Tyrosine kinase inhibitors are effective treatments for non-small-cell lung cancers (NSCLCs) with epidermal growth factor receptor (EGFR) mutations. However, relapse typically occurs after an average of 1 year of continuous treatment. A fundamental histological transformation from NSCLC to small-cell lung cancer (SCLC) is observed in a subset of the resistant cancers, but the molecular changes associated with this transformation remain unknown. Analysis of tumour samples and cell lines derived from resistant EGFR mutant patients revealed that Retinoblastoma (RB) is lost in 100% of these SCLC transformed cases, but rarely in those that remain NSCLC. Further, increased neuroendocrine marker and decreased EGFR expression as well as greater sensitivity to BCL2 family inhibition are observed in resistant SCLC transformed cancers compared with resistant NSCLCs. Together, these findings suggest that this subset of resistant cancers ultimately adopt many of the molecular and phenotypic characteristics of classical SCLC.
The tyrosine kinase inhibitors (TKIs) gefitinib, erlotinib and afatinib are effective therapies for non-small-cell lung cancers (NSCLCs) harbouring activating mutations in the epidermal growth factor receptor (EGFR). The majority of these patients achieve robust responses, with marked tumour shrinkage, abatement of symptoms and improved outcome compared with chemotherapy. Despite initial efficacy, resistance to TKIs invariably develops, with disease progression after an average of approximately 12 months. The implementation of repeat biopsy programmes at the time of clinically apparent resistance has been instrumental to the understanding of the molecular mechanisms underlying acquired resistance to EGFR TKIs. We previously reported the results of a cohort of patients undergoing repeat biopsy in which we identified secondary mutations in EGFR (T790M), amplification of the MET receptor tyrosine kinase and mutations in PIK3CA, all of which confer resistance to TKI via reactivation of key downstream signalling pathways. In addition, a subset of resistant tumours underwent phenotypic/histological changes, namely transformation to small-cell lung cancer (SCLC) and epithelial-to-mesenchymal transition. Importantly, the tumours that transformed to SCLC harboured the original activating EGFR mutation, suggesting direct evolution from the initial cancer, rather than a distinct, second primary cancer. The phenomenon of SCLC transformation in resistant EGFR mutant cancers had been previously identified in individual patient case reports and has subsequently been confirmed in another repeat biopsy patient cohort. However, the molecular details underlying this histological change and resistance to EGFR TKI therapy, as well as the relatedness of EGFR mutant SCLC to classical SCLC, remain unclear. Here, we characterize the molecular changes that occur in NSCLC to SCLC transformed TKI-resistant EGFR mutant cancers. Our results indicate that SCLC transformed resistant cancers take on many features of classical SCLC, including universal alterations to the Rb tumour suppressor, gene expression profiles similar to classical SCLC, which include reduced or absent EGFR expression, and heightened sensitivity to BCL-2 family inhibition.

Results
Transformed SCLC RNA profiles mimic classical SCLC. To perform these analyses, we amassed a collection of 11 EGFR mutant cancer samples (from nine patients) that underwent transformation to SCLC at the time of acquired resistance to EGFR TKI therapy under the auspices of an institutional review board (IRB)-approved protocol (Supplementary Table 1). As reported previously, all of the resistant SCLC cancers harboured the original activating EGFR mutation. Cell lines derived from resistant patient biopsies have been valuable tools to study acquired resistance to TKIs in lung cancer and, and two such models (MGH131-1 and MGH131-2) were derived from two different biopsies (taken several months apart) of an erlotinib-resistant patient whose cancer had transformed to SCLC (Patient #6, Supplementary Table 1). Before erlotinib, this patient’s cancer had NSCLC histology. As expected, these biopsy-derived cell lines continue to harbour the EGFR exon 19 deletion mutation in a majority of EGFR alleles (variant allele frequency ~60% for both cell lines) indicating that most, if not all, of the cells are EGFR mutation positive. Histological analyses of xenograft tumours derived from these cell lines confirmed SCLC histology and expression of neuroendocrine (NE) markers in contrast to xenograft tumours derived from a resistant EGFR mutant cancer that maintained NSCLC histology (Fig. 1a). Hierarchical clustering analysis of RNA expression revealed that the two cell lines derived from a resistant EGFR mutant SCLC more closely resembled classical SCLC cell lines (including expression of NE markers) than cell lines derived from resistant EGFR mutant NSCLCs (Fig. 1b,c and Supplementary Fig. 1a,b). In addition, we profiled the expression of ten microRNAs (miRNAs) that had been previously identified to be the most differentially regulated between adenocarcinoma and SCLC cell lines. The expression pattern of both the MGH131-1 and MGH131-2 cell lines more closely resembled classical SCLCs (Supplementary Fig. 1c). Notably, the MGH131-1 cells expressed miRNA that were also expressed in NSCLC. The MGH131-1 cells more closely resemble the mesenchymal subtype of SCLC described by Berns and colleagues (E-cadherin low, Vimentin high, less positive for NE markers, more adherent growth in culture) than the MGH131-2 cells (Supplementary Fig. 1d). However, altogether, these findings reveal that the EGFR mutant SCLC transformed cells resemble classical SCLC with respect to mRNA and miRNA expression.

Resistant transformed SCLCs lose EGFR expression. We next tested the MGH131-1 and MGH131-2 cells for their sensitivity to EGFR TKIs. Cell viability assays indicated that both SCLC transformed cell lines were highly resistant to gefitinib as well as the third-generation EGFR inhibitor, WZ4002, which effectively inhibits both activating mutations and the T790M resistance mutation (Fig. 2a)19. In contrast, a patient-derived resistant cell line that retained NSCLC histology and had a T790M mutation (MGH121) was exquisitely sensitive to WZ4002 (Fig. 2a). Thus, the EGFR mutant SCLC cell lines retain resistance to EGFR inhibition, similar to what is observed clinically.

To understand why SCLC transformed cells are insensitive to EGFR TKIs despite continued presence of the EGFR activating mutation, we measured the levels of EGFR to determine if transformation to SCLC had resulted in altered expression. Western blotting revealed an absence of EGFR expression specifically in the EGFR mutant SCLC transformed cell lines (Fig. 2b). To determine whether EGFR expression is commonly lost in EGFR mutant lung cancers that transform to SCLC, we performed IHC analysis on seven resistant cases of EGFR mutant cancers that had transformed to SCLC along with ten cases that retained NSCLC histology. As shown in Fig. 2c,d, there was a marked decrease in EGFR expression in the SCLC resistant tumours compared with baseline, but EGFR expression was intact in resistant EGFR mutant NSCLCs. Indeed, interrogation of the expression data from the cancer cell line encyclopedia database revealed that classical SCLC cell lines have significantly reduced levels of EGFR mRNA compared with adenocarcinoma cell lines (Supplementary Fig. 2a). Similarly, SCLC transformed EGFR mutant-resistant cell lines had lower levels of EGFR mRNA compared with NSCLC-resistant models (Supplementary Fig. 2b). These data suggest that SCLC transformed EGFR mutant cancers lose expression of EGFR, as is typical of classical SCLC, and thus it is not surprising that they are no longer sensitive to EGFR inhibition.

SCLC transformed cell lines are sensitive to ABT-263. The BCL-2, BCL-XL inhibitor, ABT-263, is one of the few therapies to date to exhibit marked efficacy against SCLC in laboratory studies, and although recent results from clinical trials with single-agent ABT-263 demonstrated responses in only a minority of SCLC patients, combinations with this agent are being explored. SCLC transformed EGFR mutant cells were highly sensitive to single-agent ABT-263 and markedly more sensitive than EGFR-TKI-resistant NSCLC cell lines harbouring the T790M resistance mutation (Fig. 2e). ABT-263 treatment induced a robust apoptotic response in EGFR mutant
SCLC compared with the resistant EGFR mutant NSCLC (Supplementary Fig. 2c). We next compared the IC50 values of ABT-263 in the SCLC transformed cell lines to a panel of 21 classical SCLC cell lines, and found that MGH131-1 and MGH131-2 were among the most sensitive to ABT-263 (Fig. 2f). Indeed, ABT-263 was significantly more active than gefitinib in MGH131-1 and MGH131-2 cells (Supplementary Fig. 2d). These results underscore the potential of ABT-263 as part of combination strategy to treat EGFR mutant patients with NSCLC to SCLC transformation. In total, the gene expression and drug sensitivity of the SCLC transformed cells more closely resembles classical SCLC than EGFR mutant NSCLC. These data are further supported by the clinical observations that EGFR mutant SCLCs are highly sensitive to SCLC chemotherapy regimens7.

DNA sequencing reveals genetic lesions specific to resistant SCLC. In our previous report7, we described a patient (Patient #7) who had been biopsied multiple times over the course of their disease. In this patient, both EGFR mutant adenocarcinoma and SCLC had been observed at different times. This patient ultimately passed away, and at autopsy, both SCLC and NSCLC were identified (Fig. 3a). The oscillating pattern of adenocarcinoma and SCLC that was observed suggested that different clones were selected depending on the selective pressure of the applied treatment (conceptual schematic shown in Supplementary Fig. 3a). The autopsy that was performed across a panel of cell lines derived from TKI-resistant patients (n = 10). NCI-H82 and NCI-H446 are classical SCLC cell lines used as controls for NE marker expression. Red indicates lower expression and blue indicates higher expression.

Figure 1 | SCLC transformed cell lines exhibit neuroendocrine (NE) features. (a) Haematoxylin and eosin (H&E) staining and IHC for NE markers chromogranin and synaptophysin were performed on xenografts derived from EGFR mutant MGH131-2 SCLC and MGH156 NSCLC cells. (b) EGFR mutation status, TKI sensitivity and resistance mechanism for the patient-derived cell lines analysed in c. (c) Gene expression array data of NE marker expression across a panel of cell lines derived from TKI-resistant patients (n = 10). NCI-H82 and NCI-H446 are classical SCLC cell lines used as controls for NE marker expression. Red indicates lower expression and blue indicates higher expression.

| Cell line | EGFR Mutation | EGFR TKI Status | Resistance Mechanism |
|-----------|----------------|-----------------|----------------------|
| MGH119    | Exon 19 del    | Sensitive       | NA                   |
| MGH119-R  | Exon 19 del    | Resistant       | T790M                |
| MGH121    | Exon 19 del    | Resistant       | T790M                |
| MGH125    | L858R          | Resistant       | T790M/EMT            |
| MGH126    | Exon 19 del    | Resistant       | EMT                  |
| MGH134    | L858R          | Resistant       | T790M                |
| MGH141    | Exon 19 del    | Resistant       | T790M                |
| MGH157    | Exon 19 del    | Resistant       | T790M                |
| MGH131-1  | Exon 19 del    | Resistant       | NSCLC --> SCLC       |
| MGH131-2* | Exon 19 del    | Resistant       | NSCLC --> SCLC       |
| NCI-H82   | None           | Insensitive     | NA                   |
| NCI-H446  | None           | Insensitive     | NA                   |

* Patient 6

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In the liver SCLC tumour, comparative genomic hybridization (CGH) array analysis revealed that there was a relatively large deletion in one copy of \(RB1\) that encompassed the entire gene and the surrounding region. This was accompanied by a focal deletion in the second copy that spanned only the middle exons of \(RB1\) but spared the beginning and end of the gene (Fig. 4a). These deletions were not observed in the resistant cancer with a T790M mutation and NSCLC histology. These results were confirmed by quantitative PCR (qPCR) of different exons of \(RB1\), which also demonstrated similar focal loss of \(RB1\) in the lung SCLC (Fig. 4b).

**RB is universally lost in resistant SCLC patients.** The cell lines established from biopsies of resistant \(EGFR\) mutant lung cancers were assessed for \(RB\) expression. Western blotting revealed loss of \(RB\) expression specifically in resistant \(EGFR\) mutant cell lines with
SCLC histology (Fig. 4c). Notably, the MGH125 cell line (patient #8) also lacks RB expression. This cell line was generated from a pleural effusion, which demonstrated NSCLC histology, however, a previous liver biopsy of this patient’s cancer revealed a metastatic lesion that had transformed to SCLC (Supplementary Fig. 4a). Thus, this cancer was particularly prone to SCLC transformation. Array CGH analysis revealed a focal deletion of both copies of RB1 in the MGH131-1 SCLC cell line (Fig. 4d). However, only one copy of RB1 was lost in the MGH125 cells (Supplementary Fig. 4b). Sequencing of RB1 from MGH125 cells revealed that the intact copy of RB1 harboured a nonsense mutation (R445*, Supplementary Fig. 4c), explaining the absence of RB protein expression in these cells (Fig. 4c). Thus, cell lines derived from cancers that either have transformed into SCLC or derived from tumours prone to transform into SCLC both demonstrated genetic loss of RB1.

To expand these analyses, we examined the collection of 10 EGFR mutant cancer samples (from 9 patients) that underwent transformation to SCLC at the time of acquired resistance as well as the 11 resistant controls that had maintained NSCLC histology (Supplementary Table 1). In one of the SCLC transformed cases, Patient #1, we had sufficient sample from two resistant lesions to harvest DNA and assess the RB1 locus by array CGH. Concordant with the findings from Patient #7, there was a bi-allelic loss with one relatively large deletion and a second highly focal deletion in both resistant SCLC samples (Fig. 4e).

Because we did not have sufficient tissue from the remaining samples to perform genetic analyses, we developed an immuno-histochemistry (IHC) assay to examine RB expression in the larger cohort of EGFR mutant, SCLC transformed samples. IHC has some potential advantages for determining RB status: (i) IHC requires minimal tumour material, which is a common obstacle in these clinical samples, (ii) RB deficiency is detected even when there is loss due to mechanisms other than bi-allelic deletion, such as nonsense mutations and (iii) direct visualization of individual cells allows precise interpretation in cases that contain a large proportion of stroma, which may confound next-generation sequencing (NGS) and CGH array analyses. Control experiments confirmed the robustness of the IHC assay. For example, it accurately detected strong expression in RB-positive tumours, weak RB expression in tumours with reduced levels mediated by short hairpin RNA (shRNA) knockdown and an absence of RB in tumours with dual copy loss (Supplementary Fig. 5). IHC analyses were completed on ten resistant EGFR mutant SCLC samples and revealed complete loss of RB expression in all cases (Fig. 5a, b and Table 1). As a control, RB IHC was performed on the 11 resistant tumours that remained NSCLC. RB expression was intact in all but one sample. These data reveal selective loss of RB expression in EGFR mutant lung cancers that transform to SCLC upon the development of resistance (P < .0001, Fisher’s exact test). Thus, EGFR mutant lung cancers that transform to SCLC invariably lose RB expression, similar to classical SCLC. In total, these findings suggest that chronic EGFR inhibition in EGFR mutant lung adenocarcinomas can lead to the development of cancers that adopt the genetic, histologic, expression and drug sensitivity profiles of classical SCLC.

The universal nature of the RB loss is suggestive that this may be a necessary event for the SCLC-resistant tumours to emerge. Although RB is lost in classical SCLC, it is not known if RB loss is necessary for NE differentiation or the growth and survival of cells that have differentiated along a NE lineage. It is notable that shRNA-mediated depletion of RB in gefitinib-sensitive NSCLC cell lines did not alter the sensitivity to gefitinib (Supplementary Fig. 6a). Furthermore, generating TKI-resistance in-vitro or in-
vivo in EGFR mutant cancer cell lines engineered to have loss of RB expression did not yield resistant cells/tumours with acquisition of NE marker expression or SCLC morphology (Supplementary Fig. 6b,c). These results suggest that loss of RB is likely necessary in order for acquired resistance via transformation to SCLC to develop, but it is not sufficient on its own to promote it. The latter point is further supported by our discovery of a few examples of RB-deficient adenocarcinomas. Indeed, two erlotinib-resistant cell lines (MGH125 and MGH141), a resistant patient sample (Patient #10) and two out of four pre-treatment adenocarcinoma samples from patients whose cancers transformed to SCLC (Patients #2 and #6), were also negative for RB. Although rare, the existence of these RB-deficient adenocarcinomas serves as further evidence that loss of RB alone is insufficient to promote transformation to SCLC.

Discussion

Acquired resistance is a major problem limiting the clinical efficacy of targeted therapies. Repeat biopsy studies have led to the identification of the resistance mechanism in a majority of EGFR mutant NSCLC patients that have progressed on EGFR TKIs7,13. One unexpected finding from these studies was the discovery that 5–15% of patient tumours undergo transformation to SCLC histology upon acquisition of resistance. From a historic perspective of lung cancer classification, this observation was a surprise, as differentiation into a NSCLC- or SCLC-type cancer was thought to occur early in tumorigenesis. Furthermore, the typical presentation of these diseases were quite distinct, with EGFR-mutant adenocarcinoma occurring primarily in never-smokers and displaying a more indolent natural history compared with classical SCLC, which occurs almost exclusively
in heavy smokers and tends to metastasize early and grow rapidly. Indeed, the SCLC transition seen in EGFRTK-mutant patients is often accompanied by a change in the clinical behaviour of the disease, with rapid acceleration in the growth rate, initial responsiveness to therapy followed by rapid clinical deterioration. However, repeat biopsy studies have consistently suggested that the SCLC transformed cancers represent an evolution from the initial adenocarcinoma rather than a second coincident cancer, because the activating driver EGFRTK mutations are identical to the original adenocarcinomas in all cases. To date, the mechanistic details regarding this transition are unknown. This study revealed genetic changes specifically associated with the transformation to SCLC, provided insight into why these tumours are no longer sensitive to EGFR TKIs, and associated with the transformation to SCLC, provided insight into the explanations for the lack of sensitivity of these cancers to TKI. We speculate that, upon transformation to SCLC, they take on many of the characteristics of classical SCLC, which normally do not express EGFR or rely on its activity for growth and survival. Thus, the treatment strategies that will provide the most benefit to this subset of cancers will likely resemble those that are most effective for classical SCLC.

Our data reveal that EGFRTK mutant cancers that transform to SCLC also undergo significant epigenetic changes. Hierarchical clustering analysis of gene expression data demonstrated that cell lines derived from SCLC transformed resistant biopsies share gene expression profiles more closely related to classical SCLC cell lines than other TKI-resistant cell lines that maintained NSCLC histology. Similarly, microRNA analyses revealed that SCLC transformed cells express miRNAs that are commonly upregulated in classical SCLC. It is notable, however, that the SCLC transformed cells also express a subset of miRNAs that are typically expressed in adenocarcinoma but not SCLC. Furthermore, DNA methylation analysis of resistant SCLC tumours from patient # 7 revealed a methylation pattern more consistent with adenocarcinoma than SCLC (Supplementary Fig. 8).

The finding that all EGFRTK mutant SCLC transformed samples have low/absent EGFRTK expression compared with TKI-resistant controls provides insight into the explanations for the lack of sensitivity of these cancers to TKI. We speculate that, upon transformation to SCLC, they take on many of the characteristics of classical SCLC, which normally do not express EGFR or rely on its activity for growth and survival. Thus, the treatment strategies that will provide the most benefit to this subset of cancers will likely resemble those that are most effective for classical SCLC.

EGFR activity has been associated with promoting alveolar differentiation (Supplementary Fig. 7). Following treatment with EGFRTKI, the resistant pluripotent cells, which may have accumulated additional genetic alterations (such as loss of RB1 and TP53) and maintain a different epigenetic state, are able to differentiate and subsequently expand along a lineage (SCLC) that does not require EGFR signalling (Supplementary Fig. 7). It is also interesting to note that the absence of EGFR signalling induced by the TKI may remove the impetus to differentiate along the NSCLC lineage, thereby facilitating differentiation along the other lineage. Along these lines, there have been case reports of treatment naive EGFRTK mutant SCLC, reinforcing the notion that the cell of origin of EGFRTK mutant lung cancers may have the potential to differentiate along a NE lineage. Notably, we assessed one such case (Patient #19, Table 1), and this cancer had loss of RB and EGFR expression, similar to the cases of EGFRTK mutant SCLC observed in the setting of acquired resistance to EGFRTKI.

We cannot rule out that EGFRTK mutant SCLC pre-existed before treatment with the EGFRTKI. We have carefully reviewed the histology of these samples and we do not observe a mix of NSCLC and SCLC histology in the pre-treatment tumours. Of course, this does not rule out the possibility that a very small percentage of SCLC cells that are below our detection limit do pre-exist (especially, as the biopsies only sample a minute fraction of the patients’ total cancer burden). However, from a clinical perspective, we feel that it is unlikely that these SCLCs were present from the onset of the disease in a majority of these cases because when the SCLC surfaces in the clinic, it progresses quite rapidly (like classical SCLC). In many of these cases, the TKI-induced remissions last for years and then suddenly the patient develops explosive SCLC. It seems unlikely (but, not impossible) that the same explosive cancer was present for all of those years while the patients were in remission. In such cases, we favour a model in which the cells that survived treatment undergo further ‘evolution’ to become the bona fide SCLC that ultimately presents in the clinic (as described above and shown in Supplementary Fig. 7).

The data from this study reveals the key molecular changes associated with EGFRTK mutant lung adenocarcinomas that...
transform to SCLC upon acquisition of resistance to EGFR TKI. As novel therapeutic approaches that inhibit EGFR more efficiently are widely implemented30–32, we speculate that the relative novelty of these approaches may contribute to the development of resistance in SCLC upon acquisition of resistance to EGFR TKI. As a result, the relative novelty of these approaches may contribute to the development of resistance in SCLC upon acquisition of resistance to EGFR TKI.

**Table 1 | RB status of TKI-resistant patients.**

| Patient | Cancer type | Resistance | Histology | RB status | Detection method |
|---------|-------------|------------|-----------|-----------|-----------------|
| 1       | Lung        | Pre Adeno  | Pos       | IHC       |                 |
| 2       | Lung        | Post NE    | Neg       | IHC/ genetic |                 |
| 3       | Lung        | Pre Adeno  | Pos       | IHC       |                 |
| 4       | Lung        | Post NE    | Neg       | IHC       |                 |
| 5       | Lung        | Post NE    | Neg       | IHC       |                 |
| 6       | Lung        | Post NE    | Neg       | IHC/ genetic* |               |
| 7       | Lung        | Post Adeno | Pos       | IHC/ genetic |                 |
| 8       | Lung        | Post NE    | Neg       | IHC/ genetic |                 |
| 9       | Lung        | Pre Adeno  | Pos       | IHC       |                 |
| 10      | Lung        | Post Adeno | Neg       | IHC       |                 |
| 11      | Lung        | Pre Adeno  | Pos       | IHC       |                 |
| 12      | Lung        | Pre Adeno  | Pos       | IHC       |                 |
| 13      | Lung        | Post Adeno | Pos       | IHC       |                 |
| 14      | Lung        | Pre Adeno  | Pos       | IHC       |                 |
| 15      | Lung        | Post Adeno | Pos       | IHC       |                 |
| 16      | Lung        | Pre Adeno  | Pos       | IHC       |                 |
| 17      | Lung        | Post Adeno | Pos       | IHC       |                 |
| 18      | Lung        | Post Adeno | Pos       | IHC       |                 |
| 19      | Lung        | Intrinsically NE | Neg       | IHC     |                 |

EGFR, epidermal growth factor receptor; IHC, immunohistochemistry; NE, neuroendocrine carcinoma; Neg, negative; Pos, positive; TKI, tyrosine kinase inhibitor.

*RB status in pre/post-TKI-resistant EGFR mutant lung cancers. EGFR TKI sensitivity, histology, RB expression and the detection method are listed for tumours from nine patients with resistant small-cell lung cancer (SCLC) and nine patients with resistant non-small-cell lung cancer. Patient 19 presented with classical SCLC with an EGFR mutant classical NE carcinoma and failed to respond to TKI.

The total RB IHC (Rabbit monoclonal Abcam catalogue no. ab22393) was used for each sample to serve as a loading control. A standard curve with normal female genetic DNA was generated for each primer pair in order to compare the tumour/cell line samples to a normal diploid sample.

**DNA extraction library construction and WES.** Genomic DNA (gDNA) from normal liver, diaphragmatic tumour (NSCLC), lung tumour (SCLC) and liver tumour (SCLC) from patient 7 was extracted from OCT-embedded frozen tissue blocks using the DNAAdvantage kit from Agencourt. Three micrograms of gDNA from each sample were fragmented to approximately 150–200 bp by sonication and subjected to exome enrichment using the SureSelect12. Human All Exon Target Enrichment System. Barcoded deep sequencing libraries for the exome-enriched gDNA fragments were constructed using Applied Biosystems SOLiD 5500 Fragment Library Core Kit. WES was performed with an Applied Biosystems SOLiD 5500 deep sequencer to generate paired-end colour space reads (50 nucleotides forward and 35 nucleotides reverse) by a multiplexed operation. The colour-space data were aligned to the human hg19 reference genome sequence by the Applied Biosystems LifeScope software to generate BAM files. Mutation calls were made using the muTect calling software.

**Quantiﬁcation of RB1 gDNA levels by qPCR.** RB1 gene copy number was measured via a quantitative PCR assay that has been previously described34. Briefly, reaction samples containing 10 ng of gDNA with SYBR green master mix (Roche) were run on a LightCycler 480 (Roche) for quantification. Primer pairs amplifying exons 1 (5′-TGGGAGATGAGAGGAGTTCA-3′) and 35 (5′-TGGTGGATGGCGGCAGTGTGGG-3′) were used to identify genome-wide copy number alterations. Briefly, 1 μg of tumour and control DNA (normal female gDNA, Cornell Institute) were heated to 95°C for 5 min. Random priming was used to label DNA with Cy3-DUTP (control) and Cy5-DUTP (tumour) dyes from the Agilent SureTag DNA Labeling kit. The labelled DNA was then purified over columns (Agilent) and mixed in equal proportion along with Cot-1 Human DNA (Agilent) for the hybridization steps. To hybridize the DNA to the array, incubation occurred first at 95°C for 3 min for denaturation, followed by a 30-min pre-hybridization step at 37°C and then a hybridization step for 35–40 h at 37°C. Slides were then heated to 95°C for 3 min and cooled to 4°C. Paraformaldehyde ArrayCGH wash buffer 1 for 5 min at room temperature and wash buffer 2 for 1 min at 37°C. Upon completion of the washes, slides were scanned using the G2505C Microarray Scanner (Agilent). The data were analysed using the Agilent Cytogenomics software v 2.0. CGH array data are available at GEO under accession number GSE64765 (super-series GSE64766).

**Immunohistochemistry.** RB: The total RB IHC (Rabbit monoclonal Abcam #E182, #5500) was performed on formalin-fixed, paraffin-embedded tissue sections using the Leica RX Bond Autostainer (Leica Biosystems). The sections cut 4–5 μm were baked off-line for 30 min in a 60°C oven and then loaded onto the machine. The machine then de-waxed and hydrated online. Antigen retrieval was performed in EN 2 (Citrate buffer) for 20 min and stained using the Bond Polymer Refine Detection Kit. The IHC protocol was performed with a 5 min Peroside Block, a 15-min antibody/labeler incubation, an 8-min post primary incubation, an 8-min polymer incubation, a 10-min DAB (diaminobenzidine) incubation and a 5-min AEC chromogen incubation.
5-min haematoxylin incubation. Slides were then dehydrated, cleared, cover slipped and scored by a pathologist. IHC for EGFR was performed using EGFR D38B1 antibody (Cell Signaling #4267, 1:50 dilution in SignalStain Antibody Diluent) according to the manufacturer’s protocol. EGFR expression was evaluated using H score: 3× percentage of tumour cells with high staining + 2× percentage of tumour cells with intermediate staining + 1× percentage of tumour cells with low staining, giving a range of 0–300. The expression in the normal bronchial epithelium was considered as a standard for a score of 2.

**Gene expression analysis.** RNA from the MGH119, MGH119-R, MGH121, MGH126, MGH134, MGH141, MGH157, MGH131-1 and MGH131-2 was isolated using the RNeasy kit (Qiagen). One microgram of RNA was submitted to the Dana Farber Cancer Institute Microarray Core Facility and was hybridized onto Affymetrix Human U133plus DNA microarrays using standard procedures. Expression data in the form of CEL files were obtained (ten samples). In parallel, cells were cultured in gefitinib-containing media containing polybrene (8 μg/ml) and infected with virus diluted 1 to 4 in media containing polybrene (8 μg/ml). Viral constructs expressing shRNA targeting GFP and RB (targeting sequence—5′-GGTTGTGTC-3′) were obtained from Dr Nick Dyson and production and production and delivery of lentiviral vectors were carried out as described previously. Briefly, 293T cells were transduced with vesicular stomatitis virus G (VSVG) pseudotyped vectors and infected with virus diluted 1 to 4 in media containing polybrene (8 μg/ml). Following incubation with drug for the indicated concentration/time, CellTiter-Glo assay was used to quantify the luciferase activity and to calculate the percentage of tumour cells with high staining.

**Cell viability assays.** Cell viability assays were carried out as a 96-well format with at least four replicates per condition. Cells were plated at a density of 2,000–4,000 cells per well depending on their respective size and growth rates: MGH125-2,000, MGH126-1,000, MGH134-1,000, MGH141-2,000 and the rest at 3,000 cells per well. Following incubation with drug for the indicated concentration/time, CellTitre-Glo assay reagent (Promega) was added for 10 min and plates were read on a Centro LB960 microplate luminometer (Berthold Technologies).

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Author contributions

M.I.N. designed and performed the experiments, analysed the data and wrote the manuscript. J.A.E. and L.V.S. collected patient samples, designed experiments, analysed data and wrote the manuscript. M.M.K. performed pathological analysis, designed experiments and analysed data. J.T.P., E.L.I., A.R.G., R.K.C., H.E.M., L.A.B., F.M. and N.M. performed the experiments and analysed data. C.H.M. and K.N.R. analysed data. T.M., E.H. and L.E.F. coordinated patient sample collection and testing. P.A.V. performed pathological analysis. D.B.C. collected patient samples. P.A.I. and C.M.R. collected patient samples and analysed data. D.R.B., S.R., T.S., A.J.I. and G.G. carried out data analysis.

Additional information

Accession codes: Accession codes for data sets are as follows: microarray and array CGH are at GEO (GSE64322, GSE64765, super-series GSE64766) and WES is at European Genomics Association (EGAS0000100102).

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Competing financial interests: J.A.E. is a consultant for Novartis, Sanofi-Aventis, Genentech and Astra Zeneca; owns equity in Gatekeeper Pharmaceuticals, which has interest in T790M inhibitors; is a Scientific Advisory Board member for Sanofi-Aventis; has research agreements with Novartis, Sanofi-Aventis and Astra Zeneca. A.J.I. is a consultant for Pfizer and Bioreference Laboratories. P.A.I. is a consultant for AstraZeneca, Boehringer Ingelheim, Chugai Pharma, Clovis, Genentech, Merrimack Pharmaceuticals, Pfizer and Sanofi; owns stock in Gatekeeper Pharmaceutical; receives other remuneration from LabCorp. C.M.R. has been a recent consultant for AbbVie, Biodexis, Boehringer Ingelheim, Glaxo Smith Kline and Merck regarding cancer drug development. The remaining authors declare no competing financial interests.

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