Cancer genomics: integrating form and function

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The sequencing of the human genome has formed the foundation with which to develop technologies and reagents to perform true genome-scale biological studies. In particular, the development and increasing application of these high-throughput genome-scale technologies have fundamentally altered the way one can approach the analysis of cancer. It is now possible to imagine studies that interrogate the structure, expression and function of every gene in a comprehensive, highly parallel fashion, permitting the development of multidimensional, global views of cancer. In this review, we focus on recent advances in the application of genomic strategies to the study of cancer, with an emphasis on functional genomics and the prospects for integrating the knowledge gained from these approaches to further develop our understanding of cancer and design better therapeutic strategies.

Introduction

Cancer results from the accumulation of genetic and epigenetic changes that drive cells into states of unregulated proliferation and altered morphology. Tumorigenesis is a multi-step process involving multiple genetic lesions that leads to the evasion of proliferative controls as well as the activation of survival mechanisms (1,2). In colorectal cancers, previous studies suggest that at least four to five genetic alterations are required to form a fully malignant tumor (3). Similarly, deregulation of at least five distinct pathways appears to be sufficient to transform most normal primary cells into tumorigenic cells (4). Several genetic mechanisms destabilize the normal cellular homeostasis including gains, losses or translocations of large regions of chromosomes, single-nucleotide substitutions and methylation events, all of which contribute to programming the transformed state (Figure 1). Although much of our knowledge of these cancer-promoting alterations was initially derived from the study of single genes in specific cancers, a variety of techniques have now been developed to profile these alterations and their cellular consequences comprehensively, providing means to survey the landscapes of the cancer genome, transcriptome, proteome and phenome.

The anatomy of cancer genomes

Initial genomic studies focused on characterizing changes in global expression levels, using microarray or serial analysis of gene expression analysis. By identifying gene expression signatures composed of representative transcripts, this method has proven useful in classifying tumors and predicting patient outcomes (5–7). For example, in breast cancer patients, a 70-gene signature has been identified that predicts long-term prognosis more effectively than standard clinical and histopathological markers (7). This signature is the basis of a diagnostic test recently approved by the U.S. Food and Drug Administration to assess risk and optimize treatment strategy for individual patients (8). Similarly, transcriptional profiling was used to identify a gene expression signature that predicts which primary tumors are more likely to form metastases, suggesting that the metastatic program is an early, rather than a late, stochastic event in tumor progression (9).

Abbreviations: miRNA, microRNA; siRNA, small interfering RNA.

Beyond transcriptional profiling, two whole-genome techniques, comparative genome hybridization and high-density single-nucleotide polymorphism arrays, permit the detection of chromosomal regions exhibiting decreased or increased copy number at high resolution (10). Physical loss or gain of chromosomal regions occurs in the majority of epithelial cancers; these regions often contain tumor suppressors or oncogenes, respectively. Prominent examples include amplifications of HER2 in breast cancer and N-MYC in neuroblastoma or loss of PTEN in prostate cancer or glioblastoma. Both array comparative genome hybridization and single-nucleotide polymorphism array techniques have recently been used successfully to identify genes and regions commonly altered in lung cancer (11), breast cancer (12) and melanoma (13), as well as to demonstrate correlation between the presence of recurrent alterations and patient prognosis for breast cancer (14–16).

Somatic mutations in gene-coding sequences are another common tumor-promoting mechanism. These somatic mutations can cause deregulated activation of oncogenes, as in the case of MYC or RAS, or loss of function of tumor suppressors, as in the case of TP53 or APC. More recent high-throughput sequencing efforts have uncovered novel oncogenic mutations in known oncogenes, including EGFR, B-RAF and PIK3CA (17–20), as well as identifying putative novel oncogenes (21). Of particular clinical interest, in the cases of EGFR and B-RAF, the presence of specific oncogenic mutations have been linked with enhanced sensitivity to certain drug therapies and can be used as prognostic markers to predict patient response (18,19,22).

Further advances in technologies to detect specific gene sequence changes now allow high-throughput profiling of the most common oncogenic mutations, which can be readily adapted for use in the clinic to provide information on patient genotype to guide therapeutic strategy (23).

In addition to transcriptomics and structural genomics, recent work suggests that high-throughput methods to characterize the epigenetic and proteomic changes associated with cancer promise to further refine our ability to generate molecular descriptions of the state of cancer. Epigenetic changes are clearly associated with tumorigenesis, as cancer genomes are globally hypomethylated whereas promoters at certain tumor suppressors, such as PTEN and p16INK4a, are known to undergo hypermethylation, leading to gene silencing (24–26). Proteomics offer the ability to comprehensively detect changes in expression levels, post-translational modifications as well as enzymatic function of proteins (27). Understanding these differences is critical to the development of molecularly targeted therapeutics.

Taken together, these approaches have already proven to be useful in characterizing human cancers and will undoubtedly lead to the development of diagnostic and prognostic applications that will refine our ability to diagnose and treat cancer. Importantly, these approaches can provide insight into the functional, molecular and cellular events that are ultimately responsible for the transition to the tumorogenic state. However, alterations identified by each of these approaches require further studies to determine whether such changes are truly relevant to tumorigenesis. For example, since cancer genomes often exhibit widespread and sometimes ongoing genetic instability, it is essential to differentiate between actual oncogenic events and mere bystander events, necessitating careful statistical analysis and functional validation to establish significance. With chromosomal alterations, it is often difficult to identify the genes responsible for driving the loss or amplification events, as these regions can be quite large, encompassing many genes or whole-chromosome arms. For expression profiling, it may be difficult to gain biological insight into the underlying essential changes responsible for the tumor phenotype from the many genes exhibiting altered expression levels, although there have been significant advances in this area (28).

Ultimately, each of these cancer-profiling approaches is primarily descriptive, providing a snapshot of a given tumor at a given time.
In addition, imaging-based approaches permit the detection of subtle changes in phenotype and may be particularly useful in detecting phenotypes that are not highly penetrant or that would be missed using population-based assays. Whether using population- or single cell-based approaches, performing multiplexed assays, i.e. performing several assays on the same sample simultaneously greatly increases the information obtained from such screens. By measuring multiple phenotypic parameters, increasingly refined and multidimensional descriptions of the functional consequences of gene perturbation can be discovered.

The mode of delivery of the perturbation agent, most commonly transfection or retroviral transduction, is another important consideration for any functional screen. DNA plasmids or small interfering RNA (siRNA) oligonucleotides can be introduced into the cell by transfection, resulting in transient effects, useful for rapidly assaying short-term phenotypes, such as cell cycle distribution or apoptosis (33,35). Retroviral delivery of plasmid vectors results in stable integration and expression, making possible the interrogation of phenotypes that require longer time courses to assay, including those typically used to assess oncogenic potential, such as soft agar colony formation or in vivo tumor formation. Moreover, retroviral delivery of cDNA or RNAi reagents permits a much wider range of cells to be studied.

There are two types of screening formats for in vitro cell-based assays, arrayed screens and pooled screens, each of which offers advantages for investigating a different subset of phenotypes. The arrayed screen systematically interrogates each construct or gene separately and allows one to assign a phenotype for every single well tested. A useful alternate to arrayed screens where samples are physically separated by wells is the method of reverse transfection, in which arrays of constructs are spotted onto glass slides with transfection reagent, onto which cells are plated, resulting in their transfection (36). This approach allows the screening process to be further miniaturized and testing of many constructs simultaneously can be achieved quickly and economically. Arrayed screens can be used with oligonucleotide-based, vector-based or chemical compound-based strategies, allowing greatest flexibility in the mode of delivery of the perturbation agent. However, to achieve true high-throughput potential, arrayed formats require robotic automation of many steps in order to provide the consistent liquid handling necessary for reproducible results.

The pooled format allows testing of many thousands of constructs in a single experimental sample, and generally requires a vector-based strategy of delivery, as well as a strong selection process to enable isolation of the subpopulation of cells that exhibit the desired phenotype. Identification of the selected constructs can be achieved through sequencing of the resulting individual clones or through hybridization of unique sequences or barcodes within each construct to microarrays to determine relative abundance of any particular construct. Importantly, the pooled approach permits one to investigate more complex phenotypes for which the arrayed format is not amenable, including in vivo selection strategies. To date, the pooled approach has been used primarily with strategies that employ positive selection, such as bypass of senescence or in vitro transformation assays (37–39). In such positive selection screens, the majority of cells fail to pass the selection barrier, leaving behind only the rare escapers, from which the responsible constructs can be isolated and sequenced. Negative selection strategies, in which the population of cells harboring the introduced reagent is eliminated from the population, require the use of barcode hybridization technology and remain technically more challenging, although there has been one reported success using a pool of inducible retroviral RNAi constructs to identify genes essential for cell viability (40).

**Gain-of-function approaches**

Most gain-of-function screens involve introduction of a cDNA library into cells either transiently or stably, ideally resulting in the hyper-activation of pathways positively regulated by the gene corresponding

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**Fig. 1.** Overview of genomic alterations associated with tumorigenesis, including (A) chromosomal loss, (B) chromosomal gain, (C) promoter methylation, (D) somatic mutation and for comparison (E) the unaltered state. Shown at bottom are techniques used to profile both these changes and their downstream cellular consequences, such as changes in transcript or protein level and changes in protein activity.

When mutations, genes or pathways are implicated in cancer using these approaches, detailed functional studies are required to establish the physiological relevance of identified changes in cancer. Traditionally, this process of systematically investigating one or a few genes at a time in order to determine their cellular functions and the roles they play in tumorigenesis is both slow and labor intensive. An alternative approach is to identify cancer-relevant genes by perturbation of gene expression and then validating its relevance to human cancer.

**Functional genomics**

With recent advances, the functional characterization of genes can now be performed at high throughput and high resolution using a wide variety of assays to interrogate any number of interesting and informative cancer-relevant phenotypes. In recent years, many groups have generated genome-scale reagents to facilitate functional genomic studies, using either gain- or loss-of-function approaches to investigate gene function. The basic tenet of these functional genomics studies is that by perturbing the activity of a gene, one can gain insight into its biological functions by assessment in phenotypic assays (Figure 2). This can be done in highly parallel fashion, systematically screening many thousands of genes simultaneously, allowing rapid and straightforward identification of the genes whose functions are involved in the phenotype of interest. Importantly, unlike the profiling approaches used to characterize cancer mentioned above, functional approaches allow the establishment of causal relationships between genotypes and phenotypes, rather than simple correlation.

The success of any large-scale functional screen depends largely on how well the chosen phenotypic assay performs, i.e. robustness and reproducibility. These two characteristics are essential in order to identify the genes that generate the phenotype of interest with a high degree of confidence. Recently reported functional genomics screens have used an amazing range and depth in the types of phenotypes that were interrogated in high-throughput fashion, such as cellular morphology, apoptosis, endocytic trafficking, host–pathogen interactions and Wnt signaling (29–32). Assays used in functional screens can be either population-based, homogenous, low-content approaches (30,33) or high-resolution profiling of single cells using automated microscopy imaging approaches (34) or fluorescence-activated cell sorting analysis (Figure 2). Imaging techniques are preferred when looking at phenotypes based on measurements from individual cells, such as cellular morphology or subcellular localization of molecules.
miRNAs have been described, most of which do not have identified degradation or translational inhibition. Nearly 500 annotated human expression of their target genes, either through induction of transcript (miRNA) expression libraries to screen using phenotypic assays. miRNAs improved annotation, may be realized in the not so distant future.

Building the ideal these are common modes of activation for these classes of proteins. These motifs are especially useful if one is interested in signal transduction molecules or receptor tyrosine kinases, respectively, as these are common modes of activation for these classes of proteins. Building the ideal ‘activation’ library would require tailoring the context of expression to the individual gene, which in turn requires knowledge of the activation process for all the genes within the library. Currently, this aim would be achievable only for small subsets of gene families but, with accumulating experimental evidence and improved annotation, may be realized in the not so distant future.

Another type of gain-of-function approach utilizes microRNA (miRNA) expression libraries to screen using phenotypic assays. miRNAs are endogenous small RNAs that function by down-regulating expression of their target genes, either through induction of transcript degradation or translational inhibition. Nearly 500 annotated human miRNAs have been described, most of which do not have identified targets or functions (46). miRNAs implicated in cancer include let-7, a negative regulator of RAS found up-regulated in lung cancers (47), the miR-17-92 cluster, which is up-regulated in lymphomas and can promote lymphomagenesis (48) and miR-15 and miR-16, negative regulators of BCL2, that are down-regulated in chronic lymphocytic leukemia (49). Recent work using a retroviral expression library of miRNAs identified miR-372 and miR-373 in a Ras-induced senescence bypass screen, suggesting possible oncogenic function for these miRNAs (50). Future applications of this approach will likely yield many more cancer-relevant miRNAs and the identification of their respective targets are also likely to provide further insight into the oncogenic process.

Loss-of-function approaches

Determining the consequences of gene loss of function is a classic means of elucidating gene function. RNAi technology has revolutionized mammalian biology by allowing researchers to generate loss-of-function phenotypes in mammalian cells with the precision and ease once available only to those studying lower organisms. Previous loss-of-function approaches for mammalian cells have been based on recombination-mediated gene deletion or random mutagenesis, highly effective but slow and laborious processes. Other options, including ribozymes and antisense techniques, have been useful but are less amenable to automation or high-throughput applications. Similar to the cDNA libraries used for gain-of-function approaches, RNAi libraries can be introduced into cells either stably or transiently. However, unlike cDNA over-expression, RNAi is more reliable in that loss of function can be verified by assessing efficacy of gene knockdown, either at the transcript or protein level, whereas in over-expression approaches, over-expression of a gene can be detected without necessarily indicating its activation, and non-physiological functions induced by over-expression can confound this approach.

RNAi was first described in Caenorhabditis elegans (51) and plants (52) and later in Drosophila melanogaster (53). In these model organisms, the introduction of long double-stranded RNA molecules results in potent and efficient knockdown of the target gene expression in a sequence-specific manner. Subsequently, it was discovered that short RNA duplexes (19–29 nucleotides), known as siRNAs, could bypass the interferon responses induced by long double-stranded RNAs yet still lead to potent silencing of the target genes in mammalian cells as well (54). Since this initial discovery, RNAi has been widely adopted, for applications ranging from inhibiting the function.

Fig. 2. Schematic of functional genomics approaches, giving an overview on types of perturbation agents, screening formats and several examples of possible phenotypic assays that can be used for screening.
of single genes in cell culture to developing gene therapy techniques *in vivo* to specifically target disease-associated alleles (55).

In mammalian cells, RNAi-mediated gene suppression can be induced by the introduction of chemically synthesized siRNAs or plasmids expressing RNA hairpins, known as shRNAs, which get processed to siRNAs by Dicer (56). In either case, the siRNA becomes incorporated into the RNA-induced silencing complex and directs sequence-specific mediated degradation or translational suppression of the target mRNA, resulting in decreased protein expression (57). Although siRNAs are easily synthesized and highly effective in inducing gene knockdown, such oligonucleotide reagents are relatively expensive and can only be used for transient loss-of-function experiments. Vector-based systems offer the possibility of adding selectable markers, such as green fluorescent protein (GFP) or drug resistance, stable expression of the RNAi construct, as well as being a renewable resource through propagation in *Escherichia coli*. More recently, inducible RNAi vectors have also been developed, allowing fine temporal and spatial regulation of RNAi-induced gene knockdown (40,58).

Both siRNA and shRNA libraries have been used successfully in transfection-based arrayed screens looking at phenotypes that develop shortly after gene suppression, such as apoptosis, cell-signaling events or cell cycle disruption (30,33,35). For many other cancer-related phenotypic assays, such as anchorage-independent colony formation, bypass of senescence or tumor xenografts, long-term gene suppression is essential, requiring stable integration and expression of the RNAi vector (37,39). An additional significant advantage of the retroviral-based libraries is the ability to work with cells that are refractory to transfection. This is particularly true for the lentiviral-based systems, which can even be used to infect post-mitotic and other difficult to transduce cells, including primary cells or differentiated cells (34).

Despite improvements to algorithms used to design RNAi sequences, it remains impossible to consistently and confidently predict the ability of any particular sequence to induce gene knockdown, meaning that each RNAi construct must be tested empirically for its efficacy (59). Another issue specific to the use of RNAi technology is the possible induction of undesirable off-target effects, leading to suppression of genes other than the intended target (60). This phenomenon can be either sequence dependent or sequence independent and appears to be largely unavoidable, necessitating careful post-screen analysis to identify genes whose suppression tracts to the phenotype of interest (60). One direct way to avoid off-target effects is to require that two or more independent RNAi sequences targeting different sequences within the same gene induce the same phenotype. Because of the inability to guarantee efficient knockdown with any single hairpin as well as the desirability of multiple phenotype-inducing hairpins, most of the RNAi libraries currently available have hairpin redundancy built into their designs, with multiple RNAi sequences targeting each gene. This redundancy increases the likelihood of having multiple effective RNAi constructs per gene.

As in any loss-of-function approach, suppression of any single gene may not result in an altered phenotype if there is functional redundancy with multiple other genes. This functional redundancy makes it unlikely to identify these genes from an array-based screen, but a pooled screen optimized for multiple RNAi integration events may be able to pick up phenotypes resulting from combinatorial knockdown effects. Another complicating issue that typically arises with loss-of-function approaches, particularly with engineered mouse deletion mutants, is the effect of compensatory changes that develop over time. However, with RNAi, gene suppression is acutely induced, thus making it possible to look at the consequence of loss of gene function in the absence of such compensatory changes. One additional characteristic unique to the use of RNAi is the ability to generate a series of hypomorphic phenotypes, either through different doses of a single RNAi construct or different efficacies of multiple RNAi constructs (61). This ability to fine-tune the degree of loss of function can be useful in the determination of gene function, especially in situations where complete loss of function is lethal. For the reasons stated above, as well as experimental ease and cost effectiveness, RNAi-mediated gene suppression is the current method of choice for loss-of-function studies for many investigators.

### Integration of genomic approaches

With the development of technologies designed to provide unbiased genomic, transcriptomic and functional characterizations of any normal or disease state, it is now possible to generate comprehensive molecular views of cancer with high resolution and detail previously unattainable. As these technologies evolve, it is becoming increasingly evident that the integration of multiple approaches will be far more efficient and effective in identifying and validating novel cancer genes than any single approach alone. Since the introduction of ‘omics’ technology a mere decade ago (62), there has been an explosion of huge datasets generated using these approaches. However, only more recently have we begun to see large-scale efforts to integrate these datasets to complement each other in the search for new genes and pathways involved in cancer.

Correlative studies of structural changes in the cancer genome detected by array comparative genome hybridization or high-density single-nucleotide polymorphism techniques and gene expression level changes detected by microarray techniques have demonstrated at most ~60% correlation between highly amplified genes and increased expression levels (63) and more typically 20–40% correlation (16). These observations suggest that observed amplifications or copy number gains of any gene should not be automatically assumed to have functional consequences. One way to help identify genes that are causally involved in driving the amplifications or deletions seen for any particular tumor is to determine which of these genes are also altered at the transcriptional level, or better yet, at the protein level. In this way, copy number alteration events can be filtered by comparison with transcriptomic or proteomic data to find those most likely to have some biological impact on the cancer phenotype. Recent successful applications of this strategy include the identification of *MITF* as a *bona fide* melanoma oncogene (13) and identification of candidate breast cancer genes (12,16).

Functional genomics is a key component in the integration of genome-scale analyses of cancer, as it provides a means to link perturbation of gene function to causality in different aspects of the tumor phenotype. This step is essential in establishing the role of a specific gene in tumorigenesis, including those of candidate cancer genes identified by genomic, transcriptomic or proteomic approaches. Further increasing the utility of functional screens is the ability to integrate phenotypic information from multiple screens, which can be used to generate a multidimensional understanding of gene function. To this end, publicly available phenotype databases have been established online, several of which incorporate genome-wide RNAi datasets from worms, flies and mice (64–66).

Depending on the phenotypic readout upon which any functional screen is based, hypotheses can be generated regarding the role of any particular gene in cancer. To strengthen the case for any candidate cancer genes isolated using functional approaches, evidence of actual involvement in human tumor initiation and progression is required. For example, *PITX1* was identified in an RNAi screen for genes whose suppression can replace Ras activation in *in vitro* transformation assays, suggesting possible tumor suppressor function for this gene. This hypothesis was further supported by evidence that *PITX1* is also found to be transcriptionally down-regulated in prostate, bladder and colon cancer (37). Similarly, *REST* emerged from an RNAi screen for suppressors of epithelial cell transformation and was found to lie within a chromosomal region commonly deleted in colon cancers, suggesting tumor suppressor function for this gene as well (39). miRNA-372 and miRNA-373, identified in the miRNA senescence bypass screen discussed above, were also found to be over-expressed in testicular germ cell tumors, with concomitant loss of expression of the putative miRNA target *LATS2*, supporting a positive role for these miRNAs in tumorigenesis (51). Lastly, *IKBKE* was identified simultaneously from two functional screens, a gain-of-function screen for
the ability to promote transformation in vitro and a loss-of-function screen for genes essential for viability of cancer cells, two hallmark characteristics of typical oncogenes (45). Subsequently, IKBKE was found to lie within a region commonly amplified in primary breast tumors and breast cancer cell lines, as well as being over-expressed at the protein level, suggesting that it functions as a breast cancer oncogene.

Comparative oncogenomics, the use of genomic information from genetically defined mouse models of cancer to generate hypotheses around the genetics of human cancer, promises to be of particular utility in uncovering novel cancer genes. Two recent demonstrations of successes using this approach include the discoveries of NEDD9 as a mediator of metastasis from a melanoma mouse model (67) and cIAP1 and YAP as candidate oncogenes from a hepatocellular carcinoma mouse model (68). Of course, once potential novel cancer genes have been identified using any of these unbiased profiling or functional approaches, it still remains necessary to characterize these genes individually to elucidate the cellular and molecular roles they play both in the normal and diseased context.

The genes mentioned here provide just a few examples of success in the integration of genomic, transcriptomic and functional techniques in discovering novel cancer genes and demonstrate the potential utility and power of such an integrated approach. Future efforts are likely to benefit from coordinated efforts to merge complete datasets to facilitate maximum discovery potential. Importantly, as we begin to identify new genes, pathways and molecular markers that can be used to characterize tumorigenesis and tumor progression, this information will guide us in the designing of new diagnostic, prognostic and therapeutic strategies with which to understand and treat cancer.

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References


Future directions

One therapeutically promising application of functional genomics is the identification of cancer vulnerabilities that can serve as therapeutic drug targets. By determining the set of genes whose activation or loss of function specifically promotes the death of cancer cells, we can identify new therapeutic targets or design new therapeutic strategies with which to treat cancer. As small molecule inhibitors are more easily generated than molecules to activate proteins, RNAi screens are especially useful for this purpose, as inhibition of gene function by RNAi knockdown should in many cases phenocopy inhibition by a small molecule. The discovery of new drug targets using functional genomics can be greatly aided by the information yielded from cancer genome profiling, as previous experience has shown us that many of the most specific drug targets undergo some form of detectable alteration during tumorigenesis, such as translocation (BCR-ABL), somatic mutation (EGFR) or amplification (HER2). Further, if it is possible to link the identified vulnerability to a specific molecular marker, whether it is a gene amplification event, a missense mutation or expression signature, one can then use this information to predict the subpopulation of patients most likely to benefit from this particular drug treatment. In this case, it may not even be necessary to understand the underlying molecular cause for the vulnerability to develop a clinically useful therapeutic (Figure 3). Whether or not we understand the mechanism behind the vulnerability, being able to link it with a diagnostic marker offers the possibility of truly personalized molecular-targeted therapy, an approach which many hope will ultimately prove more effective than current therapeutic strategies.

Although efforts in the past to identify cancer genes using either forward or reverse genetics approaches have been successful in identifying many tumor suppressors and oncogenes, these were largely accomplished in slow and laborious fashion, one gene at a time and generally only for the most common cancer genes. We now have the capability to systematically and comprehensively interrogate the entire human genome structurally, transcriptionally and functionally, generating datasets that can be analyzed in an integrated manner to facilitate maximum discovery potential. Importantly, as we begin to identify new genes, pathways and molecular markers that can be used to characterize tumorigenesis and tumor progression, this information will guide us in the designing of new diagnostic, prognostic and therapeutic strategies with which to understand and treat cancer.

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