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Quest for Cancer Biomarkers: Assaying Mutant Proteins and RNA That Provides the Much Needed Specificity

Suresh Mathivanan*

Department of Biochemistry, La Trobe Institute for Molecular Science, La Trobe University, Melbourne, Victoria 3086, Australia

Cancer is the leading cause of death worldwide [1]. In 2008, it was estimated that almost 12.7 million new cancer cases were diagnosed and 7.6 million cancer deaths occurred worldwide [1,2]. Cancers of lung, colon, breast, liver and prostate alone account for almost half of all the deaths [1,2]. Despite advances in surgical procedures, diagnostic and therapeutic monitoring technologies, the patient survival rate is poor for most cancers [3]. Early diagnosis of cancer can increase patient survival rate [4] and is the most promising way to cure cancer [5]. Non-invasive plasma or serum based screening of cancer specific biomarkers can be an effective diagnostic tool [6,7].

Biomarkers

Biomarkers are measurable indicators of a specific biological state [8] and have the potential to enhance patient survival rate dramatically. Ideally, biomarkers can be used for diagnostic, prognostic, predictive and pharmacodynamic purposes [7,9]. Whilst diagnostic biomarkers would aid in the identification of a disease state, prognostic biomarkers would predict the course of a disease, including its severity. Predictive biomarkers would gauge response to therapy and pharmacodynamic biomarkers would allow measurement of near-term effects of drugs, and assist in dosage selection. While metabolites and antibodies are used as biomarkers, primarily they are of genomic, transcriptomic and proteomic origin. Genomic biomarkers exploit DNA aberrations [10] at the level of chromosomes (e.g., Philadelphia chromosome) [11], gene amplification events (e.g., ERBB2 amplification in primary breast cancer tumors [12] and MYCN amplification in neuroblastoma [13]) and somatic mutations affecting individual base pairs (AKT1 mutations in many cancer types [14]). Even though genomic analyses are useful, in many instances, changes at these levels are not reflected at the RNA and protein levels. In addition, epigenetic changes, post-transcriptional and post-translational modifications have the potential to drastically alter RNA and protein amounts. Since proteins and RNAs to a certain extent can be detected in circulating bodily fluids and are most readily affected by disease, medication and recovery; they are considered to be promising candidates as biomarkers [3,15]. As the majority of biomarker analysis are based on the proteins/RNA that can be detected in bodily fluids, the mechanisms by which proteins and RNA are secreted/released by cancer cells are discussed below.

Cancer Secretome and Protein Secretion Pathways

Cancer secretome can be defined as the total set of proteins released by classical and non-classical secretory pathways from cancer cells into the surrounding microenvironment. In the classical secretory pathway [16-18], proteins are synthesized with signal peptides which target them to lumen of the endoplasmic reticulum (ER). After the protein is translocated through the ER membrane, its folding, sorting and covalent modifications occur in the ER and Golgi [19]. Secretory vesicles containing the protein bud off from the Golgi apparatus and fuse with the plasma membrane (PM) to release the contents through the process of exocytosis (Figure 1). In contrast to the classical secretory pathway, proteins released by the non-classical secretory pathways (Figure 1) are devoid of signal peptides and are independent of the ER and Golgi [20]. Currently, at least five non-classical secretory mechanisms are characterized i, exosomes ii, shedding microvesicles iii, ectodomain shedding iv, membrane transport channels, and v, flip flop.

Exosomes are 40-100 diameter membranous nanovesicles that are released into the microenvironment upon fusion of the multivesicular bodies (MVBs) with the PM [21]. They are involved in intercellular signaling and contain both luminal and membrane proteins from the host cell [22]. Ectosomes or shedding microvesicles (SMVs) are large membranous vesicles (50-1000 nm diameter) that are shed directly from the PM of a wide variety of cell types [23-26]. Following blebbing (outward protrusion) of the PM, fission of the PM stalk detaches the cytoplasmic protrusions resulting in the formation of SMVs [27]. Another well documented mechanism of extracellular protein release is ectodomain shedding [28]. Proteolysis on the exosomes, SMVs and cell surface, releases ectodomains of membrane proteins into the extracellular microenvironment [29,30]. In an unconventional manner, proteins such as fibroblast growth factors-1 and -2 are capable of translocating from the cytoplasm directly through the PM into the extracellular space [31-33]. Finally, the flip flop mechanism mediates secretion of proteins (e.g., HASPB) anchored to membrane through dual acylation in the N-terminus [34,35]. Proteins translocating to the extracellular space through the flip flop mechanism lacks transmembrane spanning domains or glycosylphosphatidylinositol anchor [34]. Even though the unconventional PM based transport channel and the flip flop mechanisms are documented in the literature, they are still not studied thoroughly in different cell models.

Current State of Biomarker Studies

Since the ultimate goal of biomarker discovery is to develop a non-invasive blood test, it is only natural that blood be the sample of choice. As blood is thought to contain a sampling of every single tissue in the body, it is considered ideal for mining biomarkers [7]. Unfortunately, for the very same reason, blood is complex and difficult to work with as they contain an abundance of core proteins from albumin to cytokines which must be depleted prior analysis [8]. To overcome the complexity of blood, alternative biomarker discovery approaches have been pursued, including proteomic analysis of cancer

***Corresponding author:** Suresh Mathivanan, Department of Biochemistry, La Trobe Institute for Molecular Science, La Trobe University, Bundoora, Victoria 3086, Australia, Tel: +61 03 9479 2565; Fax: +61 03 9479 1226; E-mail: S.Mathivanan@latrobe.edu.au

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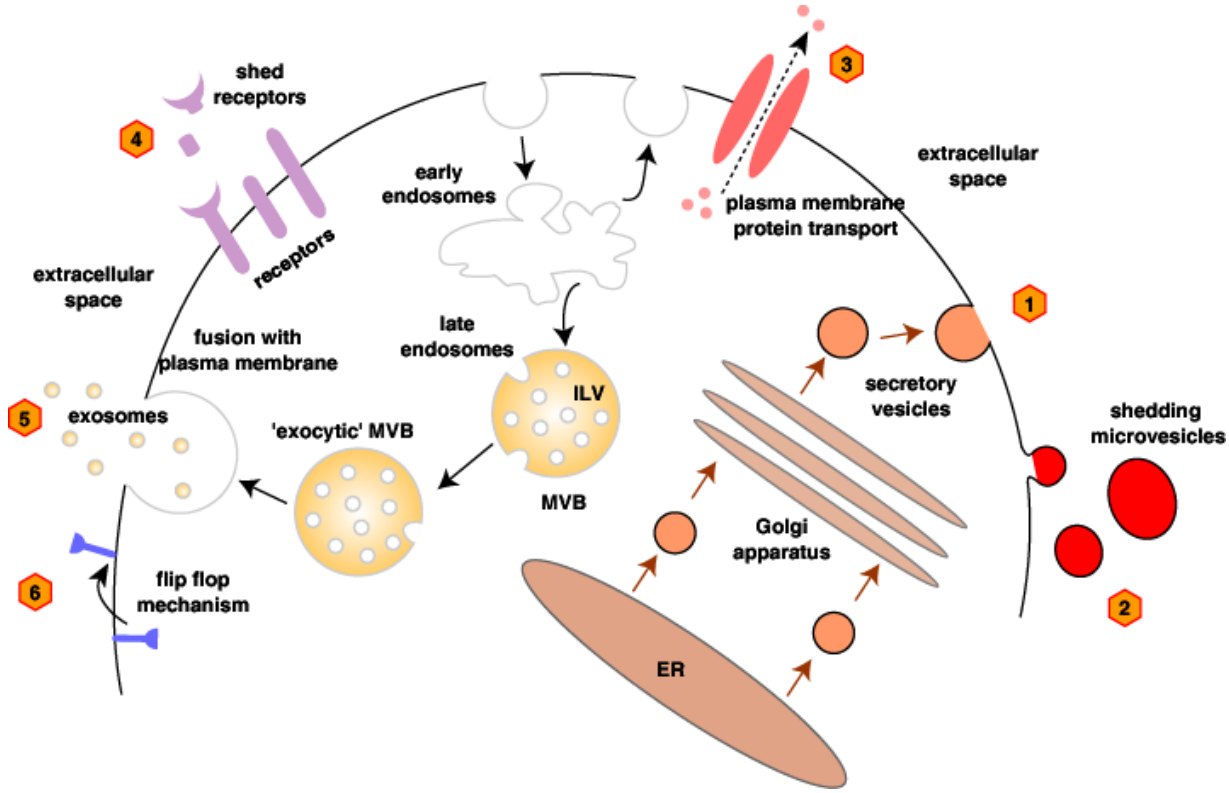

Figure 1: Classical and non-classical protein secretion pathways. Classical and non-classical modes of protein secretion are depicted. In the ER/Golgi dependent classical secretory pathway (denoted as 1), proteins are synthesized with signal peptides which target them to lumen of the ER. Secretory vesicles containing the protein bud off from the Golgi apparatus and fuse with the PM to release the contents through the process of exocytosis. Proteins released by the non-classical secretory pathways are devoid of signal peptides and are ER/Golgi independent. SMVs are large membranous vesicles (50-1000 nm diameter) that are shed directly from the PM of a wide variety of cell types (denoted as 2). Proteins such as fibroblast growth factors-1 and -2 are capable of translocating from the cytoplasm directly through the PM into the extracellular space in an unconventional manner [31-33] (denoted as 3). Proteolysis on the exosomes, SMVs and cell surface, releases ectodomains of membrane proteins into the extracellular microenvironment (denoted as 4). Exosomes, 40-100 diameter membranous nanovesicles, are released into the microenvironment upon fusion of the MVBs with the PM (denoted as 5). Flip flop mechanism mediates secretion of proteins (e.g., HASPB) anchored to membrane through dual acylation in the N-terminus [34,35] (denoted as 6).

cell lines, cancer tissues and proximal body fluids. Cancer secretome studies are often performed for the quest of identifying biomarkers that can potentially allow clinicians to diagnose cancer patients in non-invasive manners. In case of colorectal cancer (CRC), CEA is the most widely used biomarker associated with CRC screening. However, the lack of sensitivity and specificity of the test renders it unsuitable for clinical screening. Elevated serum levels of CEA are not only detected in CRC patients but also in lung, cervix [36], breast [37], gastric [38] and pancreatic [39,40] cancer patients. With the increase in diagnostic screening efficiency, these studies prompt the use of biomarker panels rather than one protein marker to improve the sensitivity and specificity of the test.

Wu et al. [41], profiled the secretome of 21 cancer cell lines from 12 cancer types including leukemia, nasopharyngeal, cervical, epidermoid, ovarian, uterine, pancreatic, colon, bladder, hepatocellular, lung and breast cancers. Comparative proteomic analysis of 21 cancer cell line secretomes resulted in the identification of collapsin response mediator protein-2 (CRMP2) as a potential biomarker for CRC. Sensitivity of CRMP2 in CRC patient plasma was found to be 60.5% superior to the sensitivity of CEA which was found to be 42.9%. Interestingly, combination of the CEA and CRMP2 increased the sensitivity from 42.9% to 76.8%. Similarly, Kulasingam et al. [42], compared the secretome profile of three breast cancer cell lines (MCF-10A, BT-474 and MDA-MB-468). Comprehensive comparative proteomic analysis resulted in the identification of activated leukocyte cell adhesion molecule (ALCAM) as a potential biomarker. Validations were performed on a set of 150 normal and 150 cancer patient serum samples. Area under the curve (AUC) was recorded as 0.78 for ALCAM outperforming classical markers such as CA15-3 (AUC:0.70) and CEA (AUC:0.63). Again in this study, combination of CA1503 and ALCAM increased the diagnostic efficiency (AUC:0.81) compared to the use of one marker alone.

Exploiting the Topography of a Cancer Cell - Driver and Passenger Mutations

Cancer arises as a consequence of accumulated mutations in key proteins that regulate cell proliferation, differentiation and death [43,44]. Recent studies involving genome-wide analysis for all somatic mutations revealed that ~40-100 amino acid mutations can be detected in a complex cancer genome [40,45,46]. When plotted on a two-dimensional map where each gene is represented by one point, these formed 'mountains'. The majority of mutated genes however, only formed 'hills' (mutated in only a few cancers). This discovery leant weight to the theory that most cancers are not caused by just a few mutations, rather by a large number of mutations, and each one brought its own fitness advantage [47]. Such somatic mutations are popularly

referred to as 'drivers'. Cancerous cells also accumulate somatic mutations which do not confer any particular growth advantage to the cell, but are present merely because they happened to be present when a driver became active. Such mutations are termed 'passengers'. Most importantly, driver mutations, by their very role in tumor progression, are uniquely positioned to be the promising biomarkers. They give the much needed specificity for cancer, only tumor cells will produce mutant proteins or RNA, and by virtue of their function, they can potentially direct attention to malfunctioning pathways.

Mutant Proteins as Biomarkers

Recently, Wang et al. showed that altered protein products of somatic mutations could be identified and quantified by mass spectrometry (MS) [48]. Using MS-based selected reaction monitoring (SRM), they were able to distinguish between normal and mutant forms of the KRAS protein (G12D) without the use of mutant-specific antibodies in cell lines, tissues samples and bodily fluids. The study showed that the SRM technique could easily be used on complex biological samples which are routinely encountered in clinical setups. In addition, the study revealed that the technique is sensitive enough to detect minute quantities of mutant forms, as little as 10 fmol. The advantages of SRM include high specificity, readily available reagents and allows for analysis of multiple proteins, unlike DNA based approaches, thereby saving precious sample material. Moreover, the technique requires no antibody against the mutant form and can be used to assay proteins with multiple mutations. One of the drawbacks of the technique is the amount of sample required which may be too high for practical use in clinical settings. While the technique can be reproduced using many different proteins, the authors quantified mutant KRAS, an intracellular protein, in biopsies. However, the use of secreted mutant proteins might increase the usability as it can be assayed in non-invasive manners.

Another recent study by our group focussed on the identification of mutated proteins from the secretome of 18 cell lines representing different stages and underlying mutation status of colorectal cancer, using a technique that we termed 'iMASp' [49]. Because MS-based protein identifications often rely on search engines with existing sequence databases [50], if the mutated sequence is not present in the database, the mutation will be undetected. For this reason, we employed an integrated genomics and proteomics strategy to create a Human Protein Mutant Database (HPMD), against which experimentally-derived secretome peptide spectra were searched. HPMD was created using publicly available datasets of known functional mutations (31,479) and SNPs (140,440 mutations) from UniProt [51], Protein Mutation Database, OMIM and SysPIMP [52]. Using iMASp, we detected 112 putative mutated tryptic peptides (corresponding to 57 proteins) from a panel of 18 human CRC cell line secretome data. The technique, when applied to publicly available MS-based CRC tissue homogenates, was able to detect 3 mutations that were also detected in CRC cell lines. Among the 112 mutations, 8 mutations were validated by RT-PCR and cDNA sequencing. Whilst the method has the potential to uncover many functional mutations at the protein level, we emphasise caution in interpreting the results as the use of large sequence databases and MS datasets has a very high correlation with increased false discovery rates [53]. Validations at the genomic/transcriptomic levels are needed to confirm the identified mutations. The iMASp technique is transferable to the analysis of cancer tissue samples as well. Though it is a proof-of-principle study, the study showed that many of the mutant proteins are secreted by cancer cells. The possibility of an altered extracellular localization of a mutated protein affords unparalleled opportunity to exploit such mutant proteins as cancer biomarkers. If such mutant proteins are also drivers, it provides the specificity that seems to lack from wild type proteins.

Exosomes and Ectosomes are Treasure Chests for Biomarker Discovery

Extracellular vesicles (EVs) are membraneous vesicles released by a variety of cells into the extracellular microenvironment [21,54]. Based on the mode of biogenesis, EVs can be classified primarily into ectosomes or SMVs and exosomes [21]. Exosomes are 40-100 nm diameter extracellular organelles of endocytic origin that are released by various cell types [55]. Inward budding of endosomal membranes results in the progressive accumulation of intraluminal vesicles (ILVs) within large MVBs. Whilst the transmembrane proteins are incorporated into the invaginating membrane, the cytosolic components are engulfed within the ILVs [56]. The MVBs can either traffic to lysosomes where they are subjected to proteosomal degradation or fuse with the PM to release their contents (ILVs) into the extracellular space as 'exosomes'. The density of exosomes varies from 1.10 - 1.21 g/ml and the commonly found markers of exosomes are Alix, TSG101, tetraspanins and heat shock proteins [57]. SMVs are large EVs ranging between 50 – 1000 nm in diameter [58]. They are shed from cells by outward protrusion (or budding) of a plasma membrane followed by fission of their membrane stalk [23,27]. SMVs are released by a variety of cells including tumour cells, polymorphonuclear leucocytes and erythrocytes [27]. The expression of phosphatidylserine (PS) on the membrane surface has been shown to be one of the key characteristic features of SMVs [27,58].

As exosomes and SMVs are secreted/released into the extracellular microenvironment and can be assessed in bodily fluids, significant interest have been created on these bioactive vesicles as possible reservoirs of biomarkers [15,59,60]. Additionally, exosomes and SMVs contain proteins, RNA and lipids that are reflective of the host cell [22,58]. Exosomes released by CRC cells have been shown to contain a CRC tissue-specific signature and also contained proteins of the cancer hallmarks [22,61]. Exosomes were observed *in vivo* in blood plasma of ovarian cancer [59] and lung cancer [62] patients. Interestingly, these studies revealed that plasma-exosome levels were increased in patients with advanced disease (e.g., mean 2.85 mg/mL exosomes for lung cancer adenocarcinoma patients compared with 0.77 mg/mL exosomes in the blood of normal volunteers [62]). In a recent study, Nilsson *et al.* detected the fusion gene TMPRSS2:ERG in exosomes isolated from the urine of prostate cancer patients [60]. Similarly, oncogenic receptor EGFRvIII is shown to be released by microvesicles [15,25,63].

Conclusions

Decades of discovery phase biomarker studies have identified numerous potential biomarkers for cancer. Some of the biomarkers translated into clinical settings often lack the required specificity and sensitivity for a routine clinical test. Whilst there is general agreed consensus that a panel of biomarkers will increase the diagnostic efficiency, the current protein/RNA based biomarker analyses often assay for wild type forms. Except for two recent studies, the mutant forms of the proteins that are indicative of oncogenesis are assayed in limited conditions. Assaying for protein mutations as disease biomarkers provides the required specificity for a biomarker test as mutant proteins are encoded by disease cells. Such mutant proteins can be assayed non-invasively in bodily fluids (in soluble secreted and EV fractions) and in tissue biopsies for intracellular mutant proteins.

Additionally, mutant RNA can be assayed in EVs in bodily fluids. Perhaps already used biomarkers such as CEA and PSA can be used in conjunction with mutant proteins/RNA to increase the specificity and sensitivity of the test.

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