MUC1 MARKER FOR THE DETECTION OF OVARIAN CANCER. A REVIEW

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Abstract

Cancer is one of the leading causes of death, ovarian cancer being the seventh leading cause of cancer-related death among women and is associated with the highest morbidity of all the of gynecologic malignancies. Early stage tumor diagnosis is essential for a positive outcome for the patients, therefore it is of paramount importance to develop new, sensitive detection methods for tumor markers found in serum in early stages, in order to improve cancer survival rate.

Mucin 1 (MUC1) is a transmembrane protein, heavily O-glycosylated, found on the apical plasma membrane of most secretory epithelia. In case of malignant processes MUC1 loses its apical distribution, is underglycosylated and overexpressed and is secreted into the blood circulation [1].

MUC1 has been identified as a marker for preneoplastic lesions, elevated levels of MUC1 protein being involved in tumor progression, especially in the process of metastasis [2].

The detection of low levels of MUC1 tumor marker plays an important role in the diagnosis, screening and prognosis of the ovarian cancer, therefore improvement of detection methods for this cancer biomarker and development of new, sensitive methods of detection, such as biosensors, is needed.

Rezumat

Cancerul reprezintă una dintre principale cauze ale mortalităţii, cancerul ovarian fiind a şaptea cauză a deceselor prin cancer în rândul femeilor şi totodată cea mai agresivă formă de cancer din sfera ginecologică. Diagnosticul precoce al tumorilor maligne este esenţial pentru un bun prognostic al bolii, de aceea pentru o rată crescută a supravieţuirii este deosebit de importantă dezvoltarea unor metode noi, sensibile de detecţie a markerilor tumorali prezenţi în sânge în stadiile incipiente de cancer.

Mucina 1 (MUC1) este o proteină transmembranară, puternic O-glicozilată, care se găseşte pe suprafaţa apicală a celulelor din majoritatea ţesuturilor epiteliale secretorii. În cazul unui proces malign proteina MUC1 este supraexprimată şi hipoglicozilată, aceasta fiind secretată în sânge [1], făcând astfel posibilă utilizarea ei ca marker în cazul leziunilor preneoplazice [2]. Posibilitatea de a detecta concentraţii scăzute de MUC1 joacă un rol important în diagnosticul, *screeningul* şi prognosticul cancerului ovarian. De aceea, optimizarea metodelor de detecţie deja existente sau dezvoltarea unor metode noi şi sensibile de detecţie a acestui marker tumoral, prezintă un interes deosebit.

Keywords: ovarian cancer, tumor markers, mucin1, MUC1

Introduction

Cancer is one of the leading causes of death in both developed and developing countries. It is estimated that one of 72 women will be diagnosed with ovarian cancer at some time during their lifetime [3], ovarian malignancies counting as the seventh cause of cancer-related death among women [4]. The survival rate is the lowest among malignancies of the female reproductive system, partially due to its late detection. Ovarian neoplasms diagnosed in early stages of the disease have a survival rate of over 90%, but early detection only occurs in 20% of all cases. The 5 year survival rate is 11% if the disease is diagnosed in advanced stages [5].

It is estimated that in 2013, about 22,240 new cases of ovarian cancer will be diagnosed and 14,030 women will die of ovarian cancer in the United States alone [3].

Ovarian malignancies can be categorized based on their origin and anatomic-pathological characteristics in three groups: epithelial carcinoma, germ cell tumors and sex-cord stromal tumors. Around 90% of ovarian cancer cases have an epithelial origin. Ovarian surface epithelial tumors are divided in five main categories: serous, endometrioid, mucinous, clear cell and Brenner type. Metastatic processes are common, breast, colon, stomach and endometrium being the most frequent locations.

Advanced stage tumors require complex treatment protocols that include surgical intervention, chemo- or polychemotherapy, hormone therapy and signal transduction inhibitors. Despite good responses to these treatments, disease relapses are common and usually refractory to further therapy. Therefore, finding markers that can predict the tumor development and new therapy methods are requirements of better outcomes in the management of the disease.

MUC1 is a transmembrane glycoprotein, a member of the mucin family, physiologically present in ductal epithelial cells of some secretory organs, including uterus, testicles, breast, kidney, digestive tract [6]. Overexpression can be found in all types of ovarian epithelial cancer, thus leading to extensive studies of MUC1 as a tumor marker and even its use in developing a potential cancer vaccine [2, 7].

Increased unpolarized expression of MUC1 has also been identified in carcinomas located in pancreas [8], lung [9], colon [10], liver [11], esophagus [12] and breast [13]. Epitopes otherwise located in the protein core, are revealed due to altered glycosylation. Overexpression can be found as early as the preneoplastic stage of the carcinoma, increasing as the disease advances.

Ovarian tumor markers found in biological fluids, like MUC1, are important for early screening because ovarian cancer is usually asymptomatic until advanced stages when the prognosis of survival is poor. Therefore research needs to focus on developing reliable immunoassays and immunosensing devices.

Many immunological and biochemical methods have been developed for the detection of markers (cancer markers and other pathologies markers like cardiovascular diseases) in biological fluids [14-17]. The most used clinical test methods are immunoassay techniques, due to their advantages of being highly specific, taking in consideration that they are based on an immunoreaction of biorecognition between an antigen and its antibody. These methods include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay, chemiluminescent immunoassay, electrochemi-luminescent immunoassay. Even though they have the advantages of being selective and sensitive, these techniques have the disadvantages of a high cost, long analysis time, the need for qualified personnel and sophisticated instrumentation; also their sensitivity is not high enough to detect tumors at an early stage. In recent years, immunosensors have attracted considerable interest because, compared to the traditional immunoassay, they are rapid and easy to use, have a low cost, higher sensitivity and specificity, possibility of miniaturization and can be used for multianalyte detection of tumor markers [18]. Immunosensors have already been developed for other tumor markers, such as prostate specific antigen (PSA) [19], carbohydrate antigel CA 19-9 [20], CA125 [21], alpha-fetoprotein [22], carcinoembryonic antigen **(**CEA) [23] and human chorionic gonadothrophin [24].

This paper is a review on MUC1 protein and the methods currently used in the detection of MUC1 protein in ovarian carcinomas, demonstrating the importance of developing easy, rapid and sensitive methods able to detect the presence of the protein in early stage carcinomas. Special attention is given to the development of biosensors that can be used as point-of-care devices for sensitive detection of the tumor marker in early stages.

MUC1. Structure and functions

The MUC1 gene encodes a type-I transmembrane glycoprotein that is expressed on the apical plasma membrane of most secretory epithelia, including female reproductive tract, mammary gland, stomach, lung, kidney, gall bladder and pancreas.

The protein consists of 2 subunits that form a stable dimmer: an $–NH_2$ terminal large extracelular region and a –COOH terminal intracelular

cytoplasmatic tail. The extracellular domain in humans contains 20–125 tandem repeats of 20 amino acids enriched in serine, threonine, and proline residues (HGVTSAPDTRPAPGSTAPPA) [13, 25]. Due to these features, the tandem repeat domain has the potential for extensive O-glycosylation; within each tandem repeat, two serines and three threonines represent five potential O-glycosylation sites that are extensively glycosylated, glycosilation depending on the availability of glycosiltransferases. The extracellular domain of MUC-1 can be released into to the extracellular matrix by an enzymatic cleavage, and thus found into the serum. The amino terminal subunit is attached to the membrane *via* non-covalent binding to the carboxyl terminal subunit. Like the amino terminal, the carboxyl terminal subunit can also undergo glycosylation. The carboxyl terminal peptide has been shown to play a role in cell signaling [13].

The core protein has a weight of 120–225 kDa, though the mature glycosylated form's weight is 250–500 kDa.

In the secretory phase of the menstrual cycle an up-regulated expression of MUC1 occurs, its level remaining high during the period of receptivity when implantation of the embryo occurs [26, 27]. MUC1 levels are also increased in normal differentiated state of lactation [27].

Mucins can be grouped in two main categories, taking in consideration their subcellular localization: membrane associated (MUC1, MUC3, MUC4, MUC12-17 and MUC20) and secreted proteins (MUC2, MUC5AC, MUC5B, MUC6-8 and MUC19). Many of these mucins are very large molecules with extended structures due to the abundance of proline residues and high degree of glycosylation. Some have comparable size to other cell surface proteins, for example MUC13, MUC1 and MUC4 have many similar features and proposed functions.

Ovarian cancer implications of MUC1

Ovarian cancer is considered to be the most lethal of gynecological neoplasm. The poor prognosis is partly due to the disease's asymptomatic evolution in the first stages and partly due to the lack of reliable prognostic markers and incomplete understanding of the tumor's pathogenesis. There are currently no available methods to indicate the malignant transformation of the ovarian surface epithelium. Pathologically modified epithelium can also be linked to malignant transformation of endometriosis. Previous studies have shown that atypical epithelium in endometriosis is a precursor of ovarian neoplasm. Due to MUC1's overexpression in all types of ovarian cancer, this glycoprotein is currently studied as a tumor related antigen and as a candidate in the development of a potential cancer vaccine. MUC1

present in tumor processes can be characterized by overexpression and hypoglycosylation, being a potential tumor associated antigen with the possibility of using it as a diagnostic marker [2, 28, 29].

MUC1, previously known as episialin, CA15-3 and others is expressed at low levels or even absent in normal tissues, but during disease progression its levels increases [30].

MUC1 protein found in tumors has a different structure compared to normal MUC1 with shorter and less dense O-glycan chains leading to an exposure of protein core regions [31]. Hipoglycosylation allows to reveal epitopes of MUC1 that are otherwise masked by conferring easier access of the immune system to the peptide core of MUC1 antigen. This property permits the design of antibodies that can discriminate between normal and tumor cells. The extended rigid structure and large size of MUC1 confers its biological functions. Increased levels of MUC1 in cancer processes mask the extracellular domains from the immune system promoting the survival of cancer cells and playing an important role in metastasis [32, 33]. In tumor tissues, mucins have the same role as in normal epithelial cells: to protect against unfavorable growth conditions and to assure the local microenvironment. This is favorable to the tumor cell growth and survival and also for the metastasis process. Also the mucin layers are involved in capturing molecules like the growth factor or mediators of inflammation that contribute to the proliferation of the tumor.

The expression of MUC1 is mainly hormonally regulated [34].

MUC1 can be present in several isoforms: MUC1/TM, MUC1/X, MUC1/Y, MUC1/Z, MUC1/SEC. The implications of MUC1 in different aspects of physiological and pathological processes could be explained by the large diversity in these MUC1 isoforms functions and properties. They play an important role in signal transduction and cell adhesion, epithelial cell growth, blastocyst implantation, immunosupression mediated by T-cells, as well as tumor growth and metastasis processes. The MUC1/SEC isoform is mainly expressed in normal cells, whereas MUC1/X, MUC1/Y, MUC1/Z and MUC1/TM can be found in tumor cells [35].

MUC1 is also involved in chemotherapeutic drug resistance. The heavy glycosylation in the extracellular domain of MUC1 prevents the chemotherapeutic agents to reach their target within the tumor cells by creating a hydrophilic layer which repels the hydrophobic drugs used in chemotherapy. Glycosylation and elevated production of MUC1 in tumor cells allows growth factors to concentrate in the proximity of their receptors and furthermore to increase the growth of cancer cells. Anti-tumor immune

response is also inhibited by MUC1 as it prevents the access of immune cells to the receptors on the cell's surface by steric hindrance [31].

The immune system was shown to exhibit a response to cancer in humans, by secreting self antibodies to tumor antigens in serum [36]. Approximately one quarter of ovarian tumors exhibit circulating self antibodies to MUC1 [37]. High levels of anti-MUC1 autoantibody are correlated with a lower risk for ovarian cancer, underlying a more favorable prognosis. The evaluation of antibodies against MUC1, several years before the diagnosis, could lead to a lower risk for ovarian cancer, due to the protective immunity, while suppression of MUC1 specific immunity could be associated with a higher risk for ovarian cancer [38, 39]. Some factors such as young age at first birth, breast-feeding, cycle length higher than 30 days or the use of oral contraceptives are more likely to increase the risk for developing ovarian cancer [40].

Self antibodies for specific tumor antigens found in serum can be detected in early stages of cancer, when the disease is asymptomatic, therefore having the potential to be used in screening and early stage diagnosis. They could represent a novel, promising diagnostic tool, if they are integrated in an assay for the detection of several tumor antigens [36, 37].

Detection methods for MUC1

Immunohistochemistry is one of the most widely used methods to detect the expression of MUC1 in cells. The method is based on the specific interaction between monoclonal and polyclonal MUC1 antibodies and the antigen, showing the expression of the antigen in tissue sections, by either using an enzyme-labeled antibody which converts a substrate into a colored product or a fluorophore-labeled antibody.

Recent studies reported that MUC1 levels were found to be increased in endometriosis, women with endometriosis having an increased risk of some malignancies, particularly ovarian cancer [41]. The presence of MUC1 in lesions of ovarian endometriosis was confirmed by immuno-histochemistry studies [2].

Another immunohistochemistry study showed that 92% of the primary ovarian tumors and 90% of metastasis lesions in analyzed samples were positive for MUC1. For this study, paraffin section from primary tumors and metastasis were incubated with normal rabbit serum followed by subsequent incubation steps with mouse C595 monoclonal antibody, antimouse biotinylated IgG and avidin/biotinylated horseradish peroxidase. Sections were developed with 3, 3` diaminobenzidine substrate and counterstained

with hematoxylin. The results showed a positive, homogeneous staining for MUC1 in tumor sections of epithelial cells but not in stromal cells, without a positive staining in negative control in primary tumors or in metastasis lesions [33].

Immunohistochemical methods are rapid, routinely available and relatively inexpensive, but the difficult standardization and invasiveness are just a few drawbacks that lead to the need of developing new techniques that are able to detect tumor markers in biological samples.

MUC1, as other tumor markers, can be found in blood in trace levels when cancer is absent. Tumor processes are followed by an increase of tumor markers levels. The detection of low levels of MUC1 is important for the diagnosis, screening and prognosis of ovarian cancer in early stages and also in predicting the outcome to a specific therapy. Biomarkers can be found in biological fluids like blood, saliva, urine, seminal plasma, pancreatic fluid, cerebrospinal fluid, as well as in circulating tumor cells and tissues [42]. The most widely used are serum markers, which have the advantage of being minimally invasive and easy to procure and stabilize. Most markers are present in more than one type of cancer (e.g. MUC1 is associated with ovarian cancer and breast cancer) and most tumors express more than one tumor marker (e.g. ovarian cancer exhibits CA125, MUC1, Lewis X mucin determinant OVX, human chorionic gonadotropin, HCG) so the use of multianalyte tests for detecting multiple tumor markers for the early stage diagnoses of cancer is of interest.

Among the methods used for tumor marker detection in serum the most used are immunoassay techniques, including ELISA, radioimmunoassay, fluoroimmunoassay or chemiluminescent immunoassay.

The most tested method for quantitative detection of MUC1 in liquid samples is ELISA. ELISA is a heterogeneous solid-phase enzyme immunoassay used to detect the presence of a substance in a liquid sample. In ELISA tests the sample containing the antigen of interest is immobilized on a solid support, specifically, *via* capture by a specific antibody, or non-specifically, *via* adsorption. The detection antibody is then added, forming a complex with the antigen; the detection antibody is either labeled with an enzyme or conjugated with another antibody labeled with an enzyme. The plate is then developed by adding the enzymatic substrate. ELISA tests can be performed in three different configurations/formats: indirect, competitive and Sandwich assay.

In a Sandwich assay the antigen is captured between a capture antibody immobilized on the plate and a secondary antibody which is linked to a detection antibody labeled with an enzyme. The advantage of this

format is that it reduces the need of a highly purified antigen, separated from the other proteins in serum that could also bind to the ELISA plate reducing therefore the amount of bonded antigen. The third antibody binds to the AbFc region, non-specifically. The use of a third antibody has the advantage of avoiding the use of labeled secondary antibodies specific for each protein and therefore reducing the costs of the assay.

Another type of immunoassay based on fluorescence was developed for simultaneous detection of MUC1 antigen CA15-3 and carcinoma antigen CA125 using fluorescein isothiocyanate-labeled monoclonal antibodies and laser-induced fluorescence as a detection method. In this case the limit of detection for MUC1 antigen was 0.09 U/mL [43].

Despite the high specificity of the mentioned immunoassays, due to the binding of a specific antigen to its antibody, these techniques are not sensitive enough to be used as screening methods, because they can only detect high concentrations of serum markers, found in late stages and not low levels corresponding to early stage tumor. Also these methods require qualified personnel, long time analysis, high costs and imply radiation hazard. Lately, efforts have been made for the development of rapid and reliable diagnostic devices for the detection of tumor markers in serum such as biosensors [44].

A biosensor is an analytical device that integrates a biological element, such as enzymes, peptides or DNA, on a solid-state surface, enabling a reversible biospecific interaction with the analyte, and a signal transducer. If antibodies or antibody fragments are used as biorecognition element the device is called immunosensor [45]. Immunosensors have the advantage of combining the specificity of the affinity immunoreaction between an antibody and its specific antigen with the sensitivity of different physical transducers such as optical, electrochemical or piezoelectrical transducers [46]. The analyte, in terms of cancer, is represented by a tumor marker**.**

The development of sensors, in particular electrochemical sensors, for cancer markers detection has been widely reported in the past years due to their several advantages over more conventional techniques such as less time and reagent consuming, ease of manipulation and possibility of miniaturization and automatization [44]. The state of the art sensing devices developed for the detection of MUC1 antigen are summarized in Table I.

Table I
Sensing devices for the detection of MUC1

Detection method	Sensing mechanism	Sensor characteristics for MUC1 detection	Ref.
Electrochemical	1) ferrocene-labeled aptamer-complementary DNA was used 2) complementary DNA immobilized on the electrode surface has the ability to hybridize both MUC1 and VEGF165 aptamers increasing the distance between ferrocene used to label aptamers and the electrode surface.	Linear range: 1-20 nM Limit of detection (LOD): 0.33 nM	[44]
Electrochemical	A gold interdigitated capacitor transducer was modified using magnetic beads for simultaneous detection of multiple tumor markers	Linear range:1-200 U/mL LOD: 50 U/mL	[48]
Electrochemical	1) N-doped graphene sheets modified electrode was used 2) Detection of MUC1 in saliva	Linear range: 0.1-20 U/mL LOD: 0.012 U/mL	[49]
Optical	Functionalization of the sensing surface with Au/ZnO films	Linear range: 2.5-20 U/mL	[50]
Electrochemical	MUC1-binding aptamer is used to recognize MCF-7 human breast cancer cells, in a Sandwich aptamer-cell-aptamer approach with an enzymatic label	Detection range: 100-10^{-7} cells LOD: 100 cells	[51]
Electrochemical	Thiolated MUC1 aptamers tethered with methylene blue are immobilized on gold electrodes	Linear range: 0-1.5 μM LOD: 50 nM	[52]
Electrochemical	Ferrocene carboxylic-doped silica nanoparticles were used as an immobilized affinity support	Linear range: 2-240 U/mL LOD: 0.64 U/mL	[53]
Electrochemical	Thiolated MUC1 aptamers are immobilized on gold nanoparticles for label free detection of the antigen	Linear range: 0-10 ng/mL LOD: 0.95 ng/mL and 3.6 ng/mL	[54]

Other clinical applications of MUC1

MUC1 is also currently explored as a potential cancer vaccine candidate. A phase I trial of idiotypic vaccination with human milk fat globule HMFG1 , an anti-MUC1 antibody, for ovarian cancer was reported. Modified ELISA protocols were used to demonstrate that a three dose regimen of intradermal vaccination with HMFG1 antibody produces a tumoral immune response and the antibody responses of individual patients were monitored using a biosensor [55].

MUC1 has also been used for tumor-targeted gene delivery, a self-assemble gene delivery system being constructed using a complex of plasmid DNA, polyethyleneimine and a MUC1 aptamer [56].

Conclusions

Tumor markers are important indicators of tumor growth and are used for diagnosis, screening and prognosis of cancer.

MUC1, a transmembrane glycoprotein of the mucin family, is physiologically present in ductal epithelial cells of some organs and is hypoglycosylated and overexpressed in the case of a malignant process, being secreted into the blood circulation serving as a potential tumor marker.

MUC1 is involved in tumor proliferation and metastasis through several mechanisms, capturing molecules like the growth factor or the mediators of inflammation that contribute to the proliferation of the tumor or masking the extracellular domains from the immune system promoting in this way the survival of cancer cells and playing an important role in metastasis processes.

Self antibodies for MUC1 can also be detected in serum in early stages of cancer, therefore having the potential to be used in screening and early stage diagnosis if they are integrated in an assay for the detection of tumor antigens.

The methods currently used to detect tumoral MUC1 include immunohistochemistry, ELISA, radioimmunoassay, chemiluminescent immunoassay and electrochemiluminescent immunoassay. The attention has been focused in recent years on the development of bio- and immunosensors, which allow an easy and rapid diagnosis on the patient bedside, significantly reducing the costs. A few biosensors have already been developed for the sensitive, selective detection of MUC1 protein, however in order to move biosensors toward point-of-care devices, further studies are necessary, in particular in the field of multitarget detection of multiple biomarkers and the application on real biological samples.

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