Molecular Mapping for Personalized Cancer Therapeutics

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Our understanding of the biomolecular basis of cancer has virtually exploded over the last 10 years. Developments in genetics, molecular biology, and molecular pharmacology promise to dramatically alter strategies of cancer treatment [1,2]. Clearly, previous therapeutic principles derived from cytotoxic based therapy now provide diminishing return with respect to patient benefit [3].

Targeted therapeutics, a "new wave" of cancer treatment, are directed against amplified genes and/or overexpressed protein kinases in malignant cells. However, the presence of function redundancy in a robust, predominantly scale-free network such as cancer buffers the effect of any single gene/target modification on the malignant process, with rare exception (e.g., CML) [4-6]. The hierarchy of cancer scale-free networks does not have a threshold for stochastic single target mediated network disruption insofar as random pathway component failure predominantly affects targets with low connectivity within the network, thereby having limited functional impact [7]. However, highly connected targets do allow for "attack vulnerability" [5,7,8]. The disordered cancer circuitry can become, almost paradoxically, more highly dependent on a specific rewired pathway (i.e., pathway addiction) [9]. The disruption of pathways that

produce robustness to certain insults are often associated with enhanced fragility to other perturbations thereby exposing an "Achilles' heel" of cancer. [9]

Therapeutic target identification based on the degree of connectivity of dynamic genomic-proteomic nodes (or hubs) in a patient-data-based network model, as opposed to targeting the more highly active metabolic pathways in rapidly proliferating cancer cells in accord with traditional chemotherapy principles, may be a more effective rationale for anti-tumor target prioritization [4,7,10,11].

In such an approach, the most intriguing targets derived from a patient's differential genomic-proteomic profile will be those highly interconnected "hub" genes which control cancer cell competitive survival, metastagenicity and/or cancer stem-cell renewal [7,10]. We herein demonstrate that semi-quantitative molecular data proteomic [12-14] and genomic profiling [15-18] derived by comparing malignant and non-malignant tissue from patients with progressive cancer can be analyzed in the context of global protein interaction networks in order to generate a prioritized list of potential protein and gene targets. Moreover, the differential overexpression of these putative targets in tumor versus normal tissue supports an RNA interference knockdown strategy [19-22], further justifying evaluation in the individual patient.

We have previously published results of seven patients who underwent complete protein and microarray analysis [23]. Priority proteins were identified in all seven patients and the selection of a single primary protein for each patient was based on degree connectivity to other known cancer stimulatory factors. Further studies were done following synthesis of siRNA to two mRNA targets identified (RACK 1 and Stathmin 1) and subsequent validation of siRNA knockdown activity.

Five proteins adequately fulfilled selection criteria for patient RW001 (Table 1). An example of comparison of protein profiles for patient RW001 between non malignant and malignant tissue is

demonstrated in Figure 1. Based on connectivity to known first order cancer growth molecules we chose RACK1 as the highest priority protein target for RNAi validation. An additional six cancer patients underwent the same process as RW001. Primary targets of these patients were as follows: JJ002, Ras related nuclear protein; LSN003, Superoxide dismutase; SK004, Heat shock 27kDa protein 1; JAG005, Enolase 1; DCL006, Stathmin 1 and HW007, Cofilin 1. These are demonstrated within the priority protein connectivity maps in Figure 2.

Antibodies to RACK1, and Stathmin 1 were demonstrated in cancer cells lines (HCT 116, CCL-247; and MDA-MB-435S HTB-129) by western blot analysis. Expression of RACK1 in tumor and normal tissue by western blot analysis is shown in Figure 3A.

siRNA inhibition demonstrated > 80% knockdown of RACK 1 and Stathmin 1 (example of RACK1 is shown in Figure 3B). A cell kill of > 50% was correlated to RACK1 (Figure 3C) and Stathmin 1 inhibition. Optimization of siRNA knockdown to demonstrate greater cell kill was not performed.

Our study results demonstrate consistent ability to identify patient-unique overexpressed protein targets in biopsied cancer specimens without corresponding expression in normal tissue through a process involving genomic and proteomic assay, and network mapping.

The culling of unique tumor genetic features illustrate the appeal of our systematic approach for the development of personalized cancer therapeutics, especially when coupled with confirmatory functional analysis for individual candidate genes. Correlation of gene expression patterns with disease outcome (survival) has been demonstrated in a variety of cancers [16-18,24-26]. However, gene transcript levels often show poor correlation with protein levels and they cannot predict post translational modifications. For Stathmin 1, TPI 1, RACK1 and Syntenin identified in RW001, there was remarkable concurrence in the

magnitude of upregulation at the mRNA and proteomic level, suggesting genetic defects at the transcription level. By using the criteria of (1) differential expression, (2) linkage to essential oncogenic processes, and (3) high connectivity, it is feasible to reduce a finite number of overexpressed proteins in malignant tissue into a smaller subset of candidate of targets, for which potentially therapeutic siRNA or shRNA agents can be constructed. These products can then be used to enable a systemic loss-of-function analysis, in order to validate a complex of "target gene targets" for trial investigation. It is envisioned that future RNAi based gene therapy for cancer can be prescribed based on the integrated mRNA-proteomic expression profile of each individual's tumor. Preliminary comparisons between siRNA and shRNA indicate that shRNA induced knockdown is more durable and efficient than siRNA [27,28] and, furthermore, more amenable to a second layer of tumor specificity via tumor-targeted vector control thereby minimizing non malignant cell uptake and potential toxic effect to non target agents [29-34].

The time required to determine high-probability protein targets (3-6 weeks) and to manufacture and validate safety of an shRNA product (6 months) may limit the use of this technology in patients with rapidly progressive disease, but conservation of the priority protein targets in tumor samples as demonstrated from patient RW001 with samples harvested nearly 10 months apart suggest an opportunity for application in patients with earlier stage, slowly progressive disease, or recurrent disease following a long latent period.

We are currently pursuing development of shRNA nanoparticle delivery vehicles for each of the top priority targets identified in the seven patients tested.

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Figure Legend

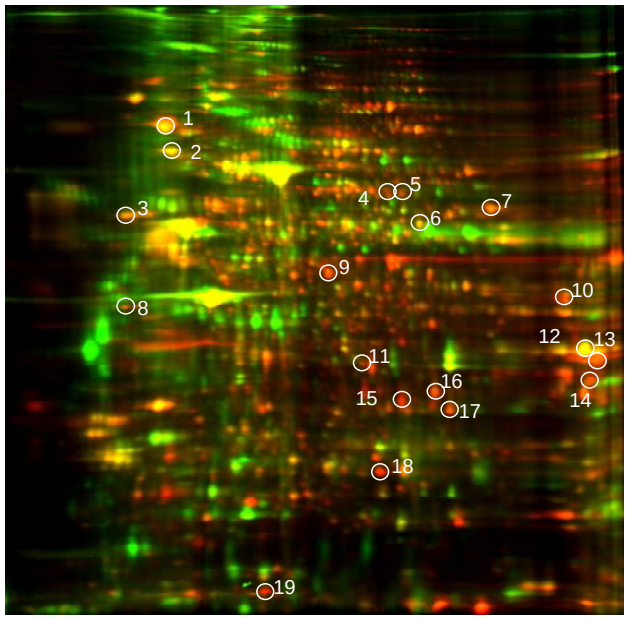
Figure 1: Analysis of the 2-D DIGE images by DeCyder Software and mass spectrometry protein identification. Panel A shows an overlaid image with proteins from the normal lymph node labeled with Cy3 (green) and proteins from the malignant lymph node labeled with Cy5 (red) from RW001. Circles indicate protein spots with significant expression level changes. Panel B shows the 3D view of protein spot 17 change between the normal and malignant tissue. Mass spectrometry (not shown) subsequently (following robotic spot picking) identified this protein as RACK1.

Figure 2: Nearest neighbor protein-protein interactions (first order) of the 7 patients that underwent our target prioritization assessment. Network connectivity maps are displayed using the VisualCell™ software.

Figure 3: **(A)** Western blot demonstrating upregulated expression of RACK 1 in RW malignant tissue and malignant cell line CCL247. Limited expression in normal tissue for RW001 (lymph node shown, skin and peripheral blood cells also negative) is also shown for comparison. **(B)** siRNA knockdown of RACK 1 lane 1 mock transfected, lane 2 24 hr post siRNA knockdown, and lane 3 24 hr post siRNA RACK 1 knockdown (sense 5' - CCUUUACACGCUAGAUGGUtt, antisense 5' – ACCAUCUAGCGUGUAAAGGtg sequence) by Western blot **(C)** cell kill in response to siRNA RACK 1 over time in colon cancer cell line CCL247. RACK1siRNA transfected cells showed significant reduction in live cell numbers compared to Untreated and Mock (Negative siRNA) treated cells, Day 7 and Day 10 $p = 0.0002$.

Figure 1.

A


1
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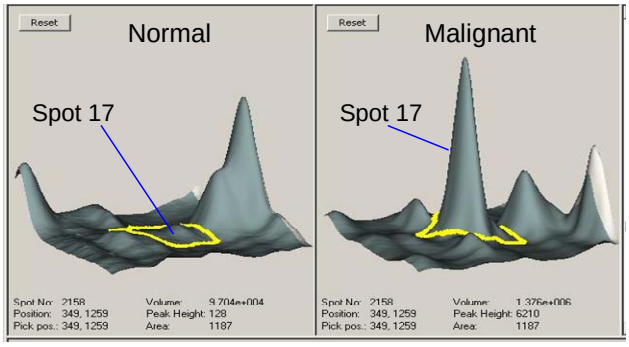
B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Normal
Spot 17
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Pick pos.: 349, 1259
Volume: 9.704e+004
Peak Height: 128
Area: 1187
Reset
Malignant
Spot 17
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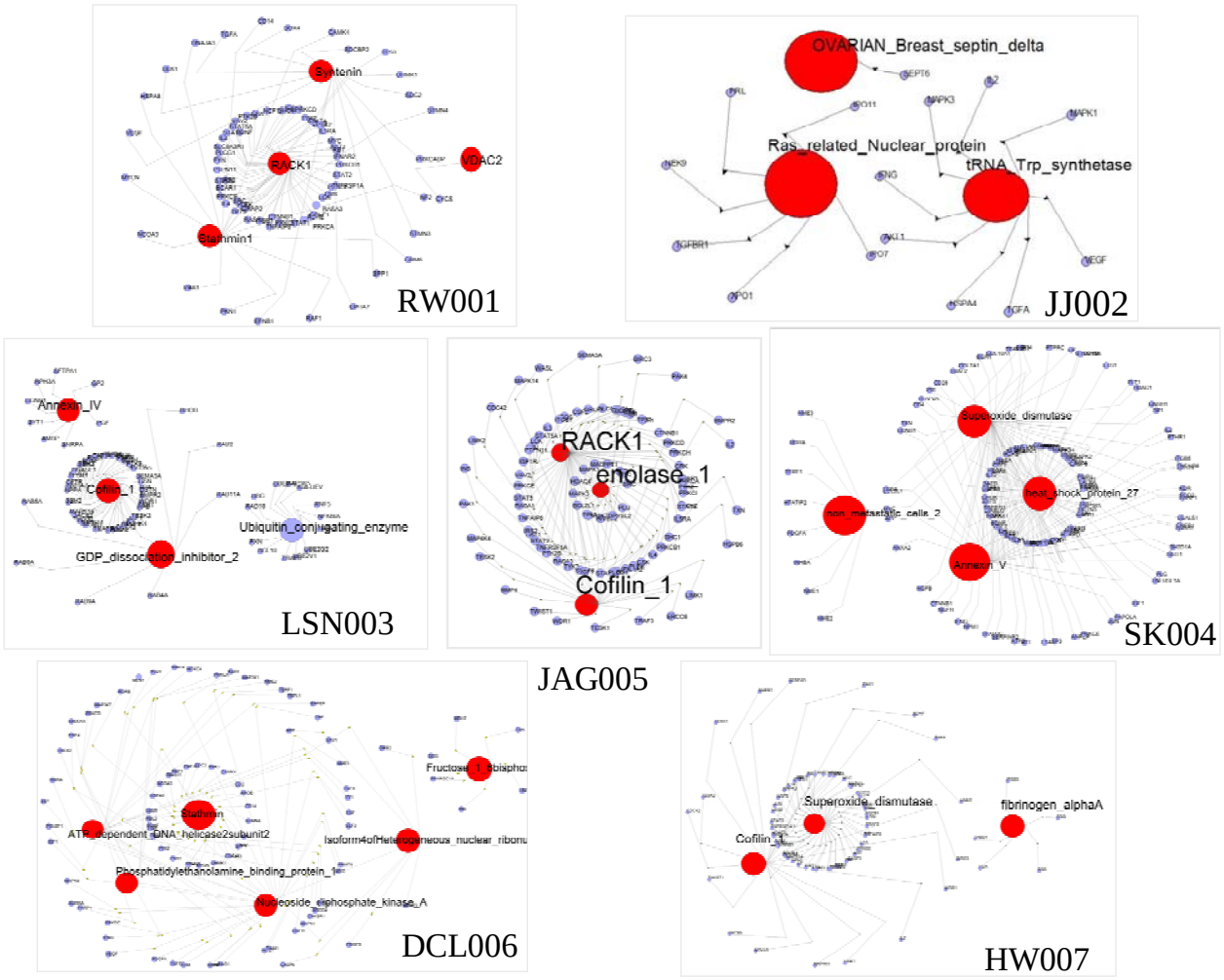
Area: 1187

Figure 2.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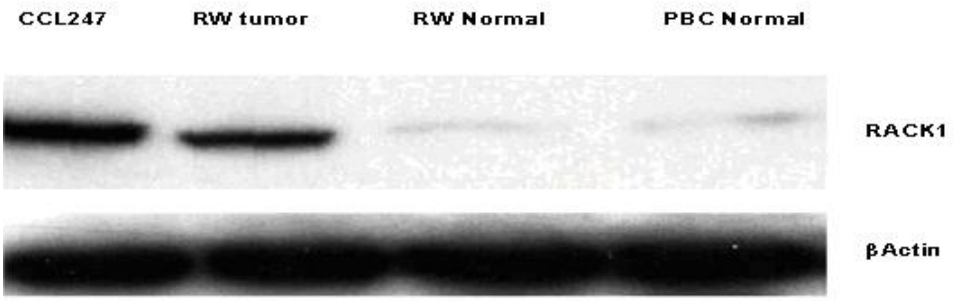
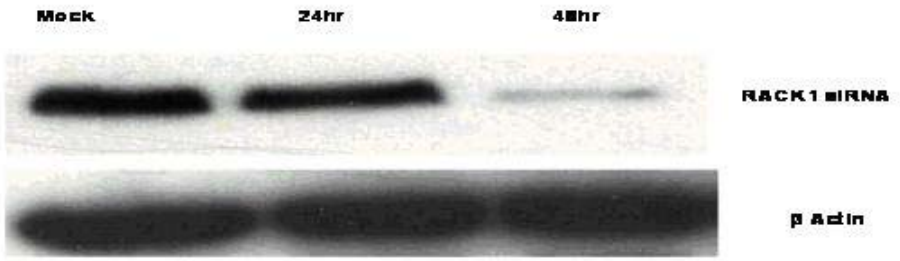
VDAC2
Stathmin1
RW001
OVARIAN_Breast_septin_delta
Ras_related_Nuclear_protein
tRNA_Trp_synthetase
JJ002
Annexin_IV
Cofilin_1
Ubiquitin_conjugating_enzyme
GDP_dissociation_inhibitor_2
LSN003
RACK1
enolase_1
Cofilin_1
JAG005
Superoxide_dismutase
heat_shock_protein_27
Annexin_V
SK004
Stathmin
Phosphatidylethanolamine_binding_protein_1
Nucleoside_diphosphate_kinase_A
DCL006
Superoxide_dismutase
Cofilin_1
fibrinogen_alphaA
HW007

Figure 3.

A. Expression of RACK1 in RW001 tumor and normal tissue



B. siRNA knockdown of RACK1 in cell line CCL247 - >80% at 48hr



C. Effect of transfection with RACK1 siRNA on cell numbers.

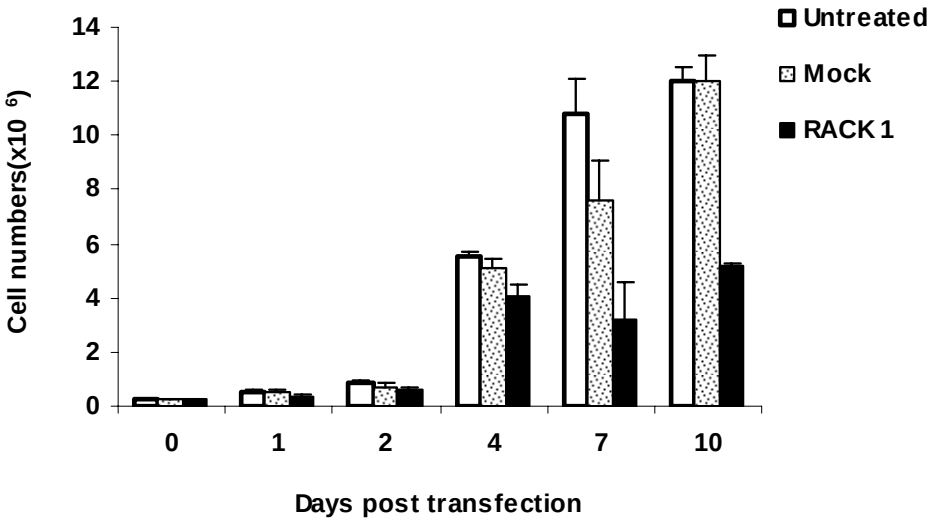


Table 1: Six priority proteins of RW001

Protein name	Key Function	Cancer Association	Reference	Protein Malignant/Normal levels (biopsy #1)	mRNA malignant/normal levels (biopsy #1)	DNA cross species preservation percent
Stathmin 1 (Oncoprotein 18)	Cell-cycle regulating protein. Regulates cell migration	SCCHN, Hepatocellular, renal cell, prostate	[35-39]	7.04	4.21	32.9% amino acid identity with Xenopus tropicalis AAH73451
TPI 1	Regulates glucose metabolism	Breast, bladder, prostate	[40-44]	3.61	1.84	33.9% amino acid identity with D. melanogaster AAS77472
RACK1 (lung cancer oncogene 7)	A scaffold protein which coordinates multiple intracellular signals associated with cell growth	Colon, pancreas, lung, breast	[45-55]	2.99	2.07	69.5% amino acid identity with D. melanogaster AAB72148
Syntenin	Regulates tumor cell growth, development and differentiation	Melanoma, breast, gastric cancer	[56-58]	7.07	4.12	78.2% amino acid identity with Xenopus tropicalis NP_001006801
Voltage-dependent anion channel 2	Regulates apoptosis	---	[59,60]	3.61	1.48	56.5% amino acid indentity with D. melanogaster CAA63413