

Cell-derived microvesicles and cancer

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ABSTRACT

Blood and other body fluids contain cell-derived microvesicles. The presence of microvesicles in cancer patients was already noticed in the late 1970s. Since then, the prothrombotic state in cancer patients has invariably been associated with the presence of such microvesicles. More recently, a growing body of evidence supports an important contribution of microvesicles to cancer cell survival, invasiveness and metastases. Here, we will present an overview of the many contributions of microvesicles to cancer development and progression. In addition, their role in risk stratification and treatment of cancer patients is discussed.

KEYWORDS

Anti-cancer treatment, cancer, exosomes, microparticles, microvesicles

INTRODUCTION

Compared with healthy controls, blood from cancer patients contains elevated levels of cell-derived microvesicles. What are these microvesicles and why are their levels elevated in cancer patients?

Types of microvesicles

Human body fluids contain two different types of cell-derived microvesicles: microparticles and exosomes. Eukaryotic cells, including blood cells, endothelial cells and cancer cells, release *microparticles* by budding off parts of their outer cell membrane. Based on electron microscopy, microparticles range in size from 100 nm to 1.0 μm .¹ *Exosomes* arise from endosomes, which are initially formed by plasma membrane invagination.

Endosomes release vesicles into their lumen, 'intraluminal vesicles'. Endosomes containing 'intraluminal vesicles' are called multivesicular bodies (MVBs). Finally, when MVB membranes fuse with the plasma membrane, these 'intraluminal vesicles' become secreted and are then called exosomes. Exosomes range in size from 30 to 100 nm and all cell types containing MVBs can be expected to secrete exosomes. Such cell types include haematopoietic cells, cancer cells, and epithelial cells.² At present, there is no generally accepted definition of microparticles and exosomes. Not only theoretical issues but especially methodological problems hamper the achievement of consensus. In this review, we will use the general term microvesicles.

General effects of microvesicles

Microvesicles are involved in many (patho) physiological processes in the human body (*table 1*). Membranes of microvesicles contain phospholipids and proteins that often originate from membrane lipid rafts of the parental cell, including functional transmembrane receptors such as tissue factor (TF). Furthermore, intracellular proteins, second messengers and genetic material can be enclosed and specifically sorted into microvesicles. As a consequence of sorting, the functional properties and biological role of microvesicles may differ from their parental cells.³ Microvesicles interact with cells by binding to cell-type specific adhesion receptors. After this initial interaction, membranes of microvesicles may fuse with the plasma membrane of the target cell, thereby transferring receptors that can induce cell signalling or even transformation, genetic information and second messengers.⁴ Microvesicles are not only involved in intercellular communication, but also in other processes including regulation of programmed cell death, modulation of the immune response, inflammation, angiogenesis and coagulation.⁵⁻⁷

Table 1. *Effects of cancer cell derived microvesicles*

Effect	Role of microvesicles	References
Improves cellular survival	Removal apoptosis inducing proteins	5
	Chemotherapy resistance	17, 18
Escape immune surveillance	Complement resistance	20-22
	T-cell suppression	23-28
	Mimic environment	13,29,30
Environmental degradation	Transport active matrix metalloproteinases	31-34
Angiogenesis	Activation coagulation system: fibrin matrix formation and PAR signalling by thrombin formation	13,38,40
	Intercellular transfer mRNA coding for growth factors	45,46
Metastasising	Intercellular transfer oncogenes	47-49

PAR=protease activated receptor.

The release of microparticles is a physiological phenomenon. All sorts of biochemical triggers induce release of microvesicles, such as cytokines and chemotherapeutics, as do physical triggers such as hypoxia and shear stress. In diseases, aberrant levels of microvesicles are observed, and their numbers, cellular origin and composition are disease (state) dependent.

Microvesicles in cancer patients

The presence of microvesicles in cancer patients was already noticed in the late 1970s.⁸ The underlying mechanism leading to the release of microvesicles from cancer cells, however, is still unknown. In a mouse model, the loss of the tumour suppressor gene p53 leads to an increased release of TF-bearing microvesicles, indicating involvement of p53 in this process.⁹

Blood from cancer patients contains not only microvesicles from cancer cells but especially high levels of procoagulant platelet-derived microvesicles. The procoagulant state of cancer patients has at least partly been attributed to these microvesicles.^{10,11} Recent studies have shown that cancer patients with venous thromboembolism have higher levels of TF-bearing microvesicles compared with cancer patients without thrombosis.^{12,13} In our opinion, this procoagulant phenotype of microvesicles is merely a side effect of a more important role they may have in cancer patients, i.e. by facilitating cancer progression. This review summarises the effects of cancer cell-derived microvesicles in cancer biology. Finally, the possible value of these vesicles in clinical practice will be discussed.

ROLE OF MICROVESICLES IN CANCER PROGRESSION

Cellular survival

Escape from apoptosis

Cells release microvesicles as a protective mechanism against *intracellular* stress. In nucleated mammalian cells, caspase 3 is one of the main executioner enzymes of

apoptosis. Microvesicles containing substantial quantities of caspase 3 are present in conditioned medium of viable cell cultures,^{14,15} but caspase 3 is not detectable within the cells from which these microvesicles originate. Various investigators have postulated that cells may escape from apoptosis by releasing caspase 3-containing microvesicles, thus preventing intracellular accumulation of the potentially dangerous caspase 3. Recently, this hypothesis was strengthened by the observation that cells indeed accumulate caspase 3 and undergo apoptosis when the release of microvesicles is inhibited.⁵ Thus, the release of caspase 3-containing microvesicles contributes to cellular survival. In addition, caspase 3 itself is also involved in the release of microvesicles. MCF-7 cells, a human breast cancer cell line lacking caspase 3, do not release any or hardly any microvesicles. Their ability to release microvesicles, however, can be restored by transfection with functional caspase 3.¹⁶ Since these microvesicles also contain caspase 3, it appears that caspase 3 contributes to its own removal (A.N. Böing, unpublished observation).

A second example, illustrating how the release of cancer cell-derived microvesicles contributes to cellular survival, comes from studies demonstrating an association between their release and multidrug resistance. Shedden and colleagues, who quantified membrane shedding-related gene expression, observed that chemo-insensitive cancer cell lines express more membrane shedding-related genes compared with chemo-sensitive cells. Furthermore, the microvesicles contained high levels of the chemotherapeutic agent doxorubicin.¹⁷ The most convincing evidence comes from a study by Safeaei and colleagues, who demonstrated that microvesicles of cisplatin-insensitive cancer cells contained 2.6-fold more cisplatin than microvesicles released from the cisplatin-sensitive cells.¹⁸

Escape from immune surveillance

In a pioneering study published in 1988, Sims and co-workers showed that complement activation triggers the release of microvesicles. When human platelets were incubated with a lytic concentration of the complement C5b-9 complex,

platelets simply survived by releasing C5b-9-enriched microvesicles. This mechanism was called 'complement resistance', and this release can be considered a form of protection against *external* stress.¹⁹ Similarly, cancer cells use the release of microvesicles to escape from complement-induced lysis.^{20,21} A recent study showed that cancer cells can inactivate the complement complexes by shedding vesicles containing the complement inhibitor membrane cofactor protein CD46, which promotes inactivation of complement C4b and C3b. Liberation of CD46 minimalises inflammation in the microenvironment. Although a solid tumour is well protected from complement attacks, micro tumours are attacked by the complement system.²²

A more indirect way to improve survival of cancer cells is by suppressing the immune response, i.e. via the release of microvesicles bearing immune modulatory molecules. Microvesicles from various cancer cells expose Fas ligand (FasL, CD95L), a ligand of the death receptor Fas (CD95), which induces T-cell apoptosis and diminishes the function of adaptive immune cells.^{23,24} Kim and colleagues showed a modest correlation between lymph node infiltration and tumour burden and the numbers of circulating FasL-exposing microvesicles in blood from patients with oral squamous cell cancer.²⁵ Microvesicles from lymphoblastoma cells exposed latent membrane protein-1 (LMP-1), another immune suppressing transmembrane protein, thereby inhibiting leucocyte proliferation. This finding may explain the observed inhibition of T-cell proliferation in patients with Epstein-Barr virus associated tumours.^{26,27} Microvesicles not only suppress effector T lymphocytes but also target antigen-presenting cells, the latter also known as dendritic cells. Valenti *et al.* showed that cancer cell-derived microvesicles are able to fuse with plasma membranes of monocytes, thereby impairing their differentiation to antigen-presenting cells.²⁸ Finally, cancer cells may hide from the immune system by mimicking the host environment. In a study by Tesselaar *et al.*, a low number of circulating microvesicles were present in cancer patients that stained for MUC1, a cancer cell antigen, and glycoprotein IIIa (integrin β_3), which is mainly present on platelets and platelet-derived microvesicles. Based on these data, they suggested that such microvesicles are released after fusion of microvesicles from malignant epithelial cells with platelets.¹³ Alternatively, platelet-derived microvesicles were shown to transfer integrins to breast and lung cancer cells.^{29,30} Thus, cancer cells can fuse with non-cancer cell-derived microvesicles, thereby receiving lipids and membrane-specific proteins which may help to escape from immune surveillance. *Figure 1A* summarises the effects of microvesicles on cellular survival.

Invasive growth and metastasising

Environmental degradation

Degradation of the extracellular matrix (ECM) is essential for tumour growth.³¹ Microvesicles expose and

contain proteases, including matrix metalloproteinase (MMP)-2 and MMP-9 and its zymogens, and urokinase-type plasminogen activator (uPA). MMPs degrade basement membrane collagens, whereas uPA catalyses the conversion of plasminogen into plasmin. Plasmin, a serine protease, degrades numerous components of the ECM, including fibrin, and activates MMP zymogens. Ginestra *et al.* analysed the content of microvesicles in ascites from 33 women with different gynaecological pathologies, including benign ovarian lesions, ovarian carcinomas, and endometrial carcinomas. They showed that ascites from the cancer patients contained higher numbers of microvesicles compared with ascites from women with benign disease. Microvesicles from patients with benign serous cysts had only minimal lytic activity, whereas those from cancer patient ascites contained active MMPs.³² Similarly, the malignant potential of tumours was associated with the MMP-2 activity of microvesicles.³³ Graves *et al.*, who evaluated microvesicles in women with early-stage and late-stage ovarian carcinoma, reported increased numbers of microvesicles in late stage ascites and showed that MMP-2, MMP-9 and uPA activities are primarily concentrated within microvesicles. Inhibition of MMP-2, MMP-9 or uPA nearly abolished the ability of these microvesicles to support tumour invasiveness, which underlines the relevance of this pathway, at least *in vitro*.³⁴ The increased invasiveness of cancer cells by microvesicle formation is shown in *figure 1B*.

Angiogenesis

Fibrin, the insoluble end product of coagulation, plays an important role in tumour growth. Tumour cells can be coated with fibrin to escape from immune detection and attacks, and the fibrin matrix supports outgrowth of new blood vessels. One of the general effects of microvesicles is their support of coagulation.³⁵⁻³⁷ Especially in cancer patients, TF-bearing microvesicles are present in the peripheral blood, albeit that the cellular origin of such microvesicles is still disputed.³⁸⁻⁴⁰ A part of the TF-bearing microvesicles is likely to originate from cancer cells and probably contributes to thrombus formation equally to leucocyte-derived microvesicles, which may also expose procoagulant TF. TF-bearing microvesicles can be captured and trapped by activated platelets at the site of a wound, thereby delivering and accumulating their procoagulant TF at the site of vascular damage.^{41,42} Furthermore, TF-bearing microvesicles may fuse with (membranes of) activated platelets, thereby transferring TF to the platelet membrane, which can then not only propagate but also initiate coagulation.³ *Figure 1C* shows the contribution of microvesicles to fibrin formation.

The procoagulant effect of microvesicles also indirectly leads to the release of growth factors. Thrombin

activates cells via cleavage of protease-activated receptors (PARs), and this activation results in release of vascular endothelial growth factor (VEGF).^{43,44} Finally, platelet-derived microvesicles stimulate mRNA expression of pro-angiogenic factors in cancer cells,²⁹ and cancer cell-derived microvesicles contain mRNA encoding growth factors such as VEGF and hepatocyte growth factor. Baj *et al.* showed that such vesicles fuse with monocytes, transferring their nucleic acids and inducing production of growth factors.^{45,46} *Figure 1D* shows the influence of cancer cell-derived microvesicles on angiogenesis.

Metastasising

Cancer cell-derived microvesicles contribute to horizontal propagation of oncogenes and their associated transforming phenotype. Recently, Newadi *et al.* demonstrated the intercellular transfer of the truncated oncogenic form of the epidermal growth factor receptor (EGFRvIII) from glioma cancer cells to glioma cells lacking this receptor. After this transfer, the recipient cells became transformed and showed characteristic EGFRvIII-dependent changes in expression levels of target genes.⁴⁷ Although not studied yet, a similar intercellular transfer of other mutant oncogenes, such as MET and HER-2, may be a general mechanism operative in different tumour types which cause cancer growth at distant sites.

DNA-containing microvesicles from apoptotic cells ('apoptotic bodies') were shown to transfer DNA to other cells. In that study, apoptotic bodies from cancer cells triggered the expression of oncogenes in fibroblasts *in vitro*. After injecting these cells to SCID mice, tumours expressing the oncogene were observed. Thus, also the genetic information necessary for transformation and cells may be functionally transferred between cells by cancer cell-derived microvesicles.⁴⁸

Skog *et al.* showed that glioblastoma cancer cells release microvesicles containing mRNA, microRNA and angiogenic growth factors. After transfer of vesicular RNA by fusion of the microvesicles with endothelial cells, the mRNA was translated into functional pro-angiogenic proteins thereby promoting angiogenesis. Cells with low levels of mRNAs produced microvesicles with high levels of mRNA in a constant distribution. This supports the hypothesis that the enrichment of microvesicles with mRNA and intracellular proteins is a selective process.⁴⁹

Whether or not microvesicles promote mobilisation of tumour cells, however, has not been extensively studied. Lymphogenous spread could be enhanced by the immune-suppressive effects of cancer cell-derived microvesicles.²⁵ Activation of platelets by TF-bearing microvesicles is probably helpful in the haematological spread of cancer cells, since activated platelets expose the adhesion receptor P-selectin and cancer cells expose

the corresponding P-selectin ligands, such as P-selectin glycoprotein (PSGL) and Sialyl Lewis. As a consequence, the cancer cells will be surrounded by platelets and/or P-selectin-bearing microvesicles, thus protecting cancer cells from immune surveillance and facilitating their binding to the vessel wall.^{39,50} The procoagulant properties of cancer cell-derived microvesicles may further support intravascular fibrin formation, which will facilitate adherence of cancer cells to the vessel wall. *Figure 1E* presents the contribution of microvesicles to cancer cell migration.

FUTURE APPLICATIONS IN CANCER THERAPY

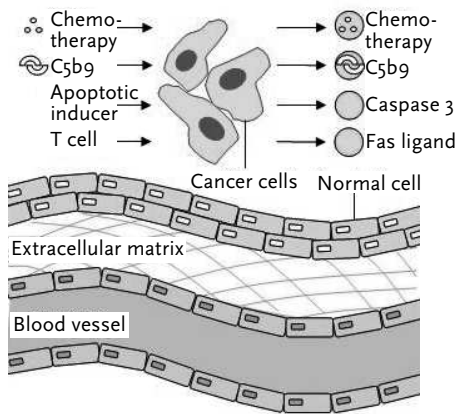
Anti-cancer treatment

Cancer cell-derived microvesicles have been used as adjuvant anti-cancer treatment. As described above, they have immunosuppressive activity due to functional alterations induced in T cells, ranging from apoptosis to defects in T-cell function.^{23-25,51} However, cancer cell-derived microvesicles may also facilitate immune attacks.^{2,52-59} Wolfers *et al.* showed that cancer cell-derived microvesicles transferred tumour antigens to antigen-presenting cells, which in turn triggered a T-cell-dependent anti-tumour response.⁵⁹ In addition, antigen-presenting cells were capable of producing microvesicles that primed cytotoxic T lymphocytes *in vivo* and even eradicated or suppressed growth of murine tumours. These autologous dendritic cell-derived microvesicles have been tested in phase I clinical trials in patients with metastatic melanoma,⁶⁰ advanced non-small cell lung cancer⁶¹ and colorectal cancer.⁶² All studies concluded that this therapy is beneficial and safe, with some patients experiencing long-term stability of disease. Currently, several studies are ongoing to optimise this autologous anti-cancer immunotherapy.^{57,63,64}

Release of microvesicles itself could be an interesting target of anti-cancer therapy, i.e. by counteracting the beneficial effects of vesicle release on cellular survival or tumour growth. Some currently used chemotherapeutics impair, at least partially, the underlying mechanisms of microvesicle release, e.g. drugs targeting at Rho-associated coiled coil-containing protein kinases (ROCK).⁶⁵ ROCK-I and II are both serine-threonine kinases which not only affect cell morphology, migration and adherence, but also markedly contribute to release of microvesicles.^{15,66} Rattan and colleagues showed that inhibition of the Rho/Rock pathway resulted in smaller tumour mass in patients with glioblastoma.⁶⁵ Because the release of microvesicles by cancer cells influences many processes associated with tumour growth, inhibition of microvesicle release is a potential target in anti-cancer treatment.

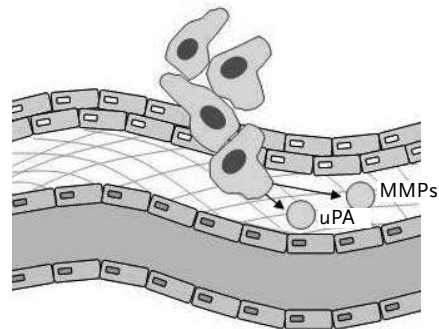
Figure 1. The role of cancer cell derived microvesicles in cancer progression

A) Cellular survival



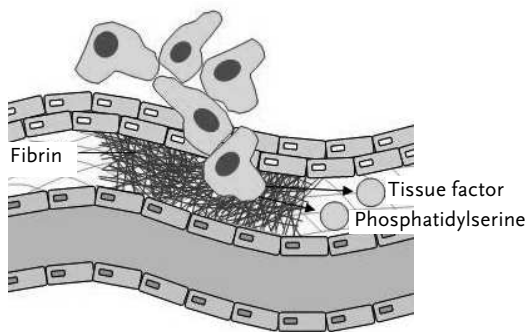
Cancer cells escape from internal (caspase 3) and external (chemotherapy, complement C5b9 complex, immune attack) stress by releasing microvesicles either containing (caspase 3, chemotherapy) or exposing C5b9 and Fas ligand. Thus, the release of microvesicles contributes to cellular survival.

B) Invasiveness



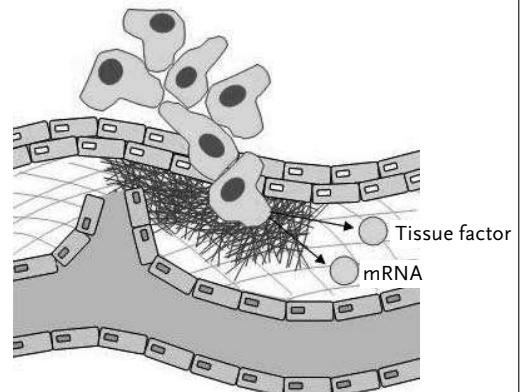
Microvesicles expose and contain several proteases, including matrix metalloproteinase (MMP)-2 and MMP-9 and its zymogens, and urokinase-type plasminogen activator (uPA). By degrading the extracellular matrix, these enzymes facilitate cancer invasiveness.

C) Fibrin formation



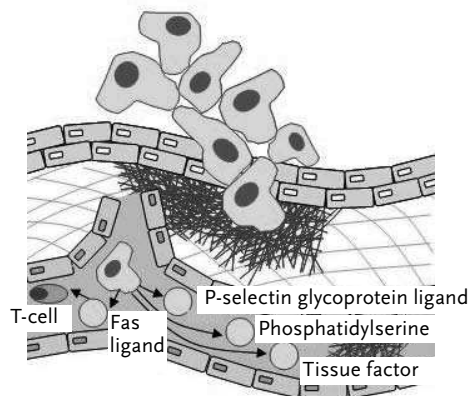
The membrane of microvesicles facilitates and initiates intravascular coagulation by exposing phosphatidylserine and tissue factor, respectively. Fibrin protects the tumour against immune attacks and forms a matrix to support angiogenesis.

D) Angiogenesis



Cancer cells induce angiogenesis by releasing microvesicles containing mRNA encoding growth factors and by exposure of tissue factor. Tissue factor not only initiates coagulation, as shown in figure 1B, but also plays a critical role in angiogenesis. Activation of the cytoplasmic tail of tissue factor and subsequent downstream signalling events induce angiogenesis. Furthermore, thrombin, the final enzyme of the coagulation cascade, cleaves several protease-activated receptors, which in turn trigger angiogenesis.

E) Metastasing



Fas ligand exposing microvesicles enhance lymph node infiltration by killing T cells. Procoagulant microvesicles facilitate intravascular fibrin formation, thus enhancing haematological spread. P-selectin glycoprotein ligand-1 bearing cancer cell-derived microvesicles contribute to clot formation by binding to P-selectin-exposing (activated) platelets.

Measurement of protein composition of microvesicles may be useful to monitor the efficacy of anti-cancer treatment. Clayton *et al.* exposed B-lymphoblastoid cell lines to external stress, i.e. 42 °C for three hours.⁶⁷ Although the number of released microvesicles was comparable with control cells, the protein composition was markedly different. Stressed cells produced microvesicles containing relatively high quantities of heat shock proteins. Since heat shock proteins form complexes with proteins containing one or more production errors, their increased presence within microvesicles could help to maintain cellular homeostasis. Thus, possibly also the protein composition of cancer-cell derived microvesicles may directly reflect the effects of anti-cancer treatment, and could be an early and noninvasive biomarker to assess the effectiveness of anti-cancer therapy.

Risk stratification

Diagnosis

Tumour-specific markers, such as mucin in adenocarcinomas, exposed on circulating microvesicles, may be useful in the early detection of cancer. In a pilot study by Smalley *et al.*, microvesicles were isolated from urine of healthy individuals and patients with bladder cancer. Eight proteins were found to be elevated in microvesicles from cancer patients compared with controls.⁶⁸ Thus, the protein composition of such microvesicles can potentially be used in the early detection of bladder cancer. Similarly, cancer-specific mRNA can be used as a marker for detection of cancer. In the study by Skog *et al.*, microvesicles were purified from serum samples from glioblastoma patients. A glioblastoma-specific mutation was observed in almost 50% of the samples, which was comparable to the percentage of this mutant in glioblastoma patients.⁴⁹ Cancer-specific microRNA was also observed in exosomes purified from plasma samples of patients with ovarian cancer. No differences in microRNA profiles were observed between early and advanced diseased patients whereas patients with benign ovarian disease and healthy women did not express these microRNA profiles. Therefore, the authors suggest that microRNA profiles could be used in patients with a high risk for ovarian cancer.⁶⁹

Prognosis

Different studies have evaluated the association between the level of microvesicles and survival of cancer patients. In the study by Tesselaar and colleagues, patients with both high microvesicle-associated procoagulant TF activity and epithelial mucin (MUC1) had a lower survival rate at three to nine months follow-up compared with patients with low TF activity and no MUC1 expression. After adjustment for other prognostic factors, the likelihood of survival for an individual with both membrane proteins present on

circulating microvesicles was 0.42 (95% CI: 0.19 to 0.94).¹³ In a prospective, nonrandomised single-centre study in hormone refractory prostate cancer patients, the impact of platelet-derived microvesicles on overall survival was assessed in 43 patients before starting chemotherapy. The overall survival was significantly shorter in patients with platelet-derived microvesicles above a certain cut-off level than in patients with values below that level.⁷⁰ Kim *et al.* performed a study in 109 patients with gastric cancer and in 29 healthy controls. Plasma levels of platelet-derived microvesicles were significantly higher in the patients than in controls, and the levels were significantly higher in patients with stage IV disease than those in patients with stage I or stage II/III without a significant difference in platelet number. Platelet-derived microvesicles predicted distant metastasis with a sensitivity and specificity of 93.3 and 91.1%, respectively.⁷¹ Thus, microvesicles may be used as a predictor of disease stage and survival in cancer patients.

Another potential application of microvesicles, especially those bearing TF, is the prediction of venous thromboembolism.^{12,13,72} Although cancer patients have four to fivefold higher risk to develop venous thromboembolism, there are currently no clinical or laboratory criteria to decide which patients warrant primary thromboprophylaxis.^{73,74} Ongoing studies are evaluating the potential of (tissue factor bearing) microvesicles levels as a marker to decide about the appropriateness of primary thromboprophylaxis.

CONCLUSION

It is now generally accepted that cell-derived microvesicles are involved in (patho) physiological processes in humans. This review supports the concept that cancer cell-derived microvesicles play an important role in cancer biology. This field requires further investigation, and additional studies are needed to establish their potential relevance as novel biomarkers in the detection of cancer and their relevance as a new target in anti-cancer therapy.

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