Low-frequency *KRAS* mutations are prevalent in lung adenocarcinomas

Aim: This study quantified low-frequency *KRAS* mutations in normal lung and lung adenocarcinomas, to understand their potential significance in the development of acquired resistance to EGFR-targeted therapies. **Materials & Methods:** Allele-specific Competitive Blocker-PCR was used to quantify *KRAS* codon 12 GAT (G12D) and GTT (G12V) mutation in 19 normal lung and 21 lung adenocarcinoma samples. **Results:** Lung adenocarcinomas had *KRAS* codon 12 GAT and GTT geometric mean mutant fractions of 1.94×10^{-4} and 1.16×10^{-3} , respectively. For 76.2% of lung adenocarcinomas, the level of *KRAS* mutation was greater than the upper 95% confidence interval of that in normal lung. **Conclusion:** *KRAS* mutant tumor subpopulations, not detectable by DNA sequencing, may drive resistance to EGFR blockade in lung adenocarcinoma patients.

Keywords: carcinogenesis • epidermal growth factor receptor • mutation • mutation detection • non-small-cell lung cancer • oncogene • oncogene-induced senescence • personalized medicine • polyclonal tumor origin • targeted molecular therapy

The dramatic expansion in gene-targeted and whole genome sequencing has improved our understanding of cancer genomes. The somatic mutations responsible for the development of lung adenocarcinomas have been identified and collected in publicly accessible databases, including the Catalogue of Somatic Mutations in Cancer (COSMIC) [1,2] and The Cancer Genome Atlas (TCGA) [3–6]. Information regarding the prevalence of potentially actionable mutations has guided the development of approaches for personalized cancer treatments [7–9]. These large-scale sequencing efforts have established that individual lung adenocarcinomas encompass large numbers of somatic mutations and copy number variations [8,10], as well as considerable intra-tumoral heterogeneity [7,11]. Tumor heterogeneity is now recognized as a major obstacle to personalized cancer treatment because it contributes to the development of acquired resistance to therapy [9,12].

The availability of molecularly targeted therapeutics has afforded non-small-cell lung

cancer (NSCLC) patients new treatment options [13,14]. Gefitinib and erlotinib represent two well-studied examples of small-molecule inhibitors of the intracellular phosphotyrosine-kinase domain of the EGFR, which have had significant clinical impact [15,16]. The efficacy of these tyrosine kinase inhibitors (TKIs) has been associated with specific genetic alterations in a patient's tumor. Specifically, mutations in the tyrosine kinase domain of the *EGFR* (predominately exon 19 deletions and point mutations in exon 21) predict response to these therapies [13,17–19].

Unfortunately, other specific tumor mutations have been associated with resistance to therapy, either primary or secondary (acquired resistance). In NSCLCs, *KRAS* mutation may cause primary resistance to EGFR blockade [20,21], as does *MET* amplification [13]. Linardou *et al.* [20] concluded from a meta-analysis of clinical studies assessing *KRAS* mutation as a biomarker of response that the overall low sensitivity observed in NSCLC patients suggests that resistance to EGFR TKIs occurs in a substantial number of patients with essentially

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wild-type *KRAS*. Because virtually all NSCLC patients who initially respond to EGFR blockade develop resistance to treatment, acquired/secondary resistance is a major obstacle to the development of effective personalized treatment of NSCLC patients [22–26]. In NSCLC patients, secondary mutation at *EGFR* codon 790 alters the binding site for TKIs and accounts for approximately 50–60% of acquired drug resistance, with *MET* amplification observed in approximately 5% of cases [27,28]. Mutations in the *RAS/RAF1/MAPK1* and *PIK3CA/AKT1* pathways have been implicated in secondary resistance to EGFR blockade [13,29]. But, in 30% of NSCLCs that develop resistance to EGFR TKIs, no secondary resistance mutations are detected [13,30]. This observation is consistent with the possibility that undetected mutant subpopulations may play a role in acquired resistance to EGFR blockade.

For advanced colorectal cancer (aCRC), it has been established that mutations in oncogenes of the mitogen-activated protein (MAP) kinase pathway predict lack of response to therapies that target the EGFR (e.g., cetuximab, and panitumumab), with mutation in *KRAS* recognized as the most frequently detected mutational driver of resistance [31]. In addition, a study by Diaz *et al.* [12] established that minor *KRAS* mutant clones, not detected by the DxS assay (QIAGEN Manchester Ltd., Manchester, UK) preexisted in aCRCs, and emergence/outgrowth of these *KRAS* mutant clones occurred predictably with EGFR blockade, driving acquired resistance to panitumumab. The prevalence of undetected *KRAS* mutant subpopulations in NSCLCs has not been investigated systematically, so the extent to which such subpopulations may contribute to the development of resistance to EGFR-targeted therapy is currently unknown. Using combination treatments to target minor mutant subpopulations in addition to predominant clones may lead to more durable patient responses.

Characterization of somatic point mutations in tumor subpopulations requires a more sensitive approach than standard Sanger sequencing, pyrosequencing, whole exome or whole genome sequencing. Sanger sequencing and pyrosequencing are capable of detecting point mutations present in approximately 10% of a DNA sample (range 5–20%, or allele frequencies of 0.05–0.2) [32,33]. An analysis of base calling algorithms and whole exome sequencing data (employing 100× coverage) concluded that existing tools missed the majority of somatic single nucleotide variants at low allelic frequencies (i.e., <0.3) [34]. Targeted deep-sequencing has the potential to detect single-nucleotide variants in subclonal tumor cell populations [35], but the sensitivity would depend on the specific methodologies employed. A reconstruction

experiment designed to characterize the tumor subclone resolution achievable by next-generation sequencing (NGS) reported that *KRAS* variant frequencies of 10, 5, 2.5 and 1% could be detected using sequencing depths of 100, 250, 500 and 1000 X, respectively [36]. Because subclonal populations of tumor cells carrying resistance-conferring point mutations may occur at lower variant frequencies than those detected by NGS, sensitive mutation detection methods may provide information that complements high-throughput sequencing data, in terms of identifying appropriate strategies for personalized cancer treatment.

A challenge associated with quantifying mutations at high sensitivity is discerning the biological significance of different levels of mutation. In the current study, therefore, quantification of mutation in normal lung and lung adenocarcinoma tissue was undertaken as an approach to ascertain the levels of mutation associated with a cancerous phenotype [37,38]. *KRAS* mutations have been detected in normal colon, pancreas, thyroid and lung tissues using high-sensitivity mutation detection methods [37–39]. The detection of *KRAS* mutation in normal tissue has been interpreted as meaning that *KRAS* mutation alone is not sufficient to initiate carcinogenesis. This conclusion is supported by studies showing that the expression of activated *KRAS* alone is not sufficient to induce cellular transformation of most cell types [40,41]. Consequently, it could be hypothesized that *KRAS* mutant tumor populations larger than those present in normal lung may be necessary to drive the development of a therapy-resistant tumor phenotype.

Allele-specific competitive blocker PCR (ACB-PCR) is a DNA-based mutation detection method that can quantify specific base pair substitutions present in DNA samples with allele frequencies as low as 1 mutant per 100,000 wild-type sequences [37,38,42–44]. Unlike approaches for mutation detection that report the presence or absence of mutation, the output of ACB-PCR is a measured mutant fraction (MF), which is the ratio of mutant allele to wild-type allele within a given DNA sample. Another technical advantage of ACB-PCR is that each assay includes a set of internal standards to confirm its sensitivity. Using the ACB-PCR method, we previously showed that *KRAS* mutant cells are present as tumor subpopulations in colon [37] and thyroid tumors [38], at levels not detectable by DNA sequencing. In the current study, we utilized the ACB-PCR approach to quantify *KRAS* codon 12 GGT to GAT mutation (*KRAS* G12D) and GGT to GTT mutation (*KRAS* G12V) in 19 normal lung samples and 25 lung tumors. We report that a majority of lung adenocarcinomas possess a level of *KRAS* mutation that exceeds the upper 95th confidence interval of that present in normal lung.

Materials & methods

Normal lung & lung tumor samples

Procedures for the acquisition and analysis of anonymous human tissues were reviewed by the FDA Research Involving Human Subjects Committee (RIHSC, FWA 00006196). Nineteen fresh-frozen normal lung samples, collected at the time of autopsy, were purchased from the National Disease Research Interchange (NDRI; PA, USA). Tissue donors died from causes other than cancer or lung disease. Twenty-one fresh-frozen primary non-small-cell lung adenocarcinomas were provided by the National Cancer Institute's, Cooperative Human Tissue Network (CHTN). Tumors from chemotherapy and radiation therapy naïve donors were requested and no evidence of chemotherapy and radiation therapy was evident in the chart reviews provided. One each squamous cell carcinoma, mesothelioma, poorly differentiated carcinoma and inflammatory myofibroblastic tumor were purchased from NDRI. All tumor specimens were histologically evaluated by board certified pathologists, who confirmed the original diagnosis.

DNA isolation

DNAs from normal lung and lung tumors were isolated on separate days, employing precautions to limit potential cross-contamination. Tissues were homogenized using an Omni THQ tissue homogenizer (Omni International, GA, USA) and 6 ml of proteinase K buffer (1 mg/ml proteinase K, 100 mM NaCl, 25 mM EDTA [pH 8], and 1% SDS) per gram of tissue. Samples were incubated approximately 16 h at 37°C, and extracted with an equal volume of a 25:24:1 phenol/chloroform/isoamyl alcohol mixture. Samples were resuspended in 200 µl of RNase buffer, comprised of 10 mg/ml RNase A (Sigma, MO, USA), 4.2 units/µl ribonuclease T1 (Sigma), 100 mM sodium acetate and 50 mM Tris-HCl (pH 8), incubated approximately 16 h at 37°C, and then extracted as described above. The DNA was ethanol precipitated and resuspended in TE buffer (5 mM Tris, 0.5 mM EDTA, pH 7.5). Finally, all genomic DNAs were restriction digested, phenol/chloroform/isoamyl alcohol extracted, ethanol-precipitated and resuspended in TE buffer at a final concentration of approximately 500 ng/µl.

First-round PCR

High-fidelity, first-round PCR reactions were performed using 1 µg of digested normal lung or lung tumor genomic DNA as template. Each 200 µl PCR reaction contained: 10 mM KCl, 10 mM (NH₄)₂SO₄, 20 mM Tris-HCl (pH 8.75), 2 mM MgSO₄, 0.1% Triton X-100, 0.1 mg/ml bovine serum albumin, 0.2 mM dNTPs, 0.2 µM RD1

(5'-TTAAGCGTCGATGGAGGAGTT-3'), 0.2 µM RD2 (5'-GTCCTGCACCAAGTAATATGC-3') and four units of cloned *PfuUltra* Hotstart DNA Polymerase (Agilent Technologies, CA, USA). The first-round PCR included a 2 min denaturation at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 56°C and 1 min at 72°C and a final 7 min extension at 72°C. The 384 bp *KRAS* PCR product (which included sequence 5' of exon 1, exon 1 and part of intron 1) was isolated following preparative agarose gel electrophoresis using a GeneClean Spin Kit (MP Biomedicals, Solon, OH), eluted with TE buffer and frozen as multiple single-use aliquots. The concentration of each DNA sample was determined by repeated measurement using an Epoch Spectrophotometer (BioTek, Winooski, VT). Final concentrations were calculated from three determinations that varied by ≤10% from the group mean.

Allele-specific competitive blocker PCR

ACB-PCR is an allele-specific PCR amplification method for mutation quantification. ACB-PCR quantification relies upon parallel analyses of a set of MF standards and first-round PCR products generated from unknown samples, all containing equal numbers of *KRAS* molecules [45]. Mutant (codon 12 GAT and GTT) and wild-type (codon 12 GGT) standards were prepared by digestion of cloned mutant or wild-type plasmid DNA with Afl II and Ava II and isolation of a DNA segment identical in sequence composition to the first-round PCR product prepared from isolated lung DNA samples.

Purified mutant and wild-type reference DNAs were mixed to generate standards with MFs of 10⁻¹, 10⁻², 10⁻³, 10⁻⁴, 10⁻⁵ and 0 (the '0' control contains only the wild-type *KRAS* codon 12 sequence and defines the technical background of the assay), at a concentration of 5 × 10⁷ copies/µl. Each ACB-PCR reaction incorporated 10 µl of each DNA mixture, for a total of 5 × 10⁸ *KRAS* copies per reaction. Each MF standard was analyzed in duplicate, along with a no-DNA control. The ACB-PCR was performed in a 96-well PCR plate (Thermo Fisher Scientific, MA, USA) using a DNA Engine thermal cycler (Bio-Rad, CA, USA).

For measurement of *KRAS* codon 12 GAT MF by ACB-PCR, each 50 µl reaction contained: 1X Stoffel buffer (10 mM KCl, 10 mM Tris-HCl, pH 8.3), 1.6 mM MgCl₂, 0.1 mg/ml gelatin, 1.0 mg/ml Triton X-100, 80 µM dNTPs, 20 mU PerfectMatch PCR Enhancer (Agilent Technologies), 500 nM primer P4 (5'-GATTACCTCTATTGTTGGA-3'), 500 nM MSP-A (5'-fluorescein-CTTGTGGTAGTTGGAGCTTA-3') and 475 nM BP-A (5'-CTTGTGGTAGTTGGAGCTTdG-3'). Each ACB-PCR included 3.33 U

of a hotstart formulation of Stoffel DNA polymerase, which was prepared by incubating *Taq* DNA polymerase Stoffel fragment (Life Technologies, NY, USA) with Platinum *Taq* antibody (Life Technologies) at a 1:1 unit ratio for 30 min on ice. Cycling conditions for the *KRAS* GAT ACB-PCR were 36 cycles of 30 s at 94°C, 45 s at 45°C and 1 min at 72°C.

For measurement of *KRAS* codon 12 GTT MF by ACB-PCR, each 50 µl reaction contained: 1X Stoffel buffer, 1.5 mM MgCl₂, 0.1 mg/ml gelatin, 1.0 mg/ml Triton X-100, 40 µM dNTPs, 90 mU PerfectMatch PCR Enhancer, 400 nM primer P3 (5'-GTTGGATCATATTCGTCCAC-3'), 400 nM MSP-T (5'-fluorescein-CTTGTGGTAGTTGGAGCTAT-3') and 400 nM BP-T (5'-CTTGTGGTAGTTGGAGCTAdG-3'). Each ACB-PCR included 3.33 U of the hotstart formulation of Stoffel DNA polymerase. Cycling conditions for the *KRAS* GTT ACB-PCR were 36 cycles of 30 s at 94°C, 45 s at 41°C and 1 min at 72°C.

Gel electrophoresis, image analysis & data collection

Equal volumes of ACB-PCR products were analyzed on nondenaturing 8% polyacrylamide gels. The fluorescent bands were visualized using a PharosFX Molecular Imager with an external blue laser (Bio-Rad). Pixel intensities of the bands (GAT ACB-PCR, 103 bp; GTT ACB-PCR, 89 bp) were quantified using Quantity One software and a locally averaged background correction (Bio-Rad). Log-linear plots relating MF to fluorescence (in pixels) were constructed and fit with a logarithmic function. For the GAT MF measurements, the average coefficient of determination (r^2) for the standard curves was 0.9936 ($n = 9$, range 0.9876–0.9965). For the GTT MF measurements, the average coefficient of determination for the standard curves was 0.9945 ($n = 9$, range 0.9905–0.9973). This function was then used to calculate the MF in each unknown sample based on the fluorescence of its ACB-PCR product [45].

Statistical analyses

The *KRAS* MF for each sample was calculated as the arithmetic average of three independent MF measurements. The average MF measurement for each sample was log₁₀-transformed. The geomean MF for mutation in each tissue type was calculated as the average log₁₀-transformed MF for each group. Log₁₀-transformed data were used for statistical analyses. Because some values were below the 10⁻⁵ level necessary for accurate ACB-PCR quantification and not all log-transformed data were normally distributed, comparisons between two groups were performed using Mann–Whitney rank sum test. Correlation analysis was performed on available data using the Spearman's rank order correlation coefficient test. Two-tailed p-values of less than 0.05 were considered significant. All statistical analyses were performed using GraphPad Prism 5 Software (GraphPad Software, Inc., CA, USA).

Results

This study focused on the quantification of *KRAS* codon 12 GGT to GAT and GGT to GTT base substitution mutations because these are the most prevalent *KRAS* mutations across tumor types. This work is part of a broad initiative to characterize the levels of these mutations in a panel of normal human tissues and tumors. In the current study, ACB-PCR was used to measure *KRAS* codon 12 GAT (G12D) and GTT (G12V) MFs in 19 normal lung samples and 25 lung tumors (21 primary lung adenocarcinomas, one squamous cell carcinoma, one mesothelioma, one poorly differentiated carcinoma and one inflammatory myofibroblastic tumor). Each unknown sample was quantified in three independent experiments, by interpolation of fluorescent intensities of unknown samples with that of a standard curve constructed using samples with defined ratios of mutant:wild type alleles (i.e., duplicate 10⁻¹, 10⁻², 10⁻³, 10⁻⁴, 10⁻⁵ and 0 standards). Representative gel images of the *KRAS* codon 12 GTT ACB-PCR analyses of normal lung and lung adenocarcinomas are

Figure 1. Allele-specific competitive blocker PCR measurement of *KRAS* codon 12 mutant fractions in normal lung and lung adenocarcinomas. Representative polyacrylamide gel images of three independent experiments. The 89 bp *KRAS* codon 12 GTT Allele-specific competitive blocker PCR products generated from mutant fraction standards (10⁻¹, 10⁻², 10⁻³, 10⁻⁴ and 10⁻⁵), no mutant control (0), normal lung (samples 10–19) and lung adenocarcinomas (samples 10–20) are shown. Exp: Experiment.

shown in Figure 1. The *KRAS* codon 12 GAT and GTT MF measurements of normal lung and lung tumor samples generated a dataset of 264 individual ACB-PCR measurements on the 44 samples. The average coefficient of variation for the triplicate *KRAS* codon 12 GAT and GTT MF measurements were 0.32 and 0.29, respectively. The average *KRAS* codon 12 GAT and GTT MF measurements are plotted in Figure 2.

KRAS MF in normal lung tissue

The normal lung samples were derived from ten men and nine women aged 41–87 years old (65.11 ± 16.23 years old, mean $\pm$ SD), including 18 Caucasians and one African American. A summary of the clinical features of each normal lung samples and their corresponding *KRAS* codon 12 GAT and GTT MFs are given in

Table 1. Seventeen of 19 and 16/19 normal lung samples had MFs greater than the lowest standard employed (i.e., $\geq 10^{-5}$) for the *KRAS* codon 12 GAT and GTT mutations, respectively. The *KRAS* codon 12 GAT geometric mean MF for normal lung was 3.25×10^{-5} (median MF, 2.90×10^{-5} ; range, 7.95×10^{-6} to 3.96×10^{-4}). The *KRAS* codon 12 GTT geometric mean MF for normal lung was 3.15×10^{-5} (median MF, 2.56×10^{-5} ; range 6.19×10^{-6} to 2.52×10^{-4}). Levels of *KRAS* codon 12 GAT and GTT mutations in normal lung were not significantly different (two-tailed *t*-test; $p = 0.93$). For normal lung, no significant correlations were found between *KRAS* codon 12 MFs and either age, gender or smoking history. The relationship between *KRAS* codon 12 MF and age is depicted in Supplementary Figure 1 (see online at: www.futuremedicine.com/doi/full/10.2217/PME.14.69).

Figure 2. *KRAS* codon 12 mutant fraction measurements for each normal lung and lung tumor sample. Circles denote the mean *KRAS* codon 12 GAT MF and the squares denote the mean *KRAS* codon 12 GTT mutant fraction, calculated from three independent allele-specific competitive blocker-PCR measurements. Error bars indicate SD of the mean. The dashed and solid horizontal lines indicate the upper 95th confidence interval of the mutant fraction present in normal lung tissue for the *KRAS* codon 12 GAT and GTT mutations, respectively. Samples analyzed included normal lung ($n = 19$), lung adenocarcinomas ($n = 21$) and other lung tumors ($n = 4$); in the order of a squamous cell carcinoma, mesothelioma, poorly differentiated carcinoma/neuroendocrine tumor and inflammatory myofibroblastic tumor).

MF: Mutant fraction (the ratio of mutant: wild-type DNA copies in a given sample).

Table 1. Clinical features of normal lung tissues and their corresponding <i>KRAS</i> codon 12 GAT and GTT mutant fractions.				
ID	Sex/age	Ever-smoker	<i>KRAS</i> GAT MF	<i>KRAS</i> GTT MF
1	F/87	N	8.86×10^{-6}	9.58×10^{-6}
2	M/71	Y	1.26×10^{-5}	6.19×10^{-6}
3	M/70	-	7.95×10^{-6}	7.40×10^{-6}
4	F/86	Y	1.83×10^{-5}	3.95×10^{-5}
5	M/70	N	1.40×10^{-5}	2.56×10^{-5}
6	M/74	N	1.51×10^{-5}	2.52×10^{-4}
7	M/41	Y	2.38×10^{-5}	3.49×10^{-5}
8	F/48	Y	1.26×10^{-5}	1.21×10^{-5}
9	M/64	Y	2.11×10^{-5}	1.99×10^{-5}
10	M/48	Y	1.51×10^{-4}	1.91×10^{-4}
11	F/86	Y	5.98×10^{-5}	2.68×10^{-5}
12	F/78	Y	4.84×10^{-5}	3.05×10^{-5}
13	M/79	Y	2.90×10^{-5}	2.51×10^{-5}
14	M/44	Y	4.87×10^{-5}	4.66×10^{-5}
15	M/43	N	3.39×10^{-5}	1.76×10^{-5}
16	F/79	N	1.05×10^{-4}	2.07×10^{-5}
17	F/70	N	5.16×10^{-5}	2.44×10^{-5}
18	F/44	Y	3.96×10^{-4}	1.84×10^{-4}
19	F/55	Y	5.45×10^{-5}	1.06×10^{-4}
MF: Mutant fraction.				

***KRAS* mutant fraction in lung adenocarcinomas**
In total, 25 lung tumors (21 lung adenocarcinomas, one squamous cell carcinoma, one mesothelioma, one poorly differentiated carcinoma and one inflammatory myofibroblastic tumor) were analyzed for *KRAS* codon 12 GAT and GTT mutation. A summary of the pathological and clinical features of each lung tumor and their corresponding *KRAS* codon 12 GAT and GTT MFs are given in Table 2. Only data obtained from lung adenocarcinomas were considered in the statistical analyses. The lung adenocarcinoma samples were derived from 13 men and 8 women aged 37–89 years old (65.67 ± 12.58 years old, mean $\pm$ SD). Nineteen were Caucasian, two African American. All lung adenocarcinomas had measurable levels ($\geq 10^{-5}$) of both *KRAS* codon 12 mutations. The *KRAS* codon 12 GAT and GTT geometric mean MFs of the lung adenocarcinomas were 1.94×10^{-4} and 1.16×10^{-3} , respectively. The frequency distributions for *KRAS* codon 12 GAT and GTT MFs measured in normal lung and lung adenocarcinomas (along with the individual measurements) are shown in Figure 3. Both *KRAS* codon 12 GAT and GTT MFs were significantly greater in lung adenocarcinomas as compared with the normal lung (two-tailed, Mann–Whitney rank sum test;

$p = 0.0065$ and $p = 0.0005$, for codon 12 GAT and GTT, respectively).
Patients were stratified as ever- or never-smokers, because the detailed information necessary to calculate pack years was not available for every subject. All 13 male lung adenocarcinomas were from ever-smokers. Of the eight female lung adenocarcinomas, five were ever-smokers and three were never-smokers. The *KRAS* codon 12 GAT and GTT MFs in lung adenocarcinomas were analyzed for associations with gender and smoking status (see Supplementary Figure 2). The analysis indicates an association between male gender and *KRAS* codon 12 GTT MF because: lung adenocarcinomas from men carried significantly greater *KRAS* codon 12 GTT MFs than *KRAS* codon 12 GAT MFs (two-tailed Mann–Whitney rank sum test; $p = 0.0159$) and lung adenocarcinomas from male ever-smokers had significantly higher GTT MFs than lung adenocarcinomas from female ever-smokers (two-tailed Mann–Whitney rank sum test; $p = 0.0137$, see Figure S2). Interestingly, the adenocarcinoma samples with the highest *KRAS* codon 12 GTT MFs were from male patients with a history of emphysema or chronic obstructive pulmonary disease (see adenocarcinoma samples 19 and 20, respectively).

Table 2. Pathological and clinical features of lung tumors and their corresponding *KRAS* codon 12 GAT and GTT mutant fractions.

ID	Sex/age	Ever-smoker	Stage; annotated pathological features	% tumor	% necrosis	Maximum dimension	LNM	<i>KRAS</i> GAT MF	<i>KRAS</i> GTT MF
Adenocarcinoma									
1	F/46	Y	IIb; poor. diff.	90	70	12.0	N	2.42 × 10 ⁻⁵	1.47 × 10 ⁻⁵
2	F/37	Y	IIIA; invasive, poor. diff.	70	20	3.5	Y	3.88 × 10 ⁻⁵	1.48 × 10 ⁻⁵
3	M/57	Y	IV; mod. diff.	100	60	5.0	N	1.02 × 10 ⁻⁵	1.19 × 10 ⁻¹
4	M/65	Y	IIb; invasive, poor. diff.	80	60	5.0	N	1.87 × 10 ⁻⁵	4.56 × 10 ⁻²
5 [†]	M/68	Y	IV; mod. diff., acinar and bronchioloalveolar type	80	50	1.7	Y	1.51 × 10 ⁻⁵	8.87 × 10 ⁻⁵
6	M/81	Y	IB; mod. diff.	70	60	4.0	N	4.05 × 10 ⁻⁵	1.16 × 10 ⁻¹
7	M/67	Y	IB; invasive, well to mod. diff., papillary and mucinous types w/ focal bronchioloalveolar features	70	30	2.8	N	5.51 × 10 ⁻⁵	3.33 × 10 ⁻⁵
8	M/78	Y	IB; mod. diff., prominent bronchioloalveolar features	80	0	4.5	-	7.06 × 10 ⁻⁵	2.81 × 10 ⁻⁵
9	F/61	Y	IIIA; mod. diff.	30	20	10.0	N	2.21 × 10 ⁻³	6.84 × 10 ⁻⁵
10	F/79	N	IB; well diff., mucinous with prominent bronchioloalveolar features	40	0	0.7	N	5.79 × 10 ⁻²	6.99 × 10 ⁻⁴
11 [†]	F/66	Y	IB; mod. diff.	80	0	3.7	N	5.27 × 10 ⁻³	2.53 × 10 ⁻⁵
12 ^{§,¶}	M/61	Y	IA; invasive, well diff.	30	0	2.0	N	4.70 × 10 ⁻⁴	8.02 × 10 ⁻⁴
13 [†]	F/79	N	IB; mod. diff.	40	0	4.5	N	6.29 × 10 ⁻⁴	9.00 × 10 ⁻⁵
14	M/62	Y	IIIA; invasive, poor. diff.	40	50	6.0	Y	5.85 × 10 ⁻²	1.28 × 10 ⁻²
15 [†]	M/89	Y	IB; invasive, mod. diff.	30	0	3.5	N	8.18 × 10 ⁻⁴	2.94 × 10 ⁻⁵
16 [†]	F/62	Y	IA; mod. diff., mixed type (papillary, acinar and nonmucinous bronchioloalveolar patterns)	15	0	2.0	N	1.37 × 10 ⁻⁴	2.68 × 10 ⁻⁴
17	M/63	Y	IIA; mod. diff., mixed acinar and papillary patterns	100	0	5.6	N	1.14 × 10 ⁻⁴	1.68 × 10 ⁻¹
18	M/80	Y	IB; invasive, mod. diff., mixed type (predominately nonmucinous bronchioloalveolar with acinar and papillary patterns)	95	0	2.8	N	6.20 × 10 ⁻⁵	8.24 × 10 ⁻⁴
19 [‡]	M/50	Y	IIIA; poor. diff.	70	70	6.6	Y	2.04 × 10 ⁻⁴	1.84 × 10 ⁻¹
20 ^{††}	M/69	Y	IIIA; well diff., predominately bronchioloalveolar type	100	0	9.5	N	2.25 × 10 ⁻⁵	4.75 × 10 ⁻¹
21 [†]	F/59	N	IB; poor. diff., mixed type (acinar and micropapillary)	50	5	3.8	N	6.44 × 10 ⁻⁵	1.92 × 10 ⁻²

[†]Sample from patient with history of NSCLC.
[‡]Sample from patient with history of breast cancer or ductal carcinoma *in situ*.
[§]Sample from patient with history of asthma.
[¶]Sample from patient with history of allergic bronchopulmonary aspergillosis.
^{‡‡}Sample from patient with history of chronic obstructive pulmonary disease.
^{††}Sample from patient with history of emphysema.
Geomean: Geometric mean; LNM: Lymph node metastasis; MF: Mutant fraction; mod. diff.: Moderately differentiated; NSCLC: Non-small-cell lung cancer; poor. diff.: Poorly differentiated; well diff.: Well differentiated.

Table 2. Pathological and clinical features of lung tumors and their corresponding KRAS codon 12 GAT and GTT mutant fractions (cont.).							
ID	Sex/age	Ever-smoker	Stage; annotated pathological features	% tumor	% necrosis	Maximum dimension	KRAS GAT MF KRAS GTT MF
Squamous cell carcinoma							
22	M/74	-	-	100	15	4.0	7.41 × 10 ⁻⁶ 8.25 × 10 ⁻⁶
Mesothelioma							
23	M/79	-	-	50	<1	-	7.85 × 10 ⁻⁶ 7.52 × 10 ⁻⁶
Poorly differentiated carcinoma/neuroendocrine							
24	F/74	-	-	80	30	2.2	8.27 × 10 ⁻⁶ 7.02 × 10 ⁻⁶
Inflammatory myofibroblastic							
25	F/71	-	-	100	0	-	7.25 × 10 ⁻⁶ 4.54 × 10 ⁻⁵
[†] Sample from patient with history of NSCLC. [‡] Sample from patient with history of breast cancer or ductal carcinoma <i>in situ</i> . [§] Sample from patient with history of asthma. Sample from patient with history of allergic bronchopulmonary aspergillosis. [¶] Sample from patient with history of chronic obstructive pulmonary disease. ^{**} Sample from patient with history of emphysema. Geomean: Geometric mean; LNM: Lymph node metastasis; MF: Mutant fraction; mod. diff.: Moderately differentiated; poor. diff.: Poorly differentiated; well diff.: Well differentiated.							

Additional correlative analyses were conducted for 11 parameters in lung adenocarcinomas using Spearman’s rank order correlation test (Table 3). With regard to *KRAS* codon 12 MFs, a few significant correlations were observed. *KRAS* codon 12 GAT was inversely associated with higher percent necrosis ($r = -0.45$, $p = 0.0404$) and percent tumor ($r = -0.65$, $p = 0.0015$). In addition, *KRAS* codon 12 GAT was inversely associated with tumor stage, although this association did not reach the $p < 0.05$ level of statistical significance ($r = -0.39$, $p = 0.0768$). Other significant associations include higher percent necrosis with loss of differentiation ($r = -0.65$, $p = 0.0013$) and advanced tumor stage ($r = 0.56$, $p = 0.0088$). Advanced tumor stage was associated with the presence of lymph node metastasis ($r = 0.60$, $p = 0.0054$) and increased maximum tumor dimension ($r = 0.48$, $p = 0.0262$), which likely reflects the staging guidelines established by the American Joint Committee on Cancer. Lung adenocarcinomas from young patients were more often advanced stage tumors ($r = -0.51$, $p = 0.0185$), consistent with a 2010 published analysis of the Surveillance, Epidemiology and End Results (SEER) database [46]. Age was inversely associated with percent necrosis ($r = -0.47$, $p = 0.0306$), and loss of differentiation, although this did not reach the $p < 0.05$ level of statistical significance ($r = -0.40$, $p = 0.0758$).

Discussion

Given the newly discovered importance of undetected mutant subpopulations in the development of resistance in aCRC patients treated with EGFR blockade, the present study analyzed whether low-frequency *KRAS* mutant subpopulations are present in lung adenocarcinomas. Our study employed a comparison between normal lung and lung tumors as an approach to identify biologically relevant levels of *KRAS* mutation. To our knowledge this is the first study to quantitatively define and statistically analyze the levels of *KRAS* codon 12 GAT and GTT mutations in both normal lung tissues (from individuals without lung cancer) and lung adenocarcinomas.

A number of studies employing sensitive mutation detection methods have detected the presence of *KRAS* mutation in normal tissues of lung cancer patients, with the results reported as the proportion of normal samples carrying *KRAS* mutation. Importantly, one study used the allele-specific mismatch amplification mutation assay to quantify *KRAS* codon 12 GTT (G12V) mutation in multiple sectors from seven normal tracheal–bronchial epithelia of healthy adults with no indication of lung pathology and observed a measurable level of *KRAS* codon 12 GTT mutation in 56% of sectors, with an average MF of 3.70×10^{-5} [39].

The current results confirm and extend this work, by showing 89.5% of normal lung samples have a *KRAS* codon 12 GAT or GTT MF $\geq 10^{-5}$, with the *KRAS* codon 12 GTT geometric mean MF measured in the current study (3.15×10^{-5}) in good agreement with that reported previously. Considering only the two *KRAS* mutations measured and assuming the mutations are heterozygous, it can be estimated that normal lung tissue contains at least one *KRAS* mutant cell per every 8,000 wild-type cells.

The presence of *KRAS* mutant cells in normal tissue is not counterintuitive, given that *KRAS* mutation alone is insufficient for cellular transformation and tumorigenesis in transgenic mouse models and *KRAS* mutation has been described as conditionally carcinogenic [40,41]. Recent studies demonstrated that oncogenic *Kras* is not constitutively active (i.e., locked in a permanent GTP bound state) [47]. In fact, mutant *Kras*, by itself, was shown to generate a level of Ras activity that does not pathologically alter most cells [48].

However, under conditions where endogenous expression of oncogenic *Kras* is augmented by upstream stimuli [47,48], oncogenic *Kras* undergoes prolonged activation and enhanced downstream signaling results in pathological disease and cancer [48]. This is in contrast to wild-type *Kras*, where upstream stimuli only induce transient effects. Taken together, this suggests that it is the level and strength of oncogenic *KRAS* activity (in conjunction with other genetic lesions, or proximate clones carrying complementary genetic lesions), not just the presence of mutant *KRAS* within a cell, that determines susceptibility to carcinogenesis [48,49].

Another interesting finding of our study is that *KRAS* MF does not increase with age. This observation is consistent with the work of Sudo *et al.* [39], who reported the lack of association between age and five point mutations, including *KRAS* codon 12 GTT and *TP53* mutations. The finding that *KRAS* mutation in normal lung does not increase with age is consistent with *KRAS* mutation potentially conferring either

Figure 3. Frequency distribution of the *KRAS* codon 12 GAT and GTT mutant fractions in normal lung and lung adenocarcinomas. For the box and whisker plots, the horizontal line indicates the median MF, the boxes indicate the 25th and 75th percentiles, and the error bars indicate the 5th and 95th percentiles. MF: Mutant fraction.

Table 3. Spearman's rank order correlation coefficients (ρ) of 11 parameters in lung adenocarcinomas.												
Variable	Mean (SD) or fraction of samples	KRAS GTT	KRAS GAT	Age	Sex	Ever- smoker	Stage	Differentiation	LNM	Maximum dimension	% tumor	% necrosis
KRAS GTT	1.16×10^{-3}	-	-0.19	0.01	0.49*	-0.03	0.26	-0.02	-0.02	0.26	0.27	0.15
KRAS GAT	1.94×10^{-4}		-	0.16	-0.31	-0.29	-0.39	0.17	-0.02	-0.09	-0.65**	-0.45*
Age	65.7 (12.6)			-	0.29	-0.16	-0.51*	0.40	-0.34	-0.35	-0.02	-0.47*
Sex (0 = female, 1 = male)	0.62 male				-	0.52*	0.19	0.11	0.15	0.06	0.36	0.16
Ever-smoker (0 = no, 1 = yes)	0.86 ever smoker					-	0.37	-0.09	0.21	0.21	0.30	0.26
Stage (1 = Stage I, 2 = Stage II, 3 = Stage III, 4 = Stage IV)	0.524 Stage I 0.143 Stage III 0.238 Stage IV 0.095 Stage IV						-	-0.24	0.60**	0.48*	0.37	0.56**
Differentiation (0 = poor, 1 = moderate, 3 = well)	0.333 poor 0.476 moderate 0.191 well						-	-	-0.42	-0.41	-0.05	-0.65**
LNM (0 = no, 1 = yes)	0.20 LNM								-	0.01	-0.02	0.41
Max. dimension	4.72 (2.85) cm									-	0.34	0.41
% tumor	0.65 (0.27)										-	0.19
% necrosis	0.24 (0.28)											-
*p ≤ 0.05												
**p ≤ 0.01												
*Geometric mean MF												
LNM: Lymph node metastasis; MF: Mutant fraction.												

positive or negative selective pressure, depending on cellular context and environmental cues. In the situation of oncogene-induced senescence, the proliferation of *KRAS* mutant cells is blocked. Nevertheless, senescent *KRAS* mutant cells may promote tumor growth through paracrine mechanisms, potentially explaining the prevalence of *KRAS* mutant tumor subclones. Dose-dependent transitions in levels of *Kras* mutation in ethylene oxide exposure mouse lung and the inverse correlation between *KRAS* MF and maximum thyroid and colon tumor dimension provide additional evidence that the fitness of *KRAS* mutant cells is context-dependent [22,50].

The standard approach for characterizing tumor mutations is DNA sequencing. *KRAS* is the third or sixth most frequently mutated gene in lung adenocarcinomas, according to the COSMIC or TCGA databases, respectively [2,3]. The frequencies of the different *KRAS* mutations in lung adenocarcinomas reported in the COSMIC database are depicted in Figure 4A. Only 3.32 and 4.07% of the lung adenocarcinomas in the COSMIC database reportedly carry a *KRAS* codon 12 GAT (G12D) or GTT (G12V) mutation, respectively [2]. Yet, using the sensitive ACB-PCR approach, we found all 21 adenocarcinomas examined had measurable levels ($\geq 10^{-5}$) of the *KRAS* codon 12 GAT and GTT mutations. Both *KRAS* codon 12 GAT and GTT MFs were significantly greater in lung adenocarcinomas than the corresponding MFs measured in the normal lung. Most importantly, 10/21 (47.6%) and 12/21 (57.1%) of lung adenocarcinomas had *KRAS* codon 12 GAT and GTT MFs greater

than the upper 95% confidence interval of that found in normal lung tissue (1.02×10^{-4} and 9.16×10^{-5} , respectively, see Figure 4B).

The current study was limited to the quantification of only two *KRAS* codon 12 mutations but found 16/21 (76.2%) of samples had a *KRAS* MF above the upper 95% confidence interval for the mutation in normal lung. The most common *KRAS* mutation detected in lung adenocarcinomas, codon 12 TGT (G12C), was not analyzed as part of the study. According to the COSMIC database, the G12C mutation accounts for 41% of reported *KRAS* mutations, while the G12D and G12V mutations together account for 38% of reported *KRAS* mutations [51]. In Figure 2, the shaded area indicates variant frequencies ≥ 0.1 , which reflects the lower limit on variant frequencies detected in the TCGA dataset (range 0.13–0.79) [3,6]. Because 11/21 (52.3%) of tumors depicted in Figure 2 had *KRAS* mutant allele frequencies above that of normal but below that detectable by DNA sequencing, it can be concluded that *KRAS* mutations are often present as subpopulations not detected by DNA sequencing. Extrapolation from the measurement of the two specific *KRAS* mutations measured by ACB-PCR (Figure 4B) to the totality of different *KRAS* mutations identified by DNA sequencing (Figure 4A) suggests that most, if not all, lung adenocarcinomas likely carry *KRAS* mutation at a level greater than that present in normal lung. Finally, the fact that 28% of the adenocarcinomas examined carried two different *KRAS* mutations, which is likely an underestimate given that only two mutations were analyzed, suggests that therapeutics that target one

Figure 4. The prevalence of *KRAS* point mutations in lung adenocarcinomas. (A) Frequency distribution of *KRAS* mutant adenocarcinomas reported in the COSMIC database (v68)[†] and **(B)** frequency distribution of *KRAS* G12D and G12V mutations (or both) in lung adenocarcinomas. Only lung adenocarcinomas with a *KRAS* mutation at a level greater than the upper 95% confidence interval of normal lung were considered mutant.

[†]Data taken from [2].

specific mutant form of the *KRAS* protein should not be employed as monotherapies.

Given the prevalence of *KRAS* mutant subpopulations in lung adenocarcinomas, it seems likely that these subpopulations are driving the development of secondary resistance to EGFR blockade, as has been shown for aCRC. However, other mechanisms of acquired resistance to EGFR TKIs have been suggested, including mutations in downstream effectors of EGFR signaling. Unlike aCRC, where there is definitive evidence that mutant *KRAS* is a predictive marker of resistance to anti-EGFR monoclonal antibodies (mAbs), the predictive value of *KRAS* in NSCLC is equivocal. Some studies report a lack of response [20,52], or even worse outcomes [53] for NSCLC patients who harbor *KRAS*-mutant tumors, while other studies report no influence of *KRAS* mutation on response or outcome to EGFR TKIs [17,19,54] or anti-EGFR monoclonal antibodies [55,56]. These apparent discrepancies may be due in part to the presence of undetected *KRAS* mutant subpopulations and/or the use of different methods for mutation detection, which vary in sensitivity [31]. In support of this idea, Marchetti *et al.* [52] demonstrated that patient response to EGFR TKIs was more accurately predicted using a more sensitive mutant-enriched sequencing method as compared with traditional, lower sensitivity PCR sequencing. The authors concluded that minor mutant clones influence patient outcome and that more sensitive mutation detection methods enable better predictions of patient response. Additional studies supporting the hypothesis that *KRAS* mutant subpopulations are impacting patient response to therapies directed at the EGFR have been reviewed [22].

KRAS mutations are more often found in lung tumors of smokers than lung tumors of nonsmokers. In lung adenocarcinomas, the most frequent mutations are G→T transversions, which have been associated with a history of smoking [57]. Consistent with that, we observed that the *KRAS* codon 12 GTT geomean MF was approximately 10-fold greater than the *KRAS* codon 12 GAT geomean MF in lung adenocarcinomas from a study population that was comprised primarily of ever-smokers (18/21). In addition, this study found a gender difference in *KRAS* codon 12 GTT MF, with significantly greater MFs in adenocarcinomas from male smokers as compared with female smokers (as well as all adenocarcinomas from females, see [Supplementary Figure 2](#)). This is consistent with a report by Dogan *et al.* [58] in which sequencing of 2529 lung adenocarcinomas revealed a greater percentage of *KRAS* G12V positive tumors in males versus females. Interestingly, high *KRAS* codon 12 GTT MFs were observed in samples of patients with a history of emphysema or

chronic obstructive pulmonary disease (see [Figure 2](#), samples 19 and 20). While this observation is only anecdotal, it may warrant investigation into the role of *KRAS* mutation in chronic inflammatory lung disease.

No significant association was found between *KRAS* codon 12 GAT or GTT MF and age, tumor size, stage, tumor differentiation, lymph node metastasis or smoking status. Surprisingly, *KRAS* codon 12 GAT mutation was inversely correlated with percent tumor cells and percent necrosis. Lung adenocarcinomas are comprised of multiple cell types and tissues including both viable and necrotic tumor cells, normal epithelial cells and stromal components such as fibroblasts, smooth muscle cells, vascular cells and inflammatory cells, all of which were represented in the DNA extracted and analyzed in this study. Thus, the *KRAS* codon 12 MFs quantified in the current study represent the entire tumor mass. The finding that greater *KRAS* codon 12 GAT MFs exist in lung adenocarcinomas with relatively lower tumor cell content suggests that the mutation may be present in cells other than those classified as tumor cells by histopathology. *KRAS* mutations have been found in atypical adenomatous hyperplasia, a precursor to certain lung adenocarcinomas, indicating they are early events in lung carcinogenesis [59]. Yet, *KRAS* mutation alone is insufficient for the complete transformation of cells and neoplastic growth [40,41]. Thus, it is possible that *KRAS* codon 12 GAT mutant cells within a tumor appear normal. The fact that *KRAS* codon 12 mutations have been observed frequently in normal tumor adjacent epithelia, which may or may not be the same *KRAS* mutation present in the lung adenocarcinoma cells, may provide additional support for this idea [60]. If true, this has clinical significance, because analyzing microdissected tumor samples by DNA sequencing (rather than considering a larger tumor sample using a high-sensitivity method) could potentially decrease the likelihood of *KRAS* mutation detection in clinical samples.

Conclusion

KRAS mutation is frequently present as undetected subpopulations of tumor cells in lung adenocarcinomas. Presently, it is unclear what impact these low-frequency mutant subpopulations may have on patient response to targeted therapies. Monotherapies directed against the bulk of a tumor have the potential to lead to the outgrowth of *KRAS* mutant subclones and subsequent relapse [12,31]. Furthermore, because so many lung adenocarcinomas carry *KRAS* mutations, therapies targeting *KRAS* mutant cells (directly or through downstream effectors) are needed for use in combination with therapies that target other specific pathways.

Future perspective

We expect that over the next 5–10 years evidence will continue to accumulate regarding the significance of minor mutant subpopulations in driving acquired resistance to targeted therapies. Studies incorporating serial biopsies and observing increases in minor mutant subpopulations with treatment will be critical in proving such subpopulations play an important role in the development of resistance. To accomplish this, more sensitive and quantitative analyses of tumor mutations than are currently available will need to be incorporated into clinical practice. In addition, future studies should address a limitation of the current study, which is that information regarding minor mutant subpopulations should be integrated with information regarding the genetic lesions in the predominant mutant clones.

Detection of tumor heterogeneity and sensitive molecular quantification of minor allelic fractions in tumor DNA are best achieved using fairly large amounts of DNA isolated from fresh or fresh-frozen

tissue. If the practice of oncology continues to evolve in the direction of making treatment decisions based on the molecular characteristics of a patient's tumor, rather than on tumor histology, this will require a shift from the current practice of performing genetic analyses using small quantities of formaline-fixed paraffin embedded material [31].

Although this work focused on the mechanism of undetected *KRAS* mutant subpopulations leading to relapse following treatment with EGFR-targeted therapies, mutations in signaling pathway components downstream from *KRAS* (such as *BRAF*, *MET*, *ERK*, *PIK3CA*, *PTEN*, *AKT* and *mTOR*) are undoubtedly associated with some portion of treatment failures [26]. This highlights the need for precise, quantitative information about which genetic lesions do and do not co-occur, including their occurrence as subpopulations. Going forward it will be important to learn whether other tumor mutations exist as variant subpopulations as frequently as has been shown for *KRAS*.

Executive summary

Background

- Acquired/secondary resistance is a major obstacle to the development of effective personalized treatment for NSCLC.
- In NSCLC, *KRAS* mutation may cause primary resistance to EGFR blockade.
- In aCRC, low frequency *KRAS* mutations drive the development of acquired resistance.

Materials & methods

- The ACB-PCR method was used to determine if NSCLCs carry low frequency *KRAS* mutations, which could potentially lead to the development of acquired resistance to EGFR blockade.
- ACB-PCR can quantify *KRAS* mutation when present at an allele frequency of 10^{-5} (one mutant allele per 100,000 wild-type alleles).

Results

- Normal lung tissue contains at least one *KRAS* mutant cell per 8,000 wild-type cells, which may underlie the prevalence of this gene as a driver of lung cancer.
- *KRAS* mutation in normal lung does not increase with age.
- ACB-PCR showed low frequency *KRAS* mutations are prevalent in NSCLCs.
- Abnormal levels of *KRAS* mutation were present in 76% of lung adenocarcinomas, whereas databases report 20–33% of lung adenocarcinomas are *KRAS* mutant.

Discussion

- A significant portion of the subclonal *KRAS* mutations cannot be detected by whole-genome sequencing and some are unlikely to be detected by targeted deep sequencing.
- Because the two mutations measured represent only approximately 38% of the *KRAS* mutations shown to occur in lung adenocarcinomas, it is likely that most, if not all, lung adenocarcinomas contain abnormal levels of *KRAS* mutant cells.
- *KRAS* mutation may lead to positive or negative selective pressure, depending on cellular context and environmental cues and negative selection pressure against *KRAS* mutant cells may explain their prevalence as mutant tumor subpopulations.

Future perspective

- Clinical studies assessing EGFR targeted therapies should quantify *KRAS* mutant allele frequencies in conjunction with clinical response.
- The time to relapse may be related to the level of *KRAS* mutation in a tumor at the time of treatment; without quantification this relationship will not be apparent.
- More sensitive methods than DNA sequencing will likely be required.
- It will be important to determine to what extent other mutational drivers of acquired resistance are prevalent as subclonal cell populations.

Given the focus on combination therapies, progress toward improving personalized cancer treatment will rely on the development of appropriate models. The task of combining promising therapies (potentially given using different relative and absolute doses and as alternating or simultaneous treatments) grows exponentially with the number of compounds to be tested [61]. This means that preclinical models are needed to prioritize such combination treatment for clinical investigations. In its 2010 draft “Guidance for Industry Codevelopment of Two or More Unmarketed Investigational Drugs for Use in Combination,” the US FDA indicates preclinical data may be used to demonstrate that two or more investigational drugs are appropriate for codevelopment because they have additive activity when used in combination [62]. The fact that tumor subpopulations may lead to relapse highlights the urgent need for preclinical models capable of reproducing the large amount of tumor heterogeneity present in tumors. In this regard, 3D tumor explant models may be a promising approach [63,64].

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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