Analysis of cancer genomes through microarrays and next-generation sequencing

Li Liu\textsuperscript{1}, Alex Yick-Lun So\textsuperscript{1*}, Jian-Bing Fan\textsuperscript{2} \\
\textsuperscript{1}Illumina, San Diego, CA 92122, USA; \textsuperscript{2}AnchorDx Corp., Cancer Research Institute, Southern Medical University, Guangzhou 510300, China \\
*These authors contributed equally to this work.

Correspondence to: Jian-Bing Fan, PhD. CEO, AnchorDx Corp., Professor, Cancer Research Institute, Southern Medical University, Guangzhou 510300, China. A. Email: jianbing_fan@yahoo.com.

Abstract: The use of microarrays and next-generation sequencing (NGS) has made significant contributions to the understanding of cancer development. These technologies have been used to profile gene expression patterns in cancer cells, identify genomic regulatory elements bound by cancer-relevant transcription factors, and determine the genetic and epigenetic landscape of cancer cells. Importantly, scientists and clinicians have begun to apply these findings to develop personalized medicine by which an individual's cancer genomic information is used to guide the selection of cancer therapy. In this article, we will review the impact that genomics technologies have had on the cancer field.

Keywords: Next-generation sequencing (NGS); microarrays; cancer genomics

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Microarray analysis of cancer

Microarray technology has been widely used in cancer research for more than a decade. The traditional solid phase DNA microarray is a collection of DNA probes attached to a solid surface such as glass, plastic or silicon chips. Alternatively, the bead array is a collection of microscopic polystyrene beads with a specific probe attached to each bead. For instance, bead arrays were applied to quantify gene expression in formalin-fixed paraffin-embedded (FFPE) tissues (1). The specific probes on the bead arrays are usually designed from short sections of the target sequences used to hybridize to DNA or cDNA samples. The relative abundance of nucleic acid sequences in the target can be detected by probe-target hybridization and quantified by detection of fluorophore or chemiluminesence signals.

Impact of microarrays on cancer biology field

Various types of microarrays have been developed for different applications. Before the development of next-generation sequencing (NGS), microarrays have had major impacts in the field of cancer biology. One of the earliest applications of microarrays was to identify differences in gene expression between cancer and normal cells (2). For instance, an early study by DeRisi et al. utilized a density microarray of 1,160 DNA elements to demonstrate that as high as 9% of the transcriptome change in expression upon cancer cell transformation (2). Since then, numerous studies have utilized microarray approaches to profile gene expression patterns that initiate or maintain the oncogenic state of cancer cells. The development of DNA microarrays enabled the acquisition of gene expression data of virtually the entire expressed genome. Tens of thousands of genes are simultaneously monitored to study their expression levels in tumor and non-tumor tissues, which facilitate the detection of meaningful patterns in complex gene-expression patterns in cancer research (3). From the understanding that cancer cells can undergo dramatic changes in gene expression, microarrays have also been utilized to improve tumor classification, which is crucial for selecting the appropriate course of cancer therapy. A seminal study showed that profiling gene expression patterns through microarrays...
could be applied to easily distinguish between acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL), demonstrating the feasibility of cancer classification through this approach (4).

In addition to monitoring gene expression patterns, microarrays have also been broadly used to decipher signal pathways directly orchestrated by cancer-relevant transcription factors. Martone et al. combined the power of chromatin immunoprecipitation with microarrays, known as ChIP-chip, to demonstrate that the NF-κB transcription factor p65 generally bind genomic elements distal from the promoter of their target genes (5). Subsequently, many groups applied ChIP-chip to demonstrate that cancer-relevant transcription factors, such as p53, estrogen receptor, and androgen receptor, generally bind to regulatory elements within both intergenic and intragenic regions far from their target promoter (6-8). Thus, the application of microarrays has advanced the scientific understanding of how cancer-relevant transcription factors control gene networks and ultimately cancer development.

Furthermore, microarrays have also been widely used to understand the genetic and epigenetic makeup of cancer cells. Microarrays have been used to identify small genetic changes, such as single nucleotide polymorphisms (SNP), in tumor cells through the use of SNP arrays. Also, the use of array comparative genomic hybridization (aCGH) has been widely used to identify large genetic abnormalities associated with cancer development, which include genetic deletions of several kilobases or duplications of entire chromosomes (9). Moreover, microarrays have been widely applied to decode the epigenome of many types of cancer cells. For example, DNA methylation arrays detect global patterns of methylation in cancer and identification of cancer biomarkers (10,11). From many studies, it is now clear that the transformation of normal to cancer cells involve a large number histone modification and DNA methylation changes.

### NGS process

NGS technology has revolutionized our understanding of the cancer genome (12,13). Twenty years ago, sequencing one human genome took more than ten years and cost $3.8 billion (14,15). In 2008, the cost had dropped to $2 million (16). Same year, the first cancer genome was sequenced by NGS technology (17). Today, a human genome can be sequenced for $1,000 on Illumina Hiseq X platform. The dramatic increase in throughput and the drop in cost greatly improved our capability to comprehensively understand a cancer and offers opportunities to advance cancer prevention, diagnostic, prognostics and treatment.

In first-generation sequencing technologies, genomic DNA is fragmented and individual fragments are cloned into plasmids or phage to create a library with millions of individual clones. The plasmids are introduced into bacterial cells, followed by growing individual bacterial clones and isolating plasmids from each clone. Millions of individual sequencing reactions are performed on plasmid DNA to generate sequence data for each plasmid. This is a very time-consuming process and cost ~$20 million to sequence a single human genome. In second-generation sequencing, the serial process of growing and sequencing millions of individual clones is replaced by highly parallel process in which billions of DNA fragments are amplified and sequenced simultaneously. The process is composed of four major steps: library preparation, clonal amplification, sequencing, and data analysis.

To create a DNA sequencing library, the isolated DNA is fragmented into 500 bp segments by sonication. Followed by end repair and addition of a single A base, Y-shaped adaptors are ligated to the ends of the DNA fragments. Alternatively, fragmentation and adaptor ligation can be achieved by incubating genomic DNA with a transposase that carries DNA adaptor sequences. The transposase simultaneously cleaves the DNA and ligates the adaptors to create a library.

The flowcell surface is pre-coated with oligonucleotides that are complementary to the adaptor sequences on the library. The DNA library is denatured and captured onto the flowcell by hybridization to these oligonucleotides. The library is clonally amplified by a process called bridge amplification, resulting in over one billion clusters with each cluster containing ~1,000 molecules. A sequencing primer is then added to the free ends of the DNA.

The billions of clonal clusters are sequenced simultaneously and in parallel (Figure 1) (18). Reversibly terminated and fluorescently labeled nucleotides are added to the sequencing primer by an engineered DNA polymerase. The reversible terminator prevents the addition of more than one base in one sequencing cycle. Each base is labeled with one of four colors that emit a fluorescent color for imaging. After recording the color and location of each base, the reversible terminator and the fluorescent dye are removed, allowing the incorporation of the next nucleotide. This process is called sequencing by synthesis (SBS). It is repeated 150-250 times in one direction and the
Figure 1 SBS with reversible terminators. The billions of clusters generated by clonal amplification are sequenced in parallel. Fluorescently labeled and reversibly terminated nucleotides are added to the sequencing primers by an engineered polymerase (A). Only a single base can be added to the growing DNA strand by the polymerase enzyme per cycle because the terminator blocks further polymerization. Unincorporated nucleotides are washed off and the flowcell is imaged to record the color and location of the incorporated nucleotides (scale bar 10 μm) (B). After imaging, the terminator and fluorescence dye are cleaved off, allowing the incorporation of the next nucleotide. This process is repeated 250 times to build a 250 bp sequence. Reprinted with permissions from “cancer genome sequencing” by Lakdawalla et al. (18).

DNA molecules are flipped over allowing re-synthesis of the reverse strand. The forward strands are then cleaved, leaving the reverse strands to be sequenced. The paired-end sequencing strategy produces sequence information from both ends of each DNA molecule, yielding twice the sequencing information from a library and facilitating an accurate alignment of the sequenced fragments.

The raw image data is converted to a fluorescence intensity table that records the location of each cluster and the color intensity values. These values are then converted to base calls. At the end of a sequencing run, billions of clusters have each produced a 2x[150-250] bp read, which are then aligned to a reference genome and converted to BAM file format that can be imported into genome browsers, such as UCSC and IGV genome browsers, for visualization. A genomic DNA library contains fragments from multiple copies of genomic DNA, therefore each base will be read multiple times from independent clones. The reads aligned to the same region are combined to make a high confidence base call.

Targeted DNA sequencing

Although whole-genome sequencing has been widely used in cancer research, the cost is still substantial and the majority of the sequence obtained is with no known significance in cancer. Targeted sequencing has thus emerged as a cost-effective approach to tumor genetic profiling (19). In target enrichment sequencing strategies, the genomic sequences of interest are selected for sequencing. Several target enrichment methods have been developed, including PCR, molecular inversion probes, array based or in-solution hybrid capture. The choice of target enrichment method depends on a variety of factors, such as size of the target region, DNA input, and genomic architecture of the region (20). In PCR-based approach, sequences of interest are enriched and amplified with sequence-specific primers. For example, Illumina Truseq Amplicon-Cancer Panel is a predesigned panel covering 212 mutations in 48 cancer-related genes. Each amplicon is flanked by two oligonucleotide probes with the same orientation followed by a proprietary extension-ligation step. PCR is then performed to add an index and sequencing motifs. In array-based capture, oligonucleotides complementary to the sequences of interest are synthesized on a chip and hybridization occurs on the surface of the chip, whereas in solution-based capture method, hybridization between DNA and probes occurs in solution, which allows less DNA input and smaller reaction volumes (21).

Annotation and interpretation of sequencing data

The sequence reads are assembled into a consensus sequence and compared against the reference genome to derive a list of variants. Whole genome sequencing data is generally low coverage (10-40x coverage) and suitable for the detection of constitutional variants. Target sequencing of specific genomic sequences of interest may increase the coverage...
to 1,000x or higher, permitting more sensitive evaluation of variants in cancer (22). Major structure variations, such as translocations, copy number variations (CNV), and insertion/deletion (indel) can also be detected by various algorithms (23). Translocation is usually detected by split-read method, where single reads are mapped to the genome discontinuously. Changes in read depth over large regions often indicate copy number changes. Indels can be detected using discordant paired reads or split reads.

**New discoveries of cancer biology using NGS**

Many applications that previously used microarrays for genomic studies have been replaced with NGS. Similar to microarrays, NGS can also be used for RNA profiling, identifying genomic elements bound by cancer-relevant transcription factors, isolating genetic changes that occur upon cell transformation, and deciphering the epigenetic makeups of cancer cells.

The invention of NGS has revolutionized the cancer biology field by providing the ability to sequence DNA in a genomic scale at unprecedented speed. NGS has essentially been used to study cancer biology in essentially every facet. Not long ago, it was thought that the human genome was made up of mostly ‘junk’ DNA. Since the sequencing of the human genome, the application of the NGS technology has contributed significantly towards the understanding that the genome encodes many important and previously unappreciated elements critical for normal cell function. NGS has paved the road for identifying non-coding RNAs, including microRNAs, long non-coding RNAs, and circular RNAs. It is now appreciated some of these non-coding RNAs play crucial roles in tumorigenesis and tumor suppression (9,24).

Because NGS has the potential to gather DNA sequence information of individual cells from samples that contain heterogeneous cell populations, it has become the leading platform to identify somatic mutations associated with cancer. A few years ago, a number of studies reported the use of whole genome or exome sequencing to identify recurrent somatic mutations associated with various cancer types. This led to the discovery of novel signal pathways that mediate cancer development. For instance, Wang et al. sequenced tumor samples from patients with chronic lymphocytic leukemia (CLL) and identified frequent somatic mutations in the coding region of SF3B1, which is a factor belonging to the spliceosome (25). In separate studies using NGS, Graubert et al. and Yoshida et al. also found recurrent mutations in other mRNA splicing factors in myelodysplasic samples (26,27). These studies led to the discovery that abnormal mRNA splicing contributes to oncogenesis in blood neoplasm. Using similar approaches, Puente et al. also identified recurrent mutation in the NOTCH1, XPO1, MYD88, and KLHL6 genes in patients with CLL (28). Thus, NGS technology has been critical in discovering new somatic mutations and signal pathways that are associated with cancer pathology.

**Application of NGS in personalized medicine**

The NGS technology has contributed to the identification of ‘hotspot’ somatic mutations associated with particular cancer types. Thus, clinicians have begun to test for the existence of these ‘hotspots’ mutations in patients to guide therapeutic selection. For instance, patients with NSCLC are often tested for somatic mutations in the kinase domain of EGFR because it has been shown that EGFR mutational status is correlated with tumor sensitivity to the kinase inhibitors gefitinib and erlotinib (29-31). Also, it has been reported that the KRAS mutational status in patients with metastatic colorectal cancer is inversely correlated with response to panitumumab therapy (32,33). Therefore, there is also an interest in identifying KRAS mutations in these cancer patients. Because of the heterogeneous and complex nature of tumors, there is a growing demand for profiling somatic mutations in a panel of ‘hotspot’ genes rather than just at an individual gene. Thus, the need to identify somatic mutations at a number of loci simultaneously has increased the demand of using NGS to guide cancer therapy.

As a result of collaborative efforts amongst academic institutions, industries, and hospitals, multiple NGS platforms/assays that examine mutations at a panel of candidate genes have become available for clinical use. For instance, Asuragen offers the Suraseq 500 panel for clinical trials that uses NGS platforms to assess the mutational status of 17 cancer targets and 500 genomic sites in tumor tissues. Similarly, the Oncotype DX diagnostic tests (Genomic Health Inc) were developed to use the genomic information of the patients’ tumors to guide breast, colon, or prostate cancer treatment; the information can be used for assessing potential chemotherapy benefits as well as likelihood of cancer recurrence. Of note, the MiSeqDx instrument (Illumina Inc) became the first NGS platform approved by the FDA for vitro diagnostic (IVD) use. Thus, it is evident that the application of NGS in clinical settings has become more pronounced and will continue to be a key
factor in shaping personalized medicine.

It is noteworthy to mention that although targeted panels will be useful for guiding selection of cancer therapy, all the mutations that induce cancer development and maintenance have not been identified. Moreover, cancer development is complex and includes various types of mutations, including somatic mutations in coding and non-coding regions, genetic translocations, gene amplifications, and genetic deletions. The ultimate goal of personalized medicine is to be able to sequence the entire genome of cancer patients to unbiasedly identify relevant somatic mutations, which will not only enable discovery of novel and previously unappreciated mutations but also enhance the precision in using genomics to guide cancer therapeutic selection.

**Application of NGS technologies in liquid biopsies**

Although assessing somatic mutation from sequencing tumor tissue is the gold standard for clinical molecular diagnosis, it is limited by the acquisition of tumor tissue samples. The development of non-invasive methods has become essential for cancer detection and monitoring. Recent studies on circulating tumor cells (CTCs), circulating tumor DNA (ctDNA) and tumor-derived exosomes highlights the potential of monitoring tumor genome evolution from a simple blood draw—an approach known as a ‘liquid biopsy’ (34-41).

CTCs are intact tumor cells shed into the bloodstream from both primary and metastatic tumors. They can be purified from blood by cell surface markers that distinguish them from normal blood cells (42). The major challenges of utilizing CTCs are isolating rare cells and sequencing low-input material. Lohr et al. reported a method to isolate, qualify and sequence whole exomes of CTCs with high fidelity using a “census-based sequencing” strategy, in which combining multiple single CTC libraries markedly reduced the false-positive rate of called somatic single-nucleotide variants (41). Using this technique, the authors demonstrated that they could detect CTC mutations that are also present in matched tumor tissues.

ctDNA is composed of small fragments of nucleic acid that are released into the bloodstream from apoptotic and necrotic tumor cells (43). Given the fact that ctDNA is significantly more abundant and easier to purify than CTCs, it is a more preferable source for molecular diagnosis. Sequencing of ctDNA has demonstrated that ctDNA is detectable in most patients with metastatic cancers, across all major cancer types (34). The biggest technical challenge of analyzing ctDNA is its low mutant allele frequency and large dynamic range. The level of ctDNA in cancer patients ranges from <0.1% to >50% out of the total cfDNA. Therefore, the technical sensitivity and dynamic range of the assay are critical to maximizing the clinical utility of ctDNA. Bratman et al. reported an ultrasensitive method for quantifying ctDNA called “cancer personalized profiling by deep sequencing (CAPP-Seq)” (35). They implemented CAPP-Seq for a non-small cell lung cancer (NSCLC) study with a design covering 139 recurrently mutated genes, and detected ctDNA in 100% of patients with stage II-IV NSCLC and in 50% of patients with stage I, with 96% specificity for mutant allele fractions down to ~0.02% (35). It is believed that with the rapid development of highly sensitive and accurate NGS technologies, “liquid biopsies” will enhance patient care and play an essential role in personalized medicine.

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