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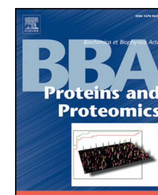

Review

Signaling pathway profiling by reverse-phase protein array for personalized cancer medicine☆,☆☆

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ABSTRACT

Deregulation of intracellular signaling through accumulation of genetic alterations is a hallmark of cancer. In the past few decades, concerted and systematic efforts have been made to identify key genetic alterations and to develop therapeutic agents targeting active signaling molecules. However, the efficacy of molecular therapeutics often varies among individuals, and precise mapping of active molecules in individual patients is now considered an essential for therapy optimization. Reverse-phase protein array or microarray (RPPA or RPPM) is an emerging antibody-based highly quantitative proteomic technology, especially suitable for profiling of expression and modification of signaling proteins in low abundance. Because the supply of clinical materials is often limited, RPPA technology is highly advantageous for clinical proteomics in view of its high sensitivity as well as accurate quantification. RPPA has now begun to be incorporated into various clinical trials employing molecular-targeted therapeutics. In this article we review and discuss the application of RPPA technology in the fields of basic, preclinical, and clinical research. The RPPA Global Workshop was recently launched to accelerate the exchange of rapidly expanding knowledge of this fascinating technology among academic laboratories and industries worldwide. This article is part of a Special Issue entitled: Medical Proteomics.

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1. Introduction

Advances in genomic and transcriptomic technologies such as gene expression microarray, comparative genomic hybridization (CGH) array and next-generation sequencing have contributed tremendously to the discovery of therapeutic targets (e.g., driver mutations, gene amplification, and fusion genes) and predictive biomarkers for cancer therapy. The major success of the HER2 antibody trastuzumab for breast cancer [1], the BCR-ABL1 inhibitor imatinib for chronic myeloid leukemia [2] and gastrointestinal stromal tumor [3], and the epidermal growth factor receptor (EGFR) inhibitors gefinitib and erlotinib for non-small-cell lung cancer (NSCLC) [4] have brought revolutionary changes to cancer treatment over the past two decades. To date, numerous molecularly targeted agents have been approved for clinical application, while at the same time several drawbacks associated with their use for cancer therapy are becoming increasingly apparent. Such shortcomings include the necessity of predictive biomarkers for the successful development and application of the targeted agents in order to select patients who are likely to respond, and emergence of drug resistance following targeted treatment. In progressing to the next stage of personalized cancer medicine, a key to increasing the success rate in designing targeted therapies will be to precisely characterize the signaling networks activated in individual tumors by using functional proteomic technologies, and integrate such data into genetic, transcriptomic and phenotypic analysis. As the majority of current targeted agents act on the components of signaling pathways that are dysregulated in cancer cells, clarification of the status of post-translational modifications, particularly phosphorylation of the targeted proteins and other signaling effectors, should provide more direct clues to the expected pharmacological responses than would be the case for genomic and transcriptional profiling.

Reverse-phase protein array (RPPA) is an antibody-based proteomic technology suitable for profiling both protein levels and post-translational modifications including phosphorylation [5–8]. It allows miniaturized 'analyte-down' immunoassay of lysates from tissues or cells, enabling concomitant monitoring of the expression of a particular protein in hundreds to thousands of samples in a quantitative or semi-quantitative manner. In addition, the throughput, sensitivity and cost effectiveness of RPPA, together with its ability to deal with

Abbreviations: ERK, extracellular signal-regulated kinase; EGFR, epidermal growth factor receptor; ELISA, enzyme-linked immunosorbent assay; FFPE, formalin-fixed paraffin-embedded; FISH, fluorescence in situ hybridization; FNA, fine-needle aspiration; F/RPPA, forward/reverse-phase protein array; HCC, hepatocellular carcinoma; HNSCC, head and neck squamous cell carcinoma; HRG, heregulin; IHC, immunohistochemistry; IC50, 50% inhibitory concentration; LCM, laser capture microdissection; MAPK, mitogen-activated protein kinase; mTOR, mammalian target of rapamycin; (N)SCLC, (non-)small cell lung cancer; TNBC, triple-negative breast cancer; VEGF, vascular endothelial growth factor receptor

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minuscule sample amounts, have accelerated the recent evolution of this technology in basic, preclinical and clinical research fields. This review focuses on the various applications of RPPA for refining personalized cancer medicine, including classification of tumors, the discovery of therapeutic targets and predictive biomarkers, and development of combined targeted therapies to overcome drug resistance.

2. Protein array technology

Protein microarray is a large-scale high-throughput "dot-blot" immunoassay, which can be generally classified into two types: forward-phase protein arrays (FPPAs) and reverse-phase protein arrays (RPPAs) [9,10] (Fig. 1). In a FPPA, each spot contains a single immobilized protein or peptide on a substratum as a capture (e.g. an antibody array), whereas each spot of a RPPA contains one test sample such as a cell lysate or a tissue lysate. RPPA is uniquely suited for quantitative or semi-quantitative detection of signaling proteins present in low abundance with high sensitivity (picomole–femtomole range) and precision (coefficient of variance <15%) [11]. Over the last decade, RPPA has been adopted for cancer research as a functional proteomic tool for characterizing dysregulated signaling networks in cancer cells and tissues. In RPPA, one protein can only be examined one slide at a time, and therefore it is difficult to examine thousands of different proteins. However, once target proteins are identified, RPPA has the power to monitor hundreds to thousands of samples in a high-throughput way.

A factor major limiting the use of RPPA is the paucity of high-quality monospecific antibodies [11]. Generally, antibodies used for RPPA must satisfy at least the following two criteria: 1) generation of explainable single or multiple bands by Western blotting, and 2) high correlation between RPPA and Western blot data in a large panel of cell lines and/or tissue samples. Additional validation methods involving various forms of manipulation such as treatment of phosphoproteins with phosphatase, knockdown of the gene of interest using siRNA, and studies of knockout mice will enhance the credibility of the antibodies. Global efforts are underway to develop well-validated databases of high-quality antibodies in the form of the Human Protein Atlas Project (www.proteinatlas.org/), Antibodypedia (http://www.antibodypedia.com/) and the Antibody Portal of the NCI (http://antibodies.cancer.gov). The RPPA research community has begun to develop a global antibody database providing universal guidelines and standardized validation methods for determining the validity of antibodies for RPPA, including a quality index for antibodies and comprehensive information regarding the verification process.

With technical improvements, RPPA has been adapted and implemented successfully by a large number of laboratories worldwide. We have also established our own RPPA platform using ProteoChip (Proteogen, Ganwon-do, Korea), a substratum coated with a cross-linker, ProLinker™ [12], and a fluorescence detection method (Fig. 2) [13]. Using this platform, we have obtained phosphoprofiles of 180 signaling nodes in 95 cell lines derived from 8 different types of cancer cultured in the presence or absence of 10% fetal calf serum [14]. Identification of potential predictive biomarkers for sorafenib response using the phosphoprofile of 23 hepatocellular carcinoma (HCC) cell lines will be discussed in the following section.

3. Application of RPPA to basic and preclinical research

In 2001, Paweletz et al. first reported the development of a RPPA platform and demonstrated its accuracy and versatility through analysis of lysates prepared from laser-capture microdissected 70% ethanol-fixed and paraffin-embedded (LCM-EFPE) prostate cancer tissues [15]. They further expanded the number of analytes for higher-density RPPA and performed proteomic profiling of the NCI-60 cell lines using 52 antibodies [7]. The study not only identified two pathological markers but also examined the correlation between the mRNA and protein expression profiles. This demonstrated that cell-structure-related proteins showed a high correlation at the mRNA and protein levels across the cell lines, whereas non-cell-structure-related proteins showed a poor correlation. Since these earlier reports, an increasing number of studies have been applying RPPA for characterization of cellular signaling networks.

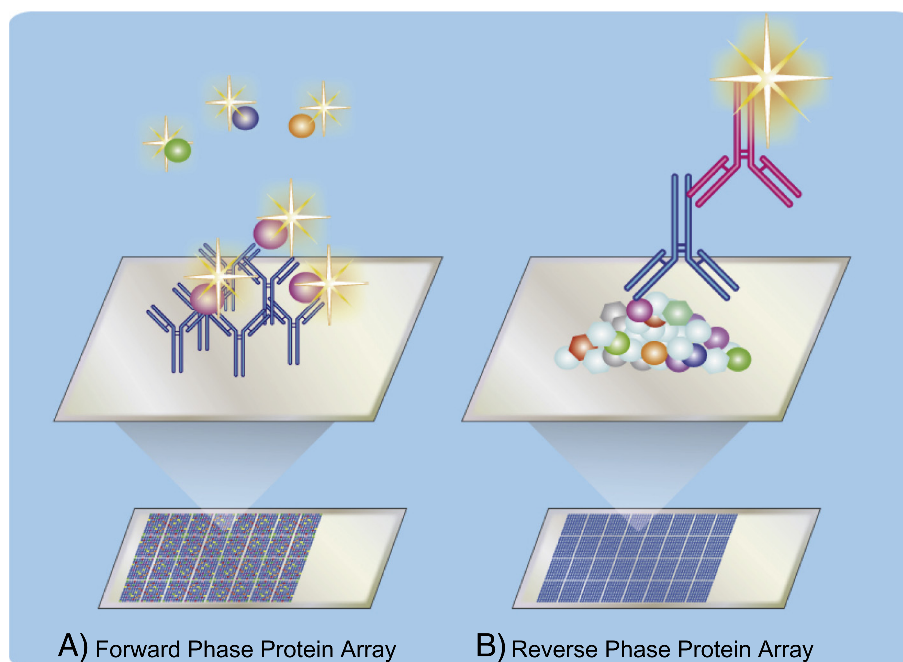

Fig. 1. Schematic representation of Protein array formats; a forward-phase protein array (FPPA) and a reverse-phase protein array (RPPA). (A) Each spot of a FPPA, exemplified by an "antibody array", contains a single antibody immobilized on the surface of a substratum to capture the protein of interest in a labeled sample (e.g. cell lysate). (B) RPPA comprises a large number of immobilized lysate spots on a substratum. An array is probed with a single antibody against the protein of interest, followed by addition of a labeled secondary antibody for detection.

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