Oncogene Mutations, Copy Number Gains and Mutant Allele Specific Imbalance (MASI) Frequently Occur Together in Tumor Cells

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Abstract

Background: Activating mutations in one allele of an oncogene (heterozygous mutations) are widely believed to be sufficient for tumorigenesis. However, mutant allele specific imbalance (MASI) has been observed in tumors and cell lines harboring mutations of oncogenes.

Methodology/Principal Findings: We determined 1) mutational status, 2) copy number gains (CNGs) and 3) relative ratio between mutant and wild type alleles of KRAS, BRAF, PIK3CA and EGFR genes by direct sequencing and quantitative PCR assay in over 400 human tumors, cell lines, and xenografts of lung, colorectal, and pancreatic cancers. Examination of a public database indicated that homozygous mutations of five oncogenes were frequent (20%) in 833 cell lines of 12 tumor types. Our data indicated two major forms of MASI: 1) MASI with CNG, either complete or partial; and 2) MASI without CNG (uniparental disomy; UPD), due to complete loss of wild type allele. MASI was a frequent event in mutant EGFR (75%) and was due mainly to CNGs, while MASI, also frequent in mutant KRAS (58%), was mainly due to UPD. Mutant: wild type allele ratios at the genomic level were precisely maintained after transcription. KRAS mutations or CNGs were significantly associated with increased ras GTPase activity, as measured by ELISA, and the two molecular changes were synergistic. Of 237 lung adenocarcinoma tumors, the small number with both KRAS mutation and CNG were associated with shortened survival.

Conclusions: MASI is frequently present in mutant EGFR and KRAS tumor cells, and is associated with increased mutant allele transcription and gene activity. The frequent finding of mutations, CNGs and MASI occurring together in tumor cells indicates that these three genetic alterations, acting together, may have a greater role in the development or maintenance of the malignant phenotype than any individual alteration.
lines harboring oncogenic mutations. As early as 1991, we reported that KRAS mutations in cancer cell lines frequently demonstrated complete or relative MASI [3] (Fig. 1b). In April 2004 just before the two initial major publications about activating mutations of epidermal growth factor receptor (EGFR) gene appeared [4,5], we examined a never smoker female with adenocarcinoma of the lung, and found a nine base pair deletion mutation in exon 19 of the EGFR gene (Fig. 1c). Even though the tumor had not been microdissected, the mutant allele appeared to be in great excess. More recently we noted the frequent presence of CNGs in tumor cells having mutant forms of the same genes [6].

Recent genome-wide approaches, especially high resolution single nucleotide polymorphism (SNP) arrays, enable evaluation of dynamic chromosomal as well as focal changes of CNG and loss of heterogeneity (LOH) with very high resolution. Within a few years, these assays have identified several novel lesions with amplification and/or LOH across several organs [7]. An important identification by SNP array was that uniparental disomy (UPD), which was originally described as a constitutional mechanism during meiosis [8], was frequently observed in several cancers [9,10,11,12]. UPD arises when an individual inherits two copies of a particular chromosome from the same parent [8]. The acquisition of UPD results in homozygosity for preexisting gene mutations with selective retention of the mutated allele. Acquired UPD in association with oncogenic mutations has been reported in hematopoietic malignancies including FLT3 and WT1 mutations in acute myeloid leukemia [10,13] and JAK2 mutations in myeloproliferative disorders [14,15]. To date, all reports of acquired UPD in solid tumors have been in association with the “two hit” inactivation of tumor suppressor genes [9,11,16].

EGFR pathway genes, including, EGFR, KRAS, BRAF, and PIK3CA genes, are well-investigated oncogenes in many tumors including lung, colorectal (CRC), and pancreatic cancers (PAC) [6,17,18,19]. Activating RAS mutations, including KRAS, are the most frequent oncogenic mutations present in human tumors, detected in about 20% of non-small-cell lung cancer (NSCLC), 40% of CRC and over 90% of PAC [19]. BRAF and PIK3CA genes are also activated by mutations in CRC [17,18,20] and occasionally in lung cancers [21,22]. Activating mutations of EGFR gene are present in 15–30% of NSCLC while they have been rarely detected in other type of human cancers [23,24]. EGFR CNGs were also reported in NSCLC and may play a role in response and survival to tyrosine kinase inhibitor therapy [6,25,26] while KRAS CNGs have not been investigated in depth in clinical tumors including NSCLCs. Taken together, the inter-relationship between mutations, CNGs and MASI is complex. The goal of the

Figure 1. Mutant allele specific imbalance (MASI) and some earlier observations. a) types of MASI. Three major types of MASI may occur. b) Complete MASI of KRAS gene as identified in 1991. We reported KRAS mutations in non-small-cell lung cancer (NSCLC) cell lines using restriction fragment length polymorphism (RFLP) method which can digest only wild type (WT) allele. We made this figure using modified methodologies from the original publication [3]. Three out of four KRAS mutant NSCLC lines showed homozygous mutations (complete MASI) of KRAS codon 12. NT, no treatment of restriction enzyme; +, presence of treatment of restriction enzyme. c) Our first EGFR mutation (exon 19 deletion) showed that the mutant allele was in great excess compared to the WT allele. WT, wild type.

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present study is to better understand the complex interrelationships between mutations, CNVs and MASI, and to clarify the biological and clinical significance of these oncogenic alterations.

**Materials and Methods**

**Frequency of homozygous mutation from the Sanger Institute public database**

We queried the zygosity status of 11 well-known and frequently mutated genes including six tumor suppressor genes (TP53, CDKN2A, PTEN, RB1, APC, and SMAD) and five oncogenes (KRAS, BRAF, PIK3CA, NRAS, and EGFR) tested in 833 cell lines from the database of the Cancer Genome Project, Sanger Institute, Cambridge, UK (www.sanger.ac.uk). We limited our examination to genes having relatively large numbers of mutations (>30) but also included the EGFR gene (7 mutations) which forms the basis of much of our work. Because of stromal cell contamination in clinical tumor samples, we limited our examination to tumor cell lines. We downloaded the free database of mutational status and zygosity status for each gene (on April 8th 2009). Zygosity status of each mutation was determined at the Institute by manual examination of the sequencing electropherograms (response to our query, Sanger #80248). We calculated the frequency of homozygosity for each of the 11 genes and for the entire oncogene or tumor suppressor groups.

**Cell lines**

We studied 114 tumor cell lines of lung cancer (n = 85), CRC (n = 19) or PAC (n = 10) origin. The details of each line are shown in Table S1. The origins of the lung lines have already been described [6,22]. We also investigated six human bronchial epithelial cell lines (HBEC lines 2KT, 3KT, 5KT, 15KT, 17KT, and 21KT), which were initiated by us [27,28]. All CRC and PAC lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA).

All cell lines were proven to have individual genetic origins by the Powerplex 1.2 system (Promega, Madison, WI) and, when available, corresponded with their original profiles as obtained from the ATCC.

**Tumor Samples**

We studied 393 tumors of NSCLC (n = 333) or CRC (n = 60) origin (Table S2a–d). DNAs from 269 NSCLC tumors from patients undergoing surgical resection in Japan, the United States or Australia having known EGFR or KRAS mutations and survival data were selected from a larger set of previously studied resected NSCLC [22,23,29]. In addition, we studied 45 DNA samples of resected lung adenocarcinomas from British Columbia Cancer Agency, Vancouver, Canada which had been studied by SNP arrays. An additional 19 resected NSCLC cases were obtained from the University of British Columbia at British Columbia Cancer Center, Vancouver, Canada [30].

**DNA and RNA extraction**

Genomic DNAs were isolated from cell lines, frozen tumors or paraffin embedded tumors (in 19 cases from Tartu University) by standard phenol-chloroform extraction [31] or by using DNeasy Tissue Kit (Qiagen, Valencia, CA, USA). Total RNAs were extracted from cell lines using RNAeasy Plus Mini Kit (Qiagen). cDNA was prepared by reverse transcription of RNA using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s protocol.

**Detection of gene mutations by direct sequencing**

We determined the mutational status of KRAS (mKRAS), BRAF (mBRAF), PIK3CA (mPIK3CA), PIK3B (mPIK3B), and EGFR (mEGFR) genes by direct sequencing as described previously [22,23] and PCR conditions are provided in Table S3. Briefly, genomic DNA or cDNA was amplified by conventional PCR. All PCR products were incubated with exonuclease I and shrimp alkaline phosphatase (Amersham Bioscience Corp., Piscataway, NJ) and sequenced directly using the Applied Biosystems PRISM dye terminator cycle sequencing method (Perkin-Elmer Corp., Foster City, CA). All sequence variants were confirmed by sequencing the products of independent PCR reactions in both directions.

**Quantification of relative ratio between mutant and wild type alleles by direct sequencing**

We quantified the relative ratios between mutant (mA) and wild type (wA) alleles by direct sequencing to determine the percent of the mutant allele (mA%) by three steps (Fig 2a): 1) magnification of electropherogram on computer screen using Finch TV software (http://www.geospiza.com/finchtv.html) which can provide sharp wave lines without boldness after maximization, 2) pixel based wave peak heights measurement using a desktop ruler software, MB-Ruler (http://www.markus-bader.de/MB-Ruler/), and 3) calculation of mA%. For point mutations, we used the following formula: mA% = Hmut/(Hmut + Hwt) (%), where Hmut is the minimum distance between midpoint of mutant wave line at peak and midpoint of baseline, and Hwt is the minimum distance between midpoint of wild type wave line at peak and midpoint of baseline. For deletion or insertion types of mutations, we used the average of mA% of the first five different waves from the beginning of mutations (Figure S1). We repeated the sequencing if the first sequencing electropherogram demonstrated high background noise.

**Plasmids construction and plasmid mixture experiment**

In order to validate mA% detected by direct sequencing, we constructed each mutant or wild type pCR2.1-TOPO plasmid from cell lines harboring 14 kinds of mutations using TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA) and QIAprep Miniprep Kit (Qiagen). We mixed mutant plasmid with corresponding wild type plasmid at various ratios and amplified the mixed plasmid as a template of PCR using paired primer sets for mutational analyses. PCR products were directly sequenced and the mA% were determined by measurement of sequencing electropherograms. Finally, we confirmed the linearity between...
the actual mixed proportion of mutant and wild type plasmids and mA% detected by direct sequencing (Fig. 2b).

Quantification of relative ratio between mutant and wild type alleles by sub-cloning

PCR products were cloned into pCR2.1-TOPO vector using TOPO TA cloning kit (Invitrogen). About 20 clones (range 15–25) were randomly selected for sequencing using either M13 forward primer or corresponding primers of each gene. mA% was calculated as the percentage of mutant clones in the total number cloned.

Quantification of relative ratio between mutant and wild type alleles by restriction fragment length polymorphism (RFLP)

Genomic DNAs from mutant samples were amplified by PCR using corresponding primers which we have previously reported (Table S3) [3,32,33]. While mA of EGFR exon 19 deletion type mutations could be distinguished from wA based on 9 to 12 base pairs differences, overnight digestion of PCR products was needed for point mutations using appropriate enzymes which can specifically digest wild type sequences (Figures S2a and b). After 12.5% polyacrylamide gel electrophoresis, the gel was stained with ethidium bromide. Band intensity of the respective mA and wA was calculated using Kodak Image Station 2000RT and Kodak 1D Image Analysis Software (Kodak, Rochester, NY) and mA% was determined from these ratios. We also confirmed that multiple control samples (wild type) were completely digested in every assay (Figures S2a and b).

Analyses of copy number by quantitative PCR assay

CNGs of KRAS, EGFR, BRAF and PIK3CA genes were determined by real-time quantitative PCR (qPCR) assay using Power SYBR® Green PCR Master Mix (Applied Biosystems) as previously reported (primer sequences are provided in Table S3) [22]. Briefly, we used LINE-1 gene, which is the most abundant autonomous retrotransposon in the human consisting of 17% of the genome [34], as a reference gene for all copy number analyses. Gene dosage of each target and reference gene was calculated using the standard curve method. Relative copy number of each sample was determined by comparing the ratio of target gene to LINE-1 in each sample with the ratio of these genes in normal human genomic DNA (EMD Biosciences, Darmstadt, Germany), made from a mixture of human blood cells from six to eight
different donors, as a diploid control. Based on our previous study [6], we defined CNG in cell lines as values greater than four.

**Single nucleotide polymorphism (SNP) array and data processing**

Samples were analyzed using the Genome-Wide Human SNP Array 6.0 platform (Affymetrix Inc., Santa Clara, CA) according to the manufacturer’s directions. GeneChip Command Console Software (GCCOS) was used to generate feature extracted intensity (.CEL) files which were subsequently processed using the Birdseed v2 algorithm in Genotyping Console 3.0.2 to create genotype (.cph) call files.

**Analysis of copy number and allelic imbalance by SNP array**

Copy number and allele status were determined using Partek Genomics Suite (Partek Inc, St. Louis, MO). All CEL files were imported using the same default parameters. Copy number values were generated by normalizing each sample’s probe set intensity to that of a reference. For tumors, paired references were used consisting of the normal lung tissue profile matching each patient. For lung cancer cell lines, an unpaired, pooled reference generated from the intensities of all 45 normal lung tissue profiles (those matching the tumors described above) was used. Regions of copy number gain and loss were then statistically detected using the Hidden Markov Model (HMM) based segmentation method of the software package with default parameters and the requirement of at least 50 contiguous probe sets.

Regions of allelic imbalance were determined using the allele specific copy number (AsCN) function of Partek. For paired analysis, only heterozygous SNPs in the reference (matched normal lung sample) were considered informative and the reference intensity for copy number creation was the allele intensity in the normal sample. In unpaired analysis, this reference intensity was taken as the average allele intensity of all reference (45 normal lung samples, see above) samples that were heterozygous for a given SNP. The ethnicity of all patients is listed in Table S2. Proportion scores for each SNP were then calculated and segmented in order to find regions of similar status and segments with a mean proportion score for all SNPs in the region >0.15 (as recommended by Partek) were considered imbalanced. Finally, adjacent regions meeting this threshold of imbalance were merged and the average proportion score calculated. The segment displaying the highest degree of imbalance across a chromosome arm (based on proportion score) is also listed for specific examples. All SNP data was visualized using SIGMA2 software (http://www.filinbox.com/technology.asp?page=3716) [35].

**mRNA expression of KRAS, EGFR, BRAF and PIK3CA genes by qPCR assay**

mRNA expression of each mutant gene was evaluated by real-time qPCR of cDNA product. Primer sequencing and PCR conditions are provided in Table S3. As an internal control, we used glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene. After quantification of each target and reference genes by the standard curve method, relative expression was calculated to compare the value of cell lines with the average value of HBEBC 15 and 21 cell lines (for NSCLCs lines) or the value of human adult normal colon RNA (BioChain Institute, CA, USA) for CRC lines, respectively.

**Ras activity by ELISA**

Ras activity was evaluated using Ras GTPase Chemi ELISA (Active Motif, CA) following the manufacturer’s protocol. Briefly, cell lysates from cell lines were quantified using BSA Protein Assay Kit (Pierce, IL). Glutathione-S-transferase (GST) fused to ras-binding-domain (RBD) of Raf which can specifically bind only to activated Ras was coated onto glutathione-coated microplates by a one hour incubation. After washing, equal amounts of cell lysates (45 µg) were applied and incubated for one hour. A primary antibody which can detect H- and K-ras was added and incubated for one hour. An hour incubation with a second antibody conjugated to horseradish peroxidase (HRP) and developing chemiluminescent reagents were used to detect activated Ras binding to the plate. The luminescent intensity, which was inversely proportional to the amount of activated Ras, was read using FLUOstar OPTIMA (BMG LABTECH GmbH, Offenburg, Germany). Each cell line was tested in duplicate. All values presented are relative light units compared with mean value of two HBEC lines that was arbitrarily assigned a value of one.

**Estimation of tumor heterogeneity by SNP array**

Tumor samples contain varying numbers of stromal and other non-malignant cells that may affect estimates of tumor cell gene copy number and allelic imbalance. To estimate tumor DNA content for clinical samples, we used a method adapted from Weir et al [7]. Briefly, we determined the log2 ratios and LOH status for each informative SNP in the tumor samples using dChip software with default settings. Regions of hemizygous deletion (i.e. one copy loss in diploid cells) in each sample were determined by identifying SNPs that displayed copy number loss (tumor vs normal log2 ratio ≤−0.2) with concordant LOH. In order to identify the lost allele in these regions, we then calculated allele-specific intensity ratios using the aroma.affymetrix package in R [36]. Since the lost allele in these regions has zero copies, any signal would be attributed to contamination by normal cells (which have one copy of each allele). Thus, this lost allele ratio represents the percent of normal cells in the sample. For each sample, the median ratio of the lost allele was then calculated for individual chromosomes and the minimum of the medians was determined. This value was then subtracting from one to determine the percentage of tumor cells in each clinical sample.

**Statistical analyses**

The differences of significance among categorized groups were compared using Chi-square or Fisher’s exact tests as appropriate for univariate analyses. Univariate analyses of overall survival (OS) were performed using the Kaplan-Meier method with a log-rank test. All data were analyzed with GraphPad Prism 5 software (GraphPad Software, San Diego, CA). All statistical tests were two-sided and probability values <0.05 were defined as being statistically significant.

**Results**

Homzygous mutations (Complete MASI) of oncogenes are frequent in tumor cell lines

For the 11 genes queried in the Sanger database, we identified a total of 1436 mutations (1157 for tumor suppressor genes, 279 for oncogenes) (Table 1, Fig. 2c and 2d). As expected, homozygous mutations were frequent in six tumor suppressor genes (81%), with the exception of APC, while the five oncogenes also had a relatively high frequency of homozygous mutations (20%). However, the frequency of homozygous mutations varied - being frequent in KRAS or EGFR mutant lines but not with PIK3CA mutations. As shown below, the true incidence of MASI is higher, as the Sanger database does not have quantitative copy number data for cell lines.
Table 1. Homozygous mutations of oncogenes are frequent in cancer cell lines.

| Genes | Sanger Institute | Our data |
|-------|-----------------|----------|
|       | % of Homozygous mutations* | % of Homozygous mutations** |
|       | (No. of mutant lines) | (No. of mutant lines) |
| KRAS | 36 (92) | 27 (75) |
| BRAF | 17 (65) | 13 (8) |
| NRAS | 10 (50) | 0 (12) |
| EGFR | 29 (7) | 20 (10) |

*Zygosity status was determined by manual examination of sequencing electropherograms at Sanger institute; **Homozygous mutations were defined as percent of mutant allele by direct sequencing greater than 90.

We used the data from our cell lines to confirm these findings for four oncogenes (total of 75 mutations) (Table 1). We found a mean incidence of 27%, range 0% for PIK3CA to 38% for KRAS. The frequencies of homozygous mutations for EGFR (20%) and BRAF (13%) were intermediate. Thus our findings are similar and complementary to the information from the Sanger database.

Determination of relative ratio between mutant and wild type alleles by direct sequencing

As described in the Methods Section, we determined the relative proportions of mutant and wild type alleles (mA%) by measurements of the direct sequencing electropherograms. To validate this approach, we applied it to mixtures of varying percentages of wild type and mutant plasmids. The results of the sequencing method were highly concordant with the actual mixture percentage of mutant and wild type plasmids for all 14 mixture experiments for all four genes tested (R² value=0.95, Fig. 2b and Table S4). Furthermore, mA% of subcloning of 48 mutant lines (R² value=0.87) and RFLP analyses of 30 mutant lines (R² value=0.89) also showed good concordance with electropherogram measurements (Figure S2c), demonstrating the accuracy of latter assay. These results fully validate the sequencing electropherogram measurement as an accurate method to determine mA%.

Estimation of tumor DNA content in clinical samples

We estimated tumor DNA content (% tumor DNA) from the SNP array data as described in Methods for 45 lung adenocarcinomas. Two control NSCLC lines (100% tumor cells) had estimated values of 99% and 95% of % tumor DNA while the median value of the tumors was 57%, range 26 to 93%. For these 45 cases, we adjusted all copy number using the % tumor DNA median value of the tumors was 57%, range 26 to 93%. For these estimated values of 89% and 95% of % tumor DNA while the mean incidence of 27%, range 0% for PIK3CA to 38% for KRAS.

Mutations and CNVs of KRAS, BRAF, PIK3CA, and EGFR genes

Details of the gene mutations and CNVs in the cell lines (n = 114) and tumors (n = 521) are provided in Tables S1, S2 and S3. Without SNP array data, the presence of UPD in tumor samples could not be determined. Because the results of cell lines and tumors were similar, a combined summary is presented in Fig. 3a. All KRAS, BRAF, and EGFR mutations were mutually exclusive across different tumor types while some PIK3CA mutant cases also harbored one of the other three mutations (Tables S1 and S2) as described previously [22,37,38].

For the three genetic alterations (mutations, CNVs or both) each gene demonstrated a distinct pattern. Most of the alterations in KRAS were mutations, with occasional CNVs or both. For EGFR, CNVs were the most frequent alteration, although mutations or both changes were present in prominent subpopulations. For BRAF, mutations and CNVs showed nearly equal frequencies, while both changes were rare. For PIK3CA, CNVs without mutations were the most frequent change (Fig. 3a).

The different patterns of MASI

The relationships between of mA% (as determined by electrophoregram measurement) and CNVs (as determined by qPCR) for the mutant genes in 68 mutant lines including seven lines with double mutations are shown in Fig. 3b. Three major patterns were observed: 1) Balanced, having a mA/wA ratio (mA/wA) of about 1 (range 0.5 to 2) without CNV (i.e. – MASI not present); 2) MASI with CNV, either complete (wA lost (mA/wA>9) or partial (mA/wA>2)); and 3) MASI without CNV (uniparental disomy, UPD), due to complete loss of wA (mA/wA>9) and selective retention/duplication of mA, respectively (Fig. 1a and 3b). Cases with UPD or complete MASI with CNVs lie off the standard curve because they lack the wA (Fig. 3b). A fourth pattern, reverse MASI, defined as wild type allele specific imbalance (mA/wA<0.5) was present in only one line (1%) having a mEGFR.

Gene specific analyses versus genome wide analyses

We evaluated MASI status in seven mAKRAS and two mEGFR lines using SNP arrays, and compared the results with MASI status determined by gene specific assays (mA% by direct sequencing and copy number by qPCR). Examples of these comparisons are shown in Fig. 4. Gene specific analyses defined the seven mAKRAS lines as one balanced type, one having MASI with CNV and five having UPD. Of the mEGFR lines, one had MASI with CNV and one had MASI with borderline CNV. Of note, the results detected by SNP array were completely concordant with those of the gene specific assays.

Individual oncogenes utilize different types of MASI

For our studies, determination of relative ratio between mA and wA (mA%) of tumor samples (in contrast to cell lines) requires SNP array analyses. As shown in Table S2b, we confirmed that there was good concordance between CNVs as estimated by SNP and qPCR methods.

For 45 adenocarcinomas having SNP array data, direct sequencing detected a high frequency of KRAS (n = 21, 47%) or EGFR (n = 14, 31%) mutations. We determined allelic imbalance (AI) and CNVs of KRAS and EGFR genes using SNP data. The percentage of tumor cell DNA in the samples was determined as described previously and we used appropriately adjusted copy numbers for further analyses. Because MASI frequencies in tumors (as determined by SNP assays) and cell lines (as determined by direct sequencing combined with qPCR) were similar (Table S6), we combined the data from 35 mutant tumors and 68 mutant cell lines.

As shown in Fig. 5a and Table 2, the frequencies for MASI (of all types) varied between individual oncogenes, being relatively high for EGFR (75%) and KRAS (58%) and lower for BRAF (38%) and PIK3CA (8%). The major type of MASI also showed gene variation (Fig. 3b and Table 2). For KRAS, UPD were more frequent than CNVs, while for EGFR the major type of MASI found in tumors and cell lines was CNVs, with UPD present in a minor subpopulation. For BRAF and PIK3CA the data were too scant to come to conclusions.
Allelic imbalance can be equally observed in wild type KRAS
We determined AI in both wild type and mutant case for KRAS and EGFR genes among the 45 lung adenocarcinomas with SNP data. For all 45 cases, AI was frequent in KRAS (n = 28, 56%) and EGFR (n = 18, 40%) (Table S2b). However, EGFR AI was significantly more frequent in mEGFR (71%) than wild type EGFR cases (29%, P = 0.008). By contrast, AI of KRAS was equally observed in mKRAS (62%) and wild type KRAS (63%, Table 2). While EGFR AIs in wild type EGFR cases were equally caused by CNG (50%) and UPD (50%), all KRAS AIs in wild type KRAS cases were caused by UPD.

Double mutations occur on the same chromosome (cis mutations)
Second site (double) mutations in the same gene (two examples each for EGFR and PIK3CA) were present in four cell lines (Table S1). For all four cell lines, they showed very similar mA% for both sites (less than 3.5% difference) (Table S1), even though two mutations of EGFR were detected by independent PCR reactions. These findings suggested that in all four cases both mutations occurred on the same parental chromosome and were in cis with each other. For EGFR mutant cases, a common activating mutation was associated with a second resistance associated mutation (T790M) and these two mutations have been described as usually or always being in cis [39,40].

MASI is present in xenografts
Subrenal capsule mice xenografts were directly established from primary human NSCLCs. These samples have the following advantage: 1) less manipulation than cell lines (close to clinical samples), and 2) lack of human normal stromal contamination [30]. We identified two KRAS or two EGFR mutations by cDNA sequencing using primer sets specific for the human gene in four of 27 subrenal xenograft samples (Figure S3). We confirmed the human specificity of our primers by lack of an amplicon using cDNA from healthy non-manipulated mouse liver as template (data not shown). Of note, three out of four mutant samples (two KRAS and one EGFR mutations) showed over 90% of mA%.

The ratio of mutant: wild type allele is maintained after transcription
To investigate whether CNGs were reflected in increased transcriptional activity, we compared mRNA expression with copy number for 70 mutant cell lines (with or without MASI). As shown in Fig. 6a, there was good concordance between the results of the two techniques, indicating that increased copy number was accompanied by increased transcription.

We then investigated whether the increased mA% of MASI lines were maintained after transcription. Using a subset of 35 mutant cell lines (with or without MASI), we compared the mA% of genomic DNA with the values from cDNA (Fig. 6b). There was almost perfect concordance between the values, indicating that the ratios of mutant:wild type alleles in genomic DNA were faithfully maintained after transcription.

Ras GTPase activity and KRAS MASI
We evaluated ras GTPase activity by ELISA for 36 cell lines including 26 lung, five colorectal, three pancreatic cancer lines and two HBEC lines (Figure S4). The linearity of the standard curve...
made by five different points was confirmed ($R^2 = 0.97$, data not shown). HBEC cultures and wild type tumor cell lines had comparably low levels of activity (Fig. 6c). Both lines with KRAS CNGs (without mutation) and those with balanced mutations (without CNGs) had significant 11–12 fold increases in GTPase activity. Cell lines having UPD (without CNGs) had a modest (approximately 50%) increase compared to the balanced mutant lines, although this increase was not significant. However mutant lines having MASI with CNGs had a significantly increased mean activity when compared to the other mutant groups.

**EGFR MASI and in vitro sensitivity to gefitinib**

We have previously reported the gefitinib sensitivity of NSCLC lines [6]. Seven of the 10 EGFR mutant lines were sensitive at a clinically achievable concentration (<1 μM). We correlated these data with the presence or absence of MASI (Table S7). While six out of seven sensitive cell lines (86%) harbored EGFR MASI, we could not find a convincing relationship between gefitinib sensitivity and EGFR MASI.
KRAS mutations and copy number gains in lung adenocarcinomas

We determined the mutational status and copy numbers of KRAS gene for 288 lung adenocarcinoma tumors including non-Asian (n = 127) and Asian (n = 161) populations obtained from five different institutions and correlated the data with clinical and other findings (Table 3). EGFR mutational status was available for 269 out of 288 cases [22,23,29]. We identified 57 KRAS mutations (20%) and 29 KRAS CNGs (10%). As demonstrated previously in Fig. 3a (for both cell lines and tumors), in this subset of tumors KRAS CNGs were more frequent in mKRAS than in wild type tumors. Because KRAS CNGs were closely associated with mKRAS, KRAS CNGs demonstrated similar associations as have been previously described for mKRAS (non-Asian ethnicity, smoking history, and mutual exclusivity with EGFR mutations). Gender differences were not significant for either mutations or CNGs.

We then evaluated the effect of KRAS alterations on clinical outcome of 237 resected lung adenocarcinoma tumors which were limited to stage I–III cases with survival data. Patients with mKRAS tumors (P = 0.2) or KRAS CNGs (P = 0.1) alone had a trend to be associated with poor prognosis. Tumors having both alterations, while present in a small subpopulation (n = 6), had worse prognosis of borderline significance (P = 0.04, Fig. 7).

We also identified 105 EGFR CNG in same subset of 269 lung adenocarcinomas. EGFR CNGs were significantly more frequent in never smokers, Asian ethnicity, were mutually exclusive with KRAS mutations and occurred more frequently with EGFR mutations than in wild type cases as previously described [26]. We were unable to investigate the effects of EGFR mutations and CNGs on survival as data on TKI therapy was incomplete.

Table 2. Summary of allelic imbalance of EGFR pathway genes.

| Subsets       | KRAS          | EGFR          | BRAF          | PIK3CA         |
|---------------|---------------|---------------|---------------|---------------|
| Mutant        | Frequency of MASI | Frequent     | Frequent      | Intermediate  | Rare          |
| Mechanisms of MASI | UPD (+CNG)   | CNG           | CNG (+UPD)    | CNG           |
| Wild type     | Frequency of AI in WT | Equally frequent as MASI | Rare         | -             |
| Mechanisms of AI | UPD           | Rare (CNG)    | -             | -             |

AI, allelic imbalance; MASI, mutant allele specific imbalance; WT, wild type; UPD, uniparental disomy; CNG, copy number gain.

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Discussion

Our earlier observations regarding homozygous mutations and MASI led us to question the commonly held belief that tumorigenesis requires biallelic inactivation for tumor suppressor genes while the potent effects of dominant oncogenes preclude the necessity of loss of the wild type allele product. We examined a public database of mutations (Sanger Institute). We found, as expected, that most inactivating mutations of tumor suppressor genes were frequently accompanied by loss of the wild type allele. However, our earlier observations on homozygosity of oncogenes were confirmed by the finding that 20% of five activating oncogene mutations were homozygous in cell lines derived from multiple tumor types. As discussed below, the true incidence of MASI is considerably higher as quantitative copy number data are missing in the Sanger database. Thus MASI, while a long observed and expected phenomenon for tumor suppressor genes, is also present in an important subset of cells harboring mutant oncogenes. Other published evidence supports this concept [41].

Detection of MASI of an oncogene requires three basic determinations: 1) detection of an oncogenic mutation; 2) copy number enumeration of the mutant gene in the tumor cells and 3) determination of the relative ratio of the mutant: wild type allele (mA%). Standard and widely accepted methods for the first two determinations exist including direct sequencing for mutations, and qPCR, FISH, aCGH or SNP analyses for CNGs [6]. For cell lines (consisting of pure tumor cell populations) mA% can be determined by subcloning or by the presence of homozygosity of the mutant allele. In order to avoid laborious and time intensive subcloning, we determined that mA% could be accurately estimated by measurements of the relative peak heights present.

Figure 5. Different frequencies and mechanisms of MASI of EGFR pathway genes. MASI is equally frequent in mutant KRAS and EGFR genes than others and PIK3CA MASI is rare (a). KRAS MASI is caused almost equally by uniparental disomy or copy number gain (CNG) while EGFR MASI is mainly caused by CNG (b). The prefix m- means mutant. MASI, mutant allele specific imbalance.

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Figure 6. Biological role of mutant allele specific imbalance (MASI). Gene dosage is highly associated with mRNA expression level (a). Proportion of mutant allele (mA%) determined by DNA sequencing electropherogram is significantly consistent with mA% by cDNA sequencing using different sets of primers (b). c) KRAS alterations are related to ras GTPase activity. KRAS mutations or copy number gains (CNGs) alone are related to high ras GTPase activity and the two molecular changes are synergistic. The prefix m- means mutant. HBEC, human bronchial epithelial cell; WT, wild type; UPD, uniparental disomy; *, KRAS mutation with CNG versus Others; **, KRAS mutation with CNG versus either KRAS mutation or CNG; ***, either KRAS mutation or CNG versus WT.

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Table 3. The association between KRAS alterations and clinical and other genetic factors in 288 lung adenocarcinomas.

| KRAS mut | P     | Subsets (n) | % | KRAS CNG | P     | Subsets | % | Mut or CNG | P     | Subsets | % | Mut and CNG | P     | Subsets | % |
|----------|-------|-------------|---|----------|-------|---------|---|------------|-------|---------|---|-------------|-------|---------|---|
| All      | -     | - (288)     | 19.8| All      | -     | 10.1   | - | All        | 26.1  | -       | - | 3.8         |       |         |   |
| Gender   | NS    | Male (161)  | 21.7| Gender   | 0.08  | Male   | 13 | Gender     | NS    | Male    | 29.2| Gender     | NS    | Male    | 5.6|
|          |       | Female (127)| 17.3|          | Female| 6.3   |    | Female     | 22    | Female  | 1.6|
| Smoking* | 0.0018| Never (101) | 9.9 | Smoking* | 0.013 | Never  | 4  | Smoking*   | 0.0001| Never   | 12.9| Smoking*   | NS    | Never   | 1  |
|          |       | Ever (184)  | 25.5|          | Ever   | 13    |    | Ever       | 33.2  | Ever    | 5.4|
| Ethnicity| 0.0006| Non-Asian (127)| 29.1| Ethnicity| NS    | Non-Asian| 13.4| Ethnicity  | <0.0001| Non-Asian| 37.8| Ethnicity  | NS    | Non-Asian| 4.7|
|          |       | Asian (161) | 12.4|          | Asian  | 7.5   |    | Asian      | 16.8  | Asian   | 3.1|
| EGFR mut**| <0.0001| Mutant (65) | 0  | EGFR mut**| 0.008 | Mutant | 1.5| EGFR mut** | <0.0001| Mutant  | 1.5| EGFR mut** | NS    | Mutant  | 0  |
|          |       | WT (204)    | 25 |          | WT     | 12.6  |    | WT         | 32.4  | WT      | 2.9|

Mut, mutation; WT, wild type; CNG, copy number gain; NS, not significant; *, Smoking status was not available in three cases; **, Nineteen cases were not determined mutational status and copy number of EGFR gene.

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on the electropherograms of routine sequencing for mutation detection. While mA% could be determined accurately in cell lines by these simple techniques, tumor samples present a much greater problem because of contamination with highly variable percentages of non-malignant cells. Reports of molecular studies often provide estimates of the percentage of tumor cells by histologic examination, but these are usually performed rapidly and are relatively inaccurate. In addition, because of the frequent presence of tumor cell polyplody, most genetic analyses require determination of the percentage of tumor DNA in the examined sample, rather than the percentage of tumor cells. For our studies, we used SNP array data for determinations of tumor cell DNA percentages. While this approach has been used by others [7], we refined the methodology. We found a mean value of 57% tumor DNA in the samples having SNP data, with a wide range of values. We arbitrarily used a slightly more conservative estimate for tumor cell DNA of 50% for the tumor samples lacking SNP data. While we used such estimates for copy number determinations in tumors, recognition of tumor homozygosity, including UPD, was limited to the tumor subsets with SNP data.

Four types of inter-relationships between mA and wA were found: a) balanced type, with mutant: wild type allele ratio of approximately one (MASI not present); b) MASI (either partial or complete) with CNG; c) uniparental disomy (complete MASI without CNG); and d) reverse MASI (wild type allele increased relative to mutant allele). For 75 mutations (in four genes) present in 68 cell lines the overall incidence of MASI was 48%, while only a single example of reverse MASI was identified (p<0.0001). Thus allelic imbalance almost invariably targets the mutant allele. Our previous observations regarding allelic imbalance (obtained by a variety of techniques including subcloning) are consistent with our present findings [3,6]. While MASI was convincingly demonstrated in cell lines the true incidence in tumors could only be determined with accuracy for the subset of lung tumors having SNP array data and mutational status of the KRAS and EGFR genes. The incidences of MASI in lung cancer cell lines and tumors for these two genes were not significantly different. However, the incidences of MASI for individual oncogenes showed differences, with high frequencies for EGFR and KRAS, intermediate for BRAF and low for PIK3CA. These differences may reflect variations in the oncogenic potential of the individual gene mutations. The frequencies of the two major forms of MASI also demonstrated individual gene differences. For EGFR and BRAF, the most frequent type was MASI with CNGs, while for KRAS, the frequencies of MASI with CNGs and UPD were similar.

While mutations of the KRAS and EGFR genes and CNGs of the EGFR gene are well described [6,19,25,26,29], the literature regarding KRAS CNGs in human tumors is sparse [7,42]. While less common than mutations in the present study, KRAS CNGs were relatively frequent. Of interest, KRAS CNGs showed the same clino-pathological associations as those previously described for KRAS mutations — relationship to smoking status, non-Asian ethnicity and mutual exclusivity with EGFR mutations [29].

While inherited UPD is associated with developmental disorders, the role of acquired UPD in cancer development is poorly understood [12]. Although UPD has been reported to be related to inactivation of tumor suppressor genes, its presence with activating oncogenic mutations has rarely been described in tumors. To date, UPD has been mainly reported in hematopoietic malignancies for a few oncogenes such as FAK2 [14]. Its incidence and role in solid tumors is largely unknown, although, as previously pointed out, this reflects the limits of our prior technology [12]. As discussed previously, homozygosity of tumor oncogenes in cancer cell lines is frequent, although the available information did not permit the distinction between MASI with CNGs or UPD as the mechanism. Using gene-specific and genome-wide approaches we found that UPD was frequent for three EGFR pathway genes, especially for KRAS gene (data for PIK3CA mutations were too sparse for evaluation). Relatively little data exists in the literature for KRAS CNGs in human tumors. Furthermore, KRAS homozygosity was observed independent of mutational status as previously described [43]. The wild type allele of KRAS can also inhibit lung carcinogenesis in mice [44], providing a possible explanation for the frequent finding of UPD with mutant and wild type oncogenes.

MASI has apparent biological and clinical significance. MASI at the genomic level was precisely maintained after transcription. While mutations, CNGs and allelic imbalance of mA and wA may all contribute to tumorigenesis, combinations of the three events may be more effective than any single event. Evidence for this concept was provided by our finding that the combination of mutation and CNGs acted synergistically to enhance ras GTPase activity. A recent report found that all KRAS mutations did not exert an equal effect on tumor cells [42]. Cancer cell lines harboring KRAS mutations could be broadly divided into KRAS-dependent and KRAS-independent groups. The vast majority of KRAS-dependent lines exhibited focal KRAS CNGs, in contrast to KRAS-independent lines. This study provides further evidence that the combination of KRAS mutations and CNGs act synergistically. Our previous findings that EGFR mutations were associated with tumor initiation while EGFR CNG might be more regarded as a tumor progression event, provide further evidence of their co-operative role in tumorigenesis [45]. Understanding the mechanism of MASI could elucidate new understandings of tumor biology and may contribute to the development of rational targeted therapies.

MASI in its various forms is frequently present in mutant EGFR and KRAS tumor cells, and is associated with increased mutant allele transcription and gene activity. The frequent finding of mutations, copy number gains and MASI occurring together in tumor cells indicates that these three genetic alterations, acting together, may have a greater role in the development or maintenance of the malignant phenotype than any individual alteration.

Supporting Information

Table S1 a) Mutant cell lines of KRAS, EGFR, BRAF, and/or PIK3CA genes (n = 68) mA%, mutant allele proportion (%); b) cell
line with both KRAS and PIK3CA mutations; **, cell line with both BRAF and PIK3CA mutations; ***, blanked values are mA% of second mutations of same gene (D549N for PIK3CA and T790M for EGFR). For EGFR DNA sequence, we performed independent PCR reaction to evaluate mA% of primary and second mutations). b) Wild type cell lines of KRAS, EGFR, BRAF, and PIK3CA genes (n = 46).

Table S2  a) Summary of 288 lung adenocarcinomas from five institutes b) Summary of 45 lung adenocarcinomas with SNP array data c) The association between KRAS and EGFR alterations and clinicopathological factors in 45 lung adenocarcinomas with SNP *; P value was calculated between Gain and Neutral; **, P value was calculated between Never smoker and Ever smoker. d) Summary of 60 colorectal cancer tumors

Table S3  a) Primer sequences for DNA sequencing b) Primer sequences for cDNA sequencing *; These primers were also used to detect KRAS or EGFR mutations in subrenal capsule mice xenografts of primary human NSCLCs because these primers are specific for human origin and no PCR product are amplified from mouse cDNA as PCR template. c) Primer sequences for restriction fragment length polymorphism *, The substitution of third letter in KRAS codon 61 (limited to CAT or CAC mutation) can change representative amino acid (Glxamine to Hystysine). d) Primer sequences for copy number analyses by quantitative PCR (qPCR) assay e) Relative mRNA expression analyses by qPCR

Table S4  The accuracy of proportion of mutant allele (mA%) of direct sequencing was evaluated by 14 kinds of plasmids mixture experiment. We mixed mutant plasmid with corresponding wild type plasmid at various ratios (5 to 7 points) and amplified the mixed plasmid as a template of PCR. PCR products were directly sequenced and the mA% were determined by measurement of sequencing electropherograms. Finally, we confirmed the linearity between the actual mixed proportion of mutant and wild type plasmids and mA% detected by direct sequencing. The results of the sequencing method were highly concordant with the actual mixture percentage of mutant and wild type plasmids in all 24 trend lines for four genes tested (R2 value >0.95).

Table S5  CNG, copy number gain; Both, cases with both mutations and CNGs; NS, not significant (P>0.1); *, 314 tumors were analyzed because of lack of mutational and copy number data of EGFR gene in 19 Estonia cases; **, data were combined current study and our previous studies - Yamamoto et al (Cancer Res 68: 6913–6921) and Gandhi et al (PLoS ONE 4: e4576).

Table S6  CRC, colorectal cancer; PAC, pancreatic cancer; MASI, mutant allele specific imbalance; UPD, uniparental disomy; CNG, copy number gain; *, limited to 45 lung adenocarcinomas with SNP data; **, because SNP array can not distinguish between MASI and reverse MASI and because incidence of reverse MASI in cell lines is low, we defined tumors harboring allelic imbalance with CNG as MASI with CNG.

Table S7  All other 35 cell lines tested (except for 3 EGFR or HER2 copy number gain cell lines) were resistant for gefitinib (IC50>10 mM) (Gandhi et al: PLoS ONE 4: e4576).

References
1. Vogtstein B, Kinzler KW (2004) Cancer genes and the pathways they control. Nat Med 10: 789–799.
2. Albertson DG, Collins C, McCormick F, Gray JW (2003) Chromosome aberrations in solid tumors. Nat Genet 34: 369–376.
3. Mitsudomi T, Viallet J, Mulshine JL, Minna JD, et al. (1991) Mutations of ras genes distinguish a subset of non-small-cell lung cancer cell lines from small-cell lung cancer cell lines. Oncogene 6: 1353–1362.
4. Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, et al. (2004) Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N Engl J Med 350: 2129–2139.
5. Pasz JG, Janne PA, Lee JC, Tracy S, Greulich H, et al. (2004) EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science 304: 1497–1500.
1. Gandhi J, Zhang J, Xie Y, Soh J, Shigematsu H, et al. (2009) Alterations in genes of the EGFR signaling pathway and their relationship to EGFR tyrosine kinase inhibitor sensitivity in lung cancer cell lines. PLoS ONE 4: e5376.

2. Weir BA, Woo MS, Getz G, Perera S, Ding L, et al. (2007) Characterizing the cancer genome of a K-RAS-driven mouse model of lung adenocarcinoma. Cancer Res 67: 7645–7651.

3. Engel E (1980) A new genetic concept: uniparental disomy and its potential effect, idiosomy. Am J Med Genet 6: 137–143.

4. Walsh CS, Ogawa S, Scoles DR, Miller CW, Kawamata N, et al. (2008) Genotype–gene loss of heterozygosity and uniparental disomy in BRCA1/2-associated ovarian carcinomas. Clin Cancer Res 14: 7645–7651.

5. Gupta M, Raghavan M, Gale RE, Chelala C, Allen C, et al. (2008) Novel regions of acquired uniparental disomy discovered in acute myeloid leukemia. J Natl Cancer Inst 100: 1429–1442.

6. Melcher R, Al-Taie O, Kudlich T, Hartmann E, Maisch S, et al. (2007) SNP-Array genotyping and spectral karyotyping reveal uniparental disomy as early mutational event in MSS- and MSI-colorectal cancer cell lines. Cytogenet Genome Res 118: 214–221.

7. Tuna M, Knuttila S, Mills GB (2009) Uniparental disomy in cancer. Trends Mol Med 15: 120–128.

8. Fitzgibbon J, Smith LL, Raghavan M, Smith ML, Debernardi S, et al. (2005) Association between acquired uniparental disomy and homozgyous gene mutation in acute myeloid leukaemia. Cancer Res 65: 9152–9154.

9. Kralevics R, Passamonti F, Buser AS, Teo SS, Tiedt R, et al. (2005) A gain-of-function mutation of JAK2 in myeloproliferative disorders. N Engl J Med 352: 1779–1790.

10. Soh J, Toyooka S, Ichihara S, Asano H, Kobayashi N, et al. (2008) Sequential molecular changes during multistage pathogenesis of small peripheral adenocarcinomas of the lung. J Thorac Oncol 3: 340–347.

11. Thomas RK, Debiasi RM, Winkler W, Laframboise T, et al. (2007) High-throughput oncogene mutation profiling in human cancer. Nat Genet 39: 347–351.

12. Endoh H, Yatake Y, Kusano T, Kusano H, Mitsuomori T (2006) PTEN and PIK3CA expression is associated with prolonged survival after gefitinib treatment in EGFR-mutated lung cancer patients. J Thorac Oncol 1: 629–634.

13. Pao W, Miller VA, Riely GJ, Somwar R, et al. (2005) Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain. PLoS Med 2: e73.

14. Bengtsson H, IzriaRR, Carvalho B, Speed TP (2008) Estimation and assessment of raw copy numbers at the single locus level. Bioinformatics 24: 759–767.

15. Singh A, Greninger P, Rhodes D, Koompan L, Violette S, et al. (2009) A gene expression signature associated with “K-Ras addiction” reveals regulators of EMT and tumor cell survival. Cancer Cell 15: 489–500.

16. Uchiyama M, Usami N, Kondo M, Mori S, Ho M, et al. (2003) Loss of heterozygosity of chromosome 12p does not correlate with KRAS mutation in non-small-cell lung cancer. Int J Cancer 107: 464–468.

17. Zhang Z, Wang Y, Vikis HG, Johnson L, Liu G, et al. (2001) Wildtype Kras2 can inhibit lung carcinogenesis in mice. Nat Genet 29: 25–33.

18. Greninger P, Rhodes D, Koompan L, Violette S, et al. (2009) A gene expression signature associated with “K-Ras addiction” reveals regulators of EMT and tumor cell survival. Cancer Cell 15: 489–500.