Neoplasma 2024; 71(2): 117–122

doi:10.4149/neo_2024_240208N59

# Participation of suicide gene extracellular vesicles in metastasis prevention

*Minireview*

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**Received February 8, 2024 / Accepted April 19, 2024**

The incidence of distant metastases is associated with most cancer-related mortalities. Extracellular vesicles (EVs), secreted from tumors and cancer-associated fibroblasts, are involved in the metastatic process mediating their organotropism through their involvement in the pre-metastatic niche formation. We have been developing suicide gene therapy mediated by EVs secreted from mesenchymal stem/ stromal cells, tumor cells, and cancer-associated fibroblasts. Suicide gene EVs conjugated with prodrug are tumor tropic, penetrate tumor cells, and kill them by intracellular conversion of nontoxic prodrug to an efficient anti-cancer drug. Here, we discuss findings regarding the possibility of using suicide gene EVs as a novel therapeutic approach for metastases, via pre-metastatic niche modification. The suicide gene EVs provide a future perspective for metastasis prevention.

*Key words: tumor-derived EVs; pre-metastatic niche; metastatic organotropism; suicide gene tumor-derived EVs; metastasis prevention*

A novel therapeutic strategy is needed to advance the treatment of aggressive tumors and metastases in cancer. Tumors such as glioblastoma [1], pancreatic cancer [2], and uveal melanoma [3] do not have reasonably efficient standard chemotherapy because of the early appearance of metastases. Consequently, the median overall survival time of these patients is extremely short. For patients with disseminated metastases, chemotherapy, and emerging immunotherapy remain to be the only options [4]. The fundamental obstacle to improving the prognosis of a cancer patient's survival lies generally in the difficulty of a localized therapeutic attack directed to the primary tumor and distant metastases. The primary limitations of standard therapies with anti-cancer drugs are due to their lack of tumor-specific targeting. Cytotoxic chemotherapy kills most cells in a tumor, but the slowly dividing cancer stem cells survive due to their relatively high resistance to drugs and because of their dormancy. Despite their small number, their character is sufficient to allow tumor recurrence [5]. Moreover, extracellular vesicles (EVs) released by tumor cells undergoing chemotherapy or radiation therapy are the cause of the formation of drug-resistant cell subpopulations [6, 7].

## Gene-Directed Enzyme Prodrug Therapy mediated by suicide gene

The tumor-homing character of human mesenchymal stem/stromal cells (MSCs) inspired our interest in using them as tumor cell-targeted therapy. We developed the Gene-Directed Enzyme Prodrug Therapy (GDEPT), also known as the "Trojan Horse" therapy, which is mediated by MSCs transduced with the suicide gene. The principle of GDEPT is based on the ability of retroviruses to infect cells which leads to the enrichment of cell DNA with genes present in the retroviral vector. Due to the presence of potent retroviral promoters, the viral genes are expressed and translated to protein products. In order to obtain suicide gene transduced cells as a source of EVs, we constructed a bicistronic retroviral vector containing suicide genes yeast cytosine deaminase::uracil phosphoribosyltransferase (*yCD::UPRT*)

[8]. The enzyme can convert a prodrug 5-fluorocytosine (5-FC) into a cytotoxic cancer drug 5-fluorouracil (5-FU). The presence of an internal ribosome entry site facilitates simultaneous expression of genes under retrovirus promoter. The product of the *neo* gene allows the selection of a homogenous population of transduced cells in a medium containing antibiotic G418. Similarly, we have been using in our studies another suicide gene system, namely Herpes simplex virus thymidine kinase with prodrug ganciclovir (HSVtk/GCV system) [9, 10], but this article is limited to the outcomes with *yCD::UPRT*/5-FC system only.

**EVs secreted from MSCs transduced with suicide gene *yCD::UPRT* conjugated with prodrug 5-FC act as cancer cell-targeted Trojan horse**

The tumor-specific tropism of mesenchymal stem/stromal cells (MSCs) was our reason to transduce human adipose tissue-originated MSCs (AT-MSCs) with retrovirus vector containing *yCD::UPRT* gene [11]. The *yCD::UPRT*-AT-MSCs were used successfully in the growth-inhibiting of several human tumors in animal cancer models. In a pilot preclinical study with nude mice, we have demonstrated that human *yCD::UPRT*-AT-MSCs administered intravenously were effective in inhibiting subcutaneous xenografts of human colorectal carcinoma cells [11], melanoma [12], and human bone metastatic prostate cells [13]. In addition, the positive therapeutic effect of the human *yCD::UPRT*-AT-MSCs cells was proven in the autochthonous prostate adenocarcinoma in TRAMP mice, which spontaneously develop aggressive prostate cancer [14]. Intracranial administration of the *yCD::UPRT*-AT-MSCs has been shown to lead to complete tumor regression off intracerebral rat C6 glioblastoma [15, 16]. Difficulty in finding *yCD::UPRT*-AT-MSCs in the place of the tumor, despite positive therapeutic effect, suggested a paracrine mode of outcome. The explanation arrived when we found the secretion of EVs possessing mRNA of the suicide gene that exhibited cytotoxic effects in the presence of prodrug in a dose-dependent manner [17]. Tumor targeting character of the *yCD::UPRT*-MSC-EVs conjugated with prodrug 5-FC was confirmed when repeated systemic injections (intravenous, intranasal) cured rats with intracerebral C6 glioblastoma [18]. Secretion of EVs from human cells successfully transduced with the *yCD::UPRT* gene has been found repeatedly. Putative cell waste role of EVs – exosomes excluding foreign products from cells may be an explanation.

**Role of EVs in tumor progressing and metastasis**

All cells secrete EVs as a way to communicate with other cells and body organs. EVs released from tumor cells transport information and materials not only between tumor cells but also between stromal cells, which play a key role in therapeutic resistance in pancreatic tumors. Moreover, EVs from tumor cells stimulate cancer-associated fibroblasts (CAF), which in return create a microenvironment that supports tumor growth. Tumor progression is enhanced by circulating tumor cells that escape from the primary tumor to form metastases. The metastatic spread of tumors is a coordinated process, in which small extracellular vesicles (sEVs) secreted from tumor cells play important roles [19–21]. Tumor cell-secreted EVs possess many diverse biological functions. They can support neoplastic growth, invasion, and metastasis by reprogramming recipient cells [22]. In addition, they are involved in pre-metastatic niche creation in organs predicted by the type of tumor [23]. The idea of the pre-metastatic niche as a microenvironment prepared for tumor cell dissemination to distant organ sites was first proposed by Lyden's group [24, 25]. Later, reports have shown that the formation of a pre-metastatic niche depends on tumor-derived EVs [26]. The establishment of the pre-metastatic niche appears to be caused by integrin-mediated fusion with resident cells that are particular to an organ [19]. The human tumor cells with the potential to metastasize release sEVs involved in the creation of a pre-metastatic niche at the predicted organs.

**Tumor-derived EVs determine the dissemination of metastases into organs**

The organotropism of different cancer types mediated by EVs is rather diverse. For example, EVs secreted by cutaneous skin-localized melanoma mediate lymphatic remodeling of draining lymph nodes leading to the creation of metastases in various organs [27], whereas uveal melanoma is characterized by a high incidence of liver metastases [28]. Generally, EVs secreted from tumor cells are organ-tropic, the pre-metastatic niche development in the liver is initiated by pancreatic cancer exosomes [29]. The tumor cells retain their original homing character; they possess the ability to rehome to the site of their origin. Fortunately, tumor cell-secreted EVs mimic the roles performed by mother cells. They can reach the appropriate target cells at distant sites and organs [30]. Thanks to these features, tumor-derived EVs are attractive targets for anti-cancer drug modification. EVs possessing suicide gene message secreted from either mesenchymal stem/stromal cells [17], or from tumor cells [31, 32], having the ability to convert non-toxic compound to cytotoxic drug intracellularly, are a promising treatment option for metastatic tumors that are untreatable at present.

**Supposed anti-metastatic potential of tumor cell-derived suicide gene-EVs**

The blocking of tumor-derived EVs to deliver their cargo to recipient cells and/or destroy them may be a powerful strategy for preventing tumor metastasis. We hypothesized that tumor cell-derived EVs converted to suicide gene-EVs conjugated with prodrug 5-FC when applied systematically, may be the way to eliminate circulating tumor cells and/or disrupt niche formation thus preventing the disseminating

tumor cells (Figure 1). In coordination with the working hypothesis, we transduced several tumor cell lines with retrovirus vector possessing the *yCD::UPRT* gene. Transduced tumor cells secreted EVs containing mRNA of this suicide gene in their cargo, similarly as *yCD::UPRT*-MSCs. The EVs were found to be cancer cell-targeted, internalized by recipient tumor cells, and induced cell death by converting the prodrug 5-FC into the cytotoxic drug 5-FU intracellularly [31].

## Tumor-derived EVs are tumor type particular and specify metastasis organotropism

Each type of cancer has its specific metastatic route directed by secreted EVs to the target organs. For example, uveal melanoma and cutaneous melanoma differ in metastatic organotropism despite the same embryonic origin. Cutaneous melanoma metastasizes in various organs, while uveal melanoma is characterized by a high frequency of metastases in the liver. The metastatic spread of uveal metastases is slow, about half of all patients will develop liver metastases within 5 years [33]. The observation that specific microRNAs transferred by EVs secreted from uveal melanoma differ from EVs of other cells was an earlier suggestion of the involvement of EVs in the uveal melanoma liver-specific organotropism [34]. Later on, the protein cargo of the uveal melanoma EVs was found involved in pre-metastatic niche preparation in the liver [35]. Several factors in uveal melanoma EVs were reported to be involved in the pre-metastatic niche formation. The activation of stellate cells in the liver via macrophage migration inhibitory factor was reported to be a way in which the pre-metastatic microenvironment was created [36]. On the other hand, the pancreatic cancer CAFs (PCAFs) of the pancreatic ductal adenocarcinoma (PDAC) are very active components of pancreatic carcinoma [37]. They function as a physical barrier, preventing efficient drug delivery. PCAFs secrete extracellular vesicles that realize not only the cross-talk of tumor cells with other stromal cells but are also involved in the modulation of the tumor microenvironment and can initiate PDAC pre-metastatic niche formation in the liver and lungs [38]. Therefore, the modification of EVs produced by PCAFs with suicide *yCD::UPRT* gene may serve as a tool for disruption of the process of the pre-metastatic niche creation, thus preventing metastasis.

## Participation of modified EVs in metastasis prevention

Tumor metastasis, the dissemination of tumor cells from a primary site to distant organs, is a major contributor to the deaths of cancer patients. Many efforts have been made in the treatment of cancer metastasis via chemotherapy and radiotherapy with limited effect. For example, clinical trials aimed at treating metastatic uveal melanoma using a range of targeted strategies failed [39]. There is an urgent need for novel treatment modalities of metastases to overcome drug resistance. EVs, with the ability to transfer messages to distant organs, especially when they are specifically targeted, may be a novel approach to metastasis prevention. The first evidence that EVs from tumor cells modified with toxic drug can prevent metastasis organotropism was brought by Xiaodong Xie *et al.* [40]. Authors demonstrated in mice that the EVs derived from the circulating breast cancer-derived cells loaded with doxorubicin inhibited breast cancer metastasis to the lungs. The breast cancer cells MDA-MB-231 subcutaneously implanted in mice created metastases in the lungs. Repeated intravenous injections of these EVs prevented metastatic spread by interfering with circulating MDA-MB-231 cells [40]. We believe that *yCD::UPRT* suicide gene-EVs from MSCs, tumor cells, CAFs various tumor types conjugated with nontoxic prodrug hold the potential to interfere with the metastatic process either by killing circulating tumor cells intracellularly and/or by inhibition of pre-metastatic niche

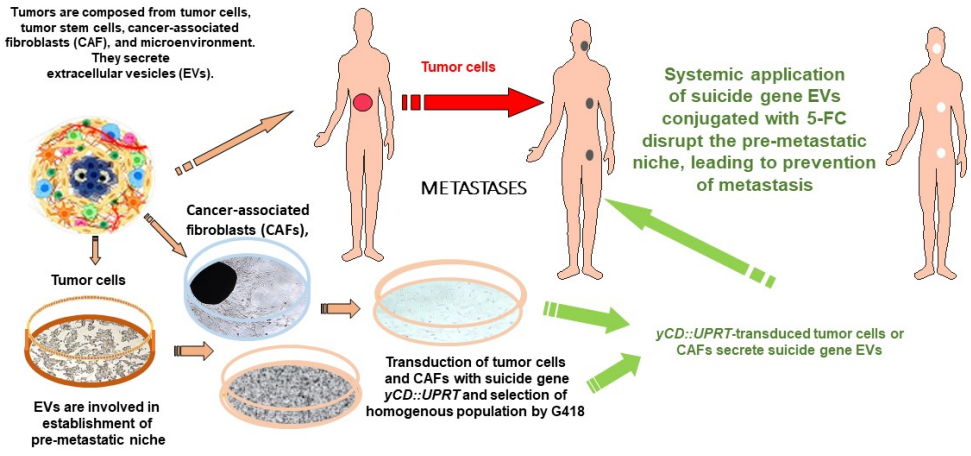


**Figure 1. The schematic workflow of tumor and CAFs cell-derived EVs isolation and their conversion to suicide gene-containing EVs.**

formation. Suicide gene EVs conjugated with 5-FC from various types of MSCs may be versatile. Tumor cell-derived *yCD::UPRT* suicide gene-EVs inhibiting pre-metastatic niche creation or damaging it, may lead to metastasis prevention [31]. Similarly, EVs secreted from uveal melanoma modified with *yCD::UPRT* suicide gene [32], when systemically repeatedly applied with 5-FC prodrug could be the way how to prevent metastasis. The inference with circulating tumor cells or prevention of proper niche creation are the mechanisms leading to metastasis prevention.

Translational research is starting to bring evidence that drug-modified tumor-released EVs can prevent the creation of metastases. The chemopreventive concept targeting the metastatic phase of the disease by exosomes releasing anticancer drug acting as a Trojan horse organotropic vesicles for specifically controlled release of active components was tested in nude mice. Exosomes from human fibrosarcoma cells HT-1080 engineered to carry Doxil systemically injected home to their original subcutaneous tumor tissues. Moreover, compared to Doxil alone, the drug-loaded exosomes showed enhanced therapeutic retention in tumor tissues and eradicated them more effectively in nude mice [30]. The inhibition of uptake of MDA-MB-231 EVs by lung fibroblasts with a selected anti-cancer drug is another protection against breast cancer metastasis. Trametinib suppressed macropinocytosis in lung fibroblasts and inhibited EVs uptake, thus preventing pre-metastatic niche formation [41]. Another example of an effective metastatic chemopreventive experimental approach was reported as a mixture of 4 drugs acting synergistic. Highly Active Metastasis Preventing Therapy (HAMPT) is a drug combination consisting of mifepristone, aspirin, lysine, and doxycycline. HAMPT within its effective concentration range (1–50 μg/ml) showed no cytotoxicity to colon cancer cells HT-29 and CT-26 but significantly inhibited adhesion and invasion of these colon cancer cell lines to human umbilical vascular endothelial cells [42].

The approach to metastasis prevention, besides many other points, is dependent on the time metastases appear. For such tumors as colon cancer and breast cancer, the chemotherapeutic way of treatment to prevent metastasis is plausible. On the other hand cancers, like PDAC where at the time of primary tumor resection the pre-metastatic niche was very likely already created, the treatment must be aggressive to have a chance to be effective. Although many studies consider PCAFs a potential therapeutic target, the lack of highly specific PCAFs markers and their high heterogeneity limit the finding of an effective anti-PDAC drug [43]. Thus far, this chemotherapeutic treatment approach has been largely unsuccessful [44]. Furthermore, PCAF-EVs are contributing to the induction of gemcitabine resistance by inhibiting the apoptosis of pancreatic cancer cells [45]. We believe the tumor-targeted suicide gene MSC-EVs and/or PCAFs EVs engineered to express suicide gene conjugated with 5-FC, both able to penetrate the PCAFs acting intracellularly hold the promise to be effective against pancreatic tumors. It may influence metastasis via modifying or destroying the pre-metastatic niche. The addition of gemcitabine to this therapeutic system may enlarge its capacity for PDAC treatment. The growth inhibition of pancreatic cancer cell lines with dental pulp MSC-EVs having incorporated gemcitabine supports our presumptions [46]. Additional support to the utility of GDEPT mediated by suicide gene EVs in metastasis prevention came from our translational study in rats with brain-implanted glioblastoma cells treated with systemic injections of suicide gene-MSC-EVs conjugated with prodrug that have shown curative effect [18].

In conclusion, neoadjuvant application of suicide gene *yCD::UPRT*-EVs conjugated with prodrug 5-FC may lead to the prevention of metastasis. Suicide gene EVs can be derived from tumor cells, tumor stromal cells, and/or from several types of human MSCs. The running translational experiments may bring needed explanations.

Acknowledgments: This work was supported by financial support No.43092023 from SPP Foundation, by the VEGA grant 1/0483/23 from the Grant Agency of the Ministry of Education, Science, Research and Sport of the Slovak Republic, and by the APVV grant 20-0143.

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