Mutant KRAS associated malic enzyme 1 expression is a predictive marker for radiation therapy response in non-small cell lung cancer

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Abstract

Background: Advanced non-small cell lung cancer (NSCLC) is an aggressive tumor that is treated with a combination of chemotherapy and radiation therapy if the patient is not a candidate for surgery. Predictive biomarkers for response to radiotherapy are lacking in this patient population, making it a non-tailored therapy regimen with unknown outcome. Twenty to 30% of NSCLC harbor an activating mutation in KRAS that may confer radioresistance. We hypothesized that mutant KRAS can regulate glutamine metabolism genes in NSCLC and maintain tumor redox balance through transamination reactions that generate cytosolic NADPH via malic enzyme 1 (ME1), which may contribute to radioresistance.

Findings: A doxycycline-inducible mouse model of KRAS G12D driven NSCLC and patient data was analyzed from multiple publicly accessible databases including TCGA, CCLE, NCBI GEO and Project Achilles. ME1 expression was found to be mutant KRAS associated in both a NSCLC mouse model and human NSCLC cancer cell lines. Perturbing glutamine metabolism sensitized mutant KRAS, but not wild-type KRAS NSCLC cell lines to radiation treatment. NSCLC survival analysis revealed that patients with elevated ME1 and GOT1 expression had significantly worse outcomes after radiotherapy, but this was not seen after chemotherapy alone.

Conclusions: KRAS driven glutamine metabolism genes, specifically ME1 and GOT1 reactions, may be a predictive marker and potential therapeutic target for radiotherapy in NSCLC.

Keywords: Radioresistance, KRAS, Glutamine, NSCLC, ME1, GOT1, ROS

Background and findings

Patients with locally advanced NSCLC that are not candidates for surgery are treated with a combination of chemotherapy and radiation therapy [1]. Clinical trials assessing the efficacy of radiation therapy in this patient population have shown mixed results [2–4]. Furthermore, 20–30% of all NSCLC harbor an activating mutation in KRAS [5]. Interestingly, several studies have demonstrated that the presence of mutant KRAS may act as a marker for radioresistance in NSCLC, yet the exact mechanism is not well understood [6–12]. Recent literature has demonstrated that mutant KRAS reprograms glutamine metabolism flux in pancreatic cancers through cytosolic aspartate transaminase (GOT1) and malic enzyme 1 (ME1) [13–18]. By synthesizing significant intracellular pools of NADPH via ME1, KRAS-reprogrammed pancreatic cancers rely on glutamine for redox balance in the face of reactive oxygen species (ROS) production from rapid proliferation and microenvironment stressors (Fig. 1a) [16]. In this context, NADPH is an essential cofactor to blunt ROS formation through the maintenance of intracellular reduced glutathione and thioredoxin [19]. However, to date, there are no studies evaluating whether mutant KRAS similarly reprograms glutamine metabolism genes in NSCLC for redox balance and whether this may be a potential mechanism to attenuate ionizing radiation (IR)-induced ROS and DNA damage. Therefore, we characterized glutamine metabolism genes in mutant vs wild-type KRAS NSCLC both in vitro and in vivo, demonstrated the necessity of ME1 in mutant, but not wild-type,
Fig. 1 (See legend on next page.)
Mutant KRAS is associated with ME1 and G0T1 expression in NSCLC

Gene set enrichment analysis (GSEA) of wild-type vs mutant KRAS NSCLC cell lines from the Cancer Cell Line Encyclopedia (CCLE) revealed that genes involved in glutamine dependent redox balance (ME1 and G0T1) were significantly upregulated in mutant KRAS cell lines with normalized enrichment scores (NES) > 1.48 (Table 1, Additional file 1: Figure S1A).

We found that 7 out of 9 mutant KRAS cell lines relied on ME1 for viability, while ME1 was dispensable in all but one of the wild-type cell lines (Fig. 1e). To verify these results, we knocked down ME1 (Fig. 1h) in H522, a wild-type KRAS cell line. Using clonogenic survival assays, we found that ME1 loss rendered HCC44, but not H522, unable to form visible colonies (Fig. 1f, g). Taken together, our analyses indicate that mutant KRAS is associated with ME1 gene expression in NSCLC and that ME1 is an essential viability gene in mutant, but not wild-type, KRAS cell lines. In support of these results, we knocked down ME1 and found both genes to be significantly upregulated in the mutant cell lines (Fig. 1d, Additional file 1: Figure S1C).

Next, we measured mRNA levels of ME1 and G0T1 in 11 mutant and 11 wild-type KRAS NSCLC cell lines and found both genes to be significantly upregulated in the mutant cell lines (Fig. 1d, Additional file 1: Figure S1C). Next, to determine if mutant KRAS NSCLC cell lines relied on ME1 for survival, we analyzed 17 NSCLC cell lines from the Project Achilles database, an openly accessible platform of large-scale functional RNAi screens of cancer cell lines to identify genes that affect cell survival [21]. We found that 7 out of 9 mutant KRAS cell lines relied on ME1 for viability, while ME1 was dispensable in all but one of the wild-type cell lines (Fig. 1e). To verify these results, we knocked down ME1 (Fig. 1h) in H522, a wild-type KRAS cell line, and in HCC44, a mutant KRAS line. Using clonogenic survival assays, we found that ME1 loss rendered HCC44, but not H522, unable to form visible colonies (Fig. 1f, g). Taken together, our analyses indicate that mutant KRAS is associated with ME1 gene expression in NSCLC and that ME1 is an essential viability gene in mutant, but not wild-type, KRAS cell lines. In support of this observation, ME1 is a known NRF2 transcriptional target, which itself is positively regulated by mutant KRAS signaling via the MAPK pathway [22, 23].

Targeting glutamine metabolism sensitizes mutant KRAS NSCLC cell lines to radiation treatment

Mutant KRAS HCC44 and wild-type KRAS H522 cells were grown in Gln-free or Gln-containing (2 mM) media for 16 h, then exposed to ionizing radiation and allowed to form colonies for 7 days. Short-term Gln deprivation did not significantly alter clonogenic survival on its own, but did sensitize HCC44 and not H522 cells to radiation, at normally sub-lethal doses (Fig. 2a, b). Using this short term glutamine deprivation protocol, we next screened the mutant KRAS NSCLC cell lines H2009, H1573 and A549; and the wild-type KRAS NSCLC cell lines H661, H322 and H596 (Fig. 2c). Interestingly, we found that upon glutamine deprivation, mutant, but not wild-type, KRAS lines were sensitized to radiation (Fig. 2c). To
pharmacologically mimic these results, we pre-treated HCC44 and H522 with the glutaminase 1 (GLS1) inhibitor, CB-839 [24], for 48 h at 1 μM followed by radiation treatment. Consistent with our glutamine deprivation results, HCC44, but not H522, was sensitized to radiation treatment (Fig. 2d).

**GOT1 and ME1 expression predicts response to radiation therapy in NSCLC patients**

To expand our *in vivo* and *in vitro* findings into a clinical context, we analyzed mutant KRAS status, tumor mRNA expression and RECIST outcomes data from the TCGA in lung adenocarcinoma (LUAD) NSCLC patients who were treated with IR (patient characteristics Additional file 2: Table S1 and Additional file 3: Table S2, https://tcga-data.nci.nih.gov/tcga/tcgaCancerDetails.jsp?diseaseType=LUAD&diseaseName=Lungadenocarcinoma) [25]. Of the 14 LUAD NSCLC patients who had a complete response (CR) to IR treatment, ~93 % (13/14) of the patient’s tumors were wild-type KRAS, while only ~7 % (1/14) of the tumors were mutant KRAS, suggesting that wild-type KRAS tumors may be more radiosensitive compared to mutant KRAS tumors, consistent with previous reports (Fig. 3a) [6–12]. _ME1_ and _GOT1_ expression levels were significantly elevated in those patients who had progressive disease (PD) when treated with IR vs patients

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**Fig. 2** Targeting glutamine metabolism sensitizes mutant KRAS NSCLC cell lines to radiation treatment. a, b Seven day clonogenic survival of HCC44 or H522 after radiation treatment after growth in either complete media or Gln deprived media for 16 h. c Clonogenic survival screen of mutant KRAS (H2009, H1573 and A549) or wild-type KRAS (H661, H322 and H596) NSCLC cell lines grown in either complete media or Gln deprived media for 16 h followed by treatment with 2 Gy of ionizing radiation. d Clonogenic survival of HCC44 and H522 pre-treated with 1 μM CB-839 for 48 h followed by treatment with various doses of ionizing radiation. All results were compared using Student’s t-tests as indicated. *p < 0.05; **p < 0.01; ***p < .001.
who demonstrated a CR after radiation therapy (Fig. 3b, c). Furthermore, we assessed overall survival outcomes in IR treated NSCLC patients (n = 73) grouped into high or low GOT1 and ME1 expressers. Interestingly, we found that patients with high expression of GOT1 or ME1 had significantly worse prognosis over a 140 month time period when compared to low GOT1 or ME1 expressers (Fig. 3d, e). Lastly, we did not observe a significant median survival difference between high and low GOT1/ME1 expressers in NSCLC patients who received chemotherapy, but not IR (Additional file 4: Figure S2A, B).

Taken together, this suggests that ME1 and GOT1 are...
predictors to radiation, but not chemotherapeutic, response in NSCLC.

Conclusions
This multi-database translational study is the first to identify mutant KRAS associated glutamine metabolism genes, GOT1 and ME1, as potential radioresistance biomarkers in NSCLC. Our study revealed that elevated expression of GOT1 or ME1 is a highly predictive biomarker in radiation treatment, but not chemotherapeutic, outcomes. Additionally, ~93% of patients with a complete response to IR treatment harbored wild-type KRAS in their tumors. To explain these observations, we hypothesize that KRAS-reprogrammed glutamine flux through GOT1 and ME1 is critical to maintain cytosolic NADPH levels for redox balance and lipid synthesis in NSCLC. In the face of ROS stress, as observed with IR treatment, NADPH is preferentially used to maintain reduced glutathione and thioredoxin 1 to protect cells from ROS damage [19]. In this context, KRAS may reprogram NSCLC glutamine metabolism similar to that observed in pancreatic cancer to maintain redox balance, thus providing an oncogene driven mechanism of radioresistance. While there are currently no known specific inhibitors of ME1 or GOT1, targeting upstream glutamine utilization via glutaminase 1 (GLS1, Fig. 1a) inhibition (with BPTES or CB-839) may blunt downstream utilization of glutamine/glutamate through GOT1 and ME1, thus depleting tumor, but not normal tissue, NADPH/GSH production, leading to tumor-specific radiosensitivity while sparing normal tissue [24].

Materials and methods
Databases
GSEA of mutant vs wild-type KRAS NSCLC cell lines was completed using the Broad Institute’s publically available Cancer Cell Line Encyclopedia (CCLE) (http://www.broadinstitute.org/ccle) [26]. Transgenic mouse data was obtained through GEO Series accession number GSE40606 at Transgenic mouse data was obtained through GEO Series accession number GSE40606. We obtained NSCLC expression, mutation, treatment and outcomes patient data from The Cancer Genome Atlas (TCGA) using the lung adenocarcinoma (LUAD) dataset (https://tcga-data.nci.nih.gov/tcga/tcgaCancerDetails.jsp?diseaseType=LUAD&diseaseName=Lung adenocarcinoma) [25]. Level 2, tumor somatic mutation data was obtained for KRAS for each patient in the analysis (Fig. 3a). Level 2, normalized gene expression data was obtained for GOT1 and ME1 for each patient in the analysis (Fig. 3b, c). Patient characteristics are shown in Additional file 2: Table S1 and Additional file 3: Table S2. Cell line gene dependency data was obtained from Broad Institute’s Project Achilles (http://www.broadinstitute.org/achilles) [21].

Kaplan-Meier statistics
Survival analysis in radiation treated NSCLC patients (n = 73) was conducted using the Kaplan-Meier Plotter webtool (kmplot.com) [27]. Briefly, kmplot segregates each gene into percentile of expression between the lower and upper quartiles and the best performing threshold is used as the final cutoff in a univariate Cox regression analysis. Kaplan-Meier survival plot and the hazard ratio with 95% confidence intervals and logrank P value is calculated with the Bioconductor package in R.

Ethical approval and consent
All human data is sourced through The Cancer Genome Atlas (http://cancergenome.nih.gov/), no patients were approached for this study. No consent and no ethical approval were required to utilize this database.

Survival assay
For clonogenic survival assays, cells were trypsinized and plated onto 6-well plates at 100, 500, or 1000 cells per well in 2 ml of complete media, Gln deprived media for 16 h or complete media containing 1 μM CB-839 for 48 h. Cells were then exposed to IR (at various doses as indicated), allowed to grow for 7 days, washed with PBS and stained with crystal violet solution. Colonies with >50 normal appearing cells were counted and percent survival calculated and graphed with dose.

RNAi transfection
For siRNA transfection, cells were plated in 10 cm plates at 2 × 10⁵ cells per plate and transfected with either control siRNA or siRNA against ME1 for 48 h followed by clonogenic survival assay.

Additional files

Additional file 1: Figure S1. (A) Raw GSEA data of mutant vs wild-type KRAS NSCLC cell lines. Red = overexpressed across all cell lines; blue = under expressed across all cell lines. Absolute top row indicates specific cell lines used in analysis. Gray = mutant KRAS; yellow = wild-type KRAS. (B) KRASG12D induction upregulated GOT1 mRNA in mouse doxycycline inducible KRASG12D embryonic fibroblasts derived from the transgenic mice. (C) mRNA expression of GOT1 in mutant KRAS vs wild-type KRAS NSCLC cell lines. Same cell lines as in Fig. 1d.

Additional file 2: Table S1. TCGA lung adenocarcinoma patient description.

Additional file 3: Table S2. GOT1 and ME1 expression in TCGA lung adenocarcinoma patient tumors with treatment response after IR.

Additional file 4: Figure S2. (A, B) Kaplan-Meier overall survival curves in chemotherapy treated NSCLC patients from TCGA database separated into high and low GOT1 and ME1 expression. Total number of chemotherapy treated NSCLC patients analyzed = 176; number of patients with high expression: ME1 = 69, GOT1 = 127; number of patients with low expression: ME1 = 107, GOT1 = 49. Logrank p-values not significant.

Competing interests
The authors declare that they have no competing interests.
Authors’ contributions
GC conceived and designed the study, performed the statistical analysis, carried out the database analyses, experimental cell culture work, programming survival analysis script in R and drafted the manuscript. All authors read and approved the final manuscript.

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