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High-throughput oncogene mutation profiling in human cancer

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Systematic efforts are underway to decipher the genetic changes associated with tumor initiation and progression[1,2]. However, widespread clinical application of this information is hampered by an inability to identify critical genetic events across the spectrum of human tumors with adequate sensitivity and scalability. Here, we have adapted high-throughput genotyping to query 238 known oncogene mutations across 1,000 human tumor samples. This approach established robust mutation distributions spanning 17 cancer types. Of 17 oncogenes analyzed, we found 14 to be mutated at least once, and 298 (30%) samples carried at least one mutation. Moreover, we identified previously unrecognized oncogene mutations in several tumor types and observed an unexpectedly high number of co-occurring mutations. These results offer a new dimension in tumor genetics, where mutations involving multiple cancer genes may be interrogated simultaneously and in 'real time' to guide cancer classification and rational therapeutic intervention.

Numerous cancer genome characterization efforts have emerged in recent years, empowered by the notion that detailed knowledge of somatic alterations will speed the development of targeted cancer therapeutics[1–3]. These initiatives have relied heavily on large-scale sequencing approaches to characterize the point mutations and short

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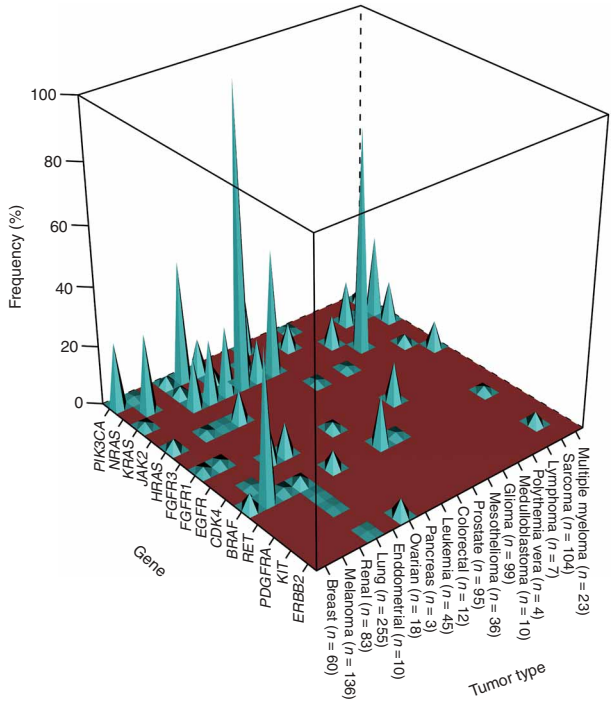

Figure 1 Frequencies of oncogene mutations across human tumor types. Frequencies (*y* axis) were calculated as percentages of tumor samples (*x* axis) from a given type that harbored an oncogene mutation (*z* axis) compared with the total number of samples of that tumor type.

insertions or deletions that represent frequent mechanisms of oncogene activation[2,4–8]. The concomitant expansion in the number of known genetic alterations in tumors has now shifted the bottleneck toward translation of such information into therapeutic benefit. Accomplishing this task will require both rigorous genetic characterization across all human tumor types and the advent of methods that detect multiple mutations with high accuracy and at acceptable cost. In this regard, systematic cancer gene mutation detection in clinical specimens has often proved difficult, particularly in the context of the ploidy alterations and admixture of non-malignant cells (stroma, lymphocytes, etc.) characteristic of tumor tissue.

Gain-of-function point mutations do not occur randomly in most known oncogenes characterized to date; instead, changes affecting a relatively small number of codons often account for the majority of somatic mutations. In principle, then, a limited number of judiciously designed genetic assays should effectively interrogate a large proportion of known oncogene mutations. For example, 16–44 assays per gene in *RAS*, *EGFR* and *BRAF* captured 90%–99% of the mutation prevalence observed thus far for these genes in human malignancies (**Supplementary Table 1** online). Therefore, we reasoned that high-throughput genotyping might provide an effective means to detect critical and/or 'targetable' cancer mutations on a large scale in clinical specimens. Accordingly, we designed 245 genotyping assays that queried 238 known somatic mutations involving 17 human oncogenes (**Supplementary Table 1**). For this proof-of-principle approach, we gave priority to mutations with high prevalence (for example, *RAS* family mutations), proven clinical implications (such as *KIT* and *EGFR*)[4,6–8] and/or strong correlation with preclinical sensitivity to targeted agents (for example, *BRAF*)[9].

To measure its sensitivity for mutation detection in tumor-derived DNA, we compared the mass spectrometric genotyping approach to both Sanger sequencing and a highly sensitive pyrosequencing-by-synthesis method (picotiter plate pyrosequencing)[10] for the detection of *EGFR* mutations in 22 primary lung tumor samples. Both genotyping and picotiter plate pyrosequencing detected 12 mutations, including three mutant alleles representing 16%, 12% and 9% of the total DNA as quantified by the pyrosequencing method (data not shown and **Supplementary Table 2** online)[10]. In contrast, Sanger sequencing detected only nine EGFR mutations, missing the three aforementioned low-frequency events[10]. We observed similar results for a panel of *KRAS* mutations in human lung adenocarcinoma samples (data not shown). Thus, the sensitivity of mass spectrometric genotyping is consistent with prior genetic association studies using pooled DNA samples[11,12], and it may exceed that of Sanger sequencing for mutation profiling in clinical tumor specimens.

In considering the specificity of mass spectrometry–based oncogene profiling, we reasoned that the distribution of the mutations identified by this method should reflect patterns observed previously in human tumors. This prediction was borne out by our results (**Fig. 1** and **Supplementary Table 2**). For example, we observed *JAK2* mutations in 3 out of 4 polycythemia vera samples[13–16], we found *FGFR3* mutations in 2 out of 23 multiple myelomas[17] and *KIT* mutations occurred in 4 out of 104 sarcoma samples[18], all of which were gastrointestinal stromal tumors (GISTs). None of these mutations occurred in any of the other tumor samples analyzed. Moreover, this high specificity was confirmed through independent validation of 393 mutation calls by Sanger sequencing or other methods (including duplicates; see **Supplementary Note** online). We found one GIST specimen carrying two *KIT* mutations, including a D816H mutation recently shown to be associated with resistance to imatinib[19] (**Supplementary Table 2**). Notably, this sample had been obtained from an individual whose tumor relapsed after imatinib treatment. Thus, our approach may facilitate prediction of clinical response and resistance to targeted cancer therapies.

Table 1 Rare or novel oncogene point mutations identified by genotyping

Sample ID	Tumor type	Assay	Mutation
RL95-2	Endometrial	OM_00067	EGFR_A289V
RL95-2	Endometrial	OM_00150	HRAS_Q61H
RPMI-8226	Multiple myeloma	OM_00190	KRAS_G12A
RPMI-8226	Multiple myeloma	OM_00079	EGFR_T751I[a]
S002039	Lung	OM_00260	RET_M918T
S002039	Lung	OM_00188	KRAS_G12V[b]
S004154	Medulloblastoma	OM_00196	KRAS_G13D
WM3682	Melanoma	OM_00127	FGFR1_S125L
WM3702	Melanoma	OM_00127	FGFR1_S125L
Meso 986	Mesothelioma	OM_00220	NRAS_G13D
Meso 713	Mesothelioma	OM_00228	NRAS_Q61K[b]
Meso 542	Mesothelioma	OM_00227	NRAS_Q61R
S003253	Multiple myeloma	OM_00246	PIK3CA_E545K
OVCAR-8	Ovarian	OM_00120	ERBB2_G776V[c]
S003195	Prostate	OM_00056	BRAF_K601E
S004480	Renal	OM_00052	BRAF_V600E
S003239	Sarcoma	OM_00052	BRAF_V600E
S006118	Sarcoma	OM_00052	BRAF_V600E
S006065	Lentigo simplex	OM_00250	PIK3CA_H1047R

[a]The detected mutation was a single-base substitution identified by an assay interrogating the deletion EGFR_E746_A750del, V ins. [b]Not confirmed by sequencing. [c]The detected mutation was a single-base substitution identified by an assay interrogating the insertion ERBB2_G776VC.

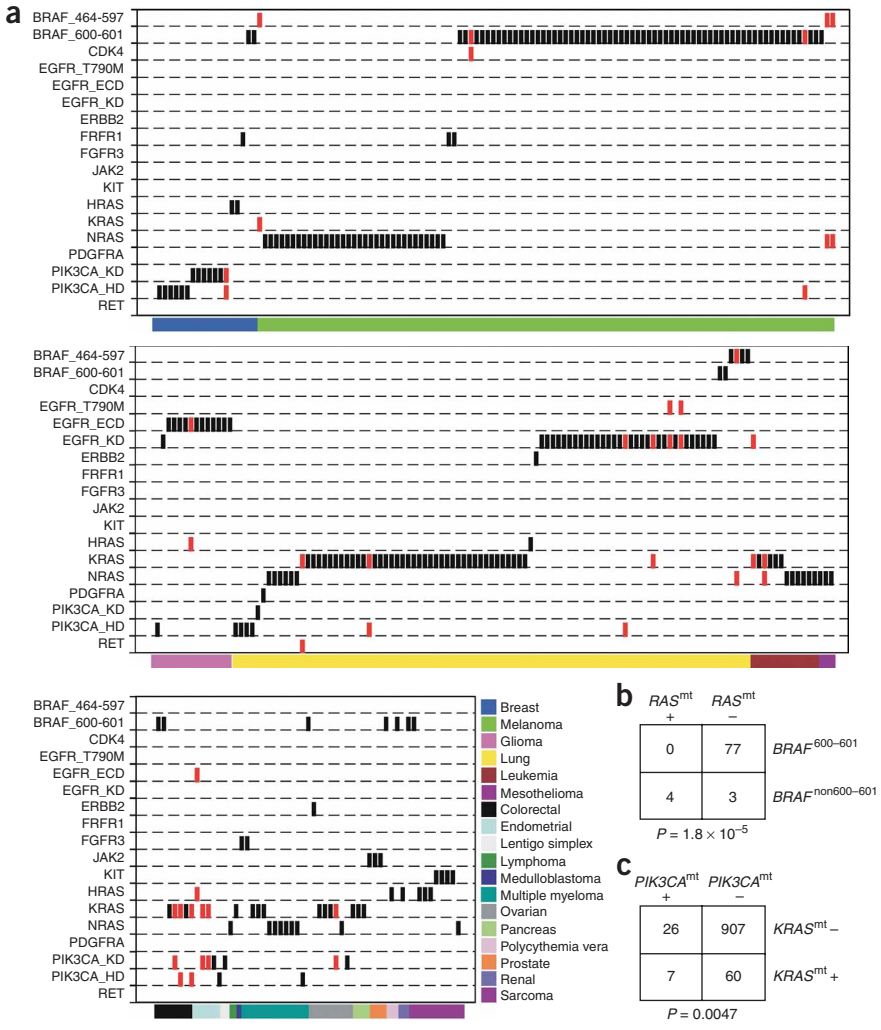

Figure 2 Mutually exclusive and co-occurring oncogene mutations in human cancer. (**a**) Oncogene mutations were grouped together when they occurred within a given gene (for example, 'KRAS' for all mutations in *KRAS*) or in the same functional domain of the encoded protein (for example, 'PIK3CA_KD' for kinase domain mutations of *PIK3CA*). When a distinct phenotype was correlated with a mutation, the mutation was grouped separately (for example, 'EGFR_T790M' for the T790M mutation of EGFR known to be correlated with resistance to EGFR inhibitors). Mutant samples (columns/black bars) are sorted by grouped oncogene mutations and by tumor type (color legend indicated). Red bars indicate co-occurring mutations. EGFR_ECD, extracellular domain mutations of *EGFR*; EGFR_KD, kinase domain mutations of *EGFR*; PIK3CA_KD, kinase domain mutation of *PIK3CA*; PIK3CA_HD, helical domain mutations of *PIK3CA*. (**b**) Incidence of *BRAF* mutations and co-occurring mutations in any *RAS* gene. (**c**) Incidence of co-occurring *KRAS* and *PIK3CA* mutations (see text for details).

In total, we performed oncogene mutation profiling on 1,000 individual tumor samples, including primary tumor specimens, cancer cell lines, short-term cultures and xenografts spanning 17 tumor lineages. We identified at least one mutation in 298 (30%) of the samples and performed confirmatory studies on approximately 90% of mutations identified, as noted above (**Supplementary Note**). Of the 238 genotyping assays employed here, 81 (34%) were called 'mutant' in at least one sample, and 14 of the 17 oncogenes queried were found mutated at least once. A 'peak-height' analysis of raw spectral data (see Methods) suggested that most of the mutations found were either heterozygous or admixed with stromal DNA; however, a subset of mutations showed spectral patterns consistent with homozygous alleles (**Supplementary Fig. 1** and **Supplementary Table 2**).

Although we generally observed a distribution of oncogene mutations that was consistent with prior literature reports (**Fig. 1**, **Supplementary Figs. 2–4** and **Supplementary Table 2** online), our approach also identified many low-frequency events involving both rare and common neoplasms (**Fig. 1**). Frequently, such mutations constituted rarely or never previously reported alterations in the associated tumors (**Table 1**). Examples include *NRAS* mutations in 3 out of 37 mesothelioma cell lines and a *PIK3CA* kinase-domain mutation in a human skin specimen that contained lentigo simplex (**Table 1**). The latter suggests that lentigo simplex might be associated with *PIK3CA* mutations, just as benign melanocytic nevi are associated with *BRAF* mutations. Additional novel mutations included an *ERBB2* (G776V) mutation in an ovarian cancer cell line[20], *PIK3CA* mutations in both a multiple myeloma and a metastatic melanoma sample, an *FGFR1* mutation in melanoma short-term cultures, an *EGFR* mutation in a multiple myeloma cell line[20], a mutation in the region encoding the extracellular domain of EGFR in an endometrial carcinoma cell line[21], a *RET* mutation in a primary non–small cell lung tumor and mutations in codons 600 or 601 of *BRAF* in sarcoma, breast, ovarian and prostate cancer specimens (see also **Supplementary Table 2**). Thus, despite the well-known uneven distribution of oncogene mutations across tumor types, these results suggest that rare and potentially 'druggable' oncogene mutations might exist in many common tumor types.

Oncogene mutations that activate common downstream pathways often occur in a mutually exclusive fashion in human cancers. While confirming this relationship among prevalent oncogene mutations (**Fig. 2a**), high-throughput mutation profiling also uncovered several co-occurring mutations that had not previously been reported (**Fig. 2a**). For example, 30% of all *PIK3CA* mutations identified were coincident with another oncogene mutation. *KRAS* was the most common partner oncogene (10% of all *KRAS* mutations co-occurred with a *PIK3CA* mutation; $P = 0.0047$; **Fig. 2**), but *EGFR* and *BRAF* mutations were also observed to co-occur with *PIK3CA* mutations (**Supplementary Table 2**). Similarly, *BRAF* mutations involving codons other than 600 or 601 were highly likely to co-occur with a *RAS* family mutation, whereas similar coincident events involving mutations in *BRAF* codons 600 or 601 were never observed ($P = 1.8 \times 10^{-5}$; **Fig. 2b**). This observation suggests that $BRAF^{V600E}$ may elicit potent oncogenic effects that are also mechanistically distinct from other BRAF kinase domain mutations[22]. Furthermore, despite the strong oncogenic potential of many *RAS*, *BRAF* and *PIK3CA* mutations, as measured by forward *in vitro* transformation assays, the observed co-occurrences suggest that alterations in the

associated pathways may often elicit complementary rather than redundant effects on tumorigenesis *in situ*.

Gain-of-function genetic alterations often cause tumor cells to become 'addicted' to the relevant oncogene or its downstream pathway[23], thereby exposing a potential therapeutic vulnerability[4,5]. Here, we have shown that high-throughput genotyping enables sensitive and accurate oncogene mutation profiling in human cancer specimens. This approach successfully identified numerous individual and co-occurring genetic alterations that promise to provide new biological and therapeutic insights in several tumor types. Given that discovery-oriented cancer gene resequencing has reached the dimension of all annotated genes in the genome[2]; large-scale mutation profiling using mass spectrometry or other methods may complement these efforts by enabling new and existing mutation panels to be queried broadly across human malignancies. Moreover, the clinical application of rapid, scalable and cost-effective mutation profiling approaches should facilitate patient stratification for the rational deployment of targeted cancer therapeutics.

METHODS

Samples. We used 1,000 tumor samples derived from the following 17 tumor types: breast cancer ($n = 60$), colorectal cancer ($n = 12$), endometrial cancer ($n = 10$), glioma ($n = 99$), leukemia ($n = 45$), lung cancer ($n = 255$), lymphoma ($n = 7$), medulloblastoma ($n = 10$), melanoma ($n = 136$), mesothelioma ($n = 36$), multiple myeloma ($n = 23$), ovarian cancer ($n = 18$), pancreatic cancer ($n = 3$), polycythemia vera ($n = 4$), prostate cancer ($n = 95$), renal cell cancer ($n = 83$) and sarcoma ($n = 104$). All primary tumor DNA samples were obtained from fresh-frozen tumor specimens based on a 70% cutoff for sample purity. For tumors that could be obtained as actual tumor biopsy specimens from collaborators (for example, all lung tumors), diagnoses were confirmed by independent histopathological review. The quality of all DNA samples was ensured by independent quantification and quantitative PCR. The study was conducted under institutional review board approval.

Selection of oncogene mutations and assay design. We queried the following databases for known somatic oncogene mutations: Cosmic[24], PubMed and an internal database of oncogene mutations discovered through our systematic resequencing efforts in human cancer specimens[6,21,25,26]. We selected only nonsynonymous coding mutations that previously had been reported to occur as somatic mutations in human cancer. The resulting list (**Supplementary Table 1**) contained 238 individual oncogene mutations, comprising single-base substitutions as well as insertions or deletions. Genomic positions for all mutations were computed using the HG16 build of the human genome and the University of California Santa Cruz (UCSC) genome annotation database. BLAT alignment information and exon structures for the National Center for Biotechnology Information (NCBI) RefSeq transcripts were downloaded from UCSC, and genomic locations for all assays were determined. Translation accuracy of all candidate mutations was determined by comparing the calculated genomic position of the candidate to the exon and BLAT alignment block information provided by the UCSC annotation information. For each mutation, the discriminating nucleotides for both wild-type and mutant alleles were determined, enabling insertions or deletions to be represented by single-base changes. Subsequently, 250 bases of neighboring DNA were added to each side of the resulting mutation assay to enable primer design. Genotyping assays (primers for PCR amplification and the extension probe) were designed using the Sequenom MassARRAY Assay Design 3.0 software, applying default parameters (maximum of six multiplexed assays per well). For complex mutations (that is, mutations defined by more than one nucleotide change, such as a deletion of bases 2345–2360 combined with a substitution of base 2364), genotyping assays were designed manually.

Mass-spectrometric genotyping. Genomic DNA from all tumor samples was purified and subjected to phi29 polymerase multiple strand-displacement whole-genome amplification, as described previously[27]. After quantification and dilution of genome-amplified DNA, multiplexed PCR was performed in 5-μl volumes containing 0.1 units of Taq polymerase, 5 ng of genome-amplified genomic DNA, 2.5 pmol of each PCR primer and 2.5 μmol of dNTP. Thermocycling was at 95 °C for 15 min followed by 45 cycles of 95 °C for 20 s, 56 °C for 30 s and 72 °C for 30 s. Unincorporated dNTPs were deactivated using 0.3 U of shrimp alkaline phosphatase, and primer extension was carried out using 5.4 pmol of each primer extension probe, 50 μmol of the appropriate dNTP/ddNTP combination and 0.5 units of Thermosequenase DNA polymerase. Reactions were cycled at 94 °C for 2 min, followed by 40 cycles of 94 °C for 5 s, 50 °C for 5 s and 72 °C for 5 s. After the addition of a cation exchange resin to remove residual salt from the reactions, 7 nl of the purified primer extension reaction was loaded onto a matrix pad (3-hydroxypicoloinic acid) of a SpectroCHIP (Sequenom). SpectroCHIPs were analyzed using a Bruker Biflex III matrix-assisted laser desorption/ionization–time of flight (MALDI-TOF) mass spectrometer (SpectroREADER, Sequenom).

Analytical and statistical methods. Mutation calls for each sample were determined using the default settings of MassArray Typer 3.4 Analyzer (Sequenom). Successful genotyping assays were defined as those in which 75% of all genotyping calls were obtained (based on 'conservative' allele calls according to the manufacturer's specifications; see below and **Supplementary Table 3** online). Unsuccessful assays were repeated after another round of primer design and testing. Automated mutation calls were generated using available computational algorithms for genotyping of diploid samples without further refinement or adaptation (Sequenom, MassArray RTTM software) ($n = 437$). These were compared with calls made by manual review of the raw mass spectra ($n = 448$), with a concordance rate of 95%. To measure assay reproducibility, a subset of tumors was interrogated in duplicate, and some mutations were detected using two independent genotyping assays (for example, mutations targeting codon 600 of *BRAF*). The statistical significance of co-occurring mutations was calculated by applying a Fisher's exact test.

To estimate mutant allele percentage and degree of heterozygosity, the heights of raw spectral peaks corresponding to the mutant and wild-type signal were quantified and compared with those from an independent dataset of germline SNPs (SNP identifiers available upon request) using 39 unique assays. For these reference SNPs, the allele status (homozygous or heterozygous) had been determined previously by mass spectrometric genotyping of 95 prostate cancer specimens (3,403 data points). Peak height ratios (mutant peak/wild-type peak) of the various mutations found in more than one tumor sample of a given tumor type were plotted and compared with the peak-height distribution of the reference SNPs (**Supplementary Fig. 1** and **Supplementary Table 2**). The relative signal was determined as (mutant peak × 100) / (mutant peak + wild-type peak). The 'positive/negative control' ranges for peak height ratios were determined from the aforementioned independent data set of 95 prostate cancer samples. Calculated peak height ratios from the reference data set were sorted by heterozygous versus homozygous calls. Although the peak height ratio boundary was not absolute between heterozygous and homozygous samples, a value of 5.53 was empirically found to be the maximum heterozygous peak height ratio (**Supplementary Fig. 1**). In total, 1,365 data points had peak-height ratios <5.53 inclusive of all heterozygous alleles (and some homozygous alleles), whereas 1,803 samples had peak-height ratios >5.53 (all homozygous alleles). Some samples were omitted ($n = 235$) because the peak height of the wild-type allele was measured as 0 (thus, the ratio would have required division by zero).

URLs. Cosmic[24]: http:/www.sanger.ac.uk/genetics/CGP/cosmic/; UCSC genome browser: http://genome.ucsc.edu.

Note: Supplementary information is available on the Nature Genetics website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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Corrigendum: High-throughput oncogene mutation profiling in human cancer

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In the version of this article initially published, the name of an author was spelled incorrectly as Laura MacConnaill. The correct spelling is Laura MacConaill. The error has been corrected in the HTML and PDF versions of the article.