 doi:10.20944/preprints201912.0386.v1

Review

Exosome Release of Drugs: Coupling with Epithelial-Mesenchymal Transition

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Abstract: Extracellular vesicles (EVs), such as exosomes or oncosomes are released with molecules unfavorable for survival from cells. In addition, accumulating evidence has shown that tumor cells often eject anti-cancer drugs such as chemotherapeutics and targeted drugs within EVs, a novel mechanism of drug resistance. The EV-releasing, drug resistance phenotype is often coupled with cellular dedifferentiation and transformation, cells undergoing epithelial-mesenchymal transition (EMT) and taking on a cancer stem cell phenotype. Recent studies have shown that the release of EVs is also involved in immunosuppression. The concept of the resistance-associated secretory phenotype (RASP) is reviewed herein.

Keywords: resistance-associated secretory phenotype (RASP); extracellular vesicle (EV); exosome; oncosome; drug resistance; epithelial-mesenchymal transition (EMT); heat shock protein (HSP); cell stress response; hypoxia; acidosis; tumor immunology

1. Introduction

Recent studies have unveiled the existence of and significant biological roles for extracellular vesicles (EVs) such as exosomes. EVs are nano-particles surrounded by lipid membranes, containing a variety of molecular cargos such as proteins, small and large RNAs, DNA, lipids, glycans, minerals, and metabolites that are thus secreted by cells [1-5]. Earlier studies have classified the range of EVs into exosomes (50-200 nm), ectosomes (100-1000 nm; also known as microvesicles) [6-8], and apoptotic bodies (1-10 μm) based on their mechanisms of generation and release, while additional types of EVs have been reported, consisting of oncosomes (oncogenic EVs) [9-11], large oncosomes (1-10 μm) [12,13], matrix vesicles [14-16], migrasomes (50 nm to 3 μm) [17,18], exopheres (~4 μm), exomeres (~35 nm), and bacterial outer membrane vesicles (OMV) [19,20] [4,21]. EVs are also classified by their size into small EVs (s-EVs; 30-500 nm) and large EVs (L-EVs; > 1 μm). These vesicles play roles in discarding unfavorable molecules from cells, while also mediating cell-to-cell communication by transferring their cargo molecules to recipient cells or organs in local and/or distant tissues [22]. Recent studies have shown that anti-cancer drugs, including chemotherapeutics and targeted drugs, can be released from cells within EVs, suggesting a novel mechanism of drug resistance. EV-mediated drug efflux is often coupled with cellular dedifferentiation involving the so-called epithelial-to-mesenchymal transition (EMT) [23].

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EMT involves a cellular transformation or dedifferentiation from an epithelial phenotype into the mesenchymal phenotype and is important in many aspects of cell biology, including development, inflammation, and cancer [24-26]. Epithelial cells are usually tightly connected to each other through intercellular adhesion and cell junction including adherence junction, desmosome, gap junction, synaptic junction, occluding/tight junction, whereas loss of these connections/adhesions is accompanied by altered cellular shapes, increased motility and migratory activities of the cells. Pre-cancerous cells often exhibit EMT, increased migration and invasion of the cells within the tumor milieu [27]. EMT is a complex process consisting of multiple sequential steps and pathways, promoted by extracellular prompts such as transforming growth factor β (TGFβ) signaling [28], epidermal growth factor (EGF) signaling [23,29], matrix metalloproteinases (MMPs) [30], intracellular signals, and transcription factors [27]. It has been shown that EMT increases the properties of cancer stem cells (CSC) or cancer-initiating cells (CIC), which are highly resistant, recurrent, and metastatic [31-33].

Recent studies have shown that increased exosome release is coupled with EMT (Figure 1). EMT enhances the exosome-releasing phenotype of cells, while, conversely, tumor-derived exosomes initiate EMT in epithelial cells as well as driving EMT in cancer cells [23]. Moreover, it has been also shown that anti-cancer drugs were released with exosomes from tumor cells, suggesting a mechanism of cancer drug resistance. The vesicle-releasing and drug-releasing phenotypes can be an aspect of the resistant-associated secretory phenotype (RASP). Studies showing EMT-coupled exosome release are reviewed as a mechanism of drug resistance and immunosuppression in cancer.

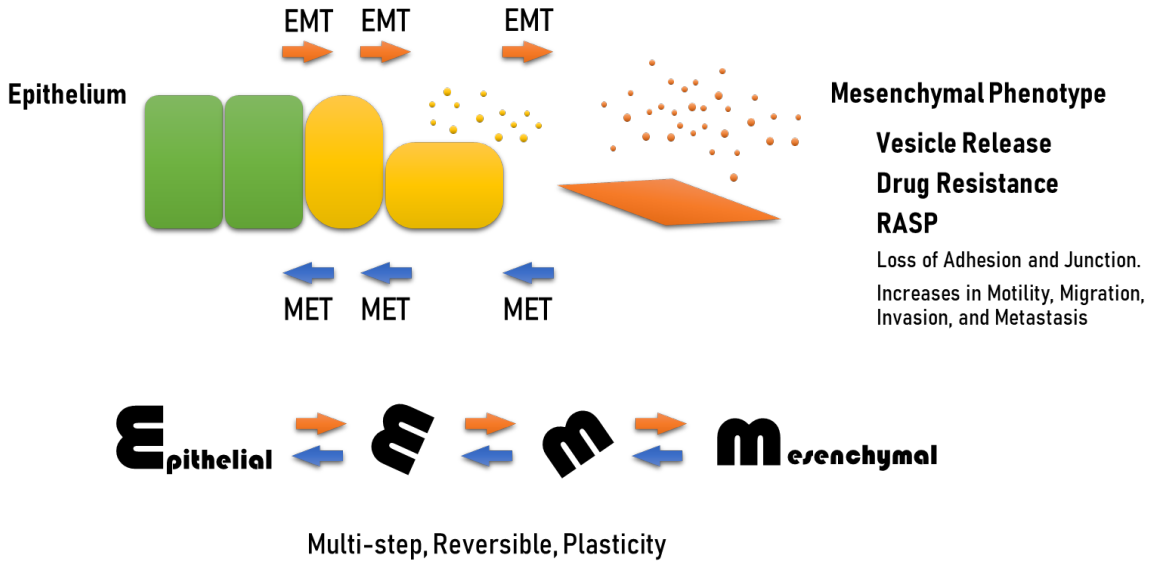

Figure 1. EMT coupled with vesicle release, drug resistance, and RASP. EMT is cellular dedifferentiation, transformation or reprogramming in which epithelial cell phenotype is switched into mesenchymal phenotype. Epithelial cells adhere to each other by epithelial intercellular adhesion molecules (i.e. E-cadherin and claudins) and desmosomes, which are often lost in EMT. The cells conferring EMT acquire increased motility, migration, invasion, and metastasis. EMT is a multi-step event and reversible, so-called plasticity. This review wraps up that EMT is often coupled with vesicle release, drug resistance, and resistance-associated secretory phenotype (RASP).

2. EV-mediated oncogenesis.

2.1. Oncosomes

 doi:10.20944/preprints201912.0386.v1

Oncosomes have been defined as oncogenic EVs or oncogenic exosomes that molecularly transfer tumor-promoting factors such as oncoproteins, oncomiR, and circulating tumor DNA (ctDNA) [11,12,34,35], while a number of studies have reported that tumor-derived exosomes played similar oncogenic roles without using the term "oncosome". In 2008, Janus Rak et al first defined oncosomes as they reported intercellular transfer of EGF receptor variant III (EGFRvIII), an oncogenic receptor, by microvesicles derived from brain tumor cells [11]. In the next year, Di Vizio et al reported that oncosome formation in prostate cancer is associated with a region of frequent chromosomal deletion in metastatic disease [35]. This group thereafter reported that large oncosomes, larger than 1 μm could be selectively sorted by flow cytometry in human prostate cancer tissues and in the circulation of mice with metastatic disease and contained MMPs, RNA, caveolin-1, and the GTPase ADP-ribosylation factor 6 [13]. The large oncosomes (or defined as L-EVs) carried most of the tumor DNA circulating in prostate cancer patient plasma [12]. In this study, whole-genome sequencing revealed that the DNA in L-EVs reflects genetic aberrations of the cell of origin, including copy number variations (CNV) of genes frequently altered in metastatic prostate cancer, i.e. MYC, AKT1, PTK2, KLF10, and PTEN. Later studies have shown that a number of additional oncogenic factors were contained in oncosomes, such as oncomiR miR-520g [36], 14-3-3 and beta-catenin [37].

A proteomic study revealed that oral cancer-derived oncosomes contain heat shock protein (HSP) family members, a number of extracellular matrix (ECM) proteins, and transcriptional regulators [38]. HSPs have been shown to assist in folding of oncoproteins essential for cancer cell survival and resistance [39-41]. Therefore, HSP-rich oncosomes and their molecular transfer can be crucial in tumor progression and resistance.

2.2. EV-mediated chaperone transfer

Human tumor cells are progressively exposed to stresses such as hypoxic stress, immune and inflammatory stress, and therapeutic stress. Such cell stresses trigger the expression of HSPs, stress-resistant cytoprotective proteins with anti-apoptotic and senescence deterring activity. Intracellular HSPs are molecular chaperones playing key roles in protein folding of normal and oncogenic proteins and balancing between proteostasis and proteolysis [39,42-44]. In addition, extracellular HSPs play key roles in cell-cell communication in cancer and immunity [45]. Extracellular HSPs and HSP-rich EVs can promote cancer progression by enhancing EMT, migration, invasion, heterogeneity, metastasis, CSC/CIC properties, and drug resistance in cancer cells and angiogenesis [46-52]. Proteomic analysis of oral cancer-derived oncosomes revealed that a number of HSP family members are contained within EVs (i.e. HSP90 homologs, large HSP members, and HSP70 family members) [38]. HSPs and oncoproteins contained within EVs could be involved in RASP, cotransferred to recipient cells leading to cancer expansion and malignant conversion of the tumor microenvironment (Figure 2).

2.3. Stromal signal-driven tumor progression

Tumor cells are surrounded by tumor stroma composed of various types of cells including cancer-associated fibroblasts (CAFs) with properties of mesenchymal stem cells (MSCs), tumor-associated immune cells including tumor-associated macrophages (TAMs), T cells, B cells, and dendritic cells (DCs), tumor endothelial cells (TECs), adipocytes, and normal epithelial cells. These stromal cells communicate with each other using cytokines, growth factors, MMPs [53], ECM, microRNAs, and EVs [54,55]. Earlier studies suggested that tumor stroma was tumor-suppressing, although recent studies have unveiled that stroma signals often drive tumor progression. Among the various stromal cells, we here review the crucial roles of CAFs, TAMs, and TECs.

CAFs secrete cytokines and growth factors such as TGFβ, HGF, FGF, NGF, IGF, and IL-6, which promote cell proliferation and migration [56,57]. CAFs also enhances cell motility and EMT through producing COX-2/PGE2 and TGFβ [58,59]. CAFs enhance angiogenesis by producing VEGF, PDGF, HGF, IL-8/CXCL8, and SDF-1/CXCL12, which acting on TECs [60]. The CAF-derived CXCL12 can act on its receptor CXCR4 on TECs and promote angiogenesis [61]. CAFs enhance inflammation by producing IL-6, IL-1, and adenosine triphosphate (ATP), while CAFs alter macrophage polarity and

 doi:10.20944/preprints201912.0386.v1

immune evasion by producing IL-6, COX-2/PGE2, and SDF-1/CXCL12. CAFs control ECM deposition and remodeling by producing fibronectin, collagen 1A1, tenascin C, osteopontin, and MMPs [62]. It has been shown that these secretory phenotypes of CAFs were often carried by EVs, including TGFβ, MMPs, microRNA, and ECMs, which alter epithelial cells, tumor cells, and tumor milieu [53,55,62-64]. Proteomics of stroma-derived EVs is important to elucidate the mechanism of tumor progression. Proteomics of secretory factors (EV and soluble) derived from CAFs identified 4247 proteins, among which a new cancer biomarker MFAP5 was discovered [65]. TAMs produce multiple immunomodulatory lipids, and several proteins involved in lipid metabolism were enriched in TAM-EVs compared to source TAMs [66].

Stromal cells, including TAMs and CAFs, are involved in drug resistance. It has been shown that exosomal miR-196a derived from CAFs conferred cisplatin resistance in head and neck cancer (HNC) through targeting CDKN1B and ING5 [63]. A concept of macrophage interference on chemotherapy was also recently suggested [67]. A new study showed that targeted elimination of macrophages elicited a type I interferon response in the tumor milieu that enhances the efficacy of platinum, but not taxane-based chemotherapy, underlining complicated regulatory roles for macrophages in chemotherapy-treated tumors [67]. TECs with high aldehyde dehydrogenase activity showed drug resistance [68]. TECs and TAMs can play key roles in drug resistance inasmuch as these tumor-associated cells often express drug resistance genes [69-74].

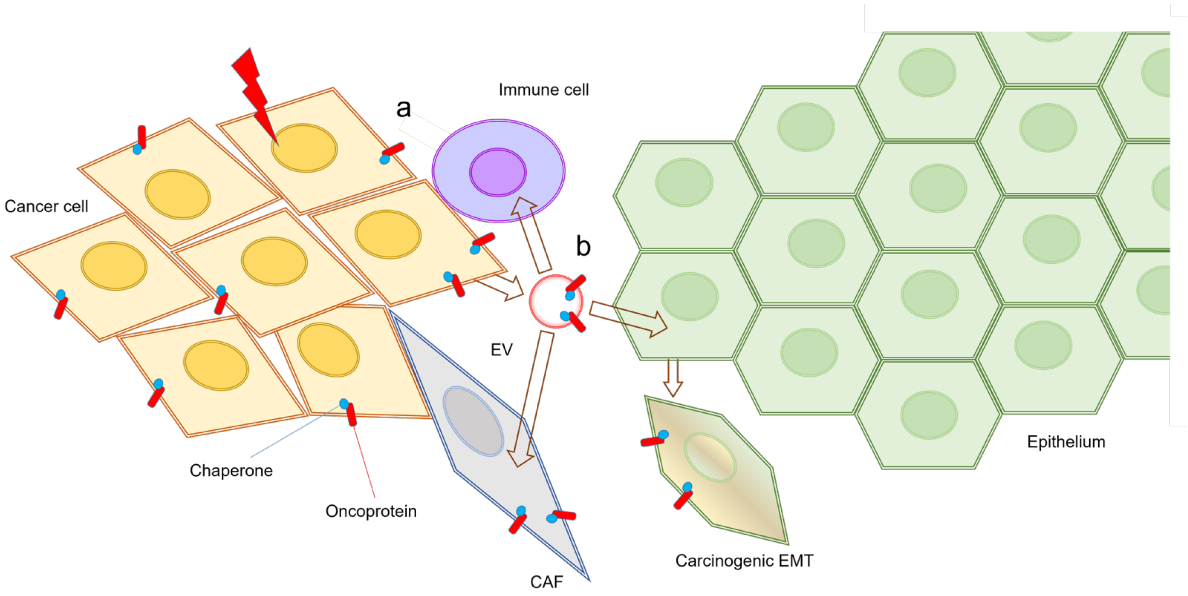

Figure 2. Exosome-mediated carcinogenic transfer. Cancer cells (orange diamonds) and/or CSCs also known as CICs can express oncoproteins (red bars) e.g. mutant or amplified receptor tyrosine kinases (RTKs) such as EGFR family members, which are functionalized by molecular chaperones HSPs (blue balls). The carcinogenic and resistance factors of EVs can be transferred to epithelial cells (green hexagons) and initiate carcinogenic EMT [23]. These factors carried by EVs can be transferred to and alter cancer-associated fibroblasts (CAFs) (gray diamond) and immune cells (shown in purple) such as TAMs. The EV-mediated transfer of carcinogenic factors and HSPs is a novel manner of cancer expansion and malignant conversion of tumor microenvironment with resistant phenotype.

3. Resistance-Associated Secretory Phenotype (RASP)

 doi:10.20944/preprints201912.0386.v1

3.1. HSP as mediators of RASP

HSPs are often carried by EVs, including exosomes, ectosomes, and oncosomes as cargos and are also potentially associated on the membrane surface of EVs [38,45,75]. Since HSPs are stress-responsive and promote stress-resistance, extracellularly released HSPs are a major aspect of RASP. Exosomal HSPs can promote the folding of oncoproteins upon molecular co-transfer to recipient cells and resultant increases in chaperoning power. High metastatic oral cancer-derived s-EVs contained significant levels of HSPs, including HSP90α, HSP90β, TRAP1, HSP110/HSPH1, and HSP70, which were coordinately increased with EGFR and EpCAM/CD326 as compared with low metastatic ones [38]. Oncosomal molecular cotransfer of oncoproteins such as mutant EGFR and amplified HSPs [42] can thus promote oncogenesis and resistance in cancer cells themselves and in the recipient cells at the local and distant milieu [23,34,38].

3.2. Exosomal ejection of drugs

There are currently two types of EV-mediated (or exosomal) mechanisms of ejection of anti-cancer drugs: (i) EV-mediated ejection of drugs targeting cell surface molecules such as EGFR-targeted cetuximab resistance, and (ii) exosomal ejection of chemotherapeutics as in cisplatin resistance. Cell surface oncoproteins, such as CD326/EpCAM, EGFR, and PD-L1, are often released from cancer cells by two mechanisms including the release of EVs and protein shedding by proteinases. The oncosomes containing such cell surface molecules can play roles as decoys against molecularly targeted drugs. Indeed, the targeted anti-EGFR antibody medication cetuximab binds to EGFR on the cell surface and inhibits EMT [23], although cetuximab was ejected by oral cancer cells within EVs containing EGFR in response to the therapeutic stress [29]. Known as a mechanism of antibody-dependent cellular cytotoxicity (ADCC), antibody drugs can recruit Fragment crystallizable region receptor (FcR)-expressed immune cells leading to cytolysis by cytotoxic T lymphocytes (CTLs) or by natural killer (NK) cells and phagocytosis by macrophages, although these antitumor immune cells can be released with EVs from cancer cells (Figure 3). The EV-mediated ejection of drugs is a new form of drug resistance in cancer cells as well as a novel aspect of RASP. Immune check point inhibitors target cell surface molecules such as programmed cell death-1 (PD-1) and Programmed cell death-ligand 1 and permit tumor cell killing by tumor-specific CTL. However, recent studies have shown that PD-L1 is often found on exosomes, playing key roles in spreading immunosuppression [76-81]. Chemotherapeutics are also reported to be secreted with exosomes. Cisplatin was secreted with exosome from ovarian cancer cells [82], melanoma cells [83], and A549 lung cancer cells [84] (see later table as well).

3.3. Ejection of toxic lipids and lipophilic drugs

Lipid efflux is also an aspect of RASP. Redundant, unfavorable lipids are evicted from cells through the release of lipid-layered EVs and lipid cholesterol efflux pumps, such as ATP-binding cassette (ABC) transporters. One such lipid efflux pump, that is overexpressed in metastatic cancer cells is ABCG1 [72]. siRNA-mediated silencing of ABCG1 triggered the accumulation of EV lipid and cell death in tumoroids, suggesting that tumor cells may release unfavorable lipids as a cell survival strategy. The most of ABC members transport lipophilic substrates such as (phospho)lipid by ABC-A1, A3, A4, A7, A12, B1, B4, and C1, sphingomyelin transported by ABC- A1 and A3, sphingolipids by ABC-B1, cholesterol by ABC- A1, A2, A5, G1, G4, and G5/G8, bile salts by ABC-B11, drugs transported by ABC-B1, C1, C2, and G2, steroids transported by ABC-C1, C10, G2, and G5/G8, and very long chain fatty acids (VLC-FAs) by ABC-D1 to D4 [73]. Notably, most drugs have been designed as lipophilic drugs in order for penetration of the drugs through lipid biomembrane. However, resistant cancer cells may eject lipophilic drugs using lipid vesicles.

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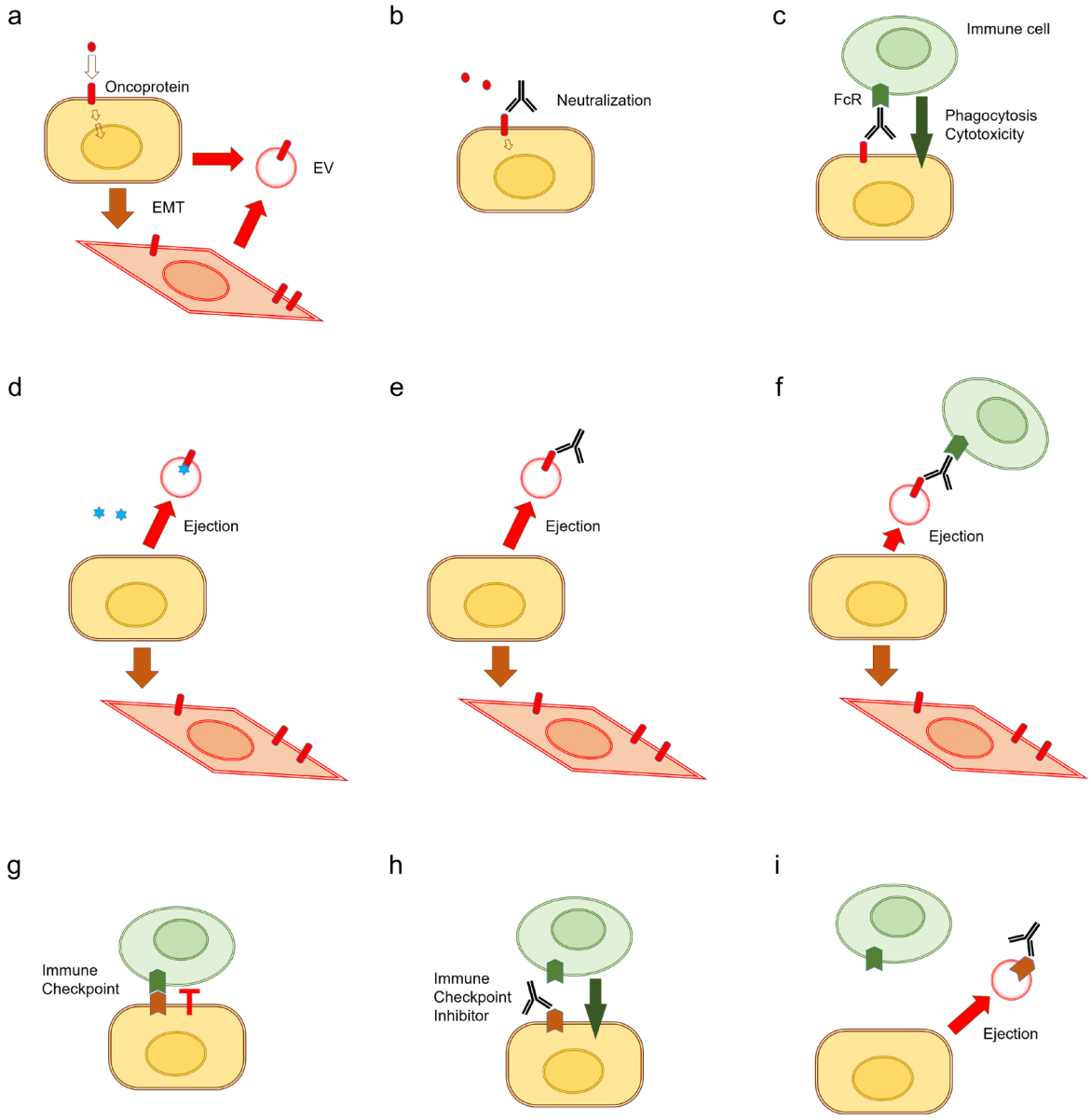

Figure 3. Exosome release in immune evasion. (a) Cancer cells and CSCs/CICs express oncoproteins such as mutant or amplified EGFR, whose signal promotes the progression of cancer cells e.g. EMT [23]. (b) Activities of cell-surface oncoproteins can be neutralized and inhibited by host-derived endogenous antibodies, molecularly targeted antibody drugs and/or small molecule inhibitors [29]. (c) The antibodies are also recognized by fragment crystallizable region receptors (FcR) of immune cells, including phagocytes and NK cells. (d, e) Cancer cells can eject molecularly targeted drugs (d, blue stars) and antibodies (e) by releasing EV containing oncoproteins as RASP [29]. Meanwhile, cancer cells can further transform and acquire resistant phenotype. (f) Cancer cells can also eject immune cells by releasing EVs. (g) The immune checkpoint enables cancer cells to evade immune cells. For example, cancer cells express PD-L1 (shown in dark brown) that binds with PD-1 (shown in dark green) on the surface of immune cells. (h) Immune checkpoint inhibitors can cancel the checkpoint and enable immune cells to attack cancer cells. (i) However, cancer cells could eject immune checkpoint inhibitors by releasing EV containing checkpoint proteins.

 doi:10.20944/preprints201912.0386.v1

4. Exosomal drug resistance

A number of studies have reported that platinum drugs such as cisplatin and carboplatin were released with exosomes as a mechanism of chemoresistance in cancer cells (Table 1). In addition, the antibody drug cetuximab was also released with oncosomes [29]. Cancer cells found in oral squamous cell carcinoma (OSCC), colorectal carcinoma (CRC), and non-small cell lung carcinoma (NSCLC) often acquire genetic amplification of EGFR, while EGFR-containing EVs are released from these cancer cells. Cetuximab bound to EGFR-EVs was co-released from OSCC cells, suggesting a mechanism of cancer drug resistance [29]. Interestingly, recent studies have shown that vesicle-releasing properties were often coupled with cellular transformation phenotypes including EMT [23,29,85,86] and CSC [44,87]. Thus, it is conceivable that the mesenchymal transition and CSC phenotype are involved with the acquisition of exosome/drug-releasing phenotypes. Anti-EMT strategies by targeting the TGFβ receptor or cyclin-dependent kinase 2 (CDK2) may inhibit exosome/oncosome release from cancer cells [28].

EMT, almost by definition, enhances the motility and migration of tumor cells. It has been recently shown that cells release migrasomes during cell migration from cellular cilia, a tail of the cell [17,88]. The proteome of migrasomes was 27% common with exosomes, while the remaining 73% was specific to migrasomes [88]. Therefore, EMT-driven migration of cancer cells may promote release of EVs including exosomes and migrasomes, which both may enhance the drug ejection, resistance phenotype of the cells.

Table 1. Exosomal drug resistance

Microenvironment	Determinant pathway	Phenotype	Type of resistance	Reference
Hypoxia	STAT3 Rab27 ↑ Rab7 ↓ Lamp1/2 ↓	Exosome release Secretory lysosome	Platinum resistance	[89]
Hypoxic tumoroids	EpCAM-exosome Extracellular HSP90α	CSC Exosome release	-	[44]
Extracellular Acidosis (Low pH)	Proton pump	Exosome release	Platinum resistance	[83]
TGFβ signal	Smad4 mutation	EMT Exosome release	Platinum resistance	[85]
EGF signal	EGFR amplification EGFR-exosome	EMT Exosome release	Cetuximab resistance	[23,29]
Stromal fibroblasts	Wnt-exosomes	CSC Exosome release	Chemoresistance	[87]
-	HSP90-exosome	Exosome release	Anti-apoptotic Survival of Metastatic cancer cell	[38,75]
-	HSF1 / HSPs	EMT ECM remodeling	Radioresistance Chemoresistance	[90]
-	miR-155-5p GATA3 ↓ TP53INP1 ↓	EMT Exosome release	Paclitaxel resistance	[86]

A number of studies reported that platinum drugs were released with exosomes from cancer cells. The antibody-drug cetuximab was also ejected with s-EVs by cancer cells [29]. Exosome-releasing phenotypes are often coupled with EMT phenotypes in cancer cells.

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Many members of the HSP family play key roles in cell survival and the promotion of drug resistance [39,45,91-94] (Table 1). Extracellular HSPs and EVs enriched with HSPs are thus a major aspect of the RASP. Molecular transfer of HSPs may increase drug resistance in cancer cells and influence the tumor microenvironment. Heat shock factor 1 (HSF1) is a master transcription factor for stress response and induction of HSPs [41,95-99]. The HSF1-HSP transcriptional system is a key axis in the stress response as well as in the stress resistance of cancer cells, although other transcription factors may be involved in such stress responses and resistant phenotypes. Indeed, the mRNA levels of HSP70 and HSP27 were upregulated by intracellular MMP3 which behaves as a moonlighting transcription factor in cancer [30,100,101]. In addition, CDC37, a kinase-specialized cochaperone of HSP90, was upregulated by myeloid zinc finger 1 (MZF1) in castration-resistant prostate cancer (CRPC) [102,103]. The mechanisms whereby these transcription factors are involved in drug resistance are under investigation.

On the other hand, it has been shown that drug-encapsulated exosomes derived from immune cells and mesenchymal stem cells (MSC) can be effectively and efficiently deliverable to cancer cells. Indeed, macrophage-derived exosome-encapsulated paclitaxel was developed to overcome multidrug resistance (MDR) in cancer cells [104]. Targeted delivery of a TLR3 agonist with single-chain antibody fragment-conjugated nanoparticles induced a type I-interferon response and apoptosis in tumor cells [105].

5. Classes of drug resistance in cancer

The mechanisms of drug resistance in cancer had previously been classified into three types, although the EV-oncosomal ejection of drugs can now be added as a fourth class (Table 2). Resistance Class I is the amplification and activation of alternative RTKs due to the known redundancy in RTK signaling pathways, e.g. ErbB/EGFR/Her family [106], IGF1R, PDGFRβ, FGFR or MET [32]. Indeed, the EGFR-S492R mutation inhibits the binding of cetuximab, an anti-EGFR antibody medication. Resistance Class II involves activating mutations in intracellular signaling proteins such as PIK3CA, RAS family, BRAF or MEK that enhance pro-tumorigenic signaling. For example, activating mutations in PIK3CA have been often detected in HNC [107]. Resistance Class III consists of stromal signals, including HGF-high stroma that activates HGF-MET signaling [108-110]. The HGF-MET signaling pathway is driven by the HSF1-HSP stress response system in triple-negative breast cancer (TNBC) [40]. Resistance Class IV is the exosome-mediated (EV-mediated) ejection of drugs discussed above.

Table 2. Classification of drug resistance in cancer

Class	Definition	Note	Ref.
Class I	Amplification and/or activation of RTK	e.g. ErbB/EGFR family, IGF1R, PDGFRβ, FGFR or MET. EGFR-S492R mutation inhibits cetuximab binding.	[106]
Class II	Activating mutations in intracellular signaling proteins	e.g. PIK3CA, RAS family, BRAF or MEK. Activating mutations in PIK3CA are frequent in HNC	[107]
Class III	Stromal signals	e.g. HGF-high stroma activates HGF-MET signaling, which is driven by HSF1-HSP stress signaling.	[40,108,110]
Class IV	EV-mediated ejection of drugs Exosomal ejection of drugs	Anti-cancer drugs are often ejected with EVs from cells.	[29]

Class I and class II drug resistance are generated by genetic alterations in tumor cells. Class III drug resistance is caused by stromal signals from the tumor microenvironment. Class IV drug resistance is EV-mediated drug ejection. The class I-III can be involved in the mechanism of class IV.

 doi:10.20944/preprints201912.0386.v1

6. Conclusions

EV-mediated ejection of anti-cancer therapeutics is a novel mechanism of drug resistance that develops in cancer. Chemotherapeutics, as well as antibody drugs, can be released with EVs derived from the tumor cells. EV/drug-releasing phenotypes are often coupled with cellular transforming processes such as EMT and CSC/CIC. RASP is a marker of resistant phenotypes and a potential target to inhibit EV release from cancer cells.

Author Contributions: conceptualization, T.E.; writing—original draft preparation, T.E.; writing—review and editing, S.K.C., K.On, and K.Ok.; visualization, T.E.; supervision, S.K.C., K.Ok, T.E.; project administration, T.E.; funding acquisition, T.E.

Funding: T.E. was funded by JSPS Kakenhi, grant numbers 17K11642-TE, 19H04051-HO, 19H03817-MT, 18K09789-KN, 17K11643-CS, 17K11669-KO, 16K11722-JM, and 16K11863-KO and by Suzuki Kenzo Memorial Foundation.

Acknowledgments: We thank Akira Sasaki for mentorable supports and Chiharu Sogawa, Yuka Okusha, Hotaka Kawai, Keisuke Nakano, and Heiichiro Udono for illuminating discussions.

Conflicts of Interest: The authors declare no conflict of interest.

 doi:10.20944/preprints201912.0386.v1

Abbreviations

ABC	ATP-binding cassette
ADCC	antibody-dependent cellular cytotoxicity
ATP	Adenosine triphosphate
CAF	Cancer-associated fibroblast
CDK	Cyclin-dependent kinase
CIC	Cancer-initiating cell
CNV	copy number variation
CRC	Colorectal cancer
CRPC	Castration-resistant prostate cancer
CSC	Cancer stem cell
CTL	Cytotoxic T-lymphocyte
ECM	Extracellular Matrix
EGF	epidermal growth factor
EGFR	Epidermal growth factor receptor
EGFRvIII	Epidermal growth factor receptor variant III
EMT	Epithelial to mesenchymal transition
EV	Extracellular vesicle
FcR	Fragment-crystallizable receptor
GIST	gastrointestinal stromal tumor
HGF	Hepatocyte growth factor
HNC	Head and neck cancer
HSF	Heat shock factor
HSP	Heat shock protein
MDR	Multidrug resistance
MMP	matrix metalloproteinase
MSC	Mesenchymal stem cell
MZF1	Myeloid zinc finger 1
NK	Natural killer
NSCLC	Non-small cell lung carcinoma
OMV	outer membrane vesicles
OSCC	Oral squamous cell carcinoma
PD-1	Programmed cell death-1
PD-L1	Programmed cell death-ligand 1
RASP	Resistance-associated secretory phenotype
RTK	Receptor tyrosine kinase
TGFβ	transforming growth factor β
TNBC	Triple negative breast cancer
Tumoroid	Tumor organoid

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