Microvesicles as a potential biomarker of neoplastic diseases and their role in development and progression of neoplasm

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Abstract

Neoplastic diseases together with cardiovascular diseases are the most frequent causes of death in the Polish population. Cancers of reproductive organs with breast cancer are responsible for the highest morbidity and mortality in women suffering from neoplasm diseases. Asymptomatic dynamics of the development of a neoplasm and no deviations from the normal level of laboratory results contribute to the fact that malignant diseases are diagnosed too late. The aim of modern medicine is to diagnose cancer at the earliest stage, however, there is no sufficiently sensitive and specific biomarker which can be used for diagnostic, prognostic and therapeutic purposes.

Cellular interactions play the main role in the development, angiogenesis and invasiveness of a tumor. Recent research suggests the possibility of microvesicles (MVs) involvement in communication between cells. The MVs ability to fuse with various cells is used in cell-to-cell contact. Microvesicles cargo may include growth factors, their receptors, protease, adhesion molecules, signaling molecules and the sequence of DNA, mRNA, and micro-RNA. Larger quantities of MVs released from neoplastic cells affect both the local environment and systematic range causing metastases and progression. The research on molecular mechanisms of MVs’ release and the presence of characteristic oncogenes in blood of patients with neoplasms is being carried out. Confirmation of MVs presence in patients’ serum can potentially serve as useful information for therapeutic purposes and as the biomarker of a neoplastic disease.

Key words: microvesicles, neoplasm, biomarker, ovarian cancer.

Introduction

Ovarian activity to produce hormones decreases in the perimenopausal period and finally the release of hormones is inhibited. Lack of estradiol in postmenopausal women causes a high risk of multiple internal diseases such as arterial hypertension, coronary artery diseases, venous thromboembolism and arteriosclerosis. Moreover, 2/3 of postmenopausal women suffer from overweight and obesity. A lower level of physical activity, improper diet and lack of ovarian hormones cause lipid disorders and changes in the level of fractions of cholesterol. That fact and a high body mass index (BMI) increase a risk of arterial hypertension and arteriosclerosis. Stroke and heart failure are typical organ complications of hypertension and arteriosclerosis. They are a frequent cause of death in women after menopause. Cardiovascular diseases rank the first among the most common causes of death in women of the Polish population. Accidents and neoplastic diseases rank the second and the third, respectively.

Cancers of reproductive organs are frequently diagnosed in the peri- and postmenopausal period. Adenocarcinoma of the uterus is the most common gynecological malignancy. Eighty percent of this type of cancer is diagnosed in women after menopause. Furthermore, hypertension, obesity and diabetes are risk factors for endometrial hyperplasias and cancer. Abnormal uterine bleeding (AUB) and abnormal sonographic features of the endometrium may suggest endometrial cancer. AUB and ET (endometrium thickness) > 4 mm indicate the need for endometrial biopsy and histological examination. When the endometrium is less than 4 mm in sonographic examination, a risk of endometrial cancer is lower. Invasive procedures allow to obtain endometrial tissue for histological examination. The result of examination makes it possible to choose appropriate treatment.

Similar proceedings are used to diagnose neoplasm of the vagina and vulva and to choose the proper treatment. Vulvae and vaginal cancer are frequently diagnosed in women after menopause. Clinical symptoms and vulvae and vaginal lesions require invasive methods of investigation such as a biopsy. Histopathological examination answers the question whether the lesion is benign or malignant. The result of histopathological
Extracellular vesicles – exosomes, apoptotic bodies, microvesicles

Microvesicles are one of several types of microparticles characterized by high levels of phosphatidylycerine in membranes. They are released from different types of eukaryotic cells as a result of their activation by growth factor, cytokines or during apoptosis [17, 18]. Microvesicles, apoptotic bodies and exosomes are the most important and well-known particles, from which merely MVs and exosomes are used by the cells as carriers of information. The beginning of studies on these structures took place in the 60s of the last century, when Wolf described the microparticles in 1967 for the first time [19]. For years MVs had been considered as redundant microparticles – the form of waste products of life processes occurring in the cell. Nowadays their potential is recognized as a useful tool in diagnosis and treatment for many diseases for example, neoplasm [2, 10, 13-15, 20-22].

Exosomes were first described in 1981 [1, 23] as a cup-shaped structures [1, 24] ranging from 30 to 100 nm in size [1]. Other studies described them as oval-shaped structures and ranging from 40 to 100 nm in diameter [24]. Differences in exosome shapes can result from different methods of visualization and analysis. Their cup-shaped morphology can be a result of fixation procedures used for transmission electron microscopy (TEM), which is a standard method used to analyze the size of these microparticles [1, 25]. Exosomes observed by cryo-electron microscopy (cryo-EM) are visualized as oval-shaped vesicles [24, 26, 27].

Exosomes are the only vesicles composed of endosomal membranes [24]. This is because exosomes are released from multivesicular bodies (MVBs) which are endosomes transformed in intracellular processes. The fusion of MVBs membrane with plasma membrane leads to the release of their contents into the extracellular space and avoidance of MVBs degradation through lysosomal pathways [24]. Exosome membranes are characterized by a low level of phosphatidylycerine exposure. However, these membranes contain other lipids, such as cholesterol, ceramide, sphingomyelin and lipids rafts [1, 24], glycolipid GM3 [24, 28, 29], CD 63, CD81, CD 9, LAMP1, TSG101, Alix and HSC70 are distinguished as typical markers for exosomes [1, 12]. Recent studies on exosomes from cancer and immune cells reveal
the ability of these structure to activate several cellular processes depending on their origin [1, 30]. A multitude of activated pathways is a result of cellular interaction with exosomal molecules, including mRNA, microRNA (miRNA) and proteins (e.g., cytoskeletal proteins, heat shock proteins, adhesion molecules, proteins involved in signal transduction, transcription regulation and antigen presentation) [1]. Exosomes can be released from platelets, dendritic cells and cancer cells in laboratory conditions as well as in vivo, which confirms their presence in many biological fluids, including urine [31-33], plasma [34], peritoneal fluid [35], saliva [36], breast milk [37], bronchoalveolar lavage liquid [38], and amniotic fluid [39]. Accurate studies of their role in cancer progression, the development and improvement of collecting methods and isolation will enable the use of exosomes as potential biomarkers in the future.

**Apoptotic bodies**

Structures formed as a result of cell degeneration in the process of programmed cell death (apoptosis) was first described in 1974 by Kerr [40] and named apoptotic bodies. Cellular organelles, fragments of DNA and histones surrounded by plasma membrane create apoptotic bodies with irregular shapes and ranging from 1 to 5 μm in size [12]. Recent research has not revealed the role of those particles in intracellular communication as exosomes and MVs have. Some studies show that apoptotic bodies’ cargo DNA and oncogenes play an important role in antigen presentation and immunosuppression [24, 41-44]. There is no appropriate and standard method of isolation that enables accurate analysis of apoptotic bodies and their role in eukaryotic organism functioning such as human body is.

**Microvesicles, ectosomes**

Many studies on MVs, which were described for the first time in 1967 by Wolf, have been carried out for the last two decades. Microvesicles are microparticles of different shapes and ranging from 100 to 1000 nm in size [1, 10, 20]. They are released from the outward surface of cells as a result of budding or blebbing of the plasma membrane [1, 10, 20]. Microvesicles are released from platelets, red blood cells, endothelial cells and carcinoma cells. This fact has been confirmed by many studies performed in many countries around the world [1, 22, 30, 45]. In tumor cells, MVs are released from the entire cell surface whereas in normal cells, there are selected areas of plasma membrane [1]. The membrane of tumor cells seems to be enriched by special lipids in the area of MVs release. Cholesterol is a lipid needed to form MVs and its lack causes inhibition of MVs production [46, 47]. Microvesicles are characterized by a high level of exposure to phosphatidylserine in their membranes, transported from the inner to outer surface of plasma membrane [1, 48]. Recent studies have not revealed typical surface markers for MVs. To signify those structures, flotillin-2, selectins, integrins, CD40, metalloproteinase can be used [1, 12, 49]. Proteins such as growth factors and their receptors, cytokines, chemokines [1, 4], enzymes, e.g. metalloproteinase, lipids, nucleic acid (mRNA, miRNA, ncRNA, DNA) [1, 21, 50-54] can be transported inside MVs. The role of MVs in several processes in the human organism depends to a great extent on the type of cargo. Microvesicles take part in angiogenesis, carcinogenesis and immune response, because of their use as information carriers in intracellular communication. Moreover, they are said to transport information locally, which has been confirmed by their presence in urine or ascites, and systematically, that has been confirmed by their presence in circulating blood. The role of MVs in intracellular communication has been mostly investigated in carcinogenesis and inflammation. Interaction of intracellular communication may occur locally, regionally and systematically, which is often characterized by direct contact between MVs surface and the surface of a target cell [1, 55] or endocytosis [46]. The influence of MVs on target cells may lead to activation and discharge of paracellular bioactive cargos [2, 15, 22, 56]. It can cause proteolytic remodeling and changes in properties of the microenvironment and changes in behavior of cells [2, 56]. Microvesicles circulating in blood stream and bodily fluids (saliva, urine, cerebrospinal fluid, peritoneal fluid) transfer onco-specified mediators called oncosomes [55]. The role of oncosomes is to transport oncoproteins [57], oncogenic transcript of mRNA [53], exchange oncogenic miRNA [2] and fragments of genomic DNA containing oncogenic sequences from cell to cell. Recent research has indicated the relationship between revealing and circulating in blood MVs and thrombosis, sepsis, coronary disease, multiple sclerosis, and some types of cancer [1, 58-62]. Researchers most accurately characterized MVs from platelet and endothelium and they noticed their increased level in several clinical disorders such as cardiovascular diseases (e.g., hypertension, atherosclerosis, congestive heart failure) [63-65], autoimmunological diseases (e.g., rheumatoid disease, vascular inflammation, diabetes type I) [66-69], hematological diseases and cerebral vessels diseases [70, 71]. Studies are still being carried out on the potential use of MVs as biomarkers which will be the gradient of the biological condition that changes according to disease progression or response to treatment. The idea of biomarkers is the ability to distinguish two similar conditions: pathological and physiological with the facility of collecting samples (e.g., saliva, blood or urine) for its identification. Routine non-invasive methods of indication should be precise, fast and potentially cheap [72].
The methods of isolation, characterization and enumeration of circulating MVs have not been validated completely. Several factors make particular steps of receiving and analysis of MVs difficult. Various studies have demonstrated that cancer-derived MVs have a lifespan of about 15-60 minutes in blood circulation [55, 73, 74]. It may result from rapid fusion and endocytosis by target cells [1]. Other research has disclosed their elimination via phagocytes due to a high level of phosphatidylserine in the outer surface leaflet [1]. The use of MVs released from tumor cells as biomarkers is restricted by a relatively small fraction of the total number of isolated MVs [1, 50]. Those facts require defining pre-analytical procedures including anticoagulants, time from collecting samples to preliminary preparation (first centrifugation), temperature and time of storage and the methods of analysis.

For isolation MVs from blood sample, researchers have used methods based on their physical properties and biochemical features. The size and density are predominantly used as parameters for isolation. Precipitate with MVs is prepared as a result of centrifugations of platelet free plasma (PFP) in ultra-centrifuge at 20 000-60 000 × g [2, 55]. Further isolation base on MVs size includes flotation in sucrose gradients and dielectrophoresis. Magnetophoretic sorting or immunofinity chromatography are used occasionally [1, 50].

The number of MVs in samples of the biological fluid is as important as their detection. Recent studies revealed the correlation between the quantity of MVs released via cells and the stage of a disease [1]. This fact may be used to make a rapid diagnosis, to assess prognosis and early detection of recurrence. That is why it is so important to form reliable and repeatable isolation methods. Flow cytometry is the most widely used method of MVs detection based on the measurement of MVs fluorescence previously marked with specific fluorescent antibodies. The imperfective aspect of this method is inability to notice vesicles (MVs and exosomes) smaller than 200 nm in diameter [1, 55], because in lower detection edges the appropriate signal is drowned out by instrumental noise. Potential difficulties may be reduced by calibration methods with special calibrations beads ranging from 0.2 to 2.0 μm in diameter which are to decrease instrumental noise [55], however, it does not eliminate it totally. Moreover, there are no characteristic antibodies for microparticles. Annexin V was used to mark MVs, however, recent studies have revealed the presence of annexin V-negative MVs [1, 30, 75]. It is necessary to use antibodies as markers for MVs on the basis of their cellular origin [1, 3, 76, 77]. The lack of a satisfactory method of analysis limits the use of ectosomes and exosomes in diagnostic and therapeutic processes. This fact and the potential of microparticles encourages to investigate and to create new methods of qualitative and quantitative evaluation.
and avoidance of apoptosis. Recent research has disclosed various mechanisms of interaction between cancer derived-MVs and the cells of the immune system. Microvesicles depending on origin (e.g., melanoma, colorectal carcinoma) indicate the ability to activate apoptosis in T-cell lymphocytes, inactivate of monocytes and inhibit cytokines release [1, 4, 86-88]. Tumor cells evade apoptosis by accumulation of factors responsible for programmed cell death in MVs and their release into extracellular matrix.

To sum up, cancer-derived MVs are unquestionably connected with the growth and progression of tumor cells. The mechanism of releasing each separate MVs generation is as important as their specific properties and composition associated with the type of cancer [2].

**Microvesicles as biomarkers of neoplastic disease**

Identification and documentation of MVs derived from spleen cells culture and lymphatic node cells from the patient suffering from Hodgkin disease in 1970 have started investigations and search for the use of those microparticles [89]. Microvesicles can be used as biomarkers for oncogenic diseases because tumor cells release high quantities of them in pathological processes including carcinogenesis and metastasis than in physiologic processes [1]. The research carried out on breast cancer cells revealed the deficiency of MVs released from normal population of breast cells – MCF10A. Cells of invasive breast carcinoma such as MDA-MB 231 produced more MVs than cells with lower invasiveness (line – MCF7) [46, 90].

Transport inside MVs, exosomes and apoptotic bodies enables stabilization of the expression of microRNA [91] in the bloodstream and protects from enzyme degradation. RNases present in plasma have the capability to degrade 99% of RNA species within 15 minutes [92]. There are several families of miRNA. Those small fragments of single-stranded nucleic acid are composed of 19 to 25 nucleotides and respond to posttranscriptional gene expression. Their regulated function affects mainly cell proliferation and differentiation and cell cycle regulation [93]. Microvesicles can be potentially useful markers for an early oncological diagnosis because of the correlation between the evaluated level of a specific subset of miRNA and the type and stage of tumor, as well as stability of miRNA in the blood and other bodily fluids [1]. miRNA and mRNA molecules were identified in oncosomes deriving from the cells of pulmonary, stomach, colorectal, ovarian and glioma cancer [2, 46, 53, 94-99]. Microvesicles released from glioma cells contain mRNA promoting tumor growth and its invasiveness. They also enable immunosuppression due to an inhibited immunoresponse [53]. mRNA activates cerebral endothelial cells to angiogenesis. The range of proangiogenic mRNA and miRNA which have some influence on progression and metastasis, has been identified in CD105 positive cells of medullary kidney carcinoma [100].

An increased level of MVs transporting oncoproteins was confirmed in patients with a diagnosed prostate cancer, urinary bladder cancer and glioma. The culture of glandular prostate carcinoma cells indicates a higher production of MVs which enables progression. The elevated production can be associated with an increased oncogenic activity of protein kinase B (PKB/Akt), or result from stimulation with growth factors (EGF) [101]. An important element of this case is an activity of regulated protein DRF 3 (diaphanous related forming 3) because its increased level may be responsible for rate inhibition of MV formation and blebbing from cell membrane [101]. Moreover, the DRF 3 expression disappears during the progression of prostate cancer [101]. Researchers have isolated MVs from patients’ urine with bladder carcinoma and have identified as their cargo eight proteins, whose level has been elevated in comparison to a sample from a healthy control group [1]. Change in protein composition of MVs isolated in urine has been observed in an early stage of a disease which could be used in earlier detection [102]. Recent studies disclose MVs, characterized by a higher concentration of CD133 and the transcript encoding the oncogenic form of EGFRvIII, in peripheral blood in a patient with glioblastoma [1, 51, 103]. Oncogenic receptors are important bioactive cargo transported by oncosomes and significantly modified microenvironment. Aggressive population of glioblastoma cells use EGFRvIII to influence non-aggressive cells. As a consequence, mitogen-activated protein kinase (MAPK) and Akt are released which leads to morphological transformation and an independent growth of a target cell [57]. Furthermore, the correlation between tumor removal, the decrease in the level of MVs and their total disappearance has been observed [1, 51, 103].

Although the receptor for AXL carried inside MVs released in chronic lymphoblastic leukemia (CLL) can be used as a biomarker in the future. AXL-positive oncosomes condition the bone marrow remodeling in order to increase the disease progression [104]. The leukocyte proliferation has been inhibited in lymphoblastoma as the consequence of activity of immune-suppressing transmembrane protein – latent membrane protein LMP-I transported via MVs [105].

Another study has shown that MVs production via LOX melanoma cells is controlled by cascade involving ARF6 GTP-ase, phospholipase D, Erk and MLCK. This pathway causes phosphorylation of myosin light chain (MLC), which leads to MVs release, proteolysis and as a result, to an increase of cell invasiveness [106]. The presence of ARF6 has been also confirmed in MVs shed from breast cancer cells population [46]. Moreover, HER2/neu oncogenic receptor can be a component of those oncosomes [50, 107, 108].
Tumor progression is possible because of a separate mechanism of MVs generation, their specific cargo and properties. Researchers have indicated the correlation between the quantity of derived-MVs and the stage of the neoplastic disease. The production of MVs which play some role in angiogenesis, cell migration and metastasis increases with its progression and invasiveness [15, 46].

In the neoplastic disease the MVs presence is as important as their concentration. An increased level of MVs has been observed in a patient with stage IV of gastric cancer in comparison with healthy controls. Moreover, it may be used in predicting metastasis formation [109]. The survival rate of patients with diffused breast cancer and pancreatic cancer has been estimated on the basis of MUC1 (epithelial mucin) expression and TF (tissue factor) activity in microvesicles identified in peripheral blood samples. The report revealed that the survival rate from 3 to 9 months was lower in patients with a higher level of TF activity and MUC1 expression when compared with a group with a low TF activity and no expression of MUC1 [110]. MVs analysis can be also useful in the detection of tumor recurrence [50, 111].

**Microvesicles as biomarkers of gynecological neoplastic diseases**

Cancer of reproductive organs together with breast cancer are the most frequent cause of death in women suffering from neoplastic diseases. 5 500 women of the Polish population die from this group of malignant tumors every year. Adenocarcinoma of the uterus is the most frequent gynecological malignancy. The incidence of uterine cancer is relatively high (7% in 2010 – Country Register of Neoplastic Diseases) and ranks the 4th among the most common neoplastic diseases diagnosed in Polish women. Morbidity of uterine cervix neoplasm and ovarian cancer is lower. However, both types of malignancy frequently cause death. The incidence of neoplastic diseases increases with age. The peak of gynecological malignancy occurs in the peri- and postmenopausal period. It is important to remember that cancer can develop in younger women. Prognosis and results of treatment depend on the stage of the disease and early diagnosis. Some gynecological neoplasms can be diagnosed on gynecological examination (i.e. vulvae cancer). Basic diagnostic investigations, such as cytology, colposcopy, ultrasonography, X-ray are frequently used to make the proper diagnosis. However, more advanced imaging methods, such as computer tomography, magnetic resonance and invasive examination (biopsy) have to be used. The use of cytology as a screening method of cervical cancer leads to a significant decrease in the incidence of this type of a neoplasm. Endometrial biopsy (pipelle, curettage) together with transvaginal ultrasound are used to diagnose uterine cancer. Endometrial biopsy is used to make histopathological diagnosis and allows to institute proper treatment quickly.

Specific diagnostic tools for identification of pre-neoplastic ovarian lesions and ovarian cancer at the lowest stage have not been found yet. Ovarian cancer is diagnosed late because the first symptoms appear in an advance stage of disease. As early diagnosis and proper treatment will prolong patients’ life, it is important to find specific and sensitive biomarkers. The use of MVs as ovarian cancer biomarkers seems to be a good direction in making diagnosis.

Recent studies revealed MVs in the peritoneal fluid collected from the patient diagnosed with ovarian cancer. Microvesicles transport proteins such as enzymes and nucleic acid. Ovarian cancer cells release lytic enzymes which damage the components of extracellular substance and reconstitute the basal membrane. It leads to increased ability of tumor cells to invade and metastasize [1, 82]. Invasion of tumor cells through the extracellular matrix is essential for hematogenous, lymphatic, and peritoneal metastases [82]. Ascites produced in the case of ovarian malignancy contain high levels of proteolytic enzymes such as matrix metalloproteinase (MMP-2, MMP-9), and urokinase-type plasminogen activator (uPA) [82, 112, 113]. Moreover, these enzymes can be carried inside MVs [1, 82]. Their activity is associated with the amount of MVs shed from tumor cells [1, 81]. Furthermore, an increased level of MVs in the peritoneal fluid correlates with tumor progression [1]. In addition, microvesicles may transport other proteinases a high level of which was confirmed in ovarian ascites. Some enzymes from the kallikreins family have invasive and stimulating properties that are used in tumor growth and metastases formation. Recent studies disclosed an overexpression of human kallikreins (hk) such as hk5, hk6, hk7, hk8, hk11, and hk14 [82, 114] in ovarian cancer. Moreover, hk5, hk6, hk10, and hk14 [82, 115-119] can be used as potential biomarkers of ovarian cancer.

Recent studies show the usefulness of MVs cargo miRNA in diagnosing ovarian cancer. The role of MVs is to protect miRNA from RNases [1, 92]. A significant number of miRNAs is deregulated and loses their suppressing function in cancer cells [1] that lead to tumor progression. miRNAs from the miR – 200 family are overexpressed in ovarian cancer [1]. Studies disclosed that a level of microparticles (MVs, exosomes) transported miRNA in the blood and peritoneal fluid is higher in women with malignant ovarian tumors in comparison to healthy patients or women with benign ovarian tumor [1, 94, 95]. Furthermore, the level of MVs and exosomes in blood correlated with the stage of diseases [1, 95]. Moreover, it has been observed that an increase in the MVs level in serum overtakes the elevation of CA 125 [120].

Exosomes carried proteins play an important role in progression of ovarian cancer. TGF-β and MAGE3/6 are
cargo of exosomes isolated from plasma of patients suffering from malignant ovarian tumor [121]. In addition, there is an observed correlation between the amount of exosomal TGF-β and MAGE3/6 and response to chemotherapy [121]. Failure of treatment and chemotherapy resistance are observed in patients with a higher level of exosomal proteins [121]. Subsequent findings demonstrated the application of MVs and exosomes as serum biomarkers of neoplastic diseases and their progression.

**Therapeutic use of microvesicles**

Although exosomes are well-known microparticles, further research on MVs is needed and may lead to a breakthrough in diagnostics and treatment of neoplastic diseases. Studies concerning the use of these two types of microparticles to create directed and personalized antineoplastic drugs are being carried out owing to their ability to transport drugs [17]. In the case of tumors indicating a higher level of oncogenic receptor HER2/neu, for example ovarian cancer, breast cancer, stomach cancer and the presence of this protein in MVs collected from peripheral blood samples, the treatment blocking HER2 receptor can be instituted [50, 107]. Moreover, some studies have been undertaken to assess the possibility of placing mRNA and microRNA in MVs, which would enable the modification of the recipient’s cell phenotype as a result of MVs fusion with its membrane.

**Conclusions**

The above results are promising and confirm the possibility to use MVs as biomarkers of a neoplastic disease. The analysis of MVs released by tumor cells into the bloodstream enables quick and repeated evaluation of carcinoma pathogenesis, biology, stage, progression, treatment response and recurrence. Noninvasiveness of the method allows to limit the number of surgical biopsies which may result in improper sample collection. Moreover, those microparticles may improve tumor treatment response and recurrence. Noninvasiveness of the bloodstream enables quick and repeated evaluation of carcinoma pathogenesis, biology, stage, progression, treatment response and recurrence.

**Disclosure**

Authors report no conflict of interest.

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