focus on EV research, and accumulating evidence has demonstrated how EVs are influential and attractive in cancer research.

EXTRACELLULAR VESICLES AS BIOMARKERS

Liquid biopsy, involving the use of circulating tumor cells (CTCs), cell-free DNA (cfDNA), and EVs, has emerged as a revolutionary technique that has provided new perspectives and dimensions to the field of cancer management and treatment (66, 90). CTCs are cancer cells that are shed by primary or metastatic sites into the circulation. The number of CTCs has been shown to correlate with the prognosis of the disease (21, 34, 91). In addition, recent studies have reported that the molecular profiling of CTCs may be useful for monitoring or predicting the responses of patients receiving specific therapies (6). The main challenge in studying CTCs is the rarity of the cells in the circulation—usually one CTC per billion blood cells (121). To date, CellSearch, which targets epithelial cell adhesion molecule (EpCAM) to capture CTCs, is the most clinically validated CTC enumeration assay (2). However, the assay cannot capture CTCs with downregulated EpCAM, and

technical improvement of the CTC assay will be required for implementation in daily clinical practice. cfDNA refers to short fragments of nucleic acids found in body fluids, such as blood or urine. cfDNA is suggested to be generated by the apoptotic degradation of cellular DNA (7), and a component of cfDNA derived from tumor cells is defined as circulating tumor DNA (ctDNA) (10). ctDNA was reported to reflect the genetic and epigenetic alterations of the original tumor and has potential as a diagnostic or prognostic biomarker for cancers. The central challenge of research is that ctDNA might make up as low as 0.01% of the total cfDNA, which makes ctDNA difficult to detect, especially at the early stages of cancers (26, 30). In addition, although both digital PCR and next-generation sequencing (NGS) are the most popular methods for investigating the genetic information in cfDNA, both techniques have some weak points for clinical application. Digital PCR can screen only known variants and investigate a limited number of variants within a single reaction. NGS, meanwhile, is still a relatively expensive and time-consuming technique. Furthermore, NGS requires bioinformatics skills for data analysis and interpretation.

As another kind of liquid biopsy, EVs have recently attracted attention. EVs have been repeatedly reported to be stably detectable in various kinds of body fluids, such as blood (12), saliva (80), urine (84), bronchoalveolar fluid (101), breast milk (53), and semen (87). In addition, they reflect the current disease state by carrying specific molecules from parental cells, such as proteins, miRNAs, mRNAs, long noncoding RNAs (lncRNAs), and lipids. Thus, targeting the cargo of EVs allows us to assess crucial molecular information about disease status. Furthermore, because of the minimally invasive nature of the sampling process and their easy accessibility, EVs are suitable for sequential collection. For these reasons, EVs have great potential as clinically useful biomarkers that can provide multiple, minimally invasive snapshots of disease status. Among the components of EVs, EV-associated proteins and RNAs have been particularly reported as tumor biomarkers for diagnosing cancer or monitoring cancer progression (Fig. 2). In the first part of this review, we discuss the current challenges and application of EV-associated proteins and RNAs as biomarkers in clinical oncology.

EXTRACELLULAR VESICLE-ASSOCIATED-ASSOCIATED PROTEINS

The molecular phenotypes of EVs can be a signature of cancer status and can be used to diagnose or predict the state of the disease. However, analysis of EVs remains challenging, as EVs are nanosized particles that are heterogeneous and sensitive to handling conditions (20, 57, 111, 113). To date, many articles have focused on methods to detect EVs (60). Among these methods, the targeting of the membrane proteins on the surface of EVs is quite useful, as we can directly target molecules on the surface of EVs without purification. Therefore, in this section, we focus on antibody assays to characterize the protein phenotype of EVs and discuss the establishment of clinically useful EV-associated protein biomarkers.

Logozzi et al. (63) first indicated that the proteins located on the surface of EVs can be useful for capturing EVs by ELISA-based methods. This group designed an in-house sandwich ELISA, named ExoTEST, to detect and quantify EVs purified from human plasma and found that the level of caveolin-1/Rab-5b double-positive EVs was significantly increased in melanoma patients compared with that in healthy donors (63). Although ExoTEST requires sample purification by ultracentrifugation, this novel noninvasive assay allows the detection and quantification of EVs and is consequently of great scientific importance.

Next, Jørgensen et al. (42, 43) modified protein array technology to establish an EV Array. The EV Array provides the opportunity to detect EVs without purification in a high-throughput manner. Captured antibodies are printed on a microarray slide, which allows EVs to be captured based on their membrane proteins. In addition, the biotinylated detection antibodies against the tetraspanins CD9, CD63, and CD81, which are antigens generally present on the surface of EVs, have been used to detect EVs (42, 43). These researchers validated the performance of the EV Array as a minimally invasive diagnostic tool by comparing plasma from non-small cell lung cancer patients with that from controls. Another study showed that a model of the combination of 30 kinds of membrane proteins had the largest area under the curve (AUC)—0.83—along with a sensitivity of 0.75 and a specificity of 0.76 (8).

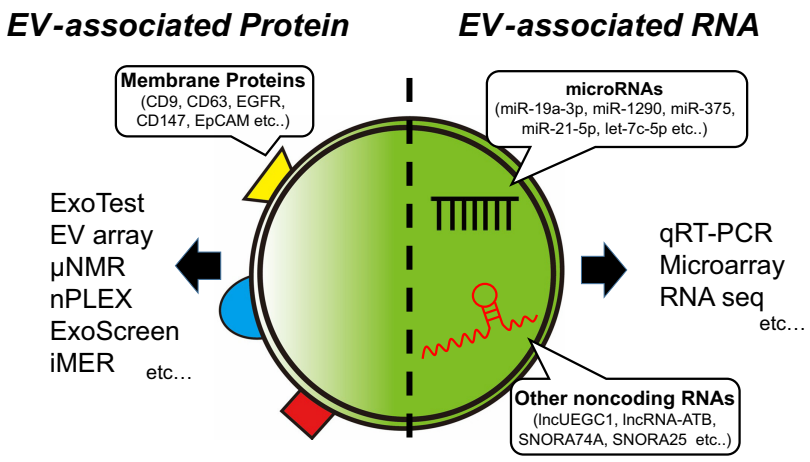

Fig. 2. Molecular components of extracellular vesicles (EVs) as cancer biomarkers. EV-associated proteins and RNAs are particularly reported as tumor biomarkers. EV-associated proteins can be directly detected without purification. On the other hand, EV-associated RNAs are stable and can be detected using several technologies, including quantitative RT-PCR (qRT-PCR), microarrays, and RNA sequencing. EGFR, epidermal growth factor receptor; EpCAM, epithelial cell adhesion molecule; iMER, immune-magnetic exosome RNA; lncRNA-ATB, long noncoding RNA-activated by transforming growth factor β; lncUEGC1, lncRNA upregulated in the exosomes of gastric cancer; nPLEX, nanoplasmonic exosome; SNORA25 and SNORA74A, small nucleolar RNAs; μNMR, micronuclear magnetic resonance.

Shao et al. (92) established a highly sensitive and rapid analytical microfluidic chip platform that quantifies the presence of glioblastoma-specific proteins, such as epidermal growth factor receptor (EGFR) and EGFR variant III, on the surface of EVs by micronuclear magnetic resonance (μNMR). This microfluidic device could differentiate patients with glioblastoma multiforme (GBM) from healthy controls. In this study, the researchers also suggested that GBM EVs reflect gene amplification or mutation and predict the response to GBM therapy. In addition, the same group reported the fabrication of a high-throughput EV screening method, a nanoplasmonic exosome (nPLEX) assay, based on transmission surface plasmon resonance through periodic nanohole arrays, which enables the analysis of proteins on the surface of EVs. This assay was able to differentiate between ovarian cancer patients and controls without cancer using ascites samples by targeting CD24 and EpCAM on the surface of EVs with an accuracy of 97% (36).

Furthermore, in 2014, our group (120) established a novel platform that can directly and easily analyze the amount of EVs. This platform, named ExoScreen, can directly quantify the amount of EVs in blood samples based on an amplified luminescent proximity homogeneous assay using two kinds of antibodies against proteins located on the surface of EVs that are detectable by photosensitizer beads (120). In this study, two types of antibodies, CD9 and CD147, were used, and this assay revealed that a greater number of CD9/CD147 double-positive EVs were contained in serum from colorectal cancer patients than in serum from healthy controls. The advantage of this assay is that it requires a very small sample volume (at least 5 μL), directly captures EVs without a complicated isolation process, and works with high-throughput analysis (it can be completed within 2 h).

The gold-standard technique for EV isolation is ultracentrifugation; unfortunately, however, this step is time consuming, and ultracentrifugation is unsuitable in clinical settings. Most of the methods that we introduced in this section (e.g., EV Array, μNMR, nPLEX, and ExoScreen) do not require an EV isolation step, and this is the greatest advantage of targeting EV-associated proteins. To date, only a limited number of samples have been examined in evaluating the diagnostic power of these platforms. To examine clearly the usefulness of these biomarkers, large-scale and interlaboratory research should be performed. Additionally, the storage conditions of EVs significantly influence the phenotype of membrane proteins on the surface of EVs; therefore, the effect of storage conditions on the surface characteristics, morphological features, and protein contents of EVs should be evaluated for reliable data (39, 65). Although further study is needed, EV-associated proteins have great potential to establish clinically useful high-throughput platforms to detect cancers.

EXTRACELLULAR VESICLE-ASSOCIATED RNAs

miRNAs are small noncoding RNAs composed of 20–25 nucleotides that posttranscriptionally control the expression of protein-coding genes. In noncancerous cells, miRNAs systematically regulate molecular RNA networks; however, in cancer cells, aberrantly expressed miRNAs disrupt the precisely regulated relationships between miRNAs and mRNAs, leading to cancer progression (88). The expression level of miRNAs in tumors is useful for tumor characterization; however, tissue sampling methods, such as biopsy, needle aspiration, or excision, are required. In 2007, Valadi et al. (108) reported a breakthrough regarding cancer diagnosis: miRNAs and mRNAs are contained in EVs and might function in recipient cells. This article suggests that miRNAs are released into the extracellular space and circulate in a stable form, leading many researchers to focus on miRNAs in EVs. Although several groups had already reported that circulating miRNAs in body fluids may serve as diagnostic biomarkers (58, 71), the stability of circulating RNAs had previously been questioned because of the presence of RNase in body fluids. Following the Valadi et al. (108) study, circulating miRNAs encapsulated within EVs were reported to be more resistant to RNase treatment than non-EV-associated circulating miRNAs (50), revealing their stability due to encapsulation in EVs and their potential as biomarkers. To date, many articles have reported EV-associated miRNAs in various kinds of body fluids as effective biomarkers (106). For instance, Ogata-Kawata et al. (77) reported that the levels of seven miRNAs in serum EVs were significantly elevated in colorectal cancer patients. The levels of these miRNAs were significantly decreased after tumor resection, suggesting their potential for identifying the tumor origin (77). Matsumura et al. (67) reported that the presence of miR-19a-3p in serum EVs could be a prognostic biomarker to predict the recurrence of colorectal cancer. Another study revealed that elevated levels of serum miR-1290 and miR-375 in EVs were associated with decreased survival in advanced-stage prostate cancer patients (35). In addition, regarding EVs in other body fluids, Foj et al. (29) reported that the levels of miR-21-5p, miR-375, and let-7c-5p were significantly elevated in EVs purified from the urine of prostate cancer patients compared with those in controls. Similar to these findings, accumulating evidence from translational studies has shown the utility of EV-associated miRNAs.

The potential of other noncoding RNAs, such as lncRNAs or small nucleolar RNAs (snoRNAs), in EVs from cancer cells as cancer biomarkers is also becoming increasingly recognized. Lin et al. (61) found that a novel lncRNA, named lncRNA upregulated in the exosomes of gastric cancer (lncUEGC1), in EVs could be a diagnostic marker for early gastric cancer, with an AUC value of 0.876. Lee et al. (59) reported that miR-21 and lncRNA-activated by transforming growth factor β (ATB) in serum EVs could be novel prognostic biomarkers for hepatocellular carcinoma. The overall survival and progression-free survival rates were significantly lower in patients with higher levels of miR-21 and lncRNA-ATB in EVs (59). Regarding snoRNAs in EVs, Kitagawa et al. (49) reported that two snoRNAs, SNORA74A and SNORA25, in serum EVs could be useful tools for the early detection of pancreatic cancer. In this study, the researchers reported that the AUCs of SNORA74A and SNORA25 in serum EVs were >0.9 for distinguishing patients in the early stages of pancreatic cancer from controls (49). In addition, Haderk et al. (32) recently reported that the expression level of the noncoding Y RNA hY4 in plasma EVs from chronic lymphocytic leukemia (CLL) patients was significantly elevated compared with that in healthy controls. Furthermore, the researchers found that hY4 in EVs derived from CLL patients regulates the expression of programmed death-ligand 1 (PD-L1) on monocytes, thus con-

tributing to cancer-related inflammation and immune escape in CLL (32).

The detection of EV-associated noncoding RNAs in biological fluids has opened a new arena in clinical chemistry for their use as diagnostic and prognostic biomarkers for cancer. Compared with the targeting of EV-associated proteins on the surface of EVs, the establishment of an effective EV-associated RNA biomarker platform requires a standardized method of EV collection. Ultracentrifugation is a conventional gold-standard method to isolate EVs from body fluids, but it is time consuming. There are several kinds of commercial EV isolation kits, but they are not ideal methods for isolating EVs because of their potential to capture soluble proteins (109). Density gradient-based purification using sucrose or iodixanol can be applied to obtain EVs with increased purity, but it takes much time to purify the EVs, and whether this method is applicable in clinical settings is currently questionable (97). However, recently, as a heterogeneous population of EVs was reported (57), a methodology for analyzing each subpopulation of EVs might be required to establish more reliable biomarkers.

Nucleic acids can be repeatedly and readily evaluated using quantitative real-time RT-PCR (qRT-PCR) with only a small sample volume. However, qRT-PCR requires a suitable reference gene or housekeeping gene, which is not easy to select in EV-associated RNAs. Especially for EV-associated miRNAs, current protocols recommend that samples be processed from identical input volumes and compensated for technical variability using spike-in, synthetic nonhuman miRNAs, such as *Caenorhabditis elegans* (cel)-miR-39 (40, 102) and *Arabidopsis thaliana* (ath)-miR-159 (24), as normalization controls. Some researchers have proposed the use of stable endogenous miRNAs as internal controls; however, because of high variability, no consensus has been reached (118).

Although some issues remain, the stability and repeatability of EV-associated RNAs in body fluids indicate great potential for their use in diagnostics and treatment design in cancers. To compensate for the limitations of EV-associated proteins and RNAs, a combination of their advantages might be valuable. Indeed, Shao et al. (93) established a microfluidic platform, termed the immune-magnetic exosome RNA (iMER) platform, which enables the enrichment of cancer-specific EVs from blood samples by targeting EV-associated proteins and on-chip analysis of their mRNA content by qPCR. This study identified key EV-associated mRNA markers potentially predictive of drug resistance. As we have indicated, combinations of EV-associated biomolecules may result in the detection of highly specific cancer biomarkers that can be used in clinically useful platforms.

EXTRACELLULAR VESICLES AS THERAPEUTIC TARGETS

Cells in the tumor microenvironment communicate with each other mainly through cytokines, chemokines, or growth factors (115). However, communication within the tumor microenvironment is also mediated by EVs derived from the cells in the tumor microenvironment. Indeed, a number of studies have reported that EVs play important roles in tumor progression (74). Hence, as the tumor microenvironment matures, the production of EVs increases exponentially, promoting tumor progression. It is not surprising that a reduction in the amount of tumor-derived EVs may provide a therapeutic opportunity for inhibiting cancer progression. To date, three kinds of EV-targeting therapeutic strategies (elimination of circulating EVs, inhibition of EV secretion, and disruption of EV absorption) have been proposed (55). Therefore, in the final part of this article, we describe the potential of these EV-targeting strategies and discuss their progress toward clinical application (Fig. 3).

ELIMINATION OF CIRCULATING EXTRACELLULAR VESICLES

One of the EV-targeting therapies is the elimination of circulating EVs secreted from cancer cells. As tumor-derived EVs generate a tumor microenvironment that is permissive for tumor growth and metastasis (55), the removal of circulating EVs could potentially provide major benefits in cancer patients. Marleau et al. (64) first reported a therapeutic strategy for the elimination of circulating EVs. In this study, researchers established a hemofiltration system that can specifically capture circulating EVs derived from cancer cells by targeting human epidermal growth factor receptor 2 (HER-2) on the surface of EVs (64). HER-2, located on EVs, has been reported to interfere with cancer therapeutics, such as trastuzumab, and to contribute to tumor progression (16); therefore, the selective removal of cancer-derived EVs by targeting HER-2 could be

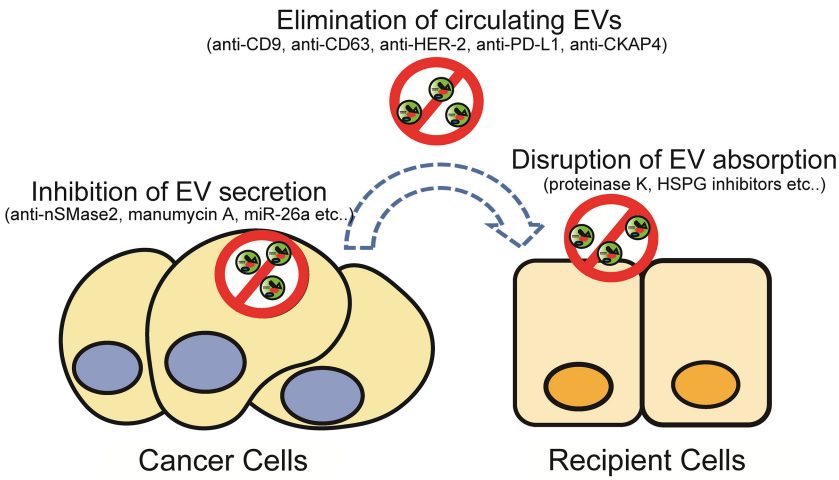

Fig. 3. Therapeutic strategies against cancer-derived extracellular vesicles (EVs). Three therapeutic applications have been proposed: elimination of circulating EVs, inhibition of EV secretion, and disruption of EV absorption. These therapeutic strategies inhibit intracellular communication via EVs, leading to the development of novel anticancer drugs. CKAP4, cytoskeleton-associated protein 4; HER-2, human epidermal growth factor receptor 2; HSPG, heparin sulfate proteoglycan; miR-26a, microRNA-26a; PD-L1, programmed death-ligand 1; nSMase2, neutral sphingomyelinase 2.

highly beneficial for cancer treatment. In addition, our group also reported the potential of eliminating circulating EVs for cancer treatment. We showed the effectiveness of the injection of specific antibodies against human CD9 and CD63, which are enriched on the surface of EVs, in a human breast cancer xenograft model. In this study, we showed that these antibodies significantly inhibited lung metastasis, although there were no obvious effects on primary tumor growth (76). As CD9 and CD63 are ubiquitous EV markers, and normal cells also secrete CD9- and CD63-positive EVs, anti-CD9 and anti-CD63 antibodies cannot specifically identify cancer-derived EVs in the human body. However, this study proposed the potential for the removal of circulating EVs using antibodies against molecules on the surface of EVs (76).

To date, several in vivo studies have reported the potential of antibodies in targeting cancer-specific molecules on the surface of EVs. In this era of immunotherapy, many researchers are interested in the molecular mechanism of immunotherapy. Programmed death ligand 1 (PD-L1) on the surface of tumor cells binds its receptor PD-1 on immune cells, thereby inhibiting their antitumor activities (13). Recently, novel mechanisms of PD-L1 expression on cancer-derived EVs were reported. In 2018, Chen et al. (14) revealed that metastatic melanoma cells release high levels of EVs carrying PD-L1 on their surface, which suppress the function of CD8 T cells and facilitate tumor progression. In the same study, these researchers also reported the potential for the clinical application of PD-L1 on the surface of EVs as a predictive biomarker for the response to anti-PD-1 therapy (14). In 2019, Poggio et al. (86) reported that PD-L1 on the surface of EVs from metastatic prostate cancer cells accelerates tumor proliferation in an immune-dependent manner. In this study, using a prostate cancer syngeneic model, the researchers revealed that resistance to anti-PD-L1 appeared to be caused by PD-L1 on the surface of EVs and showed that targeting PD-L1 on the surface of EVs could be a novel strategy to overcome resistance to current antibody approaches (86).

In addition to HER-2 and PD-L1, other cancer-specific membrane proteins on the surface of EVs could also be novel targets. Indeed, Kimura et al. (48) recently reported that cytoskeleton-associated protein 4 (CKAP4), a receptor for Dickkopf-related protein 1, is specifically located on the surface of EVs derived from pancreatic ductal adenocarcinoma and that anti-CKAP4 monoclonal antibodies (mAbs) suppressed xenograft tumor formation and extended survival. In this study, the researchers generated several different anti-CKAP4 mAbs that recognize different epitopes and selected the most efficient antibodies. This study suggested that not only identifying cancer-specific membrane proteins on the surface of EVs but also generating antibodies that can specifically and effectively detect the target molecules will be required for further development of EV-targeted therapy. Like these, targeting cancer-specific membrane proteins on the surface of EVs and eliminating circulating cancer-specific EVs might be fruitful strategies for developing not only novel biomarkers but also therapeutic targets.

INHIBITION OF EV SECRETION

Several studies have focused on other approaches that block EV production. A sphingomyelinase inhibitor drug, GW4869, is frequently used to inhibit the formation of intraluminal vesicles and the release of EVs by the fusion of MVBs to the cell membrane (52, 69). In addition, we previously reported that the attenuation of neutral sphingomyelinase 2 (nSMase2), which regulates the synthesis of ceramide, inhibited EV secretion and the transfer of miR-210-3p and suppressed angiogenesis and metastasis in a breast cancer xenograft model (51). However, downregulation of nSMase2 did not inhibit the secretion of EVs from prostate cancer cells (83), and nSMase2 has been reported to be detected in normal neural cells (122), leading to the inhibition of other crucial pathways in normal cells. Therefore, to establish EV-targeted cancer-specific therapies, the identification of cancer-specific mechanisms of EV secretion is required. Datta et al. (22) screened 4,580 compounds to identify selective inhibitors of EV biogenesis in prostate cancer. Quantitative high-throughput screened targeting of the green fluorescent protein (GFP) signal in conditioned medium from CD63-GFP-expressing prostate cancer cells treated with each compound was performed. Finally, manumycin A, a natural microbial metabolite, was repositioned as an inhibitor of EV secretion from prostate cancer cells but not normal prostate epithelial cells (22). In addition, the same group recently reported several additional inhibitors and activators of EV secretion from prostate cancer cells (23). These studies showed the potential utility of drug repositioning as a novel therapeutic strategy to target the cancer-specific EV biogenesis pathway. Furthermore, we recently modified our established high-throughput platform, ExoScreen, by performing high-throughput screening to identify novel genes related to the biogenesis of prostate cancer EVs (105). In this study, we identified miR-26a as a miRNA involved in EV secretion from prostate cancer cells and reported novel genes that are directly targeted by miR-26a and therefore regulate EV secretion in prostate cancer cells.

Recently, several studies have focused on the heterogeneity of EVs. In 2016, Kowal et al. (57) reported that EV protein markers differ across EV fractions and proposed to evaluate properly the molecular mechanisms in the secretion of each type of EV. In addition, in 2017, Tkach et al. (98) showed that different subpopulations of EVs have different effects on recipient cells. These researchers found that immature dendritic cells secrete different sizes of EVs—large EVs and small EVs—and that the former favors the secretion of T helper type 2 (Th2) cytokines, whereas the latter activates Th1 cytokine secretion (98). These findings indicate that identificating the details of the biogenesis of the EV population containing the greatest amount of oncogenic cargo that affects recipient cells will be the most effective strategy to establish therapies targeting EV biogenesis. As noted above, the biogenesis of EVs is complex, and high-throughput screening platforms will be essential to reveal all of its details. Forward genetic screens will be a powerful means for the unbiased investigation and functional characterization of specific genetic elements associated with a phenotype of interest. Recently, the clustered regularly interspaced short palindromic repeats/Cas9 (CRISPR/Cas9) approach has been adapted for conducting genome-scale screens (44, 94). Although further exploratory research will be required, this CRISPR-Cas9 screening platform may be useful to reveal novel pathways of

EV biogenesis in cancer cells and provide novel therapeutic targets.

DISRUPTION OF EV ABSORPTION

Identificating the precise mechanisms of EV absorption will provide another strategy for inhibiting cancer progression. Escrevente et al. (28) found that the uptake of EVs was significantly reduced after treatment with proteinase K. These results indicate that the proteins on the surface of EVs are associated with the uptake of EVs (28). In addition, Christianson et al. (15) reported that heparin sulfate proteoglycans (HSPGs) on the cell membrane function as receptors for EVs and that HSPG inhibitors dose dependently inhibit EV uptake. Furthermore, several other proteins located on the surface of EVs have also been reported to take part in EV uptake and could therefore contribute to the establishment of a novel strategy for EV-targeted cancer treatment (46, 72, 96). However, the precise mechanism of EV uptake is complicated and ambiguous compared with that of EV biogenesis. Indeed, no study has demonstrated an effect of the disruption of EV absorption in vivo, and progress in studies of this mechanism is expected.

As we have shown, although many challenges remain, the inhibition of EV transfer will inhibit tumor progression and has great potential as a new therapeutic strategy for cancer treatment. In the near future, EV-targeted therapy may be a novel option in addition to standard therapeutic options, such as surgery, chemotherapy, and radiotherapy.

CONCLUSIONS

In this review, we summarize current research topics on EVs as biomarkers and therapeutic targets in cancer. Because of their ease of accessibility, stability, and tumor-specific molecules, EVs have great potential as diagnostic and prognostic biomarkers. Further analysis of EVs, such as the establishment of methodologies for purifying different subpopulations of EVs and analyzing their contents easily and precisely, will be required for their clinical implementation. Indeed, the speed of EV research development is astonishing; consequently, we believe that EVs will be used as valuable biomarkers in the near future. In addition, EVs are important intercellular communication tools for promoting tumor initiation, invasion, and metastasis. Thus, an understanding of the physiology of EVs during cancer progression in detail and inhibition of this communication will provide an opportunity for the establishment of EV-targeted therapeutics.

On the other hand, the utilization of EVs as delivery vehicles or anticancer vaccines is also an attractive strategy for cancer treatment. Because of their stability in body fluids, tropism to specific organs, and minimal side effects, EVs can successfully carry anticancer molecules to target cancer cells (100). Indeed, several articles have reported the effectiveness of EVs as delivery vehicles to transport therapeutic siRNAs and antisense miRNAs through the use of in vivo models (5, 78). In addition, EVs derived from antigen-presenting cells (APCs), such as dendritic cells, transport tumor antigen complexes loaded with major histocompatibility complex molecules, which can prime naïve T cells and activate natural killer cells to shrink the cancer (124). Therefore, these EVs could be a novel strategy for cancer treatment as cell-free anticancer vaccines.

The clinical utility of EVs has been shown in a number of articles, and there is endless potential in EV research. So far, only the tip of the iceberg has been seen; most of the iceberg might be underwater and therefore unknown. We enthusiastically hope that advances in EV research will provide novel treatment strategies for cancers and be beneficial to cancer patients.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

F.U. prepared figures; F.U., N.K., K.I., T.K., S.E., and T.O. drafted manuscript; F.U., N.K., K.I., T.K., S.E., and T.O. edited and revised manuscript; F.U., N.K., K.I., T.K., S.E., and T.O. approved final version of manuscript.

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