Chapter 6

Oxidative Status Pathways: Systemic Biomarkers

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Abstract

Systemic biomarkers (i.e. biomarkers of functioning of cellular pathways) offer a broad spectrum of diagnostic capabilities. There are several approaches to using systemic biomarkers that derive from exact needs of a researcher or a clinical specialist. First, analyzing a multifunctional and multi-systemic pathway in circulating cells (e.g. leukocytes) allows to gather generalized information on functioning of the organism. Second, there are numerous pathways that, even still in circulating cells, allow to assess risks of developing or stage of development of numerous diseases, including the leaders of non-infection diseases mortality—cardiovascular diseases. Third, biopsy specimens can readily be used to assess the exact signaling type of a disease (especially cancer) thus helping in selecting the best treatment option. Due to unique properties of the human oxidative status pathways that are discussed in the present chapter, diagnostics specialists are now acquiring an all-in-one toolbox for profiling and detecting almost any non-infectious and a broad range of infectious diseases. In addition to properties of the human oxidative status pathways opening these possibilities, this chapter considers exact systemic biomarkers deriving from this approach, reveals some examples of usage of the resulting diagnostic technology and provides instances of successful clinical application of the systemic biomarker approach.

Keywords: interactomics, systems biology, personalized medicine, signaling pathways, oxidative status, systemic biomarkers

1. Introduction

The human cell is capable of receiving, processing and accordingly responding to an indefinitely wide range of stimuli [1]. These stimuli can be both exogenous and endogenous; both physical and chemical (including biochemical); both adopted to provide communication and

those requiring adaptation [2, 3]. Regardless of the classification and kind of stimuli acting upon the cell, the whole chain of events—from the signal reception and to the end-point response of the cell—follows the same logic and is guided by a given signaling pathway [4].

Signaling pathways may exploit cellular machinery to a different extent. In the simplest case, a stimulus induces a cellular component capable of performing all the necessary steps from reception and to the ultimate action [5]. In the most complex instance, the reaction toward a stimulus involves complex rearrangements of cellular components, alterations in gene expression caused by epigenetic events and induction of numerous uni-directional and opposite transcription factors, translation modulation, etc. [6–9].

These different pathways confer unequal informativeness for biomedical, mostly diagnostic and follow-up, purposes. The reason is that the "shorter" a pathway, the more difficult it is to detect changes in cellular parameters due to fast turnover and lack-to-absence of signal amplification stages. In contrast, complex pathways contain signal amplification circuits, demonstrate delayed and sustained changes in cellular parameters and, moreover, interact with other pathways (i.e. respond in dependence of cellular signaling background), thus providing a researcher with a plethora of data characterized by high signal-to-noise ratio and high informativeness [10]. Superior informativeness of complex signaling pathways renders them highly promising for clinical applications. On the other hand, from analytical point of view, responses of simpler pathways are easier to interpret, while extremely complex pathways are nearly unresolvable due to ambiguity imposed by principal limitations of our knowledge of interacting components of such pathways. Thus, it is required that clinically applicable pathways be well-studied, with the triggering stimulus and characteristic effects of the pathway known in the first place.

Generally, cellular signaling pathways are specialized toward given sets of stimuli. However, there is one group of signaling pathways that, although being specialized, too, permits diagnostics of an incredibly wide range of cellular parameters. These are oxidative status signaling pathways. In humans, oxygen participates in multitudinous processes - from spontaneous reactions of auto-oxidation of small molecules, to protein folding control, multi-step electron transfer in mitochondrial and microsomal electron transport chains and to action of NADPH oxidases tightly controlled by one of the most complex pathways of the human cell [11]. Due to this deep implication of oxygen in cellular metabolism, redox processes requiring this small molecule run in all compartments of the cell and outside the cell. Since one-electron reduction is thermodynamically favorable, all processes involving oxygen are liable to generation of reactive oxygen species (ROS) [12]. Moreover, a much more safe, thus preferred and referred to as physiological two-electron reduction of molecular oxygen produces hydrogen peroxide [13]—a molecule classified as ROS as well. A significant portion of hydrogen peroxide decomposes with the formation of free radicals, including the most short-lived and dangerous hydroxyl radical [14]. On the other hand, hydrogen peroxide serves as one of key cellular messengers—and its function in this sense include direct control of transcription factors (AP-1, NFE2L2, CREB, HSF1, HIF1, TP53, NF-κB, NOTCH, SP1, etc. [15]), direct and indirect control of higher-order kinases (CAMKII, Pka, Pkb (Akt), Pkg, Mapk, Erk [16, 17]) and epigenetic machinery [18] and direct modulation of 14–3-3 proteins function [19]. Hydrogen peroxide as well as other ROS serve as primary triggers of the oxidative status pathways, while the pathways differ in signal reception compartment, signal reception mechanisms, signal intensity

dependence and sets of modulating pathways. These differences confer significant specificity to triggering of the pathways and temporal characteristics of the response. Examples of such differences are given further.

With respect to the ultimate cellular effects and resulting oxidative status parameters, oxidative status pathways are rather well explored. The beginning of the extensive studying of oxidative status and pathways determining it dates back to the first half of XX century [20–22]. Over the time, due to implications of prooxidants and antioxidants in determination of quality of life, in aging (the two factors that significantly affected public attitude and mentality) and in numerous diseases, significant amount of experimental data has been collected. These data are detailed inasmuch as some pathways activation patterns have been characterized in a minutes to hours basis [23] and accounting signal intensity [24]. No less importantly, targets of the most complex and clinically relevant pathways are well known. And as it is shown further, these targets that may serve as biomarker panel candidates are not only mRNAs or proteins, but as well individual transcript variants, pre-mRNAs and miRNAs [25–28]. Consequently, although there are more data to be collected, these pathways may be utilized for developing systemic biomarkers for diagnostics of different health conditions using sets of individual RNAs, proteins and small molecules in analytically convenient combinations. For some of such pathways, this has already been done.

To sum up, oxygen-dependent redox processes participate in or modulate at least most cellular metabolic and signaling systems. Accordingly, these systems contain respective response circuits allowing the cell to adapt to changes in environmental and internal conditions or at least perceive these changes. As the above-mentioned metabolic and signaling systems of the cell strikingly differ in nature, cellular roles and even in action timeframes, the response circuits—termed oxidative status pathways—are rather specific in triggering stimulus, unique in sensing mechanism, subsequent events and the outcome. Among these events and results are changes in expression of target RNAs and proteins and fluctuations of some relatively stable biochemical parameters. Together these factors may (and do) serve as components of systemic biomarkers. And since the pathways are specific toward given stimuli or signals, the respective systemic biomarkers, i.e. panels of reporter target components of these pathways, allow for profiling of the activation status of the pathways. Consequently, as diseases originate and start to manifest at the bottom biological level - the cellular level - the cellular signaling pathways reflect the mode of cellular functioning. And since oxidative status pathways extraordinarily reflect and summarize most cellular systems functioning, these pathways are perfect for developing systemic biomarkers. However, there are several variables in selecting the sets of cellular factors to serve as systemic biomarker components that are to be accounted, and those are discussed in the next section.

2. Developing oxidative status pathways-based systemic biomarkers

Since systemic biomarkers are sets of cellular factors that may represent functioning of a given pathway, development of such biomarkers starts with interactomic data - signaling pathways maps. There are numerous available solutions, including Reactome [29], BioSystems [30],

GenAtlas [31], GeneGo [32], KEGG [33], etc. We have previously developed our own interactomic system dedicated exclusively to human oxidative status - Oxidative Status Interactome Map (OSIM) [34]. These interactomic systems differ in interface, data selection strategy and depth of interactome coverage. Importantly, quality of an interactomic map used for biomarker development significantly affects its specificity and sensitivity due to inherent biological overlap of targets control and multi-pathway signal reception.

2.1. Signal specificity

There are dozens of oxidative status signaling pathways operating in the human cell, and most of these pathways have hydrogen peroxide or other ROS as the primary triggering signal. However, as it was shown above, ROS originate in different cellular compartments in different cellular contexts, and this is one of the basic principles of disambiguation in signal reception [35, 36]. Further divergence of pathways is achieved by their signaling background dependence: different oxidative status pathways require different kinases for functioning, while these kinases are often redox-sensitive [37, 38]. Finally, oxidative status pathways are highly specialized toward various ROS-inducing agents, which can be both chemical and physical. For example, electrophilic compounds have critically distinct effects on the AP-1 and NFE2L2 sub-pathways of the NFE2L2/AP-1 pathway (often referred to as the NRF2 pathway) [39]. An example of physical signal specificity is thioredoxin 1 triggering by ionizing radiation, UV and ultrasound [40].

Although triggering signals significantly overlap for different pathways, cellular effects of these signals greatly depend on their cellular location - and these effects are mediated by compartment-specific sensors.

2.2. Sensor location and type

Protein sensors of oxidative status pathways are ample and extremely diverse. Different pathways are greatly dissimilar in mechanisms of activation by even the same basic stimulus - hydrogen peroxide. Moreover, the same pathway may have several sensors working in tandem. For example, the above-mentioned NFE2L2 sub-pathway has a primary hydrogen peroxide sensor in cytoplasm - it is KEAP1 protein. But in addition to this cytoplasmic pool of KEAP1, the same protein is also present in the nucleus, where it acts as the second-line sensor [41]. The same pathway is also characterized by a third-line sensor - BACH1 protein, a transcriptional repressor active in reduced state [36]. Thus, depending on cellular context, NFE2L2 activity may be attenuated to different extent and so as to result in activation of different sets of target genes, since BACH1, for instance, represses only a portion of NFE2L2-dependent genes.

Another peculiar example of sensor location-induced pathway functioning modulation is known from the NF-κB pathway. Under unstimulated conditions, NF-κB proteins are bound by I-κB proteins in the cytosol preventing NF-κB nuclear import. Upon ROS formation, NF-κB proteins are oxidized, change conformation and cannot be immobilized by the I-κBs. Further developments depend on nuclear redox context and cellular signaling background, since

oxidized NF-κB proteins are unable to transactivate the targets [42]. Interestingly, activated NF-κB protein RELA controls nuclear import of the above considered KEAP1 protein [41].

All the events of pathways activation and subsequent signal transduction have different rates in different pathways. These temporal characteristics can be utilized to collect additional diagnostic data.

2.3. Temporal considerations

Even when oxidative status pathways are activated by the same signal, ultimate effects are achieved in different time. Moreover, even within the same pathway, targets are activated at different rate. It appears that this is dictated by the complexity of signal transduction, including presence and characteristics of signal amplification circuits. It has striking effects on pathway performance. For examples, in the above-mentioned NFE2L2 pathway, some targets are activated as early as in 5 hours, while other genes fail to increase expression rate until approximately 24 hours [23].

Another example of significantly prolonged effects of a pathway is seen in HIF1A pathway run cycle. Activated HIF1A induces expression of NF-κB genes and proteins REL, RELA and NFKB1, and DICER1. In turn, activated NF-κB (and this activation is context-dependent) transactivates genes of miRNAs *MIR-93* and *MIR-199A-5P* that are further processed by DICER1. These mature miRNAs are HIF1A suppressors that eventually block the pathway activation. Due to extremely complex chain of events, this variant of HIF1A pathway run takes considerable amount of time [25].

Temporal characteristics of the pathways analyzed should be accounted when developing and applying a systemic biomarker. This can partly be achieved by selecting appropriate analytical level.

2.4. Analytical levels

Oxidative status pathways contain numerous types of molecules that can be used as biomarkers. These are protein-coding RNAs, miRNAs, proteins, and small molecules.

Just as signal reception and transduction are multi-step processes, so is the development of the cellular reaction. Upon decoding of the stimulus by transcription factors, the first step of the cellular reaction takes place - this is preparation to transcription initiation (interactions between transcription factors [43], competition [39], nuclear import of transcription factors [44], modulation of epigenetic machinery [45], etc.). Even this first step is complex, and subsequent stages occurring in the nucleus are legion [46]: these include DNA binding by transcription factors, RNA polymerase engagement, transcription, RNA-protein interactions, splicing, RNA modification, RNA stabilization, storage, degradation and cytoplasmic export. Further, numerous cytoplasmic processes provide or accompany generation of mature protein, and another plethora of events finalize the cellular reaction (e.g. the protein is modified, re-distributed within the cell or secreted). Remarkably, all these processes, dozens of them, are affected by cellular signaling background.

Generally, it is possible to take a "snapshot" of any of these phases of the cellular reaction and use the data to decode the initial signal. However, the more elaborate the product of the cellular reaction one analyzes, the harder it is to trace back the stimulus.

Thus, most existing systemic biosensors rely on mRNA level. mRNA expression analysis is a reasonable approach to assess whether a stimulus has affected the cell or there has been a dysfunction in cellular signal transduction or decoding processes: there are only few steps between transcription factor activation (signal decoding) and mRNA maturation. However, there are still more steps that can easily be affected by the cellular functional context [47–51]. Thus, pre-mRNA might serve as a valuable alternative or addition to mRNA analysis. In one of our previous studies, we assessed whether pre-mRNAs can be used for diagnostic purposes. It appeared that two of three pre-mRNAs of single transcript variant-encoding genes had sensitivity and specificity comparable to that of the respective mRNAs [52]. Comparing such diagnostic characteristics is challenging when genes coding for more than one transcript variant are considered.

In this case, individual transcripts analysis is a great alternative to standard mRNA analysis. A pathway may control transcripts' fate individually on several levels. First, transcription factors of the pathway can directly induce individual transcripts [53]. Second, transcription factors, being central to some pathways, can attract and regulate splicing machinery themselves [54]. Third, other pathway components can easily regulate splicing machinery together with promoting target gene transcription [55]. Fourth, cellular pathways have all capabilities to individually control degradation or long-term storage of mature mRNA variants of a single gene [56–58]. Individual transcript expression-based studies are rare due to technical and interpretative difficulties [59, 60], but previously we demonstrated that this approach is highly promising in case of oxidative status pathways used for development of systemic biomarkers [28].

Proteins are also sometimes used as biomarkers in systemic diagnostics and systemic pathophysiology approaches. The drawback of this approach appears to be in decreased signal-to-noise ratio leading to significant information losses. Although our lab mostly focuses on RNAs for biomarker development, we performed several attempts to use proteome as analytical level in systemic approaches [61].

2.5. Signal amplification and autoregulatory blocking

As it was mentioned above, signaling pathways are optimal for diagnostic properties only if they contain signal amplification and abruption circuits, yet they are not too complex to hinder data interpretation, and their action rate is comparatively slow.

Human oxidative status pathways are rich in signal amplification and autoregulatory blocking circuits. In one of our previous works, we discussed 15 such experimentally proven circuits of just one of the pathways - the NFE2L2/AP-1 pathway [10].

Such circuits greatly help in interpreting data and choosing time points for sample collection in repetitive measurements that greatly improve the signal decoding procedure for diagnostic purposes.

2.6. Biomarkers quantity

Careful selection of systemic biomarkers candidates and their quantity are critical, since, on average, each gene is controlled by a great number of transcription factors (both transactivators and repressors) belonging to different signaling pathways. This implies a significant limitation—so that even some well-known targets cannot be used for diagnostic purposes involving pathway activation analysis. There are many examples of genes that are controlled by multiple and functionally opposite pathways even within the oxidative status systems [62, 63].

3. Current advances in developing systemic biomarkers

Systemic biomarkers approach is a relatively new area of biomedicine. However, over two decades of its existence [64], significant advances have been made. Great effort spent in this area not only improved analytical algorithms, but also underlined the importance of personalized approach. For example, many prognostic markers have been suggested for breast cancer in the literature, in particular for predicting survival. But data collected in separate studies led to striking discovery of the lack of overlap of the predictive genes in most of these studies. This emphasized the need of personalized approach even within tumor groups that share the same histomorphology [65]. The reason for discrepancies is debatable—divergent patterns of expression profiles might have been due to several analytical factors considered in the present chapter, but the solution holds the same: systemic biomarkers are only informative when patterns of pathways activation, rather than changes in individual genes expression, are analyzed. This idea led foundation to development of several analytical tools and panels. Our lab developed an NFE2L2/AP-1 pathway-based systemic biomarker for assessing slight changes in physiological parameters of the human organism using peripheral blood leukocytes as the preferred sample type [66]. The same systemic approach utilizing another set of oxidative status markers was successfully used for unveiling features of uterine cervical incompetence patients [67]. Other labs also successfully apply pathway activation-based technologies in various field and other sample types, with special attention paid to fresh solid tumors samples and paraffin blocks [68]. Of note, Oncofinder technology [69, 70] and Oncotype DX assay [71] are among the most effective interactomics/multi-gene analysis-based tests in oncology.

In **Table 1**, some examples of suitable NFE2L2/AP-1 targets and complex markers are given along with their diagnostic properties (only area under the curve (AUC) is given, please see details in the cited publications).

As seen from **Table 1**, not only the markers may highly vary in nature, but they have different receiver operator characteristics. Notably, for each model to be studied, it is possible to choose or find a set of markers having extremely high AUCs that are hardly achievable using the traditional biomarker approaches.

Marker	ROC indices		Model	Reference
	AUC	p-value		
AKR1B1 mRNA (normalized to reference)	1.0	<0.0001	*In vitro*, HeLa cells, 24 h 400 uM hydrogen peroxide	[52]
AKR1B10 mRNA	1.0	<0.0001		
AKR1B1 mRNA/pre-mRNA ratio	0.984	<0.0001		
GSTP1 pre-mRNA	0.984	<0.0001		
AKR1B10 mRNA/pre-mRNA ratio	0.946	<0.0001		
AKR1B10 pre-mRNA	0.781	0.0284		
BACH1 tv2/*NFE2L2* mRNAs ratio	0.965	<0.0001	*In vivo*, 19–22 y.o. females, repetitive measurements, self-reported analogue-scaled psychological stress	[66]
SRXN1/*NFE2L2* mRNAs ratio	0.922	<0.0001		
NQO1/*NFE2L2* mRNAs ratio	0.902	<0.0001		
HMOX1/NFE2L2 mRNAs ratio	0.879	<0.0001		
KEAP1/*NFE2L2* mRNAs ratio	0.867	<0.0001		
PRDX6/*NFE2L2* mRNAs ratio	0.687	0.0586		
TXN tv1/*NFE2L2* mRNAs ratio	0.609	0.2891		

Table 1. The NFE2L2/AP-1 pathway functioning markers used in two in vitro and in vivo studies, with AUC in descending order within each study.

4. Conclusions

Oxidative status pathways-based interactomic profiling using the expression analysis-based methods promises a lot to the field of development of the novel diagnostic approaches and has already demonstrated great results in various areas of biomedicine. In this branch of personalized medicine, interactomics serves as a tool to select factors to be analyzed (systemic biomarkers), to suggest a method of analysis and to further account for data collected. Expression profiling then serves as the immediate molecular biological procedure used to collect biological data in the interactomic diagnostics.

Current advances in molecular biology have led to creation of numerous interactomic maps and analytical systems that can readily be used for developing novel diagnostics assays. Fast evolution of oxidative status cell biology and emerging molecular biology suggestions on cellular factors to be considered as systemic biomarkers candidate complement and promote this field. Despite the complexity of development of systemic biomarker-based assays, this novel type of diagnostic technologies appears to be inextricably intertwined with the personalized medicine era.

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Conflict of interest

The authors claim that there were no financial or other conflicts of interests related to the present chapter.

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