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Review

The cancer cell secretome: A good source for discovering biomarkers?

Maria P. Pavlou[a,b], *Eleftherios P. Diamandis*[a,b,c,*]

[a]*Department of Pathology and Laboratory Medicine, Mount Sinai Hospital, Toronto, ON, Canada*
[b]*Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, ON, Canada*
[c]*Department of Clinical Biochemistry, University Health Network, Toronto, ON, Canada*

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ABSTRACT

Cancer is a leading cause of death. Early detection is usually associated with better clinical outcomes. Recent advances in genomics and proteomics raised hopes that new biomarkers for diagnosis, prognosis or monitoring therapeutic response will soon be discovered. Proteins secreted by cancer cells, referred also as "the cancer cell secretome", is a promising source for biomarker discovery. In this review we will summarize recent advances in cancer cell secretome analysis, focusing on the five most fatal cancers (lung, breast, prostate, colorectal, and pancreatic). For each cancer type we will describe the proteomic approaches utilized for the identification of novel biomarkers. Despite progress, identification of markers that are superior to those currently used has proven to be a difficult task and very few, if any, newly discovered biomarker has entered the clinic the last 10 years.

Abbreviations: AD, adenocarcinoma; A1BG, alpha-1-beta glycoprotein; AFP, alpha-fetoprotein; ALCAM, activated leukocyte cell adhesion molecule; CA-125, cancer antigen 125; CA 15-3, cancer antigen 15-3; CA 19-9, cancer antigen 19-9; CEA, carcinoembryonic antigen; CM, conditioned medium; CRC, colorectal cancer; CRMP-2, collapsin response mediator protein 2; CT, computed tomography; CYFRA 21-1, cytokeratin 19 fragment; DDH, dihydrodiol dehydrogenase; DIGE, differential gel electrophoresis; DMBT1, deleted from malignant brain tumors 1; 2-D, two dimensional; 1-D, one dimensional; DJ1, Parkinson disease 7; DRE, digital rectal examination; ELISA, enzyme-linked immunosorbent assay; FOBT, fecal occult-blood test; HIP/PAP-I, hepatocarcinoma–intestine–pancreas/pancreatitis-associated protein I; HNP, human neutrophil peptide; ICAT, isotope-coded affinity tags; IGFBP-2, insulin growth factor binding protein 2; IHC, immunohistochemistry; IPA, ingenuity pathway analysis; i-TRAQ, isobaric tag for relative and absolute quantification; LCC, large cell carcinoma; LC-MS/MS, liquid chromatography tandem mass spectrometry; LDHB, l-lactate dehydrogenase B chain; MALDI, matrix-assisted laser desorption ionization; MALDI-MS, matrix-assisted laser desorption ionization mass spectrometry; MMP-9, metalloproteinase 9; MudPIT, multidimensional protein identification technology; NAF, nipple aspirate fluid; nano-HPLC-ESI-MS/MS, high pressure chromatography nano-electrospray ionization tandem mass spectrometry; NSCLC, non-small cell lung cancer; NSE, neuron-specific enolase; PAGE, polyacrylamide gel electrophoresis; PC, pancreatic cancer; PCa, prostate cancer; ProGRP, progastrin-releasing peptide; PGP9.5, protein gene product 9.5; PSA, prostate-specific antigen; RbAp46, retinoblastoma-associated binding protein 46; ROC, receiver operating characteristic; RP-LC-MS/MS, reverse phase liquid chromatography tandem mass spectrometry; RT-PCR, reverse transcription polymerase chain reaction; SC, squamous cell carcinoma; SCLC, small cell lung cancer; SCX, strong cation-exchange chromatography; SELDI-TOF/MS, surface enhanced laser desorption/ionization time-of-flight mass spectrometry; SILAC, stable isotope labelling with amino acids in cell culture; SRM, selected reaction monitoring; TCTP, translationally controlled tumor protein; TMA, tissue microarray; TPA, tissue polypeptide antigen; TPI, triosephosphate isomerase.

* *Corresponding author*. Mount Sinai Hospital, Joseph & Wolf Lebovic Ctr., 60 Murray St [Box 32; Flr 6 — Rm L6-201; Toronto, ON, Canada M5T 3L9. Tel.: +1 416 586 8443; fax: +1 416 619 5521.
E-mail address: ediamandis@mtsinai.on.ca (E.P. Diamandis).

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Contents

1. Introduction

More than 30% of people will develop some form of cancer during their lifetime. It is estimated that almost 1.5 million new cases will be diagnosed in USA in 2009 and approximately 600,000 of them are expected to die from cancer [1]. Four out of 200 forms of cancer (lung, prostate, colon and breast) account for about half of all deaths among men and women [1]. Detection of cancer when it is still localized and administration of early treatment usually leads to better clinical outcomes [2].

One goal of medical screening is to detect human malignancy via a simple blood test. According to the definition by the National Institutes of Health of USA, a biological marker (biomarker) is a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacological responses to a therapeutic agent. Research during the last 50 years resulted in the identification of clinically useful cancer biomarkers such as carcinoembryonic antigen (CEA), alpha-fetoprotein (AFP), prostate-specific antigen (PSA), cancer antigen 125 (CA 125), CA 15-3 and CA 19-9. However most of these biomarkers lack the necessary specificity and sensitivity for screening purposes [3]. There is currently a need for discovery of diagnostic methods with improved performance.

Over the past decade, with the completion of the Human Genome Project and the introduction of technologies that enable simultaneous examination of thousands of proteins and genes (such as mass spectrometry and protein and DNA arrays), renewed interest emerged by the proteomics and genomics community to discover novel cancer biomarkers. Proteomic analysis of a plethora of different sample types has been conducted to dig deeper into the cancer cell proteome and reveal promising biomarkers. As mentioned earlier, blood is considered the sample of choice for medical screening or diagnosis. The advantages of such simple and relatively non-invasive blood-based tests are obvious. However, biomarker discovery using plasma or serum is challenging [4,5]. Given the limitations of blood-based biomarker discovery, alternative approaches have been pursued, including proteomic analysis of proximal body fluids, cancer tissues and cancer cell lines.

The term "secretome" was introduced by Tjalsma et al. in a genome-wide study of the secreted proteins in *Bacillus subtilis* [6] and includes proteins released by a cell, a tissue or organism through different secretion mechanisms [7]. Secreted proteins constitute an important class of molecules, encoded by approximately 10% of the human genome. They participate in various physiological processes such as immune defence, blood coagulation and cell signalling and also play crucial roles in pathological processes including cancer angiogenesis, differentiation, invasion and metastasis. Proximal biological fluids (e.g. ascites fluid of ovarian cancer) that are in contact with the tumor may be enriched with proteins secreted or shed from cancer cells. Such proteins could enter the circulation and be detected in body fluids such as blood and urine.

Many reviews dealing with identification of potential biomarkers by the emerging technology of mass spectrometry have been published [8–11]. Below we will discuss cancer secretomics and focus on five common cancers (lung, breast, prostate, colorectal and pancreatic), providing examples of biomarker discovery strategies.

2. Lung cancer

Lung cancer accounts for over 30% of all cancer-related deaths [12]. The disease is clinically divided in two subtypes, small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). NSCLC accounts for 85–90% of all cases and can be further histologically subdivided in adenocarcinoma (AD), squamous cell carcinoma (SC), large cell carcinoma (LCC) and "others". The current protein-based biomarkers for lung cancer diagnosis are carcinoembryonic antigen (CEA), cytokeratin 19 fragment (CYFRA 21-1), tissue polypeptide antigen (TPA), progastrin-releasing peptide (ProGRP), neuron-specific enolase (NSE) and tumor M2 pyruvate kinase [13–16]. Recent advances in lung cancer biomarker discovery have been reviewed [17]. Other diagnostic tools include computed tomography (CT) scans, bronchoscopy and sputum analysis, which demonstrate limited efficiency, judging from the percentage (>60%) of patients diagnosed with late stage disease.

Many studies have targeted lung cancer secretome by analyzing cancer cells, pleural effusions and induced-sputum with the use of mass spectrometry. Pleural effusion is an accumulation of fluid in the pleural space and can be caused by a variety of diseases including cardiac failure, tuberculosis, bacterial pneumonia and cancer [18]. In some patients with malignant lung carcinoma, this fluid contains cancer cells which enter the pleural cavity through vascular invasion and lymphatic obstruction [19].

2.1. *Cell lines*

Using 1-dimensional (1-D) polyacrylamide gel electrophoresis (PAGE) coupled to liquid chromatography tandem mass spectrometry (LC-MS/MS), Xiao et al. delineated the secretome of primary lung cells and organ cultures. In particular, lung cancer cells and adjacent normal bronchial epithelial cells from six lung cancer patients were cultured in serum-free media. Proteomic analysis of the conditioned media revealed approximately 300 proteins, some of which showed differential expression between the normal and the cancer cells. In addition, they analyzed the conditioned media from two pairs of lung cancer and bronchus organ culture, identifying 117 proteins, 68 of which were uniquely found in the organ culture samples and not in the primary cultures. Verification studies were performed for 13 proteins using enzyme immunoassay in plasma from healthy individuals and cancer patients. They were able to detect 11 out of thirteen proteins and they generated a panel of four most promising biomarkers (CD98, fascin, polymeric immunoglobulin receptor/secretory component and 14-3-3 η) [20].

The same group deciphered the secretome of the cell line M-BE using two-dimensional electrophoresis (2D) and matrix-assisted laser desorption ionization tandem time-of-flight (MALDI-TOF/TOF) technology. M-BE cells are immortalized human bronchial epithelial cells which represent the progression of lung malignancy since they have different properties at an early and at a late passage. Forty seven proteins were found to be passage-dependent, and proteomic observations were confirmed for two of them by immunoblotting. Immunohistochemistry and ELISA for cathepsin-D were performed in tissue and serum samples respectively and an elevation was noticed in lung cancer patients compared to healthy individuals [21].

In a second study employing the model of primary culture, Chen et al. analyzed the secretomes of a primary NSCLC cell line and its brain metastatic subline (Table 1). The proteins of the conditioned media (CM) were separated by 1-D PAGE and 12 differentially expressed proteins were identified by peptide mass fingerprint and tandem mass spectrometry. L-lactate dehydrogenase B chain (LDHB) was found to associate with the metastatic phenotype and was further validated in serum samples by enzyme-linked immunosorbent assay (ELISA). The results showed that LDHB levels are elevated in the serum of lung cancer patients and correlate with the clinical stage of the disease, thus rendering LDHB a potential lung cancer biomarker [22].

Aiming to the discovery of early detection biomarkers in lung cancer, Jung et al. studied the proteomic composition of three lung cancer cell lines (Table 1) which represent the multistep nature of bronchial carcinogenesis. The analysis of the conditioned media by two-dimensional electrophoresis (2D) coupled with MALDI-MS revealed differentially secreted proteins in all three cell lines; specifically, twenty proteins showing differential expression between the semi-normal cell line and the transformed cell lines were identified by peptide mass fingerprint and were confirmed by western blot. By using immunoblotting analysis and enzyme immunoassay in cancer tissues and plasma samples, respectively, the authors confirmed that the levels of 4 of these proteins [protein gene product 9.5 (PGP9.5), translationally controlled tumor protein (TCTP), tissue inhibitor of metalloproteinase 2 (TIMP-2) and triosephosphate isomerase (TPI)] were higher in patients with lung cancer [23].

Fourteen proteins were identified in the conditioned media of the A549 lung cancer cell line by applying 2D PAGE separation and MALDI-TOF technology [24]. Dihydrodiol dehydrogenase (DDH), a previously studied protein in lung cancer, was selected for further analysis. DDH overexpression in lung cancer tissues was confirmed by reverse transcription polymerase chain reaction (RT-PCR), immunoblotting and immunohistochemistry (IHC) while serum DDH level was significantly higher in NSCLC patients than non-malignant lung tumor and healthy controls.

In a recent study of lung cancer secretome, gel LC-MS/MS was used to generate the proteomic profile of two NSCLC cell lines (CL_{1-0} and CL_{1-5}) and more than 2000 proteins were identified in total. In order to narrow down the list of the

Table 1 – Cancer cell lines that have been used in proteomic analysis of the secretome.

Cancer type	Cell lines	References
Lung cancer	M-BE	[21]
	NCIH226	[22]
	NCIH226Br	[22]
	BEAS-2B	[23]
	1799	[23]
	1198	[23]
	1170-I	[23]
	A549	[24]
	CL_{1-0}	[25]
	CL_{1-5}	[25]
	H23	[26]
	H520	[26]
	H460	[26]
	H1688	[26]
Prostate cancer	PC3	[34]
	LNCaP	[34,36]
	22Rv1	[34]
	C4-2	[35]
	C4-2B	[35]
Breast cancer	MCF10A	[41]
	MDA-MB-468	[41]
	BT-474	[41]
	MCF10AT	[43]
	MCF10DCIS.com	[43]
	MCF10CA cl.D.	[43]
	MCF-7	[44,45]
	MDA-MB-231	[44,45]
Colorectal cancer	SW480	[7]
	SW620	[63]
	Colo205	[61]
Pancreatic Cancer	Panc1	[68]
	HPDE	[68]

potential lung cancer biomarkers the authors compared the cell line secretomes to the pleural effusion proteome [25]; only twenty-two proteins overlapped between the two datasets, including 11 well-known potential lung cancer biomarkers. Retinoblastoma-associated binding protein 46 (RbAp46) was selected for further evaluation, since it has never been studied before. Both the mRNA and the protein levels of the RbAp46 were elevated in NSCLC tissues while serum RbAp46 levels were significantly increased in patients with lung cancer. It is worth mentioning that the combination of RbAp46 and CEA performed better in distinguishing healthy individuals from NSCLC patients than CEA alone.

Our group performed the extensive proteomic analysis of the conditioned media of four lung cancer cell lines of different histological types (non-small cell lung cancer: H23 (AD), H520 (SCC), H460 (LCC) and small cell lung cancer: H1688). Using a bottom-up proteomic analysis consisting of offline strong cation-exchange chromatography (SCX) and on-line reverse phase liquid chromatography-mass spectrometry (RP-LC-MS/MS) we identified approximately 1800 proteins in all four cell lines. By applying a series of selection criteria, the list of potential biomarkers was shortened and 5 proteins (ADAM-17, pentraxin 3, sTNF RI, osteoprotegerin and follisatin) were chosen for further investigation. Measurement of the levels of these proteins in serum samples from patients with lung cancer and healthy individuals by ELISA assay revealed significant differences between the two conditions [26].

2.2. *Pleural effusion*

By combining two different technologies for separation of peptides (RP and 2D) followed by HPLC-ESI-MS/MS Tyan et al. characterized pleural effusion samples from 43 lung adenocarcinoma patients. They compiled a list of approximately 1400 proteins, providing a better understanding of the lung cancer proteome [27]. In contrast to the global proteomic analysis of the previous study, Soltermann et al. chose to analyze the sub-proteome of pleural effusion samples obtained from healthy individuals and patients with lung cancer. By using glycoprotein capturing and LC-MS/MS technology, the authors isolated and identified about 170 glycoproteins. Several known lung cancer biomarkers such as CA125, CD44 and TTF-1, were present in their dataset, confirming their hypothesis that pleural effusion could be a useful source for biomarker discovery [28].

Table 2 includes the proteins identified in the conditioned media of at least two lung cancer cell lines. The proteins that have been validated are also noted.

Table 2 – Shared proteins between four studies of lung cancer cell secretome.

Protein name	Gene name	Validation	References
Alpha-enolase	ENO1	No	[21,22,24]
Glutathhione-S-transferase P	GSTP1	No	[22–24]
Heat shock protein 70	HSP70	No	[21,23]
Triosephosphate isomerase	TPI	Yes	[22,23]

3. Prostate cancer

Being the most frequently diagnosed cancer in males, prostate cancer (PCa) is a major health problem. Despite the fact that its mortality rate has been decreasing by about 4% per year since 1992 [29], this cancer still kills 30,000 men annually in the US alone. Prostate cancer presents in two distinct forms; a latent form which is found in half of the men over the age of 60 and poses no threat to patient life and an aggressive form that metastasizes quickly and eventually kills the patient.

Early detection of PCa in asymptomatic men over the age of 50 can be achieved with combination of serum levels of prostate-specific antigen (PSA) and digital rectal examination (DRE). However, recent results from two large prospective trials challenged the effectiveness of prostate cancer screening using PSA in reducing prostate cancer mortality. It appears that PSA screening does reduce prostate cancer-related mortality, but at a high cost of overdiagnosis and overtreatment. The poor specificity of PSA (20%) in intermediate ranges (4–10 ng/ml) is a problem [30], as is the inability of PSA to discriminate between aggressive and non-aggressive PCa. These pitfalls of PSA highlight the need to identify additional serum markers for diagnosis and prognosis of PCa. Reviews on candidate biomarkers for the diagnosis and management of prostate cancer have been published [31,32].

Seminal plasma can be considered as the proximal fluid of the prostate since it consists of secretions from the testis and epididymis and various male accessory glands including the prostate, seminal vesicles and Cowper's gland [33]. An extensive proteome has been compiled from the proteomic analysis of the seminal plasma from a single individual, containing more than 900 proteins [33]. This in-depth proteomic analysis can serve as a reference database for future studies in prostate and testicular cancers and male infertility.

Our group performed an extensive proteomic analysis of the conditioned media of three prostate cancer cell lines [34] (Table 1). Using a bottom-up proteomic analysis combining offline SCX and on-line RP-ESI-LC-MS/MS, we identified more than 2000 proteins. By applying a series of selection criteria, we narrowed down the list of the potential biomarkers and chose 4 proteins (follistatin, chemokine ligand 16, pentraxin 3 and spondin 2) for further investigation. Measurement of the levels of these proteins in serum samples from patients with PCa and healthy individuals by ELISA assay revealed significant differences between the two conditions.

Pang et al. compared the secretomes of androgen dependent and independent cell lines to identify differentially expressed proteins [35]. The proteins of the CM were separated by 2D electrophoresis and 5 protein spots showing differential expression were identified by MALDI-MS analysis. Immunohistochemical study of one of these proteins (uMtCK) showed significant correlation between protein levels and higher grade disease.

In the quest of proteins that are differentially expressed due to androgen stimulation, Martin et al. performed proteomic analysis of CM from the androgen dependent cell line LNCaP upon androgen stimulation [36]. Using Isotope-Coded Affinity Tags (ICAT) technology and LC-MS/MS the group identified more than 500 proteins and quantified the majority

of them. Fifty-two proteins were found to be regulated by the hormonal stimulation including PSA and human kallikrein-2. It has been postulated that these proteins may participate in the progression of carcinogenesis and can be considered as potential biomarkers.

It is worth mentioning that three out of five proteins (Table 3) found to be differentially expressed between androgen dependent and independent cell lines [35], showed also increased abundance in LNaCP conditioned media after stimulation with androgen [36]. These androgen-regulated proteins may participate in disease progression and therefore could be considered as potential prostate cancer biomarkers.

4. Breast cancer

The heterogeneity of breast cancer in terms of clinical behaviour, morphological appearance and molecular alterations is a key characteristic of the disease. Based on gene expression patterns, there are 5 subtypes with distinct clinical outcomes [37]. Currently, there are no available biomarkers for early detection of breast cancer. The most well-known serum-based markers such as CA 15-3, CEA and tissue polypeptide antigen (TPA) are used for post-operative surveillance and for monitoring therapy in advanced stage disease [38].

Several groups are studying the microenvironment of the mammary gland. Cell lines and the proximal fluid of breast (nipple aspirate fluid, NAF) are the most widely used sources for secretome analysis. The components of NAF are constantly secreted, metabolized and re-absorbed by the epithelial cells that line the ductal/alveolar system [39,40]. Therefore, NAF composition correlates with the activity of the breast microenvironment.

4.1. *Cell lines*

Our group delineated the secretomes of three breast cancer cell lines (Table 1) which roughly represent disease progression from normal to localized and finally, to a metastatic stage [41]. Using a bottom-up proteomic approach and a two-dimensional separation on a linear ion-trap, more than 1000 unique proteins in the conditioned media of the three cell lines were identified. With primary focus on the extracellular and membrane proteins, which constituted approximately 30% of all proteins, a series of selection criteria were further applied to create a shortlist with the most promising biomarkers. The shortlist included many known breast cancer biomarkers, providing some indication that this strategy is effective. One candidate, for which an ELISA was developed, it was further validated. The levels of activated leukocyte cell adhesion molecule (ALCAM), CA 15-3 and CEA in the serum of 100 healthy women, 50 healthy men and 150 breast carcinoma patients were measured by an enzyme immunoassay. The diagnostic sensitivity and specificity of the tests were calculated and the association of serum marker concentrations with various clinicopathologic variables was also examined. ALCAM outperformed both CA 15-3 and CEA in this series, suggesting that it deserves further validation as a novel biomarker for breast cancer [42].

Table 3 – Shared proteins between two studies of prostate cancer cell secretome.

Protein name	Gene name	Validation	References
Creatine kinase mitochondrial-1B	UMtCK	Yes	[35,36]
Phosphoglycerate kinase 1	PGK1	No	[35,36]
Insulin-like growth factor binding protein	IGFBP-2	No	[35,36]

Using similar techniques, Mbeunkui et al. deciphered the secretomes of 4 isogenic cell lines (Table 1) in their effort to capture the progression of breast epithelial cells towards a metastatic phenotype. They identified more than 250 proteins per cell line and focused on five proteins that showed differential expression between the different stages. Western blot analysis in the conditioned media confirmed the different abundance of these proteins while an extensive literature search highlighted their potential as breast cancer biomarkers [43].

The two previous studies used serum-free conditioned media to avoid contamination of the endogenous proteins from those of fetal bovine serum. Following a different strategy, Colzani et al., applied metabolic labelling with heavy amino acids to distinguish between the cellular and exogenous proteins [44]. Moreover, protein concentration "equalization" was used to reduce the complexity of the samples. Briefly, this method includes the incubation of complex samples with synthetic hexapeptide ligands that decrease the dynamic range of protein concentration, allowing enrichment of low abundance proteins. The combination of these methods revealed approximately 330 proteins in the conditioned media of two cell lines with different aggressiveness (MCF-7 and MDA-MB-231). Spectral counting and selected reaction monitoring (SRM) were used to validate the differentially expressed proteins.

Gel fractionation coupled with LC-MS/MS was applied to delineate the proteome of the same cell lines by Lawlor et al. [45]. Almost 500 proteins were identified in the conditioned media of the two cell lines with approximately half of them classified as extracellular or membrane bound. Similarly to the previous study, the authors focused on proteins showing differential expression between the two cell lines and they combined proteomics and gene expression data, finding poor correlation. Finally, Ingenuity Pathway Analysis (IPA) software was used to generate biological networks for proteins with at least 2-fold difference in concentration.

4.2. *Proximal fluids*

Using SELDI-TOF/MS analysis, Noble et al. generated proteomic profiles of NAF samples from cancer-bearing breasts, contralateral breasts of women with unilateral cancer and from healthy individuals. Comparison of these profiles revealed 9 statistically significant peaks between healthy individuals and patients with breast cancer and 7 discriminatory peaks between the contralateral breasts and healthy subjects [46].

Samples obtained from four individuals (three with breast cancer and one healthy) were analyzed by PAGE, speculating that NAF contains more than 1000 protein species without, though, identifying any of them [47]. In a following study, surface enhanced laser desorption ionization (SELDI) technology was

adopted to generate the proteomic profile of NAF samples from 23 women with early stage breast cancer and samples from healthy subjects. By comparing the pooled SELDI spectra they observed 17 differential peaks between normal volunteers and patients with breast cancer, 3 distinct peaks between the NAF samples from healthy individuals and the contralateral breast of the patients and no discriminatory peaks between the profiles of paired samples from one patient [48].

In a different study utilizing SELDI-MS technology, Li et al. were able to generate reproducible protein profiles from 10 subjects (5 healthy individuals and 5 patients with breast cancer) starting with 1 μg of total protein and ending-up with three discriminating peaks between the two conditions [49]. Unlike the previous studies, the authors identified these peaks as human neutrophil peptide 1 to 3 (HNP1–3).

Following a similar approach, Paweletz et al. generated the protein profiles of NAF samples from 12 women with breast cancer and 15 without and identified a signature of 2 peptides found specifically in the tumor samples and 2 peptides unique to the normal ones, with potential diagnostic importance [50]. SELDI proteomic profiling by He et al., revealed 16 peaks that distinguished between cancer and healthy samples [51]; protein identification was achieved with LC-MS/MS.

By combining ICAT technology with LC-MS/MS, Pawlik et al. identified 39 NAF proteins differentially expressed between healthy women and women with breast cancer including vitamin D binding protein, alpha-2HS-glycoprotein, lipophilin b, beta-globin and hemopexin [52].

By applying three different methodologies including SCX, immunodepletion of abundant proteins and LC-MS/MS technology, Varnum et al. characterized the NAF proteome compiling a list of 64 proteins [53]. Finally, Alexander et al. coupled two-dimensional PAGE and matrix-assisted laser desorption ionization time-of-flight mass spectrometry to analyze a single NAF sample from a healthy individual and they identified 42 proteins [54].

Table 4 depicts the proteins identified in at least two breast cancer secretome studies. Cathepsin D, a well-known breast cancer biomarker, is included in the list.

5. Colorectal cancer

Colonoscopy remains the only reliable screening tool for this major cancer site and it is partially responsible for the decrease in the observed mortality rates since 1981 [1]. However, the diagnostic value of this method is limited due to its high cost, risks and inconvenience [55,56]. On the other hand, fecal occult-blood test (FOBT) constitutes the most common non-invasive test for colon cancer detection but lacks adequate sensitivity and specificity [57]. Serum CEA has been utilized for post-operative surveillance and monitoring of late stage disease, but it is not recommended for screening [56,58].

Table 4 – Shared proteins between seven studies of breast cancer cell secretome.

	Protein name	References
Nipple aspirate fluid	Prolactin induced protein	[51–53]
	Transferrin	[51–53]
	Vitamin D binding protein	[52,53]
Cancer cell lines	Cathepsin D	[41,43]
	Galectin 3 binding protein	[41,43,44]
	L-lactate dehydrogenase B chain	[41,44]

Although the number of studies dealing with colon cancer secretomes is limited, two approaches with novel design were put forward. The first study explores the utility of tumor-derived microvesicles in biomarker discovery [59] while the second one focuses on the in vivo secretome, as can be seen through tumor explants [60].

In maybe one of the most extensive investigations of cancer cell secretomes, Wu et al. studied the conditioned media of 21 cancer cell lines derived from 12 cancer types [61]. SDS-PAGE electrophoresis coupled to MALDI-TOF MS analysis revealed approximately 320 non-redundant proteins. After generating the datasets, the authors chose to focus on colorectal cancer, for which they identified two unique proteins. One of them, collapsin response mediator protein 2 (CRMP-2), was selected for further evaluation as a potential novel biomarker for CRC. IHC and RT-PCR showed that CRMP-2 protein and mRNA levels, respectively, were elevated in tumor tissues versus normal specimens. After establishing a fluorometric ELISA assay, CRMP-2 levels in the plasma of healthy individuals and patients with colon cancer were examined. ROC analysis showed that CRMP-2 outperformed CEA, while the combination of the two markers increased the diagnostic capacity, suggesting that CRMP-2 might be a useful plasma biomarker for CRC.

In an attempt to capture the progression from premalignant stages to carcinoma, namely the adenoma–carcinoma transition, Volmer et al. studied the secretomes of a biological cell model consisting of paired Smad4-deficient tumorigenic and Smad4 re-expressing non-tumorigenic cells, established in the same cell line (SW480) [7]. Smad4 is a tumor suppressor gene primarily involved in pancreatic and colon cancer. Comparative proteome analysis by high-resolution 2-D gels coupled to MALDI-TOF MS analysis, revealed 33 proteins regulated by Smad4.

Using 1D SDS-PAGE electrophoresis coupled to nano-LC-MS/MS, Choi et al. studied the sub-proteome of microvesicular proteins [59]. Microvesicles (MV) are spherical, bilayered proteolipids containing proteins, lipids and mRNA. Several haematopoietic and non-haematopoietic cells such as mast cells, T cells, B cells, epithelial and tumor cells produce MVs which mediate intercellular communication. Tumor-derived MVs transfer molecules involved in tumor growth and metastasis including cytokines, integrins, proteases and angiogenic molecules [62]. In this study, approximately 550 proteins were identified in three independent trials, shedding some light on the biogenesis and tumorigenic properties of MV.

In a recent study by Shi et al. tumor tissue explant secretomes were used as a source for mining potential biomarkers for colorectal cancer [60]. This approach was proposed as an alternative to cell cultures, to better capture the in vivo tumor microenvironment, including cell–cell and cell–extracellular matrix-associated interactions. Metabolic labelling with [^{35}S-methionine allowed for the successful selection of the de novo synthesized and secreted proteins by the tested tumor explants, omitting all interstitial fluid proteins, originating from necrotic cells. Proteomic analyses of

these explant secretomes revealed a number of candidate biomarkers, such as desmocollin-2 and fibrinogen γ chain.

Finally, in the quest of protein biomarkers associated to metastasis, Xue et al. studied the differentially expressed proteins in a primary and its lymph node metastatic cell line [63]. Using a LC-MS/MS-based label-free quantitative approach, the authors found approximately 150 proteins showing differential expression in the secretome of the 2 cell lines; two of which were selected for further study. By using an immunoassay, the authors measured the serum levels of trefoil factor (TFF3) and growth/differentiation factor 15 (GDF15) in a large cohort of samples from healthy individuals and patients with CRC. Both proteins were shown to be significantly elevated not only in CRC patients compared to healthy individuals but also in CRC patients with metastasis compared to metastasis-free CRC patients. The results suggest that TFF3 and GDF15 hold a promise as potential biomarkers for the prediction of CRC metastasis.

6. Pancreatic cancer

Cancer antigen 19-9 (CA 19-9) is regarded as the best available serum test for pancreatic cancer [64]; however the specificity of this marker is questionable since it is also elevated in non-neoplastic conditions such as acute and chronic pancreatitis, hepatitis and biliary obstruction [65]. The diagnostic sensitivity of CA 19-9 is further compromised by the fact that patients with certain blood types do not express the antigen [66].

Pancreatic cancer cell lines and pancreatic juice are the most accessible samples for secretome analysis. Pancreatic juice is secreted by the exocrine pancreas under the control of secretin and cholecystokinin and it consists of an aqueous bicarbonate component from the duct cells and enzymes from the acinar cells. In pancreatic cancer, malignant cells are shed into the ductal lumen making juice a rich reservoir of cancer-specific proteins [67] (Table 5).

Stable isotope labelling with amino acids in cell culture (SILAC) methodology coupled to LC-MS/MS was used to compare the secretome from pancreatic ductal adenocarcinoma cells and non-neoplastic epithelial cells [68] (Table 1). Almost 200 proteins were identified with 68 of them being up-regulated. A set of 5 proteins not studied before in pancreatic cancer was selected for further validation in tissue sections (Table 4). Tissue microarray (TMA) analysis showed that these proteins are promising biomarkers. The same group delineated the proteome of pancreatic juice [69]. Juice samples from 3 patients with pancreatic adenocarcinoma were analyzed by 1D electrophoresis coupled with LC-MS/MS and 170 proteins were identified in three biological replicates. Proteins known to be synthesized by the exocrine part of the pancreas and proteins previously related to pancreatic cancer were included in their proteome.

Chen et al., performed a comprehensive analysis of pancreatic juice by ICAT technology coupled with HPLC-ESI-MS/MS [70] (Table 5). The proteomic variability of normal pancreatic juice samples was evaluated with quantitative analysis of a pooled sample and an individual sample. They identified 15 proteins showing more than 2-fold variability among the normal samples. After establishing the threshold of variability, differentially expressed proteins between juice taken from healthy individuals and patients with pancreatic cancer were sought. Thirty proteins were identified with abundance difference of at least 2-fold between the different conditions. A protein not studied before in pancreatic cancer, IGFBP-2, was selected for further validation by western blot analysis in a small number of pancreatic cancer tissues and found to be up-regulated in cancer. In a follow-up study, the same group quantified proteins in a pool of normal pancreatic juice and a pancreatitis juice sample, identifying 27 proteins that showed differential expression in pancreatitis by at least two fold. Nine proteins up-regulated in the juice from pancreatic cancer were also elevated in the pancreatitis sample [71].

Using SELDI technology, Rosty et al., screened 15 pancreatic juice samples taken from patients with adenocarcinoma and 7 samples from patients with other diseases of the pancreas [72]. The proteomic profiling revealed two discriminating peaks between the two groups. Bioinformatic analysis along with SELDI immunoassay identified one of the peaks as hepatocarcinoma–intestine–pancreas/pancreatitis-associated protein I (HIP/PAP-I). HIP/PAP-I levels were quantified by ELISA in pancreatic juice and serum samples from patients with pancreatic cancer and normal individuals showing increased levels of the protein in the cancer cases, especially in the pancreatic juice samples. Thus HIP/PAP-I may be a potential pancreatic juice cancer biomarker.

SELDI technology was also utilized to screen the conditioned media of 15 pancreatic adenocarcinoma cell lines, 2 non-malignant pancreatic cell lines and 35 cell lines representing 6 different cancer types [73] (Table 5). One peptide, corresponding to the deleted from malignant brain tumors 1 (DMBT1) protein, was present in 5 out of 15 PC cell lines but absent from the rest of the cell lines, suggesting that it could be specific to PC. It is worth mentioning that another group also identified DMBT1 as a potential biomarker for early onset of PC [74]. Immunohistochemical analysis of intraductal papillary mucinous neoplasm samples showed that DMBT1 levels were increased in comparison to normal samples.

Differential gel electrophoresis (DIGE), coupled to LC-MS/MS, was used for the identification of differentially expressed proteins in pancreatic juice from pancreatic adenocarcinoma patients and cancer-free individuals [75] (Table 5). Fourteen up-regulated and 10 down-regulated proteins were identified.

Table 5 – Proteins that have been validated as potential pancreatic cancer biomarkers in four studies of pancreatic cancer cell secretome.

Protein name	Gene name	Validation	References
CD9 antigen	CD9	Yes	[68]
Perlecan	HSPG2	Yes	[68]
Stromal cell derived factor 4	SDF4	Yes	[68]
Intergrin beta-1	ITGB1	Yes	[68]
Apolipoprotein E	APOE	Yes	[68]
Insulin-like growth factor binding protein-2	IGFBP2	Yes	[71]
Deleted in malignant brain tumors-1	DMBT1	Yes	[74]
Metalloproteinase-9	MMP9	Yes	[75]

Immunohistochemical analysis of PC and normal specimens for 3 up-regulated proteins (MMP-9, DJ-1 and A1BG) revealed that these proteins are expressed in higher levels in the cancer samples. Moreover, serum levels of MMP-9, as determined by ELISA, seemed to be able to discriminate between PC, pancreatitis and healthy individuals.

7. Is the cancer secretome a good source to mine for biomarkers?

For proteomic analysis of secretome, the preferable samples are cancer cell lines and/or proximal body fluids. Table 1 summarizes the cell lines utilized for secretome analysis included in this review. Every discovery platform is accompanied by advantages and limitations. One hypothesis is that the conditioned media of cancer cell lines should contain secreted or shed proteins that may also be found in the circulation of cancer patients. One of the major advantages of cell line systems is the immediate availability of large numbers of cell lines representing various stages and histotypes of a given cancer. Also, since the conditioned media are less complex in comparison to serum and proximal fluids, mass spectrometric analysis is straightforward, relatively cost-effective and it permits detection of low abundance proteins. In addition, due to controlled experimental conditions, the variability caused by behavioural, environmental and genetic differences in any in vivo system is minimized, thus allowing reproducible and quantifiable results. However, cell lines are an in vitro system which ignores the contributions of the host–tumor microenvironment and provides no insight into the evolution of the disease. Furthermore, no single cell line can recapitulate the heterogeneity of human tumors.

Exploring proximal biological fluids to tumors is an attractive way to search for secreted proteins. Usually, proximal fluids contain cells of tumor origin, in addition to numerous soluble growth factors released by cancer cells or the tumor microenvironment. Many proximal fluids can be obtained with minimally invasive procedures and in large amounts (e.g. ascites fluid from ovarian cancer patients). Frequent contamination by highly abundant serum proteins can increase proximal fluid complexity and data interpretation. The procedures for obtaining such fluids need to be standardized and for more informative analyses, matching non-malignant counterparts should be included.

A variety of conventional and novel proteomic methods have been utilized for the delineation of the secretome, including 2D electrophoresis, multidimensional protein identification technology (MudPIT) and proteomic pattern identification by SELDI/MALDI- TOF. Two-dimensional electrophoresis coupled with protein identification by mass spectrometry has been the workhorse of biomarker discovery over the past years [76–78]. This technique can identify differentially expressed proteins and the development of 2-D DIGE allowed enhanced reproducibility and quantification. However, 2D is a laborious and time consuming technique which requires a large amount of starting material and cannot detect low abundance proteins. MudPIT analysis displays significantly higher sensitivity and provides a broader coverage of cell proteomes. However, its low throughput, along with the need for large amounts of starting material, makes this technique unsuitable for clinical testing. Proteomic profiling of samples utilizing SELDI/MALDI-TOF technology demonstrates medium sensitivity but the highest throughput among the aforementioned technologies. It should be noted however that results with this technology have been contested as artifactual and they failed to pass independent validations as explicitly discussed elsewhere [79–81].

The "re-discovery" of proteins that are already used as cancer biomarkers provides a proof-of-principle for cancer cell secretome analysis for biomarker discovery. However, one question arises once the discovery phase is completed. "How can we narrow down a list that comprises thousands of potential biomarkers to a manageable catalogue of e.g. 5–50 candidates for further validation?" A standardized procedure for candidate selection from the discovery phase does not exist. The criteria applied are lab dependent and there is little consensus on how to filter the datasets. One popular approach is studying only proteins that show differential expression between two conditions (e.g. normal versus cancer). With relative quantification, the amount of a substance can be determined at two different conditions and expressed as a ratio. Quantitative information can be obtained through various methodologies including 2DE with fluorescence staining, isobaric tag for relative and absolute quantification (i-TRAQ), SILAC, etc., methodologies that are described in detail in several reviews [82,83].

Apart from differential expression, other filtering criteria have been used. In our breast cancer study, for instance, we focused only on the extracellular and membrane bound proteins. We hypothesized that these proteins have higher chances to enter the circulation and serve as serum biomarkers. However, this criterion raises a critical question; is it possible for an intracellular protein to be identified in the secretome? Cell lysis and cell death are unavoidable, even under highly optimized conditions. This "leakage" of proteins from damaged cells causes contamination of the conditioned media with high-abundance intracellular proteins. On the other hand, proteins can be released to the extracellular space by three different mechanisms; a classical secretion pathway, a non-classical secretion pathway and exosomes. Proteins exiting the cell through the classical secretion pathway contain a signal peptide at the N-terminus which is responsible for the prediction of a protein as being extracellular and/or secreted. During secretion through the non-classical pathway the signal sequence is missing and proteins are released by a variety of known and unknown processes. In that case, secreted proteins cannot be predicted as extracellular and could be categorized otherwise.

In the majority of the published work, the most promising biomarkers enter a pre-validation phase during which the protein levels are measured in a limited number of serum or tissue samples obtained from cancer and normal subjects. Although the levels of many of these proteins could be shown to be elevated in the cancer cases, only few were further examined in a validation phase with sufficient number of biological samples [42, 63]. This observation raises concerns about the fate of these potential biomarkers. Clearly, discovery phase comprises a critical step in the identification of novel cancer biomarkers. However, data continuously generated and accumulated without being further exploited will not yield the desirable results. We conclude that the cancer cell secretome is a good source to mine for biomarkers but this step is just the beginning of a biomarker's long journey from the bench to the clinic.

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