Effectors and potential targets selectively upregulated in human KRAS-mutant lung adenocarcinomas

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Genetic and proteomic analysis of human tumor samples can provide an important compliment to information obtained from model systems. Here we examined protein and gene expression from the Cancer Genome and Proteome Atlases (TCGA and TCPA) to characterize proteins and protein-coding genes that are selectively upregulated in KRAS-mutant lung adenocarcinomas. Phosphoprotein activation of several MAPK signaling components was considerably stronger in KRAS-mutants than any other group of tumors, even those with activating mutations in receptor tyrosine kinases (RTKs) and BRAF. Co-occurring mutations in KRAS-mutants were associated with differential activation of PDK1 and PKC-alpha. Genes showing strong activation in RNA-seq data included negative regulators of RTK/RAF/MAPK signaling along with potential oncogenic effectors including activators of Rac and Rho proteins and the receptor protein-tyrosine phosphatase genes PTPRM and PTPRE. These results corroborate RAF/MAPK signaling as an important therapeutic target in KRAS-mutant lung adenocarcinomas and pinpoint new potential targets.

How mutationally activated KRAS and other canonical RAS genes malignantly transform cells and how to block this process for therapeutic benefit has been a subject of intense investigation for over thirty years. The majority of efforts to date have relied on model systems, using established cell lines and mouse models. These studies have identified signaling pathways that are directly stimulated by biochemically active Ras proteins, including the Raf/MAPK and PI3K/Akt pathways. They also have identified pathways or processes further downstream from Ras proteins that are involved in malignant phenotypes induced by mutant RAS genes, including the NF-κB pathway, transcriptional activity of the oncogene YAP, generation of reactive oxygen species, and anabolic glucose metabolism. A third class of proposed oncogenic mediators of mutant RAS genes are induced secreted proteins including TGF-α, Vegf, IL-8, IL-6, CXCL11, and CCL5. Several members of these three classes of mediators of oncogenic Ras have been explored as potential therapeutic targets but as of yet there hasn’t been a clinically successful treatment developed for cancers with mutant RAS genes.

The recent completion of large-scale human cancer sample characterizations such as the Cancer Genome Atlas (TCGA) and the Cancer Proteome Atlas (TCPA) has enabled an altogether different approach for discovery of protein targets that are upregulated by mutant RAS genes. This approach uses a direct comparison of tumor samples containing mutant RAS genes with either corresponding normal tissue samples or with tumor samples that contain wild-type RAS genes. One chief advantage of this approach is the analysis is done using the correct in vivo physiological context, whereas with model systems there is no guarantee that the physiological context is correct although it is thought that mouse models are superior to cell culture models. Another advantage is that direct analysis of human cancer takes into account the enormous diversity of co-occurring genomic alterations. This diversity requires very large panels of human cancer cell lines to be representative and presents very difficult challenges for mouse modeling.

Recently, three discrete subtypes of human KRAS-mutant lung adenocarcinomas were discovered in a breakthrough of our understanding of the variability within this type of lung cancer. These three subtypes have both distinct RNA expression profiles and different patterns of co-occurring mutations in TP53, STK11, KEAP1, and...
Results

Raf/MAPK signaling proteins are selectively activated in KRAS-mutant lung adenocarcinomas. 230 lung adenocarcinoma samples have been comprehensively characterized for mutations and gene expression by TCGA and these same samples have been characterized by reverse-phase protein array (RPPA) for their levels of 160 different proteins and modified proteins. To determine the selective effects of mutational activation of KRAS on these proteins in lung adenocarcinomas, we compared KRAS-mutant tumors (n = 75) to other tumors with wild-type KRAS. We further divided tumors with wild-type KRAS into those with other mutations in components of the mitogenic RTK/Raf/MAPK pathway and those without, using the criteria found in the TCGA publication. The other activating mutations in the RTK/Raf/MAPK pathway strongly tended toward mutual exclusivity and included missense or inframe deletion mutations in EGFR (n = 26), ERBB2 (n = 4), or RIT1 (n = 5); inactivating mutations in NF1 (n = 19); missense mutations in BRAF (n = 16), MAP2K1 (n = 2), HRAS (n = 1), or NRAS (n = 1); exon 14 skipping mutations in MET (n = 10), gene fusions involving ROS1 (n = 4), ALK (n = 3), or RET (n = 2); and focal high-level amplification of ERBB2 (n = 5), or MET (n = 5). We performed pairwise analysis of the three tumor subsets: KRAS mutants, other RTK/Raf/MAPK mutants, and all others. Since wanted to examine the differences with the greatest potential biological impact, we ranked the differences based on the effect size, rather than p-value which can over emphasize very small changes with little variation. We used Cohen’s d statistic that is highly related to the signal-to-noise statistic used in gene expression studies. Of all the 160 measured modified proteins and native proteins, the top ranking change statistic that is highly related to the signal-to-noise statistic used in gene expression studies was activation of proteins and genes. KRAS-mutants were compared to other lung adenocarcinoma samples and were also examined for the effects of commonly co-occurring mutations.

Effect of co-occurring mutations on protein levels in KRAS-mutant lung adenocarcinomas. We next examined the effects of co-occurring mutations in TP53, STK11, and KEAP1 on protein levels in mutant-KRAS tumors. Using cBioPortal analysis of TCGA lung adenocarcinomas, we confirmed that mutations in TP53 and STK11 tend to be mutually exclusive, whereas mutations in KEAP1 and STK11 tend to co-occur. Amongst KRAS-mutant tumors, there were 22 tumors with mutations in TP53 but not STK11 or KEAP1, 20 tumors with mutations in STK11 but not in TP53, and 13 tumors with mutations in KEAP1 but not in TP53 (8 of these tumors also had mutations in STK11). There was only one KRAS-mutant tumor with mutations in both TP53 and STK11 or KEAP1. Excluding this single sample, we divided KRAS-mutant tumors into three groups: those with TP53 mutations (n = 20), those with STK11 and/or KEAP1 mutations (n = 25), and those without any of these mutations (n = 27). Pairwise analysis of these three groups revealed that the phosphorylation activation status of Raf/MAPK pathway components was not significantly affected (Supplementary Table 3). The strongest effect of co-occurring TP53 mutations was seen with increased levels of Annexin I protein (Fig. 3A). As expected, the group with mutations in the protein kinase gene STK11 had significantly lower levels of activation of its direct target, AMPK, than the other two groups (Fig. 3B). More surprising however were the significantly lower levels of phosphorylation activated PDK1 and PKC-alpha proteins in this same group (Fig. 3C,D).
Figure 1. Relative levels of select phosphoproteins in three subgroups of human lung adenocarcinomas. All values are plotted on an arbitrary log2 scale to highlight relative levels rather than absolute amounts. For each panel, the red colored group represents KRAS-mutant tumors, the orange colored group represents other Raf/MAPK pathway mutants, and the blue colored group represents all other tumors. Brackets with associated \( p \)-values indicate significant differences. (A) levels of MEK1_pS217_S221; (B) levels of MAPK_pT202_Y204; (C) levels of p90RSK_pT359_S363; (D) levels of mTOR_pS2448.

Figure 2. Relative levels of select phosphoproteins within KRAS-wild type lung adenocarcinomas with other mutations in the RTK/RAF/MAPK pathway. Pairwise comparisons were made between EGFR mutants (n = 29) and all other mutants (n = 71); NF1 mutants (n = 19) and all other mutants (n = 81); MET mutants (n = 15) and all other mutants (n = 85).
Top ranked KRAS-mutant induced genes by RNA-seq data. We then looked at RNA-seq data to find genes with the greatest induction specifically in KRAS-mutant lung adenocarcinomas. We ranked genes based on the average effect size when KRAS-mutant tumors were compared to tumors with other mutations in genes of the Raf/MAPK pathway, and when compared to tumors with no mutations in genes of the Raf/MAPK pathway. KRAS itself was the top-ranked induced gene (Fig. 4; Supplementary Table 4). Within the top-100 induced genes were five negative regulators of RTK/MAPK signaling (DUSP4, DUSP6, NF1, SPRED2, SPRY4); the canonical Raf/MAPK transcriptional targets ETV4 and ETV5 (ETS-family members) and FOS (Fig. 4; Supplementary Table 4). Also within this top-ranked group are several candidate transcriptional oncogenic effectors for mutant KRAS, including the receptor tyrosine kinase genes INSR and IGFR1; the receptor tyrosine phosphatase genes PTPRE and PTPRM; and genes encoding guanine-nucleotide exchange-factors that activate Rac/Rho/Gdc42 proteins DOCK5, DOCK1, DNMBP, and PLEKHG2; (Fig. 4). Also included in the top-100 ranked induced genes is the gene CXorf61 which encodes a tumor antigen termed Kita-Kyushu lung cancer antigen 1 (Fig. 4).

Approximately 50% of the aforementioned genes that are selectively induced in KRAS-mutant lung adenocarcinomas were also significantly affected by co-occurring mutations in TP53, STK11, or KEAP1. For the most part, co-occurring mutations in STK11 and/or KEAP1 were associated with significantly stronger expression of these genes. In only one case (DUSP6) was co-occurrence of mutant TP53 associated with stronger expression (Fig. 5).

Discussion
Several components of the Raf/MAPK pathway show strong selective activation in KRAS-mutant lung adenocarcinomas, providing further corroboration that this pathway is a key therapeutic target for KRAS-mutant lung tumors. However, a potentially important finding of this study is that many of the signaling proteins and pathways thought to be activated in KRAS-mutant human lung adenocarcinomas based on studies with models systems
are not activated, at least not at steady state levels as assayed by immunoblotting of tissue samples. This makes it less likely that these pathways or proteins correspond to KRAS-selective dependencies. These proteins include PI3-kinase (as judged by activation of AKT), AKT, and NF-κB, all of which have been proposed to be important mediators of mutant KRAS in lung adenocarcinomas. Since mTOR is the only component of the PI3-kinase pathway that was selectively activated in KRAS-mutant tumors, a greater therapeutic window could conceivably be achieved by combining Raf/MAPK inhibitors with highly selective mTOR inhibitors that do not inhibit PI3-kinase.

All three subtypes of KRAS-mutants showed approximately equivalent activation of Raf/MAPK components. However, there were significant differences in protein and phosphoprotein levels detected in the three subgroups. The subgroup comprised of KRAS/STK11 and KRAS/KEAP1 double mutants, along with KRAS/STK11/KEAP1 triple mutants, had significantly lowers levels of phosphorylated AMPK, which is to be expected since AMPK is a direct kinase target of STK11. However this group was more unexpectedly associated with lower levels of phosphorylation activation of PDK1 and PKC-alpha, both of which have been actively pursued targets for developing potentially clinically effective inhibitors. However in this case, it would appear that loss of STK11 function is likely driving this decrease in activity, and that as such this would not represent a potential selective dependency in any of the KRAS-mutant subgroups.

Genomic studies usually rank differentially expressed genes by p-value or FDR. However, this ranking method does not taking into account the size of the effect. Potential transcriptional effectors of mutant-KRAS

**Figure 4. Heatmap showing the relative RNA values of select genes in three subgroups of human lung adenocarcinomas.** The three subgroups include KRAS-mutant tumors, other Raf/MAPK pathway mutants, and all other tumors. The select genes are indicated on the right. Row values were normalized and scaled and presented as Z-scores.
would more likely be genes showing the largest consistent increase in RNA. Therefore we used Cohen’s \( d \) statistic, closely related to the signal-to-noise statistic, to rank genes selectively upregulated in KRAS-mutant tumors. Amongst the top 100 upregulated genes were several negative regulators of Raf/MAPK signaling, consistent with an important known aspect of negative feedback regulation in this pathway\(^{20,21}\), as well as canonical transcriptional targets for Raf/MAPK signaling including \( FOS \) and the ETS-family members \( ETV4 \) and \( ETV5 \). Additionally, several potential oncogenic transcriptionally activated targets were uncovered including \( IGF1R \) and \( INSR \). A connection between mutant KRAS and IGF1R in lung adenocarcinoma has previously been documented\(^{22}\). We detected transcriptional activation of several guanine-nucleotide exchange factors that activate different proteins in the Rac/Rho/Cdc42 family. Thus the effect of mutational activation of KRAS on guanine-nucleotide exchange factors for small GTP-binding proteins is broader in lung adenocarcinoma than its biochemical activation of exchange factors that activate Raf proteins. Upregulation of the receptor protein-tyrosine phosphatase gene \( PTPRE \), the third highest ranked upregulated gene, is of particular interest since it has been shown to be upregulated by RAS in a mouse model of mammary carcinoma and to possess on its own the ability to promote mammary tumor formation\(^{23,24}\). Finally, this set of upregulated genes includes a potential immunotherapy target for KRAS-mutant lung adenocarcinomas, the Kita-Kyushu lung cancer antigen 1, encoded by the \( CXorf61 \) gene\(^{25}\). This protein has also been shown to be a potential target for T cell based therapies in triple-negative breast cancer\(^{26}\).

**Methods**

We used mutational, RNAseq and protein data in our analysis from the TCGA lung adenocarcinoma project. All data downloaded was Level 3. Mutation data was downloaded from cBioPortal for Cancer Genomics (www.cbioportal.org), under Lung Adenocarcinoma (TCGA, Provisional) with “All Complete Tumors (230)”. RNAseq data was downloaded from FireBrowse by Broad Institute (www.firebrowse.org), choosing “illuminahiseq_rnaseq2-RSEM_genes”. Protein data was downloaded from The Cancer Proteome Atlas (TCPA) project in MD Anderson Cancer Center (bioinformatics.mdanderson.org/main/TCPA). To generate heatmaps, the R package “gplots” was used.

We segregated samples using cBioPortal into three mutually exclusive groups: ones with KRAS missense mutations, ones with mutations in genes encoding other components of the RTK/Raf/MAPK pathway as delineated in the text, and other samples. Those three subgroups were used to perform pairwise comparisons using protein (RPPA) and RNAseq data. We used Cohen’s \( d \) to measure effect size using the R-package “effsize”. Ranking with both effect size and t-test p-value generated top-ranked proteins and genes induced in KRAS mutants or Raf/MAPK pathway mutants (Fig. 1, Supplementary Table 1). We used a similar approach to divide KRAS mutants into three groups depending on \( TP53 \), \( STK11 \), or \( KEAP1 \) status.
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Author Contributions
Conceived and designed the analysis: S.P. Computational analysis: J.L. Analyzed data: J.L., S.P. and R.S. Wrote the paper: J.L. and S.P.

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