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The Consensus Coding Sequences of Human Breast and Colorectal Cancers

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The elucidation of the human genome sequence has made it possible to identify genetic alterations in cancers in unprecedented detail. To begin a systematic analysis of such alterations, we have determined the sequence of well-annotated human protein coding genes in two common tumor types. Analysis of 13,023 genes in 11 breast and 11 colorectal cancers revealed that individual tumors accumulate an average of ~90 mutant genes but that only a subset of these contribute to the neoplastic process. Using stringent criteria to delineate this subset, we identified 189 genes (average of 11 per tumor) that were mutated at significant frequency. The vast majority of these genes were not known to be genetically altered in tumors and are predicted to affect a wide range of cellular functions, including transcription, adhesion, and invasion. These data define the genetic landscape of two human cancer types, provide new targets for diagnostic and therapeutic intervention, and open fertile avenues for basic research in tumor biology.

It is widely accepted that human cancer is a genetic disease caused by sequential accumulation of mutations in oncogenes and tumor suppressor genes (*1*). These tumor-specific (that is, somatic) mutations provide clues to the cellular processes underlying tumorigenesis and have proven useful for diagnostic and therapeutic purposes. To date, however, only a small fraction of the genes has been analyzed and the number and type of alterations responsible for the development of common tumor types are unknown (*2*). In the past, the selection of genes chosen for mutational analyses in cancer has been guided by information from linkage studies in cancer-prone families, identification of chromosomal abnormalities in tumors, or known functional attributes of individual genes or gene families (*2*–*4*). The determination of the human genome sequence coupled with improvements in sequencing and bioinformatic approaches have now made it possible, in principle, to examine the cancer cell genome in a comprehensive and unbiased manner. Such an approach not only provides the means to discover other genes that contribute to tumorigenesis but can also lead to mechanistic insights that are only evident through a systems biological perspective. Comprehensive genetic analyses of human cancers could lead to discovery of a set of genes, linked together through a shared phenotype, that point to the importance of specific cellular processes or pathways.

To begin the systematic study of the cancer genome, we have examined a major fraction of human genes in two common tumor types, breast and colorectal cancers. These cancers were chosen for study because of their substantial clinical significance world-wide: together, they account for

~2.2 million cancer diagnoses (20% of the total) and ~940,000 cancer deaths each year (14% of the total) (*5*). For genetic evaluation of these tumors, we focused on a set of protein coding genes, termed the consensus coding sequences (CCDS) that represent the most highly curated gene set currently available (*6*). The CCDS database contains full-length protein coding genes that have been defined by extensive manual curation and computational processing and have gene annotations that are identical among reference databases.

The goals of this study were three-fold: (i) to develop a methodological strategy for conducting genome-wide analyses of cancer genes in human tumors; (ii) to determine the spectrum and extent of somatic mutations in human tumors of similar and different histologic types; and (iii) to identify new cancer genes and molecular pathways that could lead to improvements in diagnosis or therapy.

Cancer mutation discovery screen. The initial step toward achieving these goals was the development of methods for high-throughput identification of somatic mutations in cancers. These methods included those for primer design, polymerase chain reaction (PCR), sequencing, and mutational analysis (Fig. 1). The first component involved extraction of all protein coding sequences from the CCDS genes. A total of 120,839 non-redundant exons and adjacent intronic sequences were obtained from 14,661 different transcripts in CCDS. These sequences were used to design primers for PCR amplification and sequencing of exons and adjacent splice sites. Primers were designed using a number of criteria to ensure robust amplification and sequencing of template regions (*7*). While most exons could be amplified in a single PCR reaction, we found that exons larger than 350 bp were more effectively amplified as multiple overlapping amplicons. One member of every pair of PCR primers was tailed with a universal primer sequence for subsequent sequencing reactions. A total of 135,483 primer pairs encompassing ~21 Mb of genomic sequence were designed in this manner (table S1).

Eleven cell lines or xenografts of each tumor type (breast and colorectal carcinomas) were used in the Discovery Screen (table S2, A and B). Two matching normal samples were used as controls to help identify normal sequence variations and amplicon-specific sequencing artifacts such as those associated with GC-rich regions. A total of ~3 million PCR products were generated and directly sequenced, resulting in 465 Mb of tumor sequence.

Sequence data were assembled for each amplicon and evaluated for quality within the target region using software specifically designed for this purpose (*7*). The target region of each exon included all coding bases as well as the four intronic bases at both the 5' and 3' ends that serve as the major splice recognition sites. In order for an amplicon to be considered successfully analyzed, we required that ≥90% of bases in the target region have a Phred quality score (defined as $-10[\log_{10}(\text{raw per-base error})]$) of at least 20 in at least three quarters of the tumor samples analyzed (*8*). This quality cutoff was chosen to provide high sensitivity for mutation detection while minimizing false positives. Using these criteria, 93% of the 135,483 amplicons and 91% of the total targeted bases in CCDS were successfully analyzed for potential alterations.

Examination of sequence traces from these amplicons revealed a total of 816,986 putative nucleotide changes. As the vast majority of changes that did not affect the amino acid sequence (i.e., synonymous or silent substitutions) were likely to be non-functional, these changes were not analyzed further. The remaining 557,029 changes could represent germline variants, artifacts of PCR or sequencing, or *bona fide* somatic mutations. Several bioinformatic and experimental steps were employed to distinguish among these possibilities. First, any alterations that were also present in either of the two normal samples included in the Discovery Screen were removed, as these were likely to represent common germline polymorphisms or sequence artifacts. Second, as these two normal control samples would be expected to contain only a subset of known variants, any change corresponding to a validated germline polymorphism found in single nucleotide polymorphism (SNP) databases was also removed (*7*). Finally, the sequence trace of each potential alteration was visually inspected in order to remove false positive calls in the automated analysis. The combination of these data analysis efforts was efficient, removing ~96% of the potential alterations and leaving 29,281 for further scrutiny (Fig. 1).

To ensure that the observed mutations did not arise artifactually during the PCR or sequencing steps, the regions containing them were independently re-amplified and re-sequenced in the corresponding tumors. This step removed 9,295 alterations. The regions containing the putative mutations were then sequenced in matched normal DNA samples to determine whether the mutations were truly somatic: 18,414 changes were observed to be present in the germline of these patients, representing variants not currently annotated in SNP databases, and were excluded. As a final step, the remaining 1,572 putative somatic mutations were carefully examined *in silico* to ensure that the alterations did not arise from mistargeted sequencing of highly related regions occurring elsewhere in the genome (*7*). Alterations in such duplicated regions may appear to be somatic when there is loss of one or both alleles of the target region in the tumor and when the selected primers closely match and therefore amplify similar areas of the genome. A total of 265 changes in closely related regions were excluded in this fashion, resulting in a total of 1,307 confirmed somatic mutations in 1,149 genes (Table 1).

Validation screen. To evaluate the prevalence and spectrum of somatic mutations in these 1,149 genes, we determined their sequence in additional tumors of the same histologic type (Fig. 1) (table S2, A and B). Genes mutated in at least one breast or colorectal tumor in the Discovery Screen were analyzed in 24 additional breast or colorectal tumors, respectively. This effort involved 453,024 additional PCR and sequencing reactions, encompassing 77 Mb of tumor DNA. A total of 133,693 putative changes were identified in the Validation Screen. Methods similar to those employed in the Discovery Screen were used to exclude silent changes, known and novel germline variants, false positives arising from PCR or sequencing artifacts, and apparent changes that were likely due to co-amplification of highly related genes. Additionally, any changes corresponding to germline variants not found in SNP databases but identified in the Discovery Screen were excluded. The regions containing the remaining 4,948 changes were re-amplified and re-sequenced in the corresponding tumors (to ensure reproducibility) and in matched normal tissue to determine if they were somatic. An additional 365 somatic mutations in 236 genes were identified in this manner. In total, 921 and 751 somatic mutations were identified in breast and colorectal cancers, respectively (Fig. 1, Table 1, and table S4).

Mutation spectrum. The great majority of the 1,672 mutations observed in the Discovery or Validation Screens were single base substitutions: 81% of the mutations were missense, 7% were nonsense, and 4% altered splice sites (Table 1). The remaining 8% were insertions, deletions, and duplications ranging from one to 110 nucleotides in length. Though the fraction of mutations that were single base substitutions was similar in breast and colorectal cancers, the spectrum and nucleotide contexts of the substitution mutations were very different between the two tumor types. The most striking of these differences occurred at C:G base pairs: 59% of the 696 colorectal cancer mutations were C:G to T:A transitions while only 7% were C:G to G:C transversions (Table 2 and table S3). In contrast, only 35% of the mutations in breast cancers were C:G to T:A transitions, while 29% were C:G to G:C transversions. In addition, a large fraction (44%) of the mutations in colorectal cancers were at 5'-CpG-3' dinucleotide sites but only 17% of the mutations in breast cancers occurred at such sites. This 5'-CpG-3' preference led to an excess of nonsynonymous mutations resulting in changes of arginine residues in colorectal cancers though not in breast cancers (fig. S1). In contrast, 31% of mutations in breast cancers occurred at 5'-TpC-3' sites (or complementary 5'-GpA-3' sites), while only 11% of mutations in colorectal cancers occurred at these dinucleotide sites. The differences noted above were all highly significant ($P<0.0001$) (*7*) and have substantial implications for the mechanisms underlying mutagenesis in the two tumor types.

Distinction between passenger and non-passenger mutations. Somatic mutations in human tumors can arise either through selection of functionally important alterations via their effect on net cell growth or through accumulation of non-functional "passenger" alterations that arise during repeated rounds of cell division in the tumor or in its progenitor stem cell. In light of the relatively low rates of mutation in human cancer cells (*9*, *10*), distinction between selected and passenger mutations is generally not required when the number of genes and tumors analyzed is small. In large-scale studies, however, such distinctions are of paramount importance (*11*, *12*). For example, it has been estimated that nonsynonymous passenger mutations are present at a frequency no higher than ~1.2 per Mb of DNA in cancers of the breast or colon (*13–15*). As we assessed 542 Mb of tumor DNA, we would therefore have expected to observe ~650 passenger mutations. We actually observed 1,672 mutations (Table 1), many more than what would have been predicted to occur by chance ($P < 1 \times 10^{-10}$) (*7*). Moreover, the frequency of mutations in the Validation Screen was significantly higher than in the Discovery Screen (5.8 versus 3.1 mutations per Mb, $P < 1 \times 10^{-10}$, Table 1). The mutations in the Validation Screen were also enriched for nonsense, insertion, deletion, duplication, and splice site changes compared to the Discovery Screen; each of these would be expected to have a functional effect on the encoded proteins.

To distinguish genes likely to contribute to tumorigenesis from those in which passenger mutations occurred by chance, we first excluded genes that were not mutated in the Validation Screen. We next developed statistical methods to estimate the probability that the number of mutations in a given gene was greater than expected from the background mutation rate. For each gene, this analysis incorporated the number of somatic alterations observed in either the Discovery or Validation Screen, the number of tumors studied, and the number of nucleotides that were successfully analyzed (as indicated by the number of bases with Phred quality scores ≥ 20). Because the mutation frequencies varied with nucleotide type and context and were different in breast versus colorectal cancers (Table 2), these factors were included in the calculations. The output of this analysis was a *ca*ncer *m*utation *p*revalence (CaMP) score for each gene analyzed. The CaMP score reflects the probability that the number of mutations actually observed in a gene is higher than that expected to be observed by chance given the background mutation rate; its derivation is based on principles described in the Supporting Online Material. The use of the CaMP score for analysis of somatic mutations is analogous to the use of the LOD score for linkage analysis in

familial genetic settings. For example, 90% of the genes with CaMP scores > 1.0 are predicted to have mutation frequencies higher than the background mutation frequency.

Candidate cancer genes. A complete list of the somatic mutations identified in this study is provided in table S4. Validated genes with CaMP scores greater than 1.0 were considered to be *can*didate *can*cer genes (*CAN*-genes). The combination of experimental validation and statistical calculation thereby yielded four nested sets of genes: of 13,023 genes evaluated, 1,149 were mutated, 242 were validated, and 191 were *CAN*-genes. Among these, the *CAN*-genes were most likely to have been subjected to mutational selection during tumorigenesis. There were 122 and 69 *CAN*-genes identified in breast and colorectal cancers, respectively (tables S5 and S6). Individual breast cancers examined in the Discovery Screen harbored an average of 12 (range 4 to 23) mutant *CAN*-genes while the average number of *CAN*-genes in colorectal cancers was 9 (range 3 to 18) (table S3). Interestingly, each cancer specimen of a given tumor type carried its own distinct *CAN*-gene mutational signature, as no cancer had more than six mutant *CAN*-genes in common with any other cancer (tables S4 to S6).

CAN-genes could be divided into three classes: (a) genes previously observed to be mutationally altered in human cancers; (b) genes in which no previous mutations in human cancers had been discovered but had been linked to cancer through functional studies; and (c) genes with no previous strong connections to neoplasia.

(a) The re-identification of genes that had been previously shown to be somatically mutated in cancers represented a critical validation of the approach used in this study. All of the CCDS genes previously shown to be mutated in >10% of either breast or colorectal cancers were found to be *CAN*-genes in the current study. These included *TP53* (*2*), *APC* (*2*), *KRAS* (*2*), *SMAD4* (*2*), and *FBXW7* (*CDC4*) (*16*) (tables S4 to S6). In addition, we identified mutations in genes whose mutation prevalence in sporadic cancers was rather low. These genes included *EPHA3* (*17*), *MRE11A* (*18*), *NF1* (*2*), *SMAD2* (*19*, *20*), *SMAD3* (*21*), *TCF7L2* (*TCF4*) (*22*), *BRCA1* (*2*) and *TGFBRII* (*23*). We also detected mutations in genes that had been previously found to be altered in human tumors but not in the same tumor type identified in this study. These included guanine nucleotide binding protein, alpha stimulating *GNAS* (*24*), kelch-like ECH-associated protein *KEAP1* (*25*), *RET* proto-oncogene (*2*), and transcription factor *TCF1* (*26*). Finally, we found mutations in a number of genes that have been previously identified as targets of translocation or amplification in human cancers. These included nucleoporin *NUP214* (*2*), kinesin receptor *KTN1* (*27*), DEAD box polypeptide 10 *DDX10* (*28*), glioma-associated oncogene homolog 1 *GLI1* (*29*), and the translocation target gene of the runt related transcription factor 1 *RUNX1T1* (*MTG8*) (*2*). We conclude that if these genes had not already been demonstrated to play a causative role in human tumors, they would have been discovered through the approach taken in this study. By analogy, the 176 other *CAN*-genes in tables S5 and S6 are likely to play important roles in breast, colorectal, and perhaps other types of cancers.

(b) Although genetic alterations currently provide the most reliable indicator of a gene's importance in human neoplasia (*1*, *30*), there are many other genes which are thought to play key roles on the basis of functional or expression studies. Our study provides genetic evidence supporting the importance of several of these genes in neoplasia. For example, we discovered intragenic mutations in the ephrin receptor *EPHB6* (*31*), mixed-lineage leukemia 3 gene (MLL3) (*32*), gelsolin *GSN* (*33*), cadherin genes *CDH10* and *CDH20*, actin and SMAD binding protein filamin B *FLNB* (*34*), protein tyrosine phosphatase receptor *PTPRD* (*35*), and autocrine motility factor receptor *AMFR* (*36*).

(c) In addition to the genes noted above, our study revealed a large number of genes that had not been strongly suspected to be involved in cancer. These included polycystic kidney and hepatic disease 1 gene *PKHD1*, guanylate cyclase 1 *GUCY1A2*, transcription factor *TBX22*, exocyst complex component *SEC8L1*, tubulin tyrosine ligase *TTLL3*, ATP-dependent transporter *ATP8B1*, intrinsic factor-cobalamin receptor *CUBN*, actin binding protein *DBN1*, and tectorin alpha *TECTA*. In addition, seven *CAN*-genes corresponded to genes for which no biologic role has yet been established.

We examined the distribution of mutations within *CAN*-gene products to see if clustering occurred in specific regions or functional domains. In addition to the well documented hotspots in *TP53* (*37*) and *KRAS* (*38*), we identified three mutations in *GNAS* in colorectal cancers that affected a single amino acid residue (R201). Alterations of this residue have previously been shown to lead to constitutive activation of the encoded G protein α_s through inhibition of GTPase activity (*24*). Two mutations in the EGF-like gene *EGFL6* in breast tumors affected the same nucleotide position and resulted in a L508F change in the MAM adhesion domain. A total of seven genes had alterations located within five amino acid residues of each other, and an additional 12 genes had clustering of multiple mutations within a specific protein domain (13 to 78 amino acids apart). Thirty-one of 40 of these changes affected residues that were evolutionarily conserved. Although the effects of these alterations are unknown, their clustering suggests specific roles for the mutated regions in the neoplastic process.

***CAN*-gene groups.** An unbiased screen of a large set of genes can provide insights into pathogenesis that would not be apparent through single gene mutational analysis. This has been exemplified by large scale mutagenesis screens in

experimental organisms (*39–41*). We therefore attempted to assign each *CAN*-gene to a functional group based on Gene Ontology (GO) Molecular Function or Biochemical process groups, the presence of specific INTERPRO sequence domains, or previously published literature (Table 3) (Fig. 2). Several of the groups identified in this way were of special interest. For example, 22 of the 122 (18%) breast *CAN*-genes and 13 of the 69 (19%) colorectal *CAN*-genes were transcriptional regulators. At least one of these genes was mutated in more than 80% of the tumors of each type. Zinc-finger transcription factors were particularly highly represented (8 genes mutated collectively in 43% of breast cancer samples). Similarly, genes involved in cell adhesion represented ~22% of CAN-genes and affected more than two thirds of tumors of either type. Genes involved in signal transduction represented ~23% of *CAN*-genes and at least one such gene was mutated in 77% and 94% of the breast and colorectal cancer samples, respectively. Subsets of these groups were also of interest and included metalloproteinases (part of the cell adhesion and motility group and mutated in 37% of colorectal cancers), and G proteins and their regulators (part of the signal transduction group and altered in 43% of breast cancers). These data suggest that dysregulation of specific cellular processes are genetically selected during neoplasia and that distinct members of each group may serve similar roles in different tumors.

Discussion. Four important points have emerged from this comprehensive mutational analysis of human cancer. First is that a relatively large number of previously uncharacterized *CAN*-genes exist in breast and colorectal cancers and these genes can be discovered by unbiased approaches such as that used in our study. These results support the notion that large-scale mutational analyses of other tumor types will prove useful for identifying genes not previously known to be linked to human cancer.

Second, our results suggest that the number of mutational events occurring during the evolution of human tumors from a benign to a metastatic state is much larger than previously thought. We found that breast and colorectal cancers harbor an average of 52 and 67 non-synonymous somatic mutations in CCDS genes, of which an average of 9 and 12, respectively, were in *CAN*-genes (table S3). These data can be used to estimate the total number of nonsynonymous mutations in coding genes that arise in a "typical" cancer through sequential rounds of mutation and selection. Assuming that the mutation prevalence in genes that have not yet been sequenced is similar to that of the genes so far analyzed, we estimate that there are 81 and 105 mutant genes (average, 93) in the typical colorectal or breast cancer, respectively (see Supporting Online Material for details). Of these, an average of 14 and 20, respectively, would be expected to be *CAN*-genes. In addition to the *CAN*-genes, there were other mutated CCDS genes that were likely to have been selected for during tumorigenesis but were not altered at a frequency high enough to warrant confidence in their interpretation.

A third point emerging from our study is that breast and colorectal cancers show substantial differences in their mutation spectra. In colorectal cancers, a bias toward C:G to T:A transitions at 5'-CpG-3' sites has been previously noted in *TP53* (*42*). Our results suggest that this bias is genome-wide rather than representing a selection for certain nucleotides within *TP53*. This bias may reflect a more extensive methylation of 5'-CpG-3' dinucleotides in colorectal cancers than in breast cancers or the effect of dietary carcinogens (*43, 44*). In breast cancers, the fraction of mutations at 5'-TpC-3' sites was far higher in the CCDS genes examined in this study than previously reported for *TP53* (*37*). It has been noted that a small fraction of breast tumors may have a defective repair system, resulting in 5'-TpC-3' mutations (*15*). Our studies confirm that some breast cancers have higher fractions of 5'-TpC-3' mutations than others, but also show that mutations at this dinucleotide are generally more frequent than in colorectal cancers (Table 2 and table S3).

Finally, our results reveal that there are substantial differences in the panel of *CAN*-genes mutated in the two tumor types (Table 3). For example, metalloproteinase genes were mutated in a large fraction of colorectal but only in a small fraction of breast cancers (tables S5 and S6). Transcriptional regulator genes were mutated in a high fraction of both breast and colorectal tumors, but the specific genes affected varied according to tumor type (Table 3). There was also considerable heterogeneity among the *CAN*-genes mutated in different tumor specimens derived from the same tissue type (tables S4 to S6). It has been documented that virtually all biochemical, biological, and clinical attributes are heterogeneous within human cancers of the same histologic subtype (*45*). Our data suggest that differences in the *CAN*-genes mutated in various tumors could account for a major part of this heterogeneity. This might explain why it has been so difficult to correlate the behavior, prognosis, or response to therapy of common solid tumors with the presence or absence of a single gene alteration; such alterations reflect only a small component of each tumor's mutational composition. On the other hand, disparate genes contributing to cancer are often functionally equivalent, affecting net cell growth through the same molecular pathway (*1*). Thus, *TP53* and *MDM2* mutations exert comparable effects on cells, as do mutations in *RB1*, *CDKN2A* (*p16*), *CCND1* and *CDK4*. It will be of interest to determine whether a limited number of pathways include most *CAN*-genes, a possibility consistent with the groupings in Fig. 2 and Table 3.

Like a draft version of any genome project, our study has limitations. First, only genes present in the current version of CCDS were analyzed. There are ~5000 genes for which excellent supporting evidence exists but are not yet included in the CCDS database (*46*). Second, we were not able to successfully sequence ~10% of the bases within the coding sequences of the 13,023 CCDS genes (equivalent to 1,302 unsequenced genes). Third, although our screen would be expected to identify the most common types of mutations found in cancers, some genetic alterations, including mutations in non-coding genes, mutations in non-coding regions of coding genes, relatively large deletions or insertions, amplifications, and translocations, would not be detectable by the methods we used. Future studies employing a combination of different technologies, such as those envisioned by The Cancer Genome Atlas Project (TCGA) (*47*), will be able to address these issues.

The results of this study inform future cancer genome sequencing efforts in several important ways.

(i) A major technical challenge of such studies will be discerning somatic mutations from the large number of sequence alterations identified. In our study, 557,029 non-synonymous sequence alterations were detected in the Discovery Screen but after subsequent analyses only 0.23% of these were identified as legitimate somatic mutations (Fig. 1). Less than 10% of nonsynonymous alterations were known polymorphisms; many of the rest were uncommon germ-line variants or sequence artifacts that were not reproducible. Inclusion of matched normal samples and sequencing both strands of each PCR product would reduce false positives in the Discovery Screen but would increase the cost of sequencing by four-fold. Although recently developed sequencing methods could reduce the cost of such studies in the future (*48*), the higher error rates of these approaches may result in an even lower ratio of *bona fide* somatic mutations to putative alterations.

(ii) Another technical issue is that careful design of primers is important to eliminate sequence artifacts due to the inadvertent amplification and sequencing of related genes. The primer pairs that resulted in successful amplification and sequencing represent a valuable resource in this regard. Even with well-designed primers, it is essential to examine any observed mutation to ensure that it is not found as a normal variant in a related gene.

(iii) Although it is likely that studies of other solid tumor types will also identify a large number of somatic mutations, it will be important to apply rigorous approaches to identify those mutations that have been selected for during tumorigenesis. Statistical techniques, such as those used in this study or described by Greenman et al. (*11*), can provide strong evidence for selection of mutated genes. These approaches are likely to improve as more cancer genomic sequencing data is accumulated through The Cancer Genome Atlas Project (*47*) and other projects now underway.

(iv) There has been much discussion about which genes should be the focus of future sequencing efforts. Our results suggest that many genes not previously implicated in cancer are mutated at significant levels and may provide novel clues to pathogenesis. From these data, it would seem that large-scale unbiased screens of coding genes may be more informative than screens based on previously defined criteria.

(v) The results also raise questions about the optimum number of tumors of any given type that should be assessed in a cancer genome study. Our study was designed to determine the nature and types of alterations present in an "average" breast or colorectal cancer and to discover genes mutated at reasonably high frequencies. Our power to detect genes mutated in more than 20% of tumors of a given type was 90%, but only 50% of genes mutated in 6% of tumors would have been discovered. To detect genes mutated in 6% or 1% of tumors with >99% probability in a Discovery Screen would require sequence determination of at least 75 or 459 tumors, respectively. Though it will be impossible to detect all mutations that may occur in tumors, strategies that would identify the most important ones at an affordable cost can be envisioned on the basis of the data and analysis reported herein.

(vi) Ultimately, the sequences of entire cancer genomes, including intergenic regions, will be obtainable. Our studies demonstrate the inherent difficulties in determining the significance of somatic mutations, even those that alter the amino acid sequence of highly-annotated and well-studied genes. Establishing the significance of mutations in non-coding regions of the genome will likely be much more difficult. Until new tools for solving this problem become available, it is likely that gene-centric analyses of cancer will be more useful.

Our results provide a large number of future research opportunities in human cancer. For genetics, it will be of interest to elucidate the timing and extent of *CAN*-gene mutations in breast and colorectal cancers, whether these genes are mutated in other tumor types, and whether germline variants in *CAN*-genes are associated with cancer predisposition. For immunology, the finding that tumors contain an average of ~90 different amino acid substitutions not present in any normal cell can provide novel approaches to engender anti-tumor immunity. For epidemiology, the remarkable difference in mutation spectra of breast and colorectal cancers suggests the existence of organ-specific carcinogens. For cancer biology, it is clear that no current animal or *in vitro* model of cancer recapitulates the genetic landscape of an actual human tumor. Understanding and capturing this landscape and its heterogeneity may provide models that more successfully mimic the human disease. For

epigenetics, it is possible that a subset of *CAN*-genes can also be dysregulated in tumors through changes in chromatin or DNA methylation rather than through mutation. For diagnostics, the *CAN*-genes define a relatively small subset of genes that could prove useful as markers for neoplasia. Finally, some of these genes, particularly those on the cell surface or those with enzymatic activity, may prove to be good targets for therapeutic development.

References and Notes

1. B. Vogelstein, K. W. Kinzler, *Nature Med* **10**, 789 (2004).
2. P. A. Futreal *et al.*, *Nat. Rev. Cancer* **4**, 177 (2004).
3. A. Bardelli, V. E. Velculescu, *Curr. Opin. Genet. Dev.* **15**, 5 (2005).
4. B. Vogelstein, K. W. Kinzler, *The Genetic Basis of Human Cancer* (McGraw-Hill, Toronto, 2002).
5. D. M. Parkin, F. Bray, J. Ferlay, P. Pisani, *CA Cancer J. Clin.* **55**, 74 (2005).
6. www.ncbi.nlm.nih.gov/CCDS.
7. See supporting material on *Science* Online.
8. B. Ewing, P. Green, *Genome Res.* **8**, 186 (1998).
9. C. Lengauer, K. W. Kinzler, B. Vogelstein, *Nature* **396**, 643 (1998).
10. L. A. Loeb, *Cancer Res.* **61**, 3230 (2001).
11. C. Greenman, R. Wooster, P. A. Futreal, M. R. Stratton, D. F. Easton, *Genetics* **173**, 2187 (2006).
12. S. E. Kern, J. M. Winter, *Cancer Biol. Ther.* **5**, 349 (2006).
13. T. L. Wang *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **99**, 3076 (2002).
14. D. Shen *et al.*, in preparation.
15. P. Stephens *et al.*, *Nat. Genet.* **37**, 590 (2005).
16. H. Strohmaier *et al.*, *Nature* **413**, 316 (2001).
17. A. Bardelli *et al.*, *Science* **300**, 949 (2003).
18. Z. Wang *et al.*, *Cancer Res.* **64**, 2998 (2004).
19. G. J. Riggins *et al.*, *Nat. Genet.* **13**, 347 (1996).
20. K. Eppert *et al.*, *Cell* **86**, 543 (1996).
21. J. L. Ku *et al.*, *Cancer Lett.* (5 July 2006).
22. A. Duval *et al.*, *Cancer Res.* **59**, 4213 (1999).
23. S. Markowitz *et al.*, *Science* **268**, 1336 (1995).
24. C. A. Landis *et al.*, *Nature* **340**, 692 (1989).
25. B. Padmanabhan *et al.*, *Mol Cell* **21**, 689 (2006).
26. O. Bluteau *et al.*, *Nat. Genet.* **32**, 312 (2002).
27. K. Salassidis *et al.*, *Cancer Res.* **60**, 2786 (2000).
28. Y. Arai *et al.*, *Blood* **89**, 3936 (1997).
29. K. W. Kinzler *et al.*, *Science* **236**, 70 (1987).
30. H. Varmus, *Science* **312**, 1162 (2006).
31. X. X. Tang, G. M. Brodeur, B. G. Campling, N. Ikegaki, *Clin. Cancer Res.* **5**, 455 (1999).
32. M. Ruault, M. E. Brun, M. Ventura, G. Roizes, A. De Sario, *Gene* **284**, 73 (2002).
33. M. Tanaka *et al.*, *Cancer Res.* **55**, 3228 (1995).
34. A. Sasaki, Y. Masuda, Y. Ohta, K. Ikeda, K. Watanabe, *J. Biol. Chem.* **276**, 17871 (2001).
35. M. Sato *et al.*, *Genes Chromosomes Cancer* **44**, 405 (2005).
36. Y. Onishi, K. Tsukada, J. Yokota, A. Raz, *Clin. Exp. Metastasis* **20**, 51 (2003).
37. M. Hollstein, D. Sidransky, B. Vogelstein, C. C. Harris, *Science* **253**, 49 (1991).
38. J. L. Bos *et al.*, *Nature* **327**, 293 (1987).
39. R. Brent, *Cell* **100**, 169 (2000).
40. T. Ideker *et al.*, *Science* **292**, 929 (2001).
41. S. L. Ooi *et al.*, *Trends Genet.* **22**, 56 (2006).
42. T. Soussi, G. Lozano, *Biochem. Biophys. Res. Commun.* **331**, 834 (2005).
43. M. Olivier, S. P. Hussain, C. Caron de Fromentel, P. Hainaut, C. C. Harris, *IARC Sci. Publ. 247* (2004).
44. J. F. Costello *et al.*, *Nat. Genet.* **24**, 132 (2000).
45. A. H. Owens, D. S. Coffey, S. B. Baylin, Eds., *Tumor Cell Heterogeneity* (Academic Press, New York, 1982), pp. 441–460.
46. K. D. Pruitt, T. Tatusova, D. R. Maglott, *Nucleic Acids Res.* **33**, D501 (2005).
47. http://cancergenome.nih.gov/index.asp
48 Y. H. Rogers, J. C. Venter, *Nature* **437**, 326 (2005).

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Supporting Online Material

www.sciencemag.org/cgi/content/full/1133427/DC1
Materials and Methods
Figs. S1 and S2
Tables S1 to S5
References

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Fig. 1. Schematic of mutation discovery and validation screens.

Fig. 2. Mutation frequency of *CAN*-gene groups. *CAN*-genes were grouped by function using Gene Ontology groups, INTERPRO domains, and available literature. Bars indicate the fraction of tumors (35 breast or 35 colorectal) with at least one mutated gene in the functional group.

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Table 1. Summary of somatic mutations*

Screen	Tumor	Number of mutated genes	Number of mutations	Nonsynonymous mutations in coding sequences										Mutations in non-coding sequences				Nucleotides successfully analyzed (Mb)°	Mutation frequency (mutations/Mb)
				Mis-sense		Non-sense		Insertion		Deletion		Duplication		Splice site†		UTR‡			
Discovery Screen¶	Colon	519	574	482	(84.0)	35	(6.1)	3	(0.5)	18	(3.1)	17	(3.0)	17	(3.0)	2	(0.3)	208.5	2.8
	Breast	673	733	600	(81.9)	39	(5.3)	3	(0.4)	48	(6.5)	2	(0.3)	37	(5.0)	4	(0.5)	209.2	3.5
	Total	1149	1307	1082	(82.8)	74	(5.7)	6	(0.5)	66	(5.0)	19	(1.5)	54	(4.1)	6	(0.5)	417.7	3.1
Prevalence Screen#	Colon	105	177	126	(71.2)	26	(14.7)	2	(1.1)	10	(5.6)	3	(1.7)	9	(5.1)	1	(0.6)	28.7	6.2
	Breast	137	188	145	(77.1)	8	(4.3)	2	(1.1)	13	(6.9)	12	(6.4)	8	(4.3)	0	(0.0)	34.3	5.5
	Total	236	365	271	(74.2)	34	(9.3)	4	(1.1)	23	(6.3)	15	(4.1)	17	(4.7)	1	(0.3)	63.0	5.8
Both screens combined	Colon	519	751	608	(81.0)	61	(8.1)	5	(0.7)	28	(3.7)	20	(2.7)	26	(3.5)	3	(0.4)	237.2	3.2
	Breast	673	921	745	(80.9)	47	(5.1)	5	(0.5)	61	(6.6)	14	(1.5)	45	(4.9)	4	(0.4)	243.5	3.8
	Total	1149	1672	1353	(80.9)	108	(6.5)	10	(0.6)	89	(5.3)	34	(2.0)	71	(4.2)	7	(0.4)	480.7	3.5

*Numbers in parentheses refer to percentage of total mutations. ¶Coding and adjacent non-coding regions of 13,023 CCDS genes were sequenced in 11 colorectal and 11 breast cancers. #Genes mutated in the discovery screen were sequenced in 24 additional tumor samples of the affected tumor type. †Intronic mutations within 4 bp of exon/intron boundary. ‡Mutations in untranslated regions (UTR) within 4 bp 5' of initiation codon or 4 bp 3' of termination codon. °Nucleotides with Phred quality score of at least 20.

Table 2. Spectrum of single base substitutions*

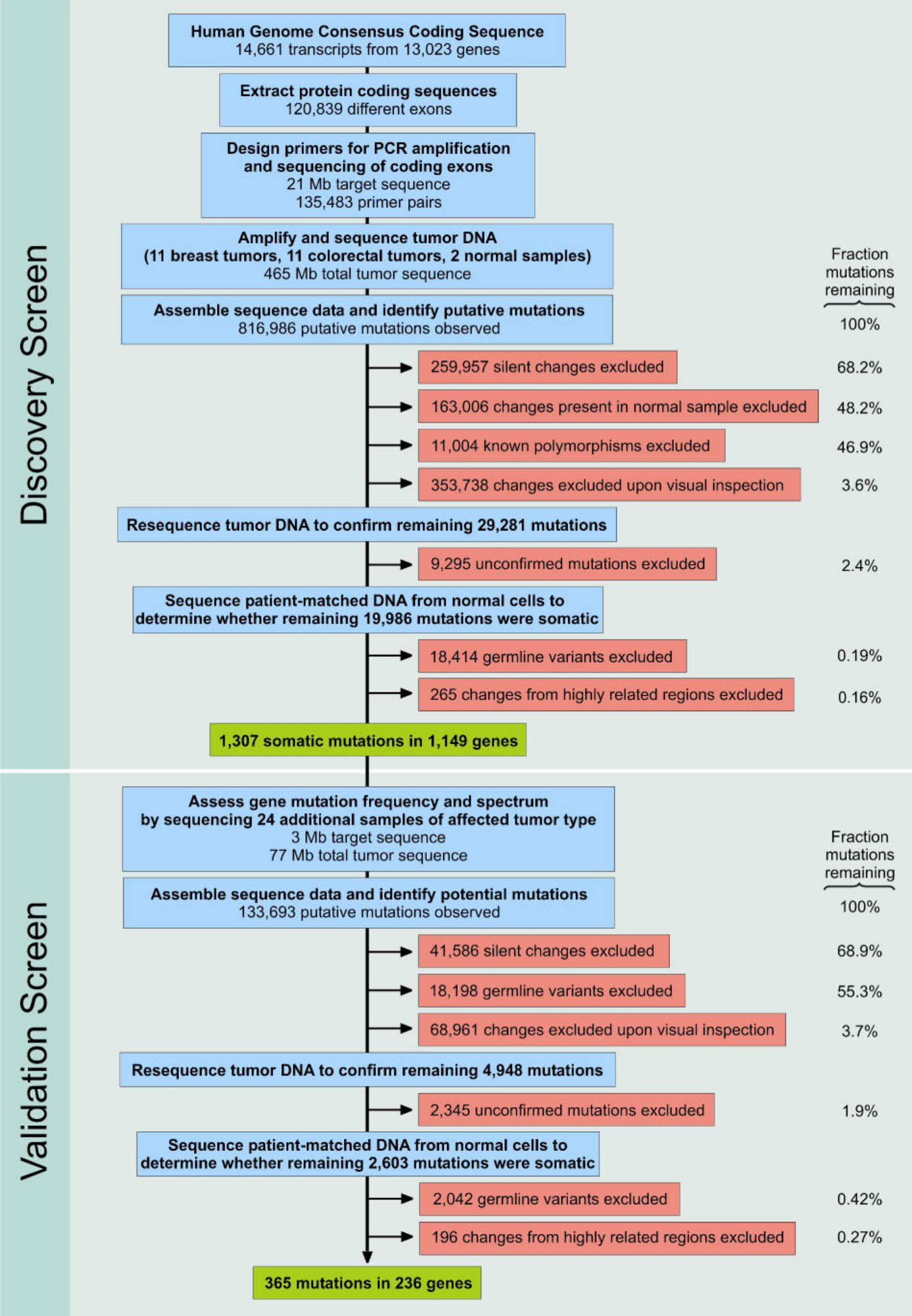
Screen	Tumor	Total number of substitutions	Substitutions at C:G base pairs						Substitutions at T:A base pairs						Substitutions at specific dinucleotides¶			
			C:G → T:A		C:G → G:C		C:G → A:T		T:A → C:G		T:A → G:C		T:A → A:T		5'-CpG-3'		5'-TpC-3'	
Discovery Screen	Colon	535	325	(60.7)	36	(6.7)	70	(13.1)	42	(7.9)	38	(7.1)	24	(4.5)	254	(47.5)	54	(10.1)
	Breast	678	230	(33.9)	207	(30.5)	110	(16.2)	54	(8.0)	30	(4.4)	47	(6.9)	115	(17.0)	235	(34.7)
	Total	1213	555	(45.8)	243	(20.0)	180	(14.8)	96	(7.9)	68	(5.6)	71	(5.9)	369	(30.4)	289	(23.8)
Prevalence Screen	Colon	161	88	(54.7)	12	(7.5)	23	(14.3)	14	(8.7)	13	(8.1)	11	(6.8)	55	(34.2)	25	(15.5)
	Breast	160	59	(36.9)	32	(20.0)	38	(23.8)	18	(11.3)	5	(3.1)	8	(5.0)	24	(15.0)	22	(13.8)
	Total	321	147	(45.8)	44	(13.7)	61	(19.0)	32	(10.0)	18	(5.6)	19	(5.9)	79	(24.6)	47	(14.6)
Both screens combined	Colon	696	413#	(59.3)	48#	(6.9)	93	(13.4)	56	(8.0)	51	(7.3)	35	(5.0)	309#	(44.4)	79#	(11.4)
	Breast	838	289#	(34.5)	239#	(28.5)	148	(17.7)	72	(8.6)	35	(4.2)	55	(6.6)	139#	(16.6)	257#	(30.7)
	Total	1534	702	(45.8)	287	(18.7)	241	(15.7)	128	(8.3)	86	(5.6)	90	(5.9)	448	(29.2)	336	(21.9)

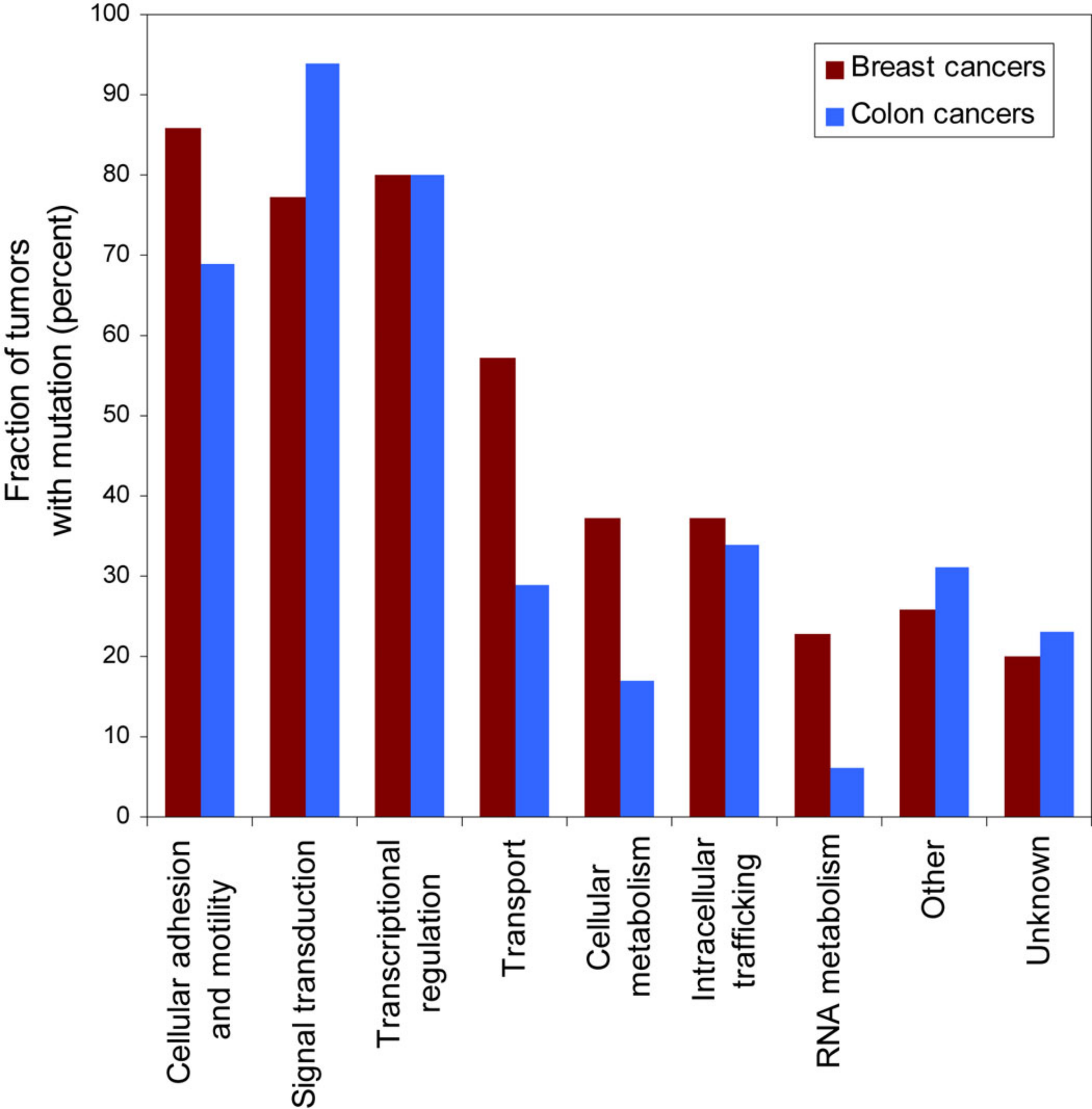
*Base substitutions in coding sequences resulting in nonsynonymous changes as well as substitutions in non-coding sequences are included (see Table 1). Numbers in parentheses indicate percentage of total mutations. # indicates that the values in this category were significantly different between breast and colorectal cancers (P <0.0001). ¶Includes substitutions at the C or G of the 5'-CpG-3' dinucleotide, the C of the 5'-TpC-3' dinucleotide, or the G of the 5'-GpA-3' dinucleotide.

Table 3. Functional classification of *CAN-* genes*

Breast cancers						Colorectal cancers			
***CAN*-genes and CaMP scores**						***CAN-* genes and CaMP scores**			
Cellular adhesion and motility (examples: cytoskeletal protein binding GO:0008092, cell adhesion GO:0007155, metallopeptidase activity GO:0008237)									
FLNB	3.4	*TMPRSS6*	2.0	*RAPH1*	1.4	*PKHD1*	3.5	*CNTN4*	1.6
MYH1	2.7	*COL11A1*	1.8	*PCDHB15*	1.4	*ADAMTSL3*	3.3	*CHL1*	1.3
SPTAN1	2.6	*DNAH9*	1.7	*CMYA1*	1.4	*OBSCN*	3.0	*HAPLN1*	1.2
DBN1	2.5	*OBSCN*	1.7	*MACF1*	1.3	*ADAMTS18*	2.7	*MGC33407*	1.2
TECTA	2.4	*COL7A1*	1.5	*SYNE2*	1.3	*MMP2*	2.3	*MAP2*	1.0
ADAM12	2.3	*MAGEE1*	1.5	*NRCAM*	1.1	*TTLL3*	2.2		
GSN	2.2	*CDH10*	1.5	*COL19A1*	1.1	*EVL*	2.0		
CDH20	2.2	*SULF2*	1.5	*SEMA5B*	1.1	*ADAM29*	2.0		
BGN	2.1	*CNTN6*	1.4	*ITGA9*	1.1	*CSMD3*	1.9		
ICAM5	2.1	*THBS3*	1.4			*ADAMTS15*	1.8		
Signal transduction (examples: intracellular signaling cascade GO:0007242, receptor activity GO:0004872, GTPase regulator GO:0030695)									
VEPH1	2.1	*PFC*	1.5	*PRPF4B*	1.3	*APC*	>10	*PTPRD*	2.2
SBNO1	2.1	*GAB1*	1.5	*CENTG1*	1.3	*KRAS*	>10	*MCP*	2.1
DNASE1L3	1.9	*ARHGEF4*	1.4	*MAP3K6*	1.3	*EPHA3*	4.2	*NF1*	1.9
RAP1GA1	1.8	*NALP8*	1.4	*APC2*	1.3	*GUCY1A2*	3.5	*PTPRU*	1.4
EGFL6	1.8	*RGL1*	1.4	*STARD8*	1.2	*EPHB6*	3.5	*CD109*	1.3
AMFR	1.7	*PPM1E*	1.4	*PTPN14*	1.1	*TGFBR2*	2.9	*PHIP*	1.2
CENTB1	1.7	*PKDREJ*	1.4	*IRTA2*	1.1	*GNAS*	2.6		
GPNMB	1.7	*CNNM4*	1.3	*RASGRF2*	1.1	*RET*	2.3		
INHBE	1.7	*ALS2CL*	1.3	*MTMR3*	1.1	*P2RY14*	2.2		
FLJ10458	1.6	*RASAL2*	1.3			*LGR6*	2.2		
Transcriptional regulation (examples: regulation of transcription GO:0045449, zinc finger C2H2-subtype IPR007086)									
TP53	>10	*CHD5*	1.8	*ZFP64*	1.4	*TP53*	>10	*ZNF442*	1.9
FLJ13479	3.4	*CIC*	1.7	*ZNF569*	1.4	*SMAD4*	4.6	*SMAD3*	1.9
SIX4	2.5	*KEAP1*	1.6	*EHMT1*	1.3	*MLL3*	3.7	*EYA4*	1.5
KIAA0934	2.5	*HOXA3*	1.6	*ZFYVE26*	1.2	*TBX22*	3.3	*PKNOX1*	1.4
LRRFIP1	2.4	*TCF1*	1.6	*BCL11A*	1.1	*SMAD2*	3.1	*MKRN3*	1.3
GLI1	2.3	*HDAC4*	1.6	*ZNF318*	1.1	*TCF7L2*	2.8		
RFX2	2.1	*MYOD1*	1.5			*HIST1H1B*	2.5		
ZCSL3	1.8	*NCOA6*	1.5			*RUNX1T1*	2.4		
Transport (examples: ion transporter activity GO:0015075, ligand-gated ion channel activity GO:0015276, carrier activity GO:0005386)									
ATP8B1	3.1	*ABCB8*	1.7	*ABCB10*	1.4	*ABCA1*	2.8	*C6orf29*	1.1
CUBN	2.5	*KPNA5*	1.7	*SCNN1B*	1.3	*SLC29A1*	1.9		
GRIN2D	2.4	*ABCA3*	1.7	*NUP133*	1.1	*SCN3B*	1.9		
HDLBP	2.2	*SLC9A2*	1.6			*P2RX7*	1.3		
NUP214	1.8	*SLC6A3*	1.5			*KCNQ5*	1.2		
Cellular metabolism (examples: aromatic compound metabolism GO:0006725, generation of precursor metabolites GO:0016445, biosynthesis GO:0009058)									
ACADM	2.0	*NCB5OR*	1.7	*PHACS*	1.4	*UQCRC2*	1.9		
PRPS1	1.8	*ASL*	1.6	*XDH*	1.3	*ACSL5*	1.6		
CYP1A1	1.7	*GALNT5*	1.4			*GALNS*	1.2		
Intracellular trafficking (examples: endoplasmic reticulum targeting sequence IPR000886, membrane fusion GO:0006944)									
OTOF	2.2	*PLEKHA8*	1.8	*KTN1*	1.5	*SYNE1*	2.3	*PRKD1*	1.9
LRBA	2.1	*LOC283849*	1.7	*GGA1*	1.4	*SEC8L1*	2.2	*LRP2*	1.2
AEGP	1.8	*SORL1*	1.7			*SDBCAG84*	2.2		
RNA metabolism (examples: RNA processing GO:0008353, RNA splice site selection GO:0006376)									
C14orf155	3.3	*RNU3IP2*	1.7	*KIAA0427*	1.5	*SFRS6*	1.3		
SP110	1.8	*C22orf19*	1.5	*DDX10*	1.3				
Other (examples: response to DNA damage stimulus GO:0006974, protein ubiquitination GO:0016567)									
FLJ40869	2.1	*SERPINB1*	1.4			*FBXW7*	5.1	*K6IRS3*	1.2
BRCA1	2.0					*UHRF2*	1.5	*CD248*	1.2
MRE11A	1.6					*LMO7*	1.3	*ERCC6*	1.0
Unknown									
KIAA1632	2.4	*KIAA0999*	1.3			*C10orf137*	2.7	*KIAA1409*	1.6
MGC24047	2.1					*LOC157697*	2.0	*C15orf2*	1.0

**CAN-* genes were assigned to functional classes using Gene Ontology (GO) groups, INTERPRO domains and available literature. Representative GO groups and INTERPRO domains are listed for each class.


Discovery Screen
Human Genome Consensus Coding Sequence
14,661 transcripts from 13,023 genes
Extract protein coding sequences
120,839 different exons
Design primers for PCR amplification and sequencing of coding exons
21 Mb target sequence
135,483 primer pairs
Amplify and sequence tumor DNA
(11 breast tumors, 11 colorectal tumors, 2 normal samples)
465 Mb total tumor sequence
Fraction mutations remaining
Assemble sequence data and identify putative mutations
816,986 putative mutations observed
100%
259,957 silent changes excluded
68.2%
163,006 changes present in normal sample excluded
48.2%
11,004 known polymorphisms excluded
46.9%
353,738 changes excluded upon visual inspection
3.6%
Resequence tumor DNA to confirm remaining 29,281 mutations
9,295 unconfirmed mutations excluded
2.4%
Sequence patient-matched DNA from normal cells to determine whether remaining 19,986 mutations were somatic
18,414 germline variants excluded
0.19%
265 changes from highly related regions excluded
0.16%
1,307 somatic mutations in 1,149 genes
Validation Screen
Assess gene mutation frequency and spectrum by sequencing 24 additional samples of affected tumor type
3 Mb target sequence
77 Mb total tumor sequence
Fraction mutations remaining
Assemble sequence data and identify potential mutations
133,693 putative mutations observed
100%
41,586 silent changes excluded
68.9%
18,198 germline variants excluded
55.3%
68,961 changes excluded upon visual inspection
3.7%
Resequence tumor DNA to confirm remaining 4,948 mutations
2,345 unconfirmed mutations excluded
1.9%
Sequence patient-matched DNA from normal cells to determine whether remaining 2,603 mutations were somatic
2,042 germline variants excluded
0.42%
196 changes from highly related regions excluded
0.27%
365 mutations in 236 genes


Breast cancers
Colon cancers
Fraction of tumors with mutation (percent)
100
90
80
70
60
50
40
30
20
10
0
Cellular adhesion and motility
Signal transduction
Transcriptional regulation
Transport
Cellular metabolism
Intracellular trafficking
RNA metabolism
Other
Unknown