LKB1/KRAS mutant lung cancers constitute a genetic subset of NSCLC with increased sensitivity to MAPK and mTOR signalling inhibition

LKB1/STK11 is a serine threonine kinase known to be involved in several cellular processes including, signal transduction, energy sensing and cell polarity (Spicer and Ashworth, 2004). Germline inactivating mutations of LKB1 are known to cause Peutz-Jeghers syndrome, a hereditary condition, which results in the development of benign (hamartomatous) polyps in gastrointestinal tract, mucocutaneous pigmentation (Hemminki et al, 1998; Mehenni et al, 1998) and a predisposition to developing cancers in a variety of tissues; colon, small intestine, breast, ovary, pancreas and lung (Giardiello et al, 1987). Approximately 30% of somatic lung adenocarcinomas harbour an inactivating mutation in LKB1 (Sanchez-Cespedes et al, 2002), and a recent study lists LKB1 as one of the four most frequently mutated genes in lung adenocarcinoma (Ding et al, 2008).

In mammalian cells, LKB1 exists in a complex with STE20-related adaptor (STRAD) and mouse protein 25 (MO25; Boudeau et al, 2003). It is a member of the calcium/calmodulin regulatory kinase-like family and is thought to phosphorylate and activate at least 13 kinases, including AMPK (Lizcano et al, 2004; Jaleel et al, 2005). AMP-activated protein kinase (AMPK) is a regulator of cellular energy metabolism, and the main function of the pathway is to restore cellular energy levels and is switched on in low ATP, high AMP conditions, caused by processes, such as hypoxia and cellular stress (Hardie et al, 2003). Activated AMPK phosphorylates TSC2 activating the TSC1-2 complex, which inhibits RAS homologue enriched in brain (RHEB), a small GTP-binding protein and prevents the activation of mammalian target of rapamycin (mTOR; Garami et al, 2003). The mammalian target of rapamycin kinase plays a central role in regulating protein synthesis and control of cell growth (cell size and mass) (Schmelze and Hall, 2000; Fingar et al, 2002). Loss of LKB1, therefore, leads to a failure to inhibit RHEB and loss of suppression of mTOR signalling, leading to increases in cell size and mass (Corradetti et al, 2004; Shaw et al, 2004).

MATERIALS AND METHODS

Cell culture

Cell lines of a known genetic background (Table 1) were grown in RPMI (Invitrogen, Life Technologies Inc., Carlsbad, CA, USA) apart from CAL12T, which was grown in DMEM (Invitrogen) supplemented with 10% FBS and 1% PSG.

Statistical analysis

Statistical analysis of the co-occurrence of LKB1 and ras-MAPK pathway mutations in 87 NSCLC cell lines was carried out by the Fisher’s exact test.

CI-1040/rapamycin treatment and proliferation assay

Cells were seeded in six replicates to 48-well plates. After 24 h, this was replaced with media containing 0, 0.1, 0.5, 1, 5, 10 μM CI-1040 or 0, 0.1, 0.5, 1, 5, 10, 25, 50, 75, 100 μM rapamycin (Sigma-Aldrich Co, St Louis, MO, USA). After 72 h, the proliferation rate was measured using the CyQuant proliferation assay kit (Invitrogen) according to the manufacturers instructions. Statistical analysis was carried out using two-tailed unpaired t-tests.
Cells were seeded to 6-well plates; 24 h later, this was replaced with media containing 0, 0.1, 0.5, 1, 5, 10 μM CI-1040 or 0, 1, 10, 50, 100, 200 nM rapamycin. Then 8 and 24 h after the addition of CI-1040 and 24 h after the addition of rapamycin, protein was harvested in RIPA buffer containing protease (Sigma) and phosphatase (Roche Applied Science, UK) inhibitors. Samples were mixed with pre-
Figure 2 Cells with inactivated LKB1 and activated KRAS are more sensitive to the MEK inhibitor CI-1040 and the mTOR inhibitor rapamycin; however, dual inhibition is neither additive nor synergistic. Cell lines tested; NCI-H460 (LKB1-/-/KRASmut), A549 (LKB1-/-/KRASmut), NCI-H1734 (LKB1-/-/KRASmut), NCI-H2030 (LKB1-/-/KRASmut), collectively labelled as LKB1-/-/KRASmut in the figure; NCI-H1838 (wt), NCI-H1975 (wt), NCI-H2009 (KRASmut), NCI-H1563 (LKB1-/-), collectively labelled as control cell lines; CAL12T (LKB1-/-/BRAFmut), and SKMEL28 (positive control; BRAF V600) (A). Cells were seeded in six replicates to 48-well plates. After 24 h, this was replaced with a medium containing 0, 0.1, 0.5, 1, 5 or 10 μM CI-1040. After 72 h, proliferation rate was determined using CyQuant proliferation assay (Invitrogen) according to the manufacturer’s instructions. The results shown here are from two independent experiments. Proliferation rates were measured relative to the untreated control. (B) Values from the two clusters shown in panel A were averaged to calculate the statistical significance between the clusters, values shown ± s.d between the cell lines within the cluster; n ≥ 4 for each cluster. Two-tailed unpaired t-test gave a P-value of < 0.001 for all CI-1040 concentrations. (C) The same protocol as in panel A was followed; however, after 24 h, this was replaced with a medium containing 0, 0.1, 0.5, 1, 5 or 10 μM rapamycin. The results shown here are again from two independent experiments. Proliferation rates were measured relative to the untreated control. Owing to the similar nature of the results of the CI-1040 experiment, the cell lines were clustered according to their mutation status (LKB1-/-/KRASmut) or control cells (LKB1-/-, WT and KRAS mut). Unpaired two-tailed t-tests carried out to determine statistical significance; P < 0.05 for concentrations of 10 μM and upwards, n = 3 for each cluster means ± s.d. (D) Dual inhibition experiments were carried out using 10 μM rapamycin and a range of CI-1040 concentrations in the same format as single-agent experiments, cell lines were again grouped by their mutation status and statistical significance calculated using two-tailed unpaired t-tests, P ≤ 0.01; results are from two independent experiments each with six replicates, mean ± s.d. (E) Comparison of dual-agent treatment to most potent single-agent treatment to determine whether the agents are additive/synergistic in the LKB1-/-/KRASmut group. Statistical significance determined using unpaired two-tailed t-tests between single-agent treatment group and each dual-treatment group. The only significant value marked *P = 0.005, n = 3, mean ± s.d. (F) Comparison of dual-agent treatment to most potent single-agent treatment in control cell lines to determine whether the agents are additive/synergistic in the control group. No statistically significant values were found, n = 3, means ± s.d.
To further explore this potential, NSCLC lines of known genetic backgrounds (Table 1) were treated with the MEK inhibitor CI-1040. Figure 2A shows that the LKB1/KRAS mutant cell lines have a uniform enhanced sensitivity to CI-1040 when compared with wild-type cell lines, LKB1 mutant lines or KRAS mutant lines (labelled control cell lines in Figure 2B). Interestingly, the LKB1/BRAF(G466V) mutant cell line (CAL12T) is insensitive to CI-1040 and falls in the top cluster. The mean relative proliferation rate of the LKB1/KRAS mutant cell lines, and compared with the control cell line cluster was statistically significant ($P < 0.001$ at all CI-1040 concentrations $> 0$; Figure 2B). LKB1/KRAS mutant cell lines have a mean IC$_{50}$ value of $5 \mu $M compared with the control cell lines, which have a mean IC$_{50} > 10 \mu $M. We next investigated the importance of mTOR signalling in the genetic subtypes of lung cancers under study. Figure 2C shows that inhibiting mTOR using rapamycin had a more pronounced affect on proliferation in LKB1/KRAS mutant cell lines; in this case, the sensitive cluster also included the LKB1/BRAF mutant cell line CAL12T. The IC$_{50}$ of the LKB1/MAPK mutant cluster was significantly different from the control cluster (40 nM vs > 100 nM, $P \leq 0.04$). Dual inhibition of MEK and mTOR (Figure 2D) leads to a statistically significant

**Figure 3** The sensitivity to the MEK inhibitor CI-1040 and the mTOR inhibitor rapamycin are not due to downstream effects on cyclin D1, but in the case of MEK inhibition are due to downstream effects on phospho-p70S6K (thr389). (A) To determine the effect of MEK inhibition on MAPK downstream pathway components cell lines were seeded to 6-well plates, and the following day, it was replaced with media containing 0, 0.1, 0.5, 1, 5, 10 $\mu $M CI-1040. After 8 and 24 h, the addition of CI-1040 protein was harvested in RIPA buffer containing protease (Sigma) and phosphatase (Roche) inhibitors. Samples were mixed with premade sample buffer and reducing agent (NUPAGE), and heated at 95°C for 5 mins. In all, 10 $\mu $g of protein was run on a 4–12% precast gel (NUPAGE), and transferred to a PVDF membrane (Invitrogen) by western blotting. Membranes were probed with either rabbit anti-ERK, antiphospho ERK (Cell Signalling, 1/1000), anticyclin D1 (Santa-cruz, 1/500) antibodies, followed by secondary antibodies (Cell Signalling, 1/2500). The bands on the membrane were displayed using enhanced chemiluminescence method (Pierce). (B) and (C) Using the same immunoblotting protocol as above, mTOR downstream pathway components were assessed after treatment with CI-1040 or rapamycin. Total p70S6K and phospho-p70S6K (thr389) and cyclin D1 protein levels were analysed in 0, 0.1, 0.5, 1, 5, 10 $\mu $M CI-1040- treated cell lines. Phospho-p70S6K (thr389) and cyclin D1 protein levels were analysed in cell lines treated with 0, 1, 10, 50, 100, and 200 $\mu $M rapamycin.

**RESULTS**

Resequencing of known cancer genes in a series of 87 NSCLC cell lines (http://www.sanger.ac.uk/genetics/CGP/CellLines/) found significant association of LKB1 inactivating mutations with KRAS mutations ($P = 0.03$). This association has recently been confirmed in an independent study (Matsumoto et al., 2007). Association of LKB1 inactivation clustering with LKB1/KRAS mutant lung cancers and MEK inhibition (Figures 1A and B) was also observed. LKB1 and RAS/BRAF/MEK (MAPK) signalling pathways are linked through RHEB, which when active, activates mTOR and inhibits wild-type BRAF, but not the mutated form (Im et al., 2002; Garami et al., 2003; Karbownikczek et al., 2004). It was recently shown that the inhibition of RAFI (cRAF) activity by RHEB prevents heterodimerisation of BRAF and RAFI (Karbownikczek et al., 2006). LKB1 mutations in NSCLC may, therefore, have a general requirement for an activation of the MAPK cascade to overcome suppression through RHEB inhibition. This interdependence suggests that the inhibition of MAPK signalling may constitute a potential opportunity for therapeutic intervention in this genetic subset of NSCLC (Figure 1C).

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decrease the proliferation rate in the LKB1/KRAS mutant cluster versus the control cell lines (P-value: ≤0.01). This effect of dual inhibition in general did not reach statistical significance for additivity nor being synergistic in either cluster (Figures 2E and F), although at the highest concentrations (5 μM CI-1040 with 10 nM rapamycin) in the LKB1/KRAS mutant cluster, the data were consistent with an additive model. However, this may be due to the combined toxic effects of higher drug concentrations.

To confirm that MEK inhibition was being achieved in the cells, we carried out immunoblot analysis of ERK, phosphorylated ERK and cyclin D1 levels at 8 and 24 h following CI-1040 treatment (Figure 3A). In all cell lines, levels of phosphorylated ERK decreased with increasing CI-1040 concentration by 8 h; however, wild-type cell lines for both genes or LKB1 mutant required higher concentrations of CI-1040 to prevent phosphorylation of ERK. The effect of MEK inhibition on cyclin D1 levels did not appear to correlate with genetic status, and interestingly, the KRAS mutant cell line NCI-H2009 showed a similar decrease in phosphorylated ERK, and perhaps the greatest decrease in cyclin D1 levels, despite the inhibitor having little effect on proliferation. Altogether, these data show that the effects of MEK inhibition on phospho-ERK are driven by the presence or absence of a KRAS mutation and are independent of LKB1 mutation status, whereas the proliferation effects are related to LKB1/KRAS combined mutation status. As there was no correlation with cyclin D1 levels and enhanced sensitivity to MEK inhibition, we carried out immunoblot analysis of p70S6K and phospho-p70S6K (thr-389) levels; phosphorylation of this residue is critical for kinase function (Pullen and Thomas, 1997). Figure 3B shows that CI-1040 treatment had no effect on total p70S6K protein levels; however, a decrease was observed in phospho-p70S6K (thr-389) levels, specifically in LKB1/KRAS mutant cell lines. This decrease in phosphorylation correlated well with the observed IC50 for this genetic subset. Figure 3c shows that rapamycin treatment had no effect on cyclin D1 protein levels, but had a potent effect on phospho-p70S6K (thr-389) levels in all cell lines regardless of the mutation status.

DISCUSSION

Here, we report that LKB1 inactivation and KRAS activation in non-small-cell lung cancer denotes a functionally distinct set of lung cancer, which display sensitivity to the single-agent treatment with the MEK inhibitor CI-1040 or rapamycin. It has been previously shown in melanomas that treatment with CI-1040 caused a dose-dependent reduction in phospho-ERK and cyclin D1 protein levels in BRAFV600E mutant cell lines, but not in NRAS mutant melanomas (Solit et al, 2006). Our results, while showing dose-dependent reduction of phospho-ERK in KRAS mutant NSCLC cell lines, show inhibition of proliferation only in the subset with both KRAS and LKB1 mutations, highlighting the importance of the cross-talk in these pathways and supporting the genetic data that the co-occurrence of these two mutations is non-random. Dose-dependent decrease in phospho-ERK and reduction in proliferation rate did not result in a corresponding decrease in cyclin D1 levels, suggesting that in this subset of NSCLC, the downstream effector pathways may be different to BRAFV600E melanomas. The effects of reduced proliferation in this genetic subset may be due to the reduction in the activity of p70S6K, which is downstream of both mTOR and ERK1/2, shown by the specific reduction of phosphorylation of thr-389 p70S6K in LKB1/KRAS cell lines following MEK inhibition. These data further highlight the importance of cross-talk between these pathways in this genetic subset. Interestingly, CAL21T, the LKB1/BRAF mutant cell line is insensitive to CI-1040 yet sensitive rapamycin, suggesting an additional, smaller genetic subset in NSCLC and possibly highlighting the difference of the non-V600 BRAF mutations found in NSCLC (Brose et al, 2002). Despite being sensitised to CI-1040 and rapamycin, dual-agent treatment did not have demonstrably additive or synergistic effects in LKB1/KRAS cell lines, suggesting the possible redundancy in the pathways. The lack of additivity may be explained by the observation that rapamycin potently inhibits p70S6K phosphorylation at thr-389, therefore precluding any additional effect of CI-1040 on the p70S6K activity, further confirming redundancy of the pathways in this genetic subset of NSCLC.

Mutation status in cancers has been shown to predict response to targeted therapies, as exemplified by the efficacy of EGFR inhibitors in EGFR mutant lung cancer (Lynch et al, 2004; Paez et al, 2004). Data presented here suggest that LKB1/KRAS mutated tumours are a genetic and functionally distinct subset of NSCLC. Further, these data suggest that investigation of this subset of lung cancers with respect to newer generation inhibitors of MAPK and mTOR signalling pathways may provide a new opportunity for investigation of targeted therapeutics in this common adult malignancy.

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