InnoScan scanner 710-IR and RPPA Technology: A robust tool to analyze cell signaling pathways

Summary

The Reverse Phase Protein Array (RPPA) platform is located within the Institut Curie in Paris (France). The RPPA platform overall objective is to provide new biological insight on the protein level and their activity through signaling pathway analysis, with the ultimate goal to improve the treatment and the outcome of cancer patients.

This platform provides a personalized proteomics analysis on small amounts of various types of samples using high-throughput automated equipment including the Innoscan scanner 710 IR.

Here, we present the basics of the RPPA technology and point out how the routine use of the Innoscan Scanner 710 IR is an essential part of our workflow. Finally, examples of Cancer research applications proceeded at the Curie institute are described to illustrate the ease-of-use and reliability of the InnoScan Scanner 710-IR.

RPPA Technology Basics and Workflow (figure1)

Reverse phase protein array (RPPA) is a high throughput miniaturized dot-blot and antibody-based technology (figure 1A.) that allows analyzing protein expression levels and activation status of signaling pathways (by studying phosphorylated or total protein expression). It combines high throughput analysis with very small amounts of sample consumption.

Indeed, as RPPA technology is a multiplexed technology, up to one thousand samples can be analyzed simultaneously on the same nitrocellulose covered microscope slides (arrays).

The RPPA workflow is described figure 1 in the panel B. Using a dedicated arrayer, only 1 to 2 ng of protein lysate is printed per sample and per spot. Printed samples can be tumor samples, xenografts, cell lines, or a combination of these and are systematically printed in serial dilutions and replicates, making the technology highly robust and quantitative.

Then, with an Autostainer, arrays are successively incubated with antibodies and fluorescent dye to detect proteins of interest (figure 1A.):

i) Target Specific Primary antibodies that have been previously validated by Western Blotting to assess their specificity for the protein of interest.

At Institut Curie, the RPPA platform has tested more than 2000 commercial antibodies and validated 640 primary antibodies for RPPA labelling so far, among which almost 150 antibodies against phosphorylated proteins. This panel of antibodies covers the majority of cell signaling pathways. For each project, the PI selects the antibodies of interest among the validated antibodies. New antibodies are continuously evaluated by the RPPA platform and specific antibodies can be validated to fulfill the requirement of collaborators.

ii) Species Secondary labelled antibody for detection.

iii) Fluorescent dye IRDye 800CW Streptavidin (LI-COR, Ref 926-32230) is used for the detection.

Positive and negative controls are performed with arrays labeled with total protein stain FCF (protocol from Grace Biolabs) for positive controls and arrays labelled without primary antibody for the negative controls.

Before quantification and data analysis (figure 1C.), arrays are scanned with the InnoScan Scanner 710 IR to detect the Infra-Red fluorescence for each spot. The scanner has two excitation lasers: 785 and 670nm. Thanks to the real time autofocus and autoloader, it is possible to automatically scan 24 arrays successively. One slide is scanned at 10µm/pixel excitation in less than 4 min.

The nitrocellulose coated on the glass slide has fluorescence in the red spectra. We choose to work with near Infra-Red fluorescence in order to decrease background fluorescence of the nitrocellulose and thus increase the signal-to-noise ratio. The Innoscan 710-IR specification fits perfectly with our needs to deliver reliable data, with a sensitivity similar to chromogenic detection but an increased linear range.

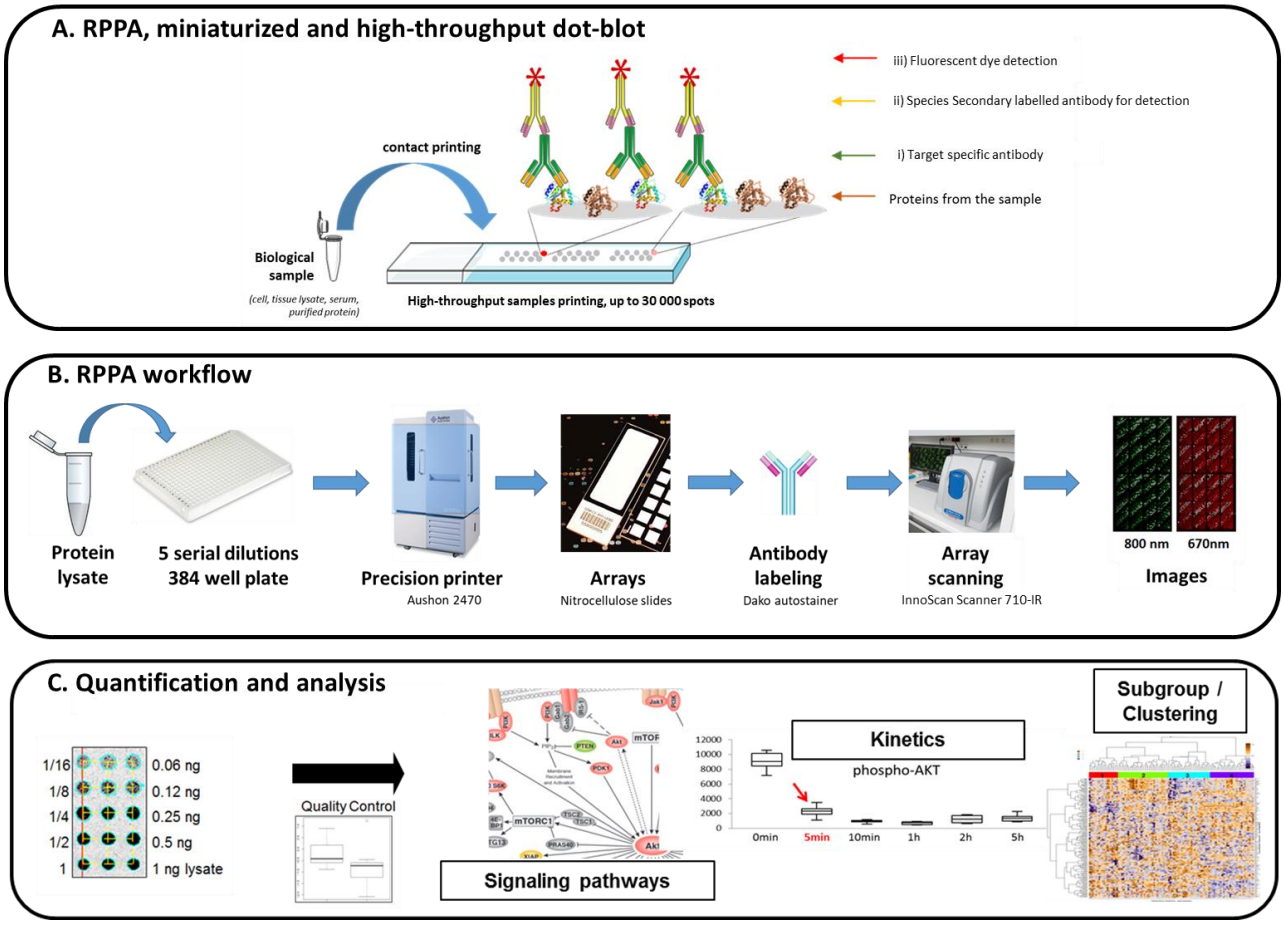

Figure 1: Reverse Phase Protein Array Workflow description. A. Principle of RPPA, a miniaturized high throughput dot-blot. Proteins are printed onto a nitrocellulose-coated glass slide prior the detection of proteins using antibodies linked to fluorescent dye. B. Workflow from protein extraction to images acquisition using Innopsys scanner 710-IR. C Data analysis of images using in-house software PARYS.

Applications for Cancer Research:

The RPPA Platform is involved in diverse projects, covering fundamental questions to clinical trials evaluation, where we offer a personalized high-throughput proteomic analysis on samples provided by our collaborators, allowing:

- The analysis of protein expression levels
- The analysis of cell signaling pathway activation status and dynamics
- The identification of prognostic or predictive biomarkers
- The identification of potential therapeutic targets
- Classification of samples based on protein profiles
- Pharmacodynamics studies

Cervical Cancer (CC): RAIDs consortium European project.

Cervical cancer (CC) remains the second most commonly diagnosed cancer in women. The majority of cervical cancers results from persistent infection of high risk human papillomavirus types (HPV). Indeed, they inactivate normal cellular controls via the viral proteins E6 and E7, resulting in genomic instability, accumulation of molecular alterations, inactivation of tumor suppressor gene and oncogene activation.

The RAIDs European Union (EU)-funded study collected, between 2013 and 2017, a prospective CC samples (tumour and blood) and clinical dataset involving 419 participant patients from 18 centers in seven EU countries. So far, Next Generation Sequencing (NGS) has been carried out, allowing defining dominant genetic alterations, and Reverse Phase Protein Arrays (RPPA) was applied to study cell signaling pathways.

The RPPA platform (Institut Curie) processed 280 samples including baseline and non-baseline tumors and cell lines). Samples were processed (protein extractions, titrations, successive dilutions, printing, labeling, scanning, quantification and analysis) as described previously (Scholl S, et al., EBioMedicine 2019, PMID: 30952619).

Arrays were labeled with 194 specific primary antibodies. The processed slides (n=355) were scanned with Innoscan 710-IR (Innopsys) at 785 nm (for antibodies or negative controls) or 670nm (for positive controls)

Unsupervised clustering of RPPA protein expression data revealed three clusters of patients, which were enriched for EMT (Epithelial Mesenchymal Transition), DNA damage signaling and MAPK (Map Kinase) / PI3K (PI3 Kinase) pathways, respectively. See the figure 2.

Interestingly, the patients within the cluster "EMT" showed shorter Progression Free Survival (PFS) as compared to the other two clusters combined (Fig. 2) (p=0.03).

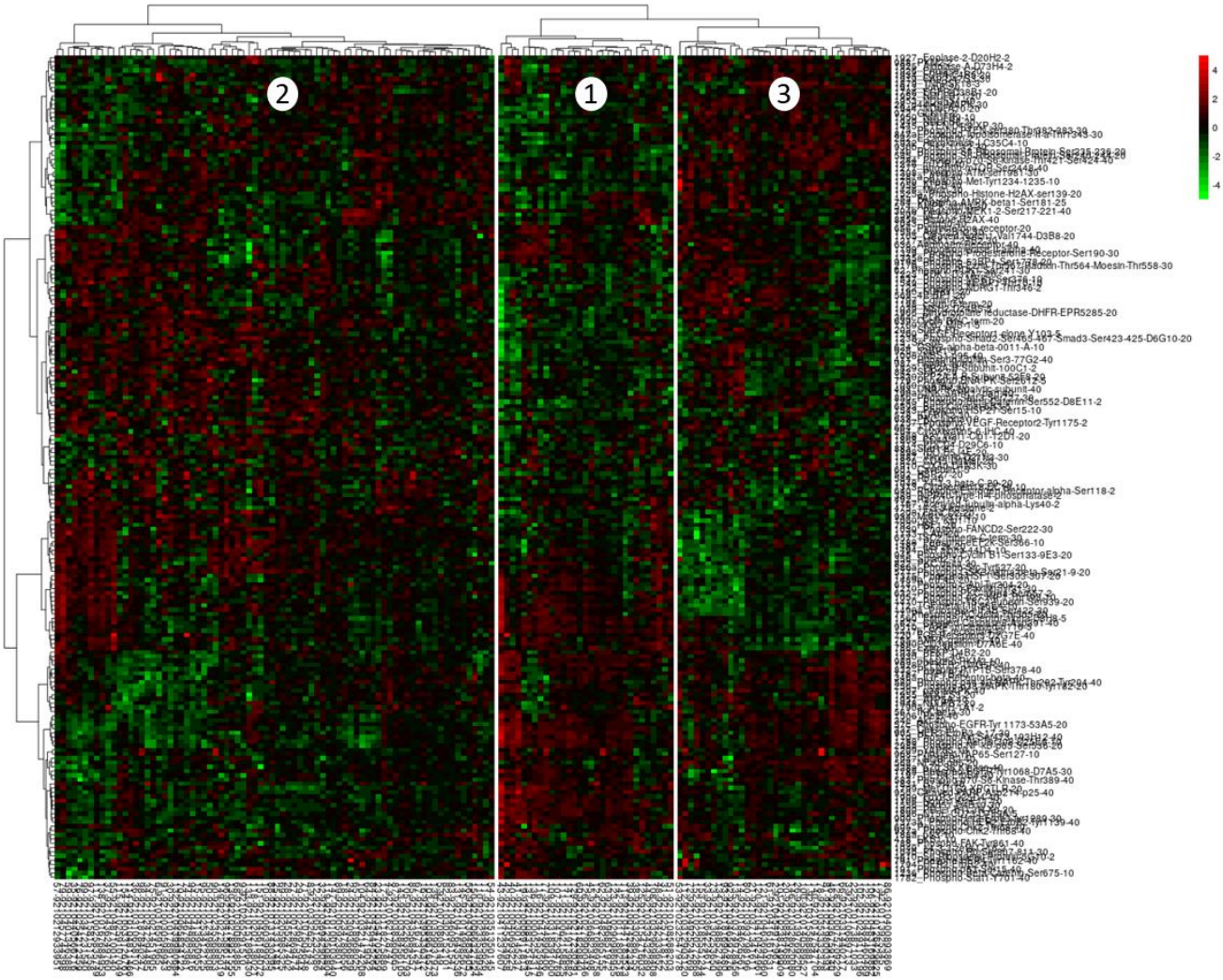

Figure 2 : Among baseline tumors, three main clusters with different protein expression profiles are identified. Proteins that characterize each cluster are enriched in: Cluster 1: EMT, ErbB signaling, Cluster 2: DNA damage signaling, Cluster 3: p38 MAPK and PI3K signaling.

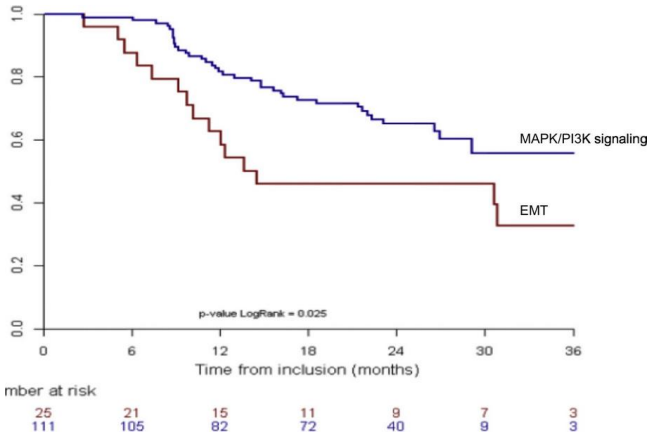

Figure 3 : Progression free survival of subgroups of cervical cancer patients defined by RPPA analysis (n = 136). Comparison of outcome of cluster "EMT" (Red) versus pooled protein expression clusters of DNA damage and MAPK/PI3K signaling (Blue), suggests a poorer PFS for the patients whose tumors showed EMT associated protein expression.

Moreover, samples from cluster 1 were characterized not only by EMT but also by upregulated expression of PD-L1 (for Programmed death-ligand 1 or B7-H1) and B7-H4 (or VTCN1 for V-set domain-containing T-cell activation inhibitor 1). See figure 4.

PD-L1 belongs to the B7 protein family. It is a protein expressed on the surface of antigen-presenting cells (and on some cancer cells) and binds to PD1 receptor (on T cells) and regulates the immune system by inhibiting T cells activation when it is linked to its receptor.

B7-H4 also belongs to the B7 protein family and negatively regulates T-cell-mediated immune response inhibiting T-cell activation, proliferation, cytokine production and development of cytotoxicity.

That's why EMT biomarkers may allow the identification of patients that could potentially benefit from Immune Check Point Inhibitors (ICI) therapy in the future.

Finally, thanks to the RPPA technology, differences in expression and activation of DNA repair proteins were observed among the three clusters. This result may be of interest for the clinic with regard to PARP inhibitor treatments.

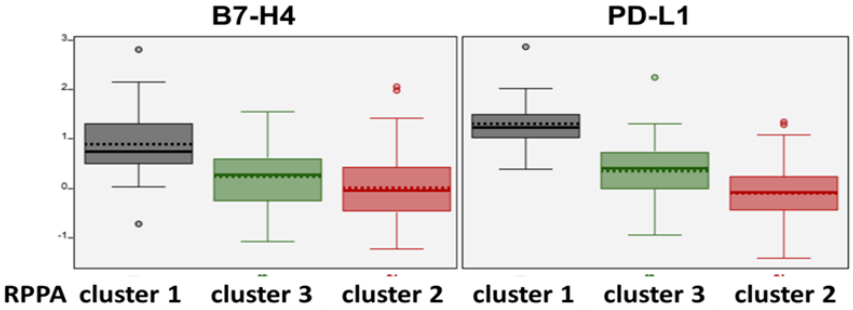

Figure 4: Relative expression level of PD-L1 and B7-H4 using RPPA. High expression level of PD-L1 and low expression level of in Cluster 1 - EMT.

Hepatocellular carcinomas (HCCs) project.

Hepatocellular carcinomas (HCCs) are heterogeneous and aggressive tumors. Due to this molecular tumor complexity, current treatments are not very efficient at advanced stages with only limited benefit in terms of survival.

The aim of this project was to identify new compounds or molecules that might be effective against HCC and biomarkers of the treatment response.

We investigated the biological activity of 400 samples (including 360 human liver tumor and non-tumor tissues and 40 liver cancer-derived cell lines (LCCL)) using RPPA (Caruso S *et al.*, Gastroenterology 2019, PMID: 31063779). We analyzed 126 proteins involved in various signaling pathways; 82 total form and 44 phospho-proteins representing a total of n= 215 slides.

Within the LCCLs data, unsupervised clustering of RPPA results separated LCCL into 3 subgroups that reflect transcriptomic subgroups and cell differentiation state (see figure 5).

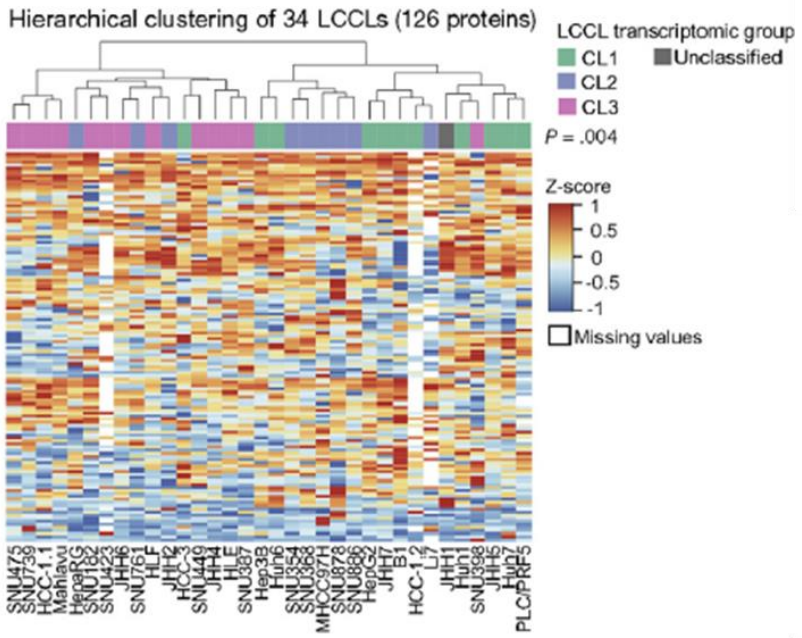

Figure 5: Hierarchical clustering of LCCL protein profiles and association with transcriptomic subgroups (c2 test). Cluster 1: Progenitor Subclass, Cluster 2: Mixed epithelial mesenchymal, Cluster 3: Mesenchymal like

In the Cluster 1 (Progenitor subclass), hepato-specific genes and fetal/progenitor markers (ALDH1A1, E-Cadherin, p190A, RSK2, Her3, FGFR4, cytokeratin19) are well expressed, whereas they are downregulated in cluster 3 (mesenchymal like).

The clusters 2 (mixed epithelial, mesenchymal) and 3 have a high expression level for TGF-beta, no canonical beta-catenin and EMT proteins which suggest that the WNT and TGF-beta pathways are activated for these cells. See figure 6.

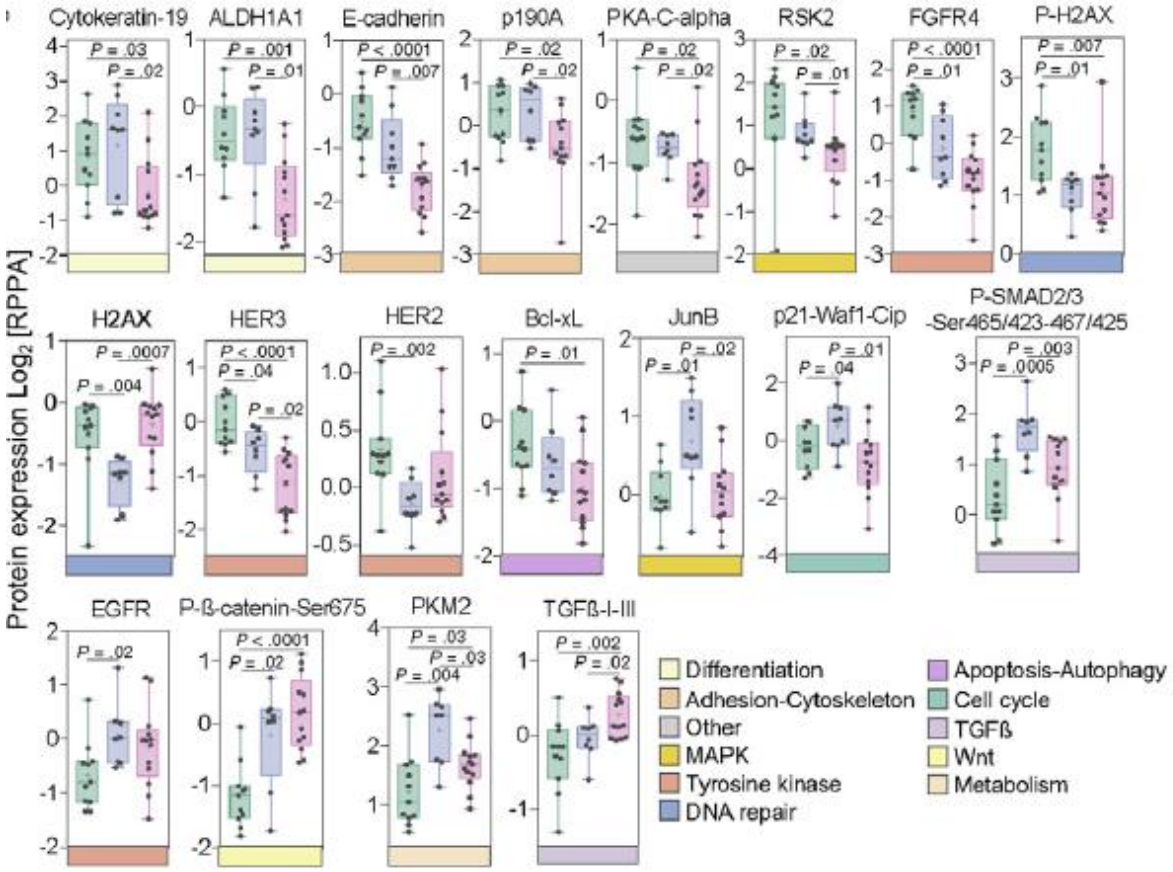

Figure 6: Relative expression level of proteins using RPPA in LCCL. Boxplots of the 19 proteins differentially expressed between LCCL transcriptomic subgroups (Mann-Whitney test).

RPPA data from the cell lines and the tumors, combined with genomics and transcriptomics data, suggest that the protein profiles of liver cancer cell lines seem representative of most aggressive HCCs from patients and would be a good tool to understand liver cancer development and drug responses. A summary is presented figure 7.

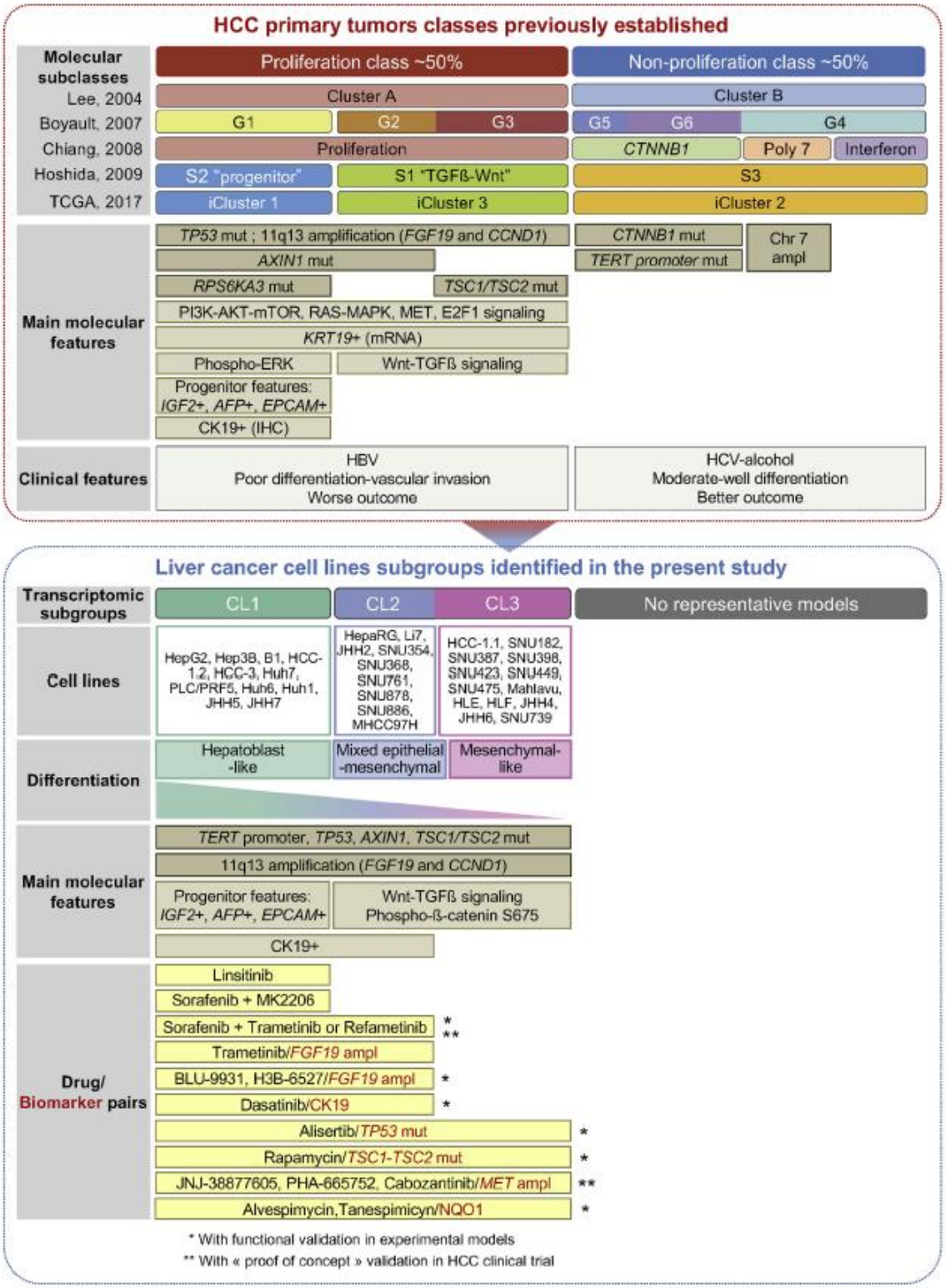

Figure 7: HCC molecular classification. Summary of HCC molecular classes previously established and their corresponding LCCL subgroups with the main drug/biomarker pairs associations identified in the present study. HCC molecular classes and associated features extracted from previous reports. Ampl, amplification; mut, mutation.

Conclusion:

The Reverse Phase Protein Array is a miniaturized high-throughput antibody based technology allowing the detection of hundreds of proteins and phopho-proteins from a small amount of biological sample. The sensitivity of RPPA combined with the Innoscan scanner 710-IR is the perfect setup to give the opportunity to combine a phospho-proteomic approach with other –omics technology for your research projects. This powerful research tool helps you to investigate signaling pathway, to understand mechanisms of action of the drugs, to find new biomarkers for prognostic or therapeutic targets.

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