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UNRAVELLING NEW PIECES OF TUMOUR METASTASIS PUZZLE: THE ROLE OF PROTEOMICS

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Summary

Despite the abundance of attention that cancer has attracted, it continues to constitute one of the deadliest scourges of the modern era. Tumour heterogeneity greatly contributes to the ineffectiveness of current therapies and hampers the study and treatment of cancer. There are two models accounting for tumour heterogeneity and propagation, namely clonal evolution model and cancer stem cell model. In particular, cancer stem theory has attracted much attention lately, as these cells with self-renewal and differentiation abilities are responsible for the initiation of tumour development, growth, and its ability to metastasize and reoccur, and provide a reasonable explanation for poor prognosis for patients in advanced stages of solid tumours. Advances in technologies such as proteomics open new avenues in metastasis research by specifically revealing complex protein networks involved in tumour progression, which should facilitate early diagnosis and provide the basis for designing more effective treatment strategies.

Keywords: cancer stem cell; metastasis; proteomics

Introduction to the clonal evolution model and the cancer stem cell model

Despite the abundance of attention that cancer has attracted, it continues to constitute one of the deadliest scourges of the modern era. Although technological improvements in screening modalities have facilitated detection of smaller tumours, current therapies for most types of cancer often fail. One of the reasons for this certainly lies in the specific feature of cancer cells to uncontrollably grow and divide (Figure 1). In addition, common characteristic of all cancers

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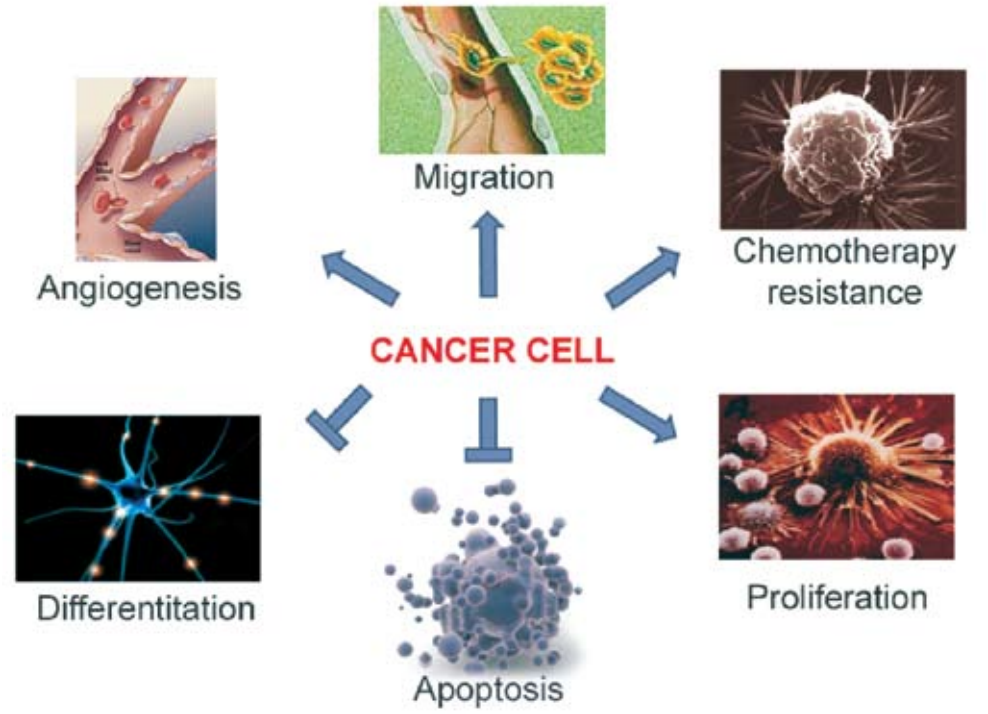

Figure 1. Cancer cell fate. Cancer cells have a selective growth advantage over adjacent normal cells and acquire uncontrolled proliferation during tumorigenesis (e.g. resistance to apoptosis). Further on, metastasis process occurs that according to clonal theory involve invasion and angiogenesis, migration and arrest in the capillary beds of distant organs.

is striking variability among the cancer cells within a single tumour, whereby these cells differ in features such as size, morphology, antigen expression and membrane composition, as well as behavioural properties such as proliferation rate, cell-cell interaction, metastatic proclivity, and sensitivity to chemotherapy [1-3. This tumour heterogeneity contributes to the ineffectiveness of current therapies and hampers the study and treatment of cancer, because tumour samples may not be representative of the whole and because its origins are not completely known [3,4].

There are two models that account for tumour heterogeneity and propagation: clonal evolution model and cancer stem cell model. According to the model of clonal evolution, cancer is formed through the accumulation of genetic changes in cells and gradual selection of clones [5]. The majority of therapeutic approaches (i.e. conventional therapies) based on the elimination of tumour ce-

lls are based on this theory [6]. According to this model, tumour is induced after multiple mutations occur in a random single cell, conferring it a selective growth advantage over adjacent normal cells [3]. As the tumour further develops, genetic instability and uncontrolled proliferation facilitate the production of cells with additional mutations and hence new characteristics, such as resistance to apoptosis. Therefore, increased proliferative capacity of cancer cells is the result of accumulated genetic mutations that enhance cellular proliferation or suppress normal growth inhibitory mechanisms and programmed cell death (apoptosis) [7]. The two main types of genes that play a key role in cancer development are oncogenes and tumour suppressor genes. Oncogenes are mutated forms of normal cellular genes generally involved in promoting cell proliferation, whereas tumour suppressor genes normally regulate proliferation in the way to stop it when necessary. Tumour suppressor gene products typified by p53 are frequently transcription factors that suppress mitosis and cell growth to allow for DNA repair. Nearly half of all cancers involve altered p53 genes. Other suppressor genes include Rb (retinoblastoma family), APC (adenomatous polyposis coli), SMAD4, TP53, p16/CDKN2A and BRCA (breast cancer susceptibility protein) types 1 and 2. Cancer results from cumulative mutations of oncogenes and tumour suppressor genes, which together allow the unregulated growth of cells. Oncogenes are typically dominant because they provide gain-of-function, whereas tumour suppressor genes are recessive and contain loss-of function mutations. Both copies of a suppressor gene need to mutate to cause loss-of-suppressor function, whereas only one copy of oncogene needs to mutate for gain-of-function.

Poor outcomes of current therapies, in particular poor prognosis for patients in advanced stages of solid tumors, opened the possibility that tumor cells include a population of cells responsible for the initiation of tumor development, growth, and its ability to metastasize and reoccur. Because these cells share some similarities with stem cells, they are referred to as cancer stem cells (CSCs). Cancer stem cells (CSCs) are rare tumor cells that have the potential to proliferate, self-renew and induce tumorigenesis. Their self-renewal and differentiation abilities lead to the production of all tumor cell types, thereby generating tumor heterogeneity. Additionally, cancer stem cells are highly resistant to chemotherapy and radiation, probably due to the fact that cancer stem cells are quiescent and have high levels of ABC transporters, as well as great capacity for DNA repair and abundant anti-apoptotic proteins [8]. Some additional specific features of CSCs include over-expression of CD44, unknown status of cell cycle, deregulated self-renewal signaling, perturbed adhesion/migration, and possibi-

lity to be blocked in undifferentiated state [9]. Evidence for the existence of such cells was first proposed for haematological malignancies (leukaemia and multiple myeloma) [10] and, more recently, for solid tumors, including breast, brain, prostate, head and neck, lung, skin, liver, ovary, colon and pancreatic cancer [11-13]. When implanted into immune-deficient mice, CSCs generate tumors that are identical to the parental tumors [14].

Major concept of CSCs model assumes that these cells arise from stem cells or progenitor cells (i.e. partially differentiated precursor cells with a limited proliferation capacity) [15]. According to the pre-tumor progression hypothesis, the development of tumor results from the clonal evolution of the CSC population [16]. The transformation of a normal stem cells into a cancer stem cell is the result of the accumulation of genetic aberrations (mutations in oncogenes, suppressor genes and miss-match repair genes) and epigenetic alterations (abnormal methylation, histone modification) [15]. Several pivotal signaling pathways, including Wnt/β-catenin, Hedgehog, and Notch pathways have been shown to play critical roles both in normal stem cells and cancer stem cells [17], and targeting these signaling pathways in cancer stem cells might lay the groundwork for new, prospective approaches in future cancer treatment [8].

Molecular basis of metastasis

The most deadly aspect of cancer is its ability to spread to other parts of the body through the blood and lymph systems in the process termed metastasis.

Metastasis is typically difficult to cure by standard surgical procedures, radiation therapy, and chemotherapy, and confers poor prognosis for the affected patient. As a result, 90% of human cancer deaths are attributed to local invasion and distant metastases [18]. The molecular mechanisms involved in this process are not completely understood but those associated with vessel formation (angiogenesis), cell attachment, invasion (matrix degradation, cell motility), and cell proliferation are generally accepted to be critical. The major molecular mediators of these processes are growth factor signaling molecules, chemokines, cell–cell adhesion molecules (cadherins, integrins) as well as extracellular proteases (matrix metalloproteinases).

Five major steps in metastasis process can be distinguished: 1. invasion and infiltration of surrounding normal host tissue with penetration of small lymphatic or vascular channels; 2. release of neoplastic cells (either single cells or small clumps) into the circulation; 3. survival in the circulation; 4. arrest in the capillary beds of distant organs; and 5. penetration of the lymphatic or blood vessel walls followed by growth of the disseminated tumour cells [19].

Traditional models of metastasis have described a process in which genomic instability causes the genesis of rare sub-populations of cells within the primary tumor that have acquired a metastatic phenotype [20,21]. This viewpoint was supported by the clinical observation that metastatic lesions are rare events despite the continual dissemination of significant numbers of tumor cells into the circulation [22,23]. However, recent gene expression profiling experiments have shown that paired primary tumors and metastases are similar, whereas a significant difference is observed when primary tumors with or without metastases are compared [18,24-26]. These studies challenged the theory that metastasis-capable cells are rare, and suggest that metastatic capacity is embedded in the majority of cells within the primary tumor and may be determined at an early stage of carcinogenesis. In the light of several recent experiments implicating that only rare cancer-initiating tumor stem cells are capable of forming distant metastasis at any significant rate [27,28], Molloy *et al.* [21] has postulated that non-transformed stromal cells adjacent to primary tumor may contribute, at least partially, to the 'metastasis' signature seen in bulk tumor. Different inflammatory molecules such as cytokines, chemokines and growth factors are produced in the local tumor environment by different cells accounting for a complex cell interaction and regulation of differentiation, activation, function and survival of multiple cell types [29]. Chemokines, secreted by both, stromal and non-tumor-derived stem cells can act as positive mediators of metastasis in both, the microenvironment of the primary tumor by stimulating migration of tumor cells away from the area, as well as in the microenvironment of the distant organs by encouraging metastatic cell homing [21]. In addition, different growth factors secreted by stromal cells can also mediate tumor initiation and progression, e.g. epithelial growth factor (EGF), fibroblast growth factor (FGF), hepatocyte growth factor (HGF), insulin-like growth factor (IGF), and transforming growth factor-β (TGF-β). The complex interaction between cytokines, chemokines, growth factors and their receptors forms an intricate network at the tumor site, which is primary responsible for an overall tumor progression and spreading.

Short overview of proteomics technologies

Most issues in modern cell biology have been tackled using the so called "reductionist methods", i.e., by studying one gene, one protein or one specific protein modification at a time. This reductionism has been necessary, given the complexity of biological systems and lack of appropriate tools for developing more integrative methodologies. With the continuous development of techno-

logy, it is now possible to get a more thorough understanding of complex biological systems by simultaneous observation of their many characteristics. In this respect, proteomics has emerged as powerful approach that provides an insight into actual cellular processes. The term "proteome" was first coined in 1994 by an Australian scientist Marc Wilkins to describe the total set of proteins expressed in a given cell at a given time. The study of proteome or proteomics covers all aspects of protein properties on a large scale, including its level of expression, protein folding and 3-D structure, function, protein interactions, cellular localization and the modifications, such as isoforms and post-translational modifications. The analysis of proteins represents a challenge as their structure, function and expression could be controlled at many cellular regulatory points starting from transcription, mRNA splicing, translation and subsequent protein modifications, such as the addition or removal of phosphate groups at specific sites, addition of carbohydrate chains (glycosylation), formation of protein complexes e.g. with lipids (HDL, LDL, VLDL), cellular translocation etc. [30]. While proteome profiling is an important and necessary step for a complete understanding of the function of a protein complex, proteomics can also be applied to study global proteome function. Unlike genomes, proteomes are dynamic and many proteomic studies focus on examining changes in proteome composition under various conditions.

The main methodological approaches in proteomics research are "shotgun" approach and classical gel-based approach. "Shotgun proteomics" refers to the direct liquid chromatography/mass spectrometry (LC-MS) analysis of complex peptide mixtures derived from proteolytic digestion of heterogenous mixtures of proteins in order to rapidly reveal a global profile of the protein complement within the mixture. LC-MS requires that the protein mixture be fractionated either in order to reduce complexity by targeted fractionation of peptides bearing specific chemical features (e.g. reactive sulfhydryl groups as at cysteine residues or phosphorylation caused by post-translational modifications) or with the aim of increasing the potential of mass spectrometer to detect all peptides in the sample mixture [31]. The latter approach, commonly known as MudPIT (multi-dimensional protein identification technique) is achieved by using several sequential separation methods (usually microcapillary cation-exchange and reverse-phase chromatography) coupled with tandem mass spectrometry.

Gel-based approach relies on two-dimensional gel electrophoresis (2-DE) separation of complex protein mixtures followed by mass spectrometric analysis of selected protein spots. In 2-DE, proteins are first resolved by isoelectric focusing according to their isoelectric points, and then according to their molecular

weights using sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE). At the core of most modern proteomic studies lies mass spectrometry (MS) [32]. Mass spectrometer consists of an ion source, a mass analyser that measures the mass-to-charge ratio (*m*/*z*) of the ionized analytes, and a detector that registers the number of ions at each *m*/*z* value. Electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) are the ionization methods most commonly used for protein and peptide samples. ESI generates ions directly from solution and is therefore coupled with liquid-chromatographic (LC) or electrophoretic instrumentation. The sample is sprayed into a fine spray in the presence of an electric field. Charge accumulates on the sample droplets leading to their explosion due to mutual repulsion of charges and formation of ions. In MALDI, the sample is mixed with a UV-absorbing matrix compound (e.g. α-cyano-4-hydroxy-*trans*-cinnamic acid, sinapinic acid) and crystallized. The mixture is then excited with a laser bringing about evaporation of the matrix compound, which carries the sample molecules into vapor phase. Sample ions are formed by the electrons and protons exchange with the matrix. MALDI-MS is usually used to analyze relatively simple peptide mixtures, whereas ESI-MS (LC-MS) is the method of choice when it comes to the analysis of complex sample mixtures [32]. Recent advances in mass spectrometry and the determination of the complete genomic sequences of several organisms have greatly facilitated the application of proteomics to many research fields.

One of the most important goals in proteomics is the ability to accurately measure quantitative changes in protein expression in response to a variety of internal and external stimuli [33]. Therefore, the development of methods that would allow accurate protein quantitation is currently one of the most challenging aspects of proteomics. One of the most common methods used in quantitative proteomics is introduction of a chemically equivalent differential mass tag that allows the comparative quantitation of proteins in one sample to another. The labels change the mass of a protein or peptide without affecting the analytical or biochemical properties [34]. Differential isotopic labels can be introduced metabolically, enzymatically or chemically, and, depending on the method used, at either the peptide or the protein level [33]. One of the most popular chemical labeling method is ICAT (isotope-coded affinity tags), in which the stable isotopes are incorporated after isolation by selective alkylation of cysteines with either a heavy (d8) or light (d0) reagent [35]. The two protein mixtures are then mixed and digested with trypsin. ICAT-labeled peptides are isolated by biotin-affinity chromatography and then analyzed by online HPLC coupled to a tandem mass spectrometer. The ratio of the ion intensities for an ICAT- labeled pair

quantifies the relative abundance of its parent protein in the original cell state. In addition, the tandem mass spectrum reveals the sequence of the peptide and unambiguously identifies the protein [35].

DIGE (Difference Gel Electrophoresis) is a method that labels protein samples with fluorescent dyes before 2-DE, enabling accurate analysis of differences in protein abundance between different samples. In DIGE, samples of interest are labeled with two (or three) spectrally distinct fluorescent dyes (Cy-2, Cy-3 and Cy-5) and run on the same gel. The three gel images corresponding to each of the Cy-Dye scans are then superimposed to generate a spot map where paired spots within the same gel can be accurately quantified.

Recently, SELDI-TOF MS (Surface Enhanced Laser Desorption/Ionization Time-of-Flight Mass Spectrometry) has gained much appreciation in cancer research for its ability to reveal potential diagnostic biomarkers in diverse complex biological specimens, such as serum, plasma, intestinal fluid, nipple aspirate fluid, urine, cell lysates, etc. The development of SELDI-TOF MS has overcome limitations of other proteomic approaches in terms of the inability to analyze hundreds of samples within a short time, which is essential for obtaining biologically and statistically relevant data. T his technique couples array-based technology (ProteinChip®, Ciphergen Biosystems Inc.) with MALDI-TOF MS. The protein chip arrays contain either chemically (anionic, cationic, hydrophobic, hydrophilic, or metal ion) or biochemically (immobilized antibody, receptor, DNA, enzyme, etc.) active surface, which retains proteins according to their specific physicochemical properties. After adding matrix solution to bound proteins, the latter are ionized with nitrogen laser and their molecular masses measured by TOF mass analyzer. As a result, unique protein abundance profiles of species bound to the chip surface are obtained. Comparisons of spectra obtained from large number of different samples reveal unique or over-expressed protein signal in a particular sample set; however, this method determines the molecular mass of differentially abundant proteins rather than their identity. For more comprehensive overview of proteomics technologies, readers are referred to dedicated literature [33,36].

Recent advances in metastasis research using proteomics tools

Two-dimensional gel electrophoresis (2-DE) (Figure 2) followed by mass spectrometry-based (MS) protein identification has been the most widely used proteomics method in the study of metastatic processes in diverse panel of cancers. By applying this approach, Wang et al. [37] found the CLIC1 protein (chloride intracellular channel 1) to be correlated with metastasis of gallbladder

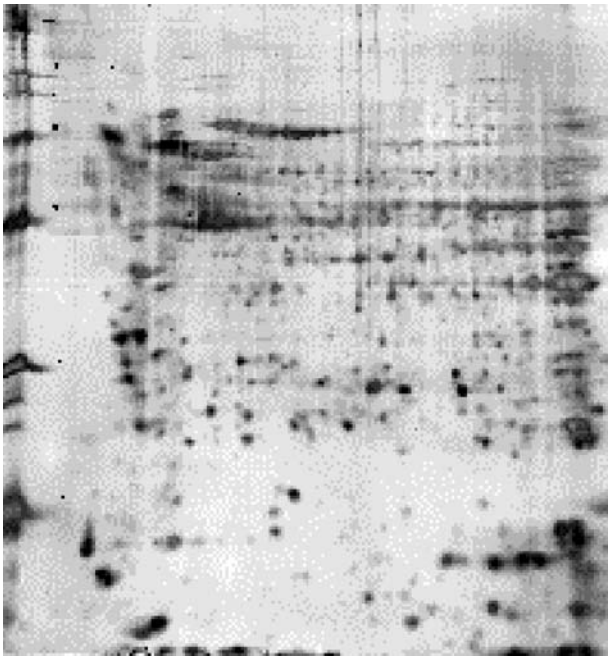

Figure 2. Example of a 2-DE gel. The picture presents a gel obtained by resolving total protein lysates from HEp-2 cells treated with metotrexate.

carcinoma, the most frequent form of bile duct cancer, as its expression was significantly up-regulated in the highly metastatic gallbladder cancer GBC-SD18H cell line when compared to the poorly metastatic GBC-SD18L cell line. Similarly, proteomics profiling of tumor tissues from gastric cancer revealed CLIC1 to be significantly up-regulated in 67.9% of the patients [38]. This study has brought into relation the elevated expression of CLIC1 with lymph node metastasis, lymphatic invasion, perineural invasion, advanced pathological stage and poor survival in gastric cancer, and highlighted the role of CLIC1 in tumor invasion and metastasis in gastric cancer. Furthermore, comparative 2-DE/MS analysis of two non-small cell lung cancer cell lines with different metastatic potentials identified unambiguously 33 differentially expressed proteins [39]. Among these, the over-expression of S100A11 in non-small cell lung cancer tissues was associated with higher tumor-node-metastasis stage and positive lymph node status, implying regulatory role of this protein in promoting invasion and metastasis of non-small cell lung cancer [39]. Involvement of this protein in metastasis was also confirmed for other tumor types as well. For example, Song et al. [40] applied 2-DE/MS approach towards analysis of metastasis-associated proteins in human hepatocellular carcinoma (HCC) tissues. Among 16 differentially expressed proteins including metabolic enzymes, subcellular trafficking

proteins, chaperones and cytoskeletal regulating proteins, these authors found S100A11 along with HSP27 and CK18 to be specifically connected with metastasis of HCC. In addition, HSP27 was proposed as a useful therapeutic target not only for HCC, but also for other types of tumors, since its elevated levels were correlated with lymph node metastasis in breast and prostate cancer [40].

Besides 2-DE, difference gel electrophoresis (DIGE) has proved a valuable tool in search of metastasis markers. A bright example is the study of lymph node metastatic prostate cancer (LNM PCa), which provided evidence that increased expression of e-FABP5, MCCC2, PPA2, Ezrin, and SLP2 and decreased expression of SM22 are useful diagnostic markers for the existence of LNM PCa [41]. The same authors also reported on the significantly higher levels of e-FABP5 in serum of patients with LNM PCa. e-FABP5, whose over-expression was previously established in prostate cancer tissues, induces invasion and metastasis by up-regulating the central player in the metastatic cascade, namely VEGF [42]. Besides e-FABP5, ezrin has been recognized as a key component in tumor metastasis due to its unprecedented role as an integrator of signals between metastasis-associated cell surface molecules (Met receptor and CD44) and signal transduction components (Rho and Akt) [43].

Although many genes have the ability to inhibit both tumorigenesis and metastasis, metastasis suppressor genes are unique in that they specifically block secondary tumor formation without affecting the primary tumor. Breast cancer metastasis suppressor 1 (BRMS1) is a protein with poorly understood molecular functions, whose over-expression decreases the metastatic potential of human breast cancer and melanoma cells, whereas tumor cell lines with low levels of BRMS1 are highly metastatic. In order to unravel the role of BRMS1 in metastasis, Rivera et al [44] used the sensitive DIGE analysis coupled with MS to compare the protein expression profiles of wild-type melanoma cells (WT), melanoma cells over-expressing BRMS1 (Mel-BRMS1), and melanoma cells in which endogenous BRMS1 was silenced by a short hairpin RNA (sh635). Interestingly, more than 75% of the identified proteins were down-regulated in Mel-BRMS1 cells compared to WT, whereas all the identified proteins in sh635 cells extracts were up-regulated compared to WT indicating that transcriptional repression might be one of the functions of BRMS1, which might occur indirectly through interactions of BRMS1 with proteins in the transcriptional machinery [44]. Based on the several identified proteins that regulate actin dynamics, the authors also proposed that BRMS1 might suppress metastasis in part by affecting cytoskeletal structures such as focal adhesions or lamellipodia. Furthermore, Nm23-H1, the first metastasis suppressor gene to be characterized, has been shown to alter both gene and

protein expression in cancer cells. To elucidate potential molecular mechanism by which Nm23-H1 mediates metastasis suppression, Lee et al. [45] performed ICAT (isotope capture affinity tag) proteomic analysis on control and Nm23-H1 stable transfected MDA-MB 435 breast cancer cells. The ICAT data revealed that 189 and 381 proteins were significantly down- and up-regulated, respectively, upon over-expression of wild-type Nm23-H1. However, the most important finding of this study was identification of several proteins involved in RNA-related functions, including GEMIN5, which plays a role in differential mRNA splicing. Based on obtained data, the authors propose that Nm23-H1, through the regulation of RNA processing proteins, may play a role in proteome stability [45].

Maspin is a tumor-suppressor protein that abolishes metastatic capacity in vivo of invasive mammary carcinoma cells [46]. Although this protein is the subject of intense study, the mechanisms by which maspin elicits its anti-tumor and anti-metastatic effects are still the not completely clear. Chen et al. [47] successfully applied shotgun proteomics based on multidimensional protein identification technology (MudPIT) to compare the proteomes of maspin-deficient and maspin-expressing tumor cells, and found that the expression of maspin has widespread effects on the tumor cell proteome. In most cases, protein expression was affected without changes in mRNA levels, indicating that maspin has a significant influence on post-transcriptional regulation of protein levels. This finding is probably a result of maspin's effects on the expression and activity of the proteasome, which may be central to maspin's tumor suppressor activity [47]. The same authors have showed that maspin causes significant alterations in proteins associated with the actin network (e.g. epithelial protein lost in neoplasm, gelsolin, etc.) necessary to support enhanced migratory ability of invasive cancer cells, and pro-apoptotic proteins (e.g. protein phosphatase 2, Acinus, etc.).

Proteomic profiling of serum and plasma is an emerging technique to identify new biomarkers indicative of disease severity and progression. In this respect, SELDI-TOF technology has emerged as a successful tool for obtaining metastasis-associated protein profiles for different cancer types including ovarian, breast, prostate, lung and colorectal cancer, as well as laryngeal squamous cell carcinoma (LSCC) [48-51]. The most exciting aspect of all of these studies was actually the ability of SELDI-TOF technology to accurately distinguish between patients with or without metastasis, which might have potential clinical use in diagnostic and prognostic purposes. However, clinically interesting study using this technology was recently presented by Gonçalves et al. [52], who performed SELDI-TOF profiling of early postoperative serum from 81 high-risk early breast cancer patients as to identify specific protein signature correlating with meta-

static relapse. These authors found that postoperative serum proteomic profiles may indeed predict metastatic relapse in high-risk primary breast cancer patients receiving adjuvant chemotherapy, providing the evidence that such an approach may have a significant prognostic value.

In conclusion, the emerging field of proteomics has opened many new chapters in cancer research. In particular, such methodical approach holds great promise in everyday clinical practice, especially in monitoring disease progression and identifying high-risk patients, which should facilitate early diagnosis and treatment of the patients (Figure 3).

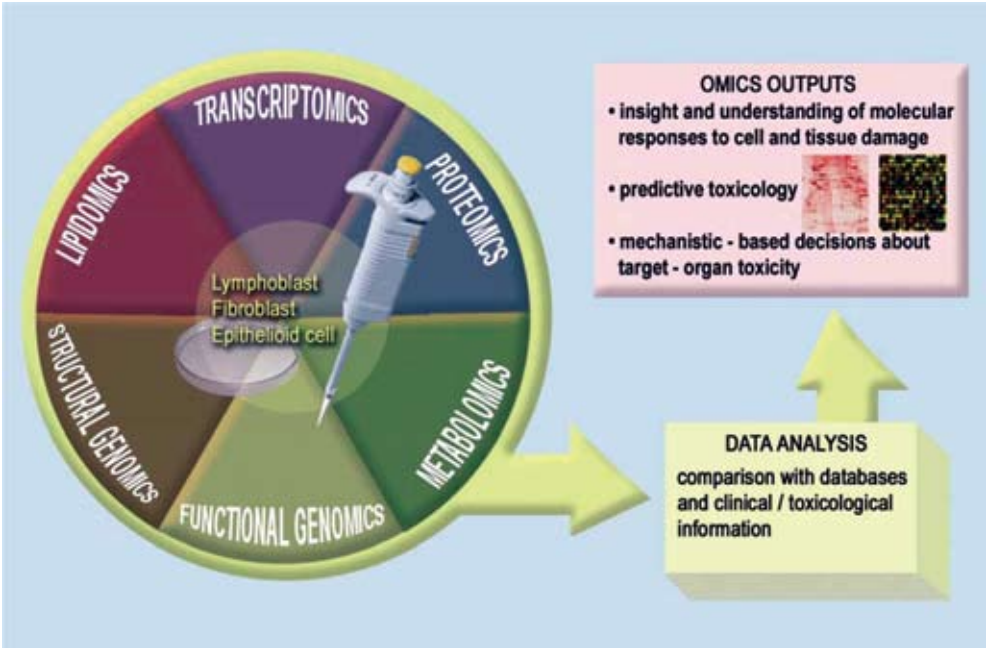

Figure 3. The ability to combine high-throughput genomic, proteomic, metabolomic and other experimental approaches with assays that address discovery of novel molecular mechanisms of cancer development and metastasis will eventually speed the emergence of safer, more effective and better-targeted therapeutic agents. Large scale (robotics) in vitro screening using representative cultured human cell lines (i.e. lymphoblast, fibroblast and epithelioid cells), and in vivo studies on "humanized" mouse models combined with functional genomic analysis of different organs might speed up both in vitro and in vivo testing.

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Sažetak

Novosti u mozaiku metastaziranja zloćudnih tumora

Unatoč napretku u istraživanju i proučavanju zloćudnih tumora, ta je bolest i dalje velik izazov modernoj medicini. Biološka raznolikost, ali i klasično poimanje mehanizama metastaziranja tumora, razlog su neučinkovitosti postojećih načina liječenja. Dva su modela kojima se objašnjava ta raznolikost – model klonske evolucije te model matičnih stanica novotvorina. Ovaj drugi u posljednje je vrijeme privukao pozornost jer su matične stanice novotvorina, zbog svojih sposobnosti samostalnog obnavljanja i diferenciranja, odgovorne za nastanak i razvoj tumora te njihovu sposobnost metastaziranja i pojave recidiva. Metode globalnih analiza poput proteomike otvaraju nove mogućnosti u istraživanju procesa metastaziranja, jer omogućuju identifikaciju složenih mreža proteina uključenih u progresiju novotvorina, što može pridonijeti ranoj dijagnostici i omogućiti razvoj učinkovitijih lijekova protiv metastaza.

Ključne riječi: matične stanice novotvorevina; metastaziranje; proteomika