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Technological advances in precision medicine and drug development

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Abstract

New technologies are rapidly becoming available to expand the arsenal of tools accessible for precision medicine and to support the development of new therapeutics. Advances in liquid biopsies, which analyze cells, DNA, RNA, proteins, or vesicles isolated from the blood, have gained particular interest for their uses in acquiring information reflecting the biology of tumors and metastatic tissues. Through advancements in DNA sequencing that have merged unprecedented accuracy with affordable cost, personalized treatments based on genetic variations are becoming a real possibility. Extraordinary progress has been achieved in the development of biological therapies aimed to even further advance personalized treatments. We provide a summary of current and future applications of blood based liquid biopsies and how new technologies are utilized for the development of biological therapeutic treatments. We discuss current and future sequencing methods with an emphasis on how technological advances will support the progress in the field of precision medicine.

Keywords

precision medicine; target sequencing; diagnostic; cell free DNA; circulating tumor cells; drug development; DNA methylation; biomarkers; immunotherapy; extracellular vesicles

1. Introduction

The five-year relative survival for cancer patients increased steadily from 48.7% in 1975 to 69% in 2007 [1]. While still a matter of debate, this success has been mainly credited to improved diagnostic methods and earlier detection [2]. Despite this extraordinary progress, the number of deaths in the United States in 2015 due to all cancer types has been estimated at 589,430 [1], corresponding to about a 66.5% five-year survival rate. Thus, development of improved early diagnostic tests has great potential to additionally increase the overall survival. Current cancer diagnostics are largely based on invasive methods (i.e. biopsies,

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Declaration of interest

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endoscopy). Through technical advances in instrumentation and the development of new contrast agents and radiolabeled tracers, diagnostic imaging has witnessed a remarkable evolution impacting virtually every aspect of clinical management of cancer. Yet there remains a need for more effective, more sensitive, and less invasive approaches. This is particularly relevant for asymptomatic cancer types such as ovarian, lung, kidney, and pancreatic cancers. Pancreatic adenocarcinomas, for example, are a rare highly aggressive form of cancer with a five-year survival rate as low as 1% [3]. They are often found after they have metastasized and are beyond opportunity of complete resection, which is the only curative option [4].

Many scientists have long desired the identification of plasma biomarkers that can reliably detect early stage cancer [5]. In this endeavor studies have focused mainly on molecules and cellular components that can be detected in the blood: circulating proteins, vesicles, tumor cells, and nucleic acids. We summarize below the known circulating biomarkers and briefly describe how they are shed into the blood stream and their current applications as diagnostic tools. In the quest for better therapeutics several new technologies are being implemented that rely on biological treatments as opposed to traditional small molecules. Additionally, new development of therapeutics is paramount to relieve patients with more advanced disease and those with tumors that do not respond to standard chemotherapies, such as pancreatic cancer.

2. Liquid Biopsy: Technologies Advancing Personalized Medicine

Advances in recent molecular technologies have enabled scientists to capitalize on the discoveries of circulating whole cells or cellular components in the blood to act as information surrogates for their tissues of origin. These emerging technologies can be of use in many disease states or in pregnancy to gain information that would otherwise require invasive tests [6]. For precision medicine this has the most exciting potential in cancer treatments and early diagnosis. Improved accessibility of molecular technologies through lower costs, and increased assay sensitivity has fueled recognition that all tumors, even of the same type, have unique properties that affect their growth, treatment response, spread, and prognosis. Furthermore, tumor heterogeneity within the same patient can undermine successful treatment. Assessment of clonal variations in solid tumors is notoriously difficult and predicting patient response to treatment is confounded by tumor heterogeneity [5]. Since it is extremely difficult to measure tumor heterogeneity with traditional biopsies due to variations in tissue sampling, scientists have long embraced the idea of a liquid biopsy as a companion diagnostic tool. Analysis of circulating components in the blood, which include whole cells, nucleic acids, and extracellular vesicles, holds the promise to define new non invasive biomarkers, better assess tumor biological variables including heterogeneity and clonal evolution, and gather new, clinically relevant evidence to make better-informed treatment decisions. A summary of current and speculative uses can be seen in Figure 1.

2.1 Circulating tumor Cells

Circulating tumor cells (CTCs) were first discovered over 150 years ago but only recently has their usefulness has been appreciated [7]. The identification and isolation of CTCs offers

an attractive alternative to traditional biopsies [8]. CTCs can originate both from primary and metastatic sites, therefore allowing for a more complete portrait of the genetic variations contributing to tumorigenesis. This feature can aid in the selection of appropriate treatments and provide an extraordinary prognostic marker [9]. For example, in a recent study of colorectal cancer, CTCs originating from metastases, defined as nucleated cells expressing CEA (a set of highly related glycoproteins involved in cell adhesion), but not CD45, were shown to be associated with increased risk of relapse [10]. However the presence of primary CTCs did not have the same strong predictive value.

For clinical applications, CTCs must be first detected and, depending on downstream application, isolated. Two main hurdles limit CTC detection: low prevalence in the blood (in some cases less than one CTC per 5 ml of blood), and need of prior knowledge of expressed surface markers [11]. There are currently several ways to detect CTCs, each with strengths and weaknesses. The most common method is antibody-based capture, with capture assays based on physical characteristics, functional assays, and other methods being less frequently used [12]. While antibody-based assays can be very sensitive, a prior knowledge of expressed surface markers is requisite. In addition, the expression of markers in CTCs may be different than in the primary tumor, and/or their expression can change with time [13]. For example, common antibody-based methods for CTC isolation reliant upon the expression of the epithelial cell adhesion molecule (EpCAM) fail to isolate cells that lose or intrinsically lack EpCAM, but are positive for a variety of other tumor-specific markers. This was documented in a study in which EpCAM-negative CTCs isolated from breast cancer patients were shown to express brain-metastasis-specific markers and had the potential to metastasize *in vivo*. [14]. While some detection assays can also isolate CTCs, many, particularly those that require fixation or tight binding to beads during isolation, kill or damage the cells in the process, decreasing their potential downstream uses [13]. In addition, CTCs are often found in clumps, and therefore assays better suited to isolate single cells are not as effective [15,16]. To overcome these major hurdles several groups are developing new technologies. Two leading collaborating groups at Harvard and MIT have made great strides in new generation chip-based technologies. These are based on CTCs' unique physical properties, such as their generally larger size, or their increased membrane flexibility, relative to other cells [17,18]. Other chips, while having the limitation of being dependent on cell surface markers, have the advantage of allowing for on-site physical capture and release of CTCs like the chips dependent on physical properties. Other marker-independent devices can capture only clusters, which have been shown to be more informative to assess risk of metastasis in some cancers [16]. Recently a spiral device, based on the microfluidic dynamics of liquid moving through the chip, has enabled CTC selection based on size and promises both rapid and label-free isolation of tumor cells from the blood with minimal equipment needed [19]. While all these technologies are promising, the CellSearch System, which provides prognostic and treatment response information for metastatic breast, colorectal, and prostate cancers, remains the only FDA-approved method for the analysis of CTCs [20].

Several methods can be employed to analyze CTCs; genomic, transcriptomic, proteomic, and imaging assays are some of the more common downstream applications [21,22]. DNA and RNA sequencing can be performed on single cells and used to identify mutations or

gene transcription changes [23]. In this respect, particularly promising is one study's reported detection of epidermal growth factor receptor (EGFR)-activating mutations, which are common in non-small cell lung cancer (NSCLC), in CTCs from 92% (11 of 12) of studied NSCLC patients [24]. Proteomics has been used to identify new CTC markers in hepatocellular carcinoma [25] and microscopy of labeled cells is often used to identify CTCs in blood samples [26]. The common goal of these assays is to extract clinically applicable information such as actionable gene mutations, protein expression levels, tumor heterogeneity, prognosis, or response to treatment [27]. Prognosis is perhaps the strongest application with multiple studies linking CTC analysis with prognosis, especially breast cancer [9]. In general, a higher CTC frequency in the blood is linked with poor prognosis as this is correlated with advanced metastasis in several cancers types [21]: this also holds true for CTC clusters [28]. More novel is the use of CTCs for personalized treatments [29]. In a variety of cancers, mutation analysis for common variants in CTCs is being evaluated to assist in treatment selection [30,31]. While still exploratory, this avenue is gaining recognition in the medical research community.

2.2 Cell Free DNA

An emerging biomarker for precision medicine and diagnostics is circulating cell free DNA (cfDNA). It was discovered in 1948 by Dr. Mandel that cfDNA concentrations increase in the blood in a variety of conditions including pregnancy, chronic diseases states, and cancer [32]. cfDNA is comprised of DNA fragments released from cells throughout the body and found circulating in the blood. These mechanisms of release are not yet fully understood. cfDNA fragment size is around 200bp, but it has long been known it contains peaks corresponding to nucleosome-associated DNA (~147 bp). In fact, recent work shows that the fragmentation patterns of an individual's cfDNA contains evidence of the epigenetic profile of the cells of origin; sequencing information can therefore be used as a footprint to trace cfDNA back to the tissue/cell of origin [33]. This represents a prime example of how technological advances in molecular biology now make it possible to harness cfDNA's potential for information mining.

There are two main applications for cfDNA: prenatal diagnosis and disease biomarkers, particularly in cancer. With the ability to detect fetal cfDNA circulating in the maternal blood stream, vast progress has been made in prenatal diagnostics. Use of commercially available cfDNA prenatal tests in the assessment of common trisomies is the best established. Less developed is the use of cfDNA to detect other disease causing genetic aberrations, such as chromosomal microdeletions [34,35]. The ability to access the fetal genome earlier (10 weeks vs. 12–16 of traditional tests) allows parents have more time to make potentially difficult decisions, such as whether to continue with the pregnancy. cfDNA-based tests have proven to be more sensitive and accurate in detecting trisomies than standard methods combining imaging and serum protein markers; this means lower rates of false positives and fewer unnecessary, costly, and potentially harmful invasive tests, such as amniocentesis or chorionic villus sampling [36]. Acknowledging the potential benefits, recognized medical bodies have released official recommendations for cfDNA prenatal testing but have also called for potential ethical issues (i.e. direct to consumer marketing), to be addressed prior to full implementation as a stand-alone diagnostic tool [37]. Furthermore,

concerns have been raised about the lack of quality control in labs testing fetal cfDNA after a blinded study revealed some labs identified fetal DNA in women who were not pregnant [38]. Additionally, these tests are not recommended for women carrying more than one fetus due to the decrease in sensitivity [39].

The clinical utility of cfDNA as a biomarker in cancer is in development, holding promise of earlier detection, mutation matched treatment guidance, prognosis assessment, and treatment response observation [40]. Both tumor-specific mutational and epigenetic changes can be detectable in cfDNA, supporting its role as a companion for traditional biopsies [41,42]. Preliminary clinical trials are underway for a handful of cancer prognostic biomarkers, such as for the methylation status of the putative oncogene *Septin 9* for early detection of colorectal cancer [43] and detection of BRAF mutations in melanoma [44]. The major limitation of using cfDNA is its low concentrations in the blood and the fact that the majority of cfDNA is derived from non-tumor cells. In order to be of clinical utility, cfDNA based-tests need to be sensitive and specific to analyze tumor DNA relative to the normal DNA [45].

2.3 Extracellular Vesicles – Exosomes and Microvesicles

Once thought to be merely a means of waste disposal, extracellular vesicles (EVs) have now been demonstrated to play a role in cellular communication. Fusion of EVs with target cells was originally thought to serve primarily to initiate immune response, however, it is now known that EVs derived from tumor cells can serve nefarious purposes, such as transfer of drug resistance mechanisms and promotion of metastasis [46] and vascular development, a key hallmark of tumor growth [47].

EVs are categorized as microvesicles (or microparticles), exosomes, and apoptotic bodies. Microvesicles and exosomes are the most extensively studied as potential biomarkers. Size and intracellular point of origin differentiate these bodies from one another, with microvesicles being larger and derived from the plasma membrane and exosomes being smaller and of endosomal origin [47]. The most common method for EV isolation from fluids is serial centrifugation, entailing multiple centrifugations at increasing speeds, to separate vesicular fractions. The first centrifugation serves to remove cell debris, the next pellets the microvesicle fraction, and the final step isolates the exosomes [48]. Given the overlap in size between some exosomes and microvesicles, this method has its limits and cross-contamination can be high. In addition, loss of material during washes and the type of rotor used for sample processing can confound the ability to obtain pure samples [47]. Other methods exist, such as flow cytometry, field-flow fractionation, spectroscopy, and nanoparticle tracking [48,49], yet no ideal method has emerged, mainly due to the heterogeneity of EVs and difficulties in distinguishing between fractions based on size, structure, and protein composition [46].

As biomarkers, extracellular vesicles are of interest in terms of the shear numbers presented as well as their contents. Both microvesicles and exosomes contain packaged material, such as DNA, RNA, and proteins, which might otherwise be subject to degradation during travel between cells. These particles can travel to nearby cells, emulating a paracrine effect, or to more distal tissue, establishing a systemic effect [50]. Shedding of EVs occurs at higher

levels in tumor cells than in normal cells [51]. It has been observed that tumor-derived EVs originating from drug-sensitive cells are smaller and more exosomal in morphology and protein expression than those produced by drug-resistant cells, which contain smaller amounts of exosome-associated proteins. In addition, multi-drug resistant cells have been reported to produce more EVs than drug-sensitive cells [46]. The contents of the vesicles may also point to the cells of origin, which can be useful in assessing tumor heterogeneity, as highlighted in a study by Skog, et al. In the study, the glioblastoma-associated type III EGFR variant (EGFRvIII), which lacks a portion of the EGFR extracellular ligand binding domain, was detected in sera EVs of glioblastoma patients whose biopsies were EGFRvIII-negative. This finding suggests that biopsies may have been retrieved from non-representative areas of heterogeneous tumors [52].

Because the cargo of tumor-derived vesicles seems to be sorted preferentially from the cellular milieu [49] and the contents are dynamic and reflective of the patient's current disease state, EVs can be used for disease surveillance and treatment response [53]. The Skog, et al. study demonstrated an overall reduction in the level of circulating tumor-derived microvesicles in glioblastoma patients after tumor resection and also demonstrated a reduction in the presence of circulating EGFRvIII, confirming the EVs were harboring EGFRvIII. Additionally, miR-21, an antiapoptotic factor known to be over expressed in human glioblastoma cells, was identified in EVs isolated from cerebral spinal fluid of glioblastoma patients; its presence correlated with the presence of cancer and its expression dropped precipitously following surgical resection [54].

The ability of EVs to function as transporters of biologically relevant material also makes them a candidate for the delivery of therapeutic agents. In 2011, Alvarez-Erviti, et al. presented the first successful use of modified exosomes as a vehicle to deliver therapeutic short interfering RNAs to the mouse brain [55]. In 2012, engendered EVs expressing membrane epidermal growth factor (EGF) on their surface showed specific binding to EGFR-expressing cancer tissues and were able to transfer therapeutic microRNAs to tumor cells [56]. In 2015, it was reported that loading Paclitaxel through passive diffusion into tumor cell-derived EVs increased the cytotoxic effect of the drug *in vitro* [48]. This study, which thoroughly characterized centrifugally-isolated EVs by transmission electron microscopy, nanoparticle tracking, and Western blot analysis, points towards a viable future harnessing the clinical utility of these particles for delivery of drugs and other potential therapeutics, such as microRNAs.

2.4 microRNA

As microRNAs (miRNA) are often transported by EVs, the two are often discussed in tandem [57]. miRNAs are a class of small, noncoding RNAs with regulatory functions on protein expression through gene silencing, either by inhibition of translation through imperfect complementarity to the target site or degradation by perfect binding [57]. Originally discovered in *Caenorhabditis elegans*, miRNAs were thought to be unique to the species until their subsequent isolation in mammals about fifteen years ago [58]. Since then, interest in miRNAs has exploded, especially in cancer research for their use as biomarkers and potential therapeutic agents.

miRNAs, particularly miR-21, have been extensively linked to cancer, through either overexpression and resultant in tumor suppressor silencing, and down-regulation which may contributing to oncogene activation via inhibited suppression [59]. miRNA expression is often tissue-specific and different tumor subtypes may present unique signatures. For example, breast cancer subtypes ER⁺PR⁺HER2⁺, ER⁻PR⁻HER2⁺, and ER⁻PR⁻HER2⁻ (known as triple negative breast cancer, TNBC), have distinct miRNA expression patterns; specifically, miR-205 expression in TNBC was shown to have a positive correlation with clinical outcome [60]. miRNA profiles can be predictive of cancer progression [60], and they can function as another means to study tumor heterogeneity.

More recent is the concept of circulating miRNAs found bound to lipoproteins, RNA-binding proteins, or encapsulated in extracellular vesicles [61]. Though most closely studied in cancer, circulating miRNAs are of interest for several other diseases and disorders including pre-eclampsia [62], diabetes [63], cardiovascular disease [64] and hypertension [65], sepsis [66], inflammatory bowel disease [67], and even depression [68].

3. New Technologies in Drug Development

Small molecules have historically been the main interest in therapeutic drug development and discovery. In fact, in 1977 the FDA approved the small molecule Tamoxifen as the first drug for the treatment of breast cancer. In the past 20 years however, interest has shifted to biological therapeutics such as antibodies, blood components, and gene therapy.

3.1 Immunotherapies

It has long been known that the human immune system plays a role in diseases, and drugs modulating the immune system have been on the market for decades. Major drawbacks with immune modulating therapies, with the exception of antigen-specific therapies, is their effects either broadly suppress or stimulate the immune system. Targeted immunotherapies use immune system components to drive the immune response against specific cells, allowing efficacy and minimizing off target effects.

One of the most revolutionizing discoveries in cancer therapy came from the finding in 1963 by Abelev and colleagues when they discovered antiphospholipid antibodies as a serum marker for hepatoma [69]. This translated to the knowledge that tumors express unique antigens and most importantly, under the correct conditions, the immune system is capable of exterminating cancer cells. This led to first identify tumor-specific antigens that could be targeted by specific antibodies and more recently to genetically engineer the immune system to attack tumor specific antigens [70].

Since 1982, when the first monoclonal antibodies against CD20 (Rituxan) were FDA-approved to treat B cell lymphomas, antibody drugs have experienced rapid growth and many still take center stage in cancer treatment today (e.g., Herceptin, which targets HER2/neu, was FDA approved in 1998 and remains a standard of care compound for the treatment for HER2 positive breast tumors) [71]. Antibody therapies account for the largest share of therapeutic biologicals, outcompeting growth factors, hormones, fusion proteins, cytokines, therapeutic enzymes, recombinant vaccines, blood factors, and anticoagulants in sales

worldwide [72]. For example, the antibody drug Humira, a TNF inhibitor used to treat several autoimmune diseases, first reached global sales of over \$1 billion in 2013 and has since continued to grow [73]. Antibody therapy is used to treat a variety of disease, from auto immune/inflammatory, to cancer, to infectious disease [74]. The majority of antibody therapies are monoclonal antibodies (mAb), with polyclonal antibodies (pAb) becoming more heavily investigated, especially for cancer therapy [75]. While mAbs target one specific epitope of an antigen, pAbs can target multiple epitopes and therefore hold the potential for increased efficacy [76]. While their use in autoimmune and transplant rejection is vital in preserving transplants and maintaining normal immune function, their use in advancing personalized medicine for cancer treatment is poised to experience great growth [77]. Polyclonal antibodies will be more likely to efficiently target heterogeneous tumors, as was demonstrated in a xenograft model of ovarian cancer. In the model, polyclonal antibodies were generated against the tumor-causing cell line injected into the mice, allowing for multiple epitopes on more than one antigen to be targeted and led to better tumor inhibition [78]. Tumor heterogeneity is possibly a patient-specific feature; therefore pAb use may increase as precision medicine becomes integrated into cancer therapies.

The most recent breakthrough in immunotherapies is in the advancement of personalized immunotherapies. In 2014 the USDA granted chimeric antigen receptor T-cell therapy (CART) the breakthrough therapy designation; trials using this approach have since offered great promise in precision medicine [79]. CART uses a patient's own modified T-cells to attack their tumors and can potentially provide protection against relapse. T-cells are isolated from a patient's blood and genetically engineered to recognize an antigen specific to their tumor. The cells are then expanded *in vitro* to therapeutic levels and reintroduced into the patient [80]. The primary focus thus far has been blood cancers, with some studies exploring the feasibility of targeting solid tumors [79]. One of the main challenges associated with CART in solid tumors is the lack of unique and identifiable cell surface markers that can be used for targeting [81]. The therapy needs to be very specific to each patient, requiring adequate mutational and expression profiling to ensure the correct markers are selected, which makes creating generalized treatments improbable. Several groups are tackling this problem in hopes of replicating the success seen in blood cancers; in leukemia, for example, remission of greater than six months has been reported in up to 67% of study participants [80]. Like most cancer treatments, CART can be associated with the phenomenon of cytokine release syndrome, which results mainly from rapid activation of the immune system leading to rapid cell death of cancer cells. This side effect, often presenting as flu-like symptoms, is generally treated with supportive care or steroids [82].

3.2 miRNA therapeutics in clinical trials

The therapeutic use of miRNAs is an attractive option, given miRNAs' ability to target multiple genes and the possibility of easy systemic delivery of miRNA therapeutics. Based upon the mechanism of miRNA function, two strategies have been employed for their therapeutic use: increase expression of a down-regulated tumor-suppressing miRNA, or block the expression of an oncogenic miRNA. miRNA-based therapeutics using each strategy are emerging, primarily through miRNA replacement therapy and through inhibition with anti-miRNA oligonucleotides (AMOs), the first generation of which was known as

antagomirs [83]. miRNA replacement therapy aims to re-express down-regulated tumor-suppressor miRNAs through delivery of synthetic miRNA mimics [84]. AMOs function by binding to the miRNA, thereby inhibiting the binding of the miRNA to the target mRNA [85]. However, this methodology faces challenges of degradation and effective delivery of the oligonucleotides and potential off-target effects. To overcome these challenges, molecules can be delivered via EV packaging and other means, and modified AMOs, including Locked Nucleic Acids (LNAs, described below), have been developed with features aimed to increase stability of the oligonucleotides [83,86].

Several companies have ongoing clinical trials using miRNA-based therapeutics, leveraging both AMO inhibitor and synthetic mimic approaches. The first miRNA-based therapeutic to enter clinical trials was Santaris Pharma's miravirsen, a miR-122 inhibitor, for treatment of chronic Hepatitis C infection (HCV), and is in ongoing Phase II trials [87,88]. Miravirsen uses proprietary LNA technology, in which the ribose ring of the nucleic acid analog is locked with a methylene bridge between the 2'O and 4'C atoms [89], thereby increasing its stability, efficiency, and specificity [85]. Santaris Pharma was acquired by Roche in 2014, and while the miravirsen clinical trials are still ongoing, it is unclear whether Roche will continue to pursue miRNA-targeting drugs, given heavy market competition and potential for more profitability in other drug markets [90].

The first miRNA mimic to enter Phase I clinical trials was Mirna Therapeutics' MRX34 in 2015, for use in solid tumors and hematological malignancies. MRX34 is a double-stranded RNA mimic of miR-34, a tumor-suppressing miRNA, encapsulated in a liposomal nanoparticle formulation [91]. miR-34, a regulator of the p53 pathway, is decreased in several cancers [85]. Expansion of the cohorts enrolled for the MRX34 trials is expected in 2016, specifically including liver, lung, and renal cancers, as well as melanoma, lymphoma and myeloma [92]. Mirna Therapeutics published *in vitro* results in 2014 suggesting a synergistic effect in pairing miR-34 with erlotinib, a tyrosine kinase inhibitor targeting EGFR, for the treatment of non-small cell lung carcinoma and hepatocellular carcinoma [93]; this work was independently validated later that same year and published in 2015 by Stahlhut, et al [94].

Later this year, Mirna Therapeutics intends to enter Phase I trials for its second candidate miRNA mimic, and the company also has miRNA mimics in the pipeline for miR-101, miR-215, miR-16, and let-7 as potential treatments for hepatocellular carcinoma, non-small cell lung cancer, colorectal cancer, and pancreatic cancer [95]. The choice of these prospective mimics is concordant with literature reports indicating down-regulation of these miRNAs, resulting in oncogene activation. Down-regulation of miR-101 was associated with a more aggressive cancer phenotype in hepatocellular carcinoma [96] and restoration of miR-101 has been associated with suppression of lung tumorigenesis in murine xenograft models via DNMT3A inhibition [97]; miR-215 was shown to be increased in non-relapsing lung adenocarcinoma cases compared to relapsing cases [98]; and increased expression BCL2 as a consequence of decreased miR-16 has been posited as a mechanism in chronic lymphocytic leukemia [99] and in lung cancer [100]. Lastly, let-7 has a long history of tumor suppressive roles through RAS and HMGA2 regulation [101,102], both of which have been implicated in pancreatic cancer [103,104].

Regulus Therapeutics has ongoing clinical trials for its prospective miRNA therapeutics, including a direct competitor of miravirsen, RG-101. RG-101, a GalNAc-conjugated antimir targeting miR-122, is currently in Phase II studies, evaluating use of the drug in combination with others for HCV treatment. In Phase I trials are RG-125(AZD4076), in collaboration with Astra-Zeneca, a miR-103/107 antimir developed with the intent of treating non-alcoholic steatohepatitis (or fatty liver disease), and RG-012, targeting miR-21, which, notably, has been granted orphan drug status for treatment of the renal fibrosis-causing Alport syndrome. The company is currently exploring therapeutics targeting miR-10b and miR-221 for glioblastoma and hepatocellular carcinoma, respectively [105].

Lastly, MiRagen Therapeutics has initiated a Phase 1 clinical trial for the company's anti-fibrosis candidate MRG-201, a synthetic miRNA mimic (promiR) to miR-29b. The study is currently being conducted in healthy individuals and may expand to patients with cutaneous scleroderma. The company's pipeline includes two miRNA-based therapeutics targeting miR-155 (MRG-106 for hematological disorders and MRG-107 for ALS) and candidates targeting miR-92a for treatment of diabetic wound healing and peripheral artery disease. In conjunction with Servier, the company is also exploring targeting miR-208 and miR-15 in the treatment of cardiac conditions [106].

3.3 CRISPR

Clustered, regularly interspaced, short palindromic repeat (CRISPR) technology is derived from an adaptive prokaryotic immune response and enables precise endonuclease activity allowing for easy, rapid, and efficient modification of endogenous genes. Originally developed to aid basic research, CRISPR's potential use for development of novel human therapeutics is gaining recognition. The number of CRISPR publications skyrocketed from just 45 in 2010 to over 1200 in 2015. The potential use of CRISPR in gene therapy to treat various diseases, such as cancer and genetic disorders, has revitalized interest in gene therapy, which has had a checkered past [107]. The potential for gene therapy as a treatment emerged in the 1990's with the successful treatment of some patients affected by X-linked severe combined immunodeficiency (SCID-X1), who harbored mutations in the gene encoding for the γ -chain protein shared by several interleukins. The lack of therapeutic options to treat these young patients lead to the development of a retroviral-based gene therapy used to replace the faulty copy of the affected gene [108]. The enthusiasm from the initial successful was overshadowed by long-term consequences in six out of the 20 patients tested [109,110]. One patient died due to lack of immune system correction, and five patients developed T-cell leukemia, one of whom died. The other 14 treated children are reconstituted, as are the four children who are in remission from leukemia treatment. These results posed a large setback that resulted in increased safety investigations and the delay of other trials [111]. Gene therapy has advanced considerably since the initial trials, largely due to improvements in the design of retroviral vector strategies.

CRISPR holds promise, through more precise integration, to further advance the field of gene therapy by reducing the major side effect of inadvertent activation of oncogenes [112,113]. One of the most promising aspects of CRISPR in terms of drug development is that, once established, the specificity and stability of the genetic change introduced should

require no further intervention to maintain the expression of the repaired gene [107]. In most studies CRISPR is used with the Cas9 endonuclease, which can be directed to the gene target using researcher-developed RNA guide sequences [114]. The guide sequences enable fast and cost effective targeting as compared to other specific endonucleases, such as zinc finger nucleases [115]. The use of CRISPR has exploded in the mouse research field where it has been used both in the generation of mouse models of disease and as a treatment of regionalized disease such as the regeneration of muscle function in *mdx* mice [116,117]. CRISPR recently made headlines when it was first used to introduce alterations in the human embryos in an effort to alter the human β -globin gene, which is mutated in β -thalassemia. While none of the treated embryos were considered viable, the application of this new technological option raised ethical issues; additionally, it uncovered unexpected technical difficulties, such as off-target alterations [118]. The extent of off-target cleavage induced by CRISPR-Cas9 and other engineered nucleases has recently been brought to the attention of the scientific community and elicits caution regarding its future use as a therapeutic option [119,120]. To avoid off-target alterations, new high-fidelity CRISPR-Cas9 endonucleases are in development with the hope of reducing rates of off-target effects to undetectable levels [121]. A major limiting factor in the advancement of CRISPR use in clinical trials is the choice of the delivery method. Gene therapy can be delivered using viral methods, including lentivirus, adeno-associated virus, and adenovirus, and non-viral based methods, including lipid-mediated transfection, electroporation, and hydrodynamic delivery [107]. The preferential method may vary based on whether the desired effects are systemic or localized, the type of cell targeted, and the gene being affected. Recent work analyzed the effect of combining both viral and non-viral methods for systemic delivery to correct defects in hepatocytes and showed promising results of increased liver function in treated mice [122]. More studies are needed to evaluate potential complications and efficacies for each of the delivery methods. Given the interest this technology has generated, these studies are expected to proceed in a timely manner.

4. Sequencing: The Next and the Next-Next Generation

4.1 History

Much of the progress made in the area of DNA sequencing is to the credit of evolving technologies and is expected to keep pace with technology evolution. There have been significant changes in the field of genomic sequencing since Sanger introduced his chain-termination sequencing approach in 1977 and Applied Biosystems' automation of the approach in 1987 using capillary electrophoresis [123]. So-called "Sanger sequencing" was adopted as the "first-generation" of DNA sequencing and has long been considered the gold standard, but the method is unwieldy, expensive, and impractical for sequencing of multiple targets. Using this technology, the Human Genome Project was completed in 13 years, with the first draft of the human genome assembly published in 2001 [124,125], at a final project cost of approximately \$2.7 billion dollars [126]. By comparison, with the advent of Massively Parallel Sequencing (MPS) technologies, an entire human genome can now be sequenced in a matter of days [127] and for a few thousand dollars, though the actual cost is greatly dependent upon the application. Illumina recently announced the reality of the \$1000-Genome with its HiSeq X Ten platform, the caveat being that this pricing is achieved

only when the platform is used at the company's stated scale, sequencing 18,000 genomes per year [128].

Next generation sequencing (NGS), which took the stage in the mid-2000s, is achieved through means of MPS and has introduced several improvements over the first generation. In contrast to Sanger sequencing, NGS offers the ability to sequence several regions of interest concurrently [129]. This advantage is heavily exploited in genetic testing and is evidenced by the number of targeted panels offered to clinicians by genetic testing companies such as Ambry, GeneDx, Myriad, and others. From a current clinical perspective, it is generally more practical to preferentially examine regions of potential interest, rather than incur the higher cost for whole genome sequencing (WGS) or whole exome sequencing (WES), as only a small percent of the human genome has been shown to encode for functional proteins.

WGS and WES currently serve a better place in a discovery setting, where the burden of the bioinformatic load does not inhibit the timely reporting of clinically relevant results [130]. Through WGS and WES, NGS has revolutionized the study of epigenetics, the heritable changes (e.g., methylation and histone modifications) that do not alter DNA sequence. By establishing a means to efficiently sequence the entire genome, NGS allows researchers to survey not only the genome and transcriptome but also to efficiently examine the non-protein coding regions, in which many epigenetic regulator and miRNA-encoding regions lie, creating a more complete picture of downstream biological function [131].

4.2 Next Generation Sequencing Platforms

Cornering the market in NGS platforms are Illumina and ThermoFisher's Ion Torrent lineup. The platforms leverage different sequencing technologies: Illumina utilizes sequencing-by-synthesis, measuring the signal as fluorescently-tagged nucleotides bind [131], while Ion employs semiconductor sequencing, in which the shift in pH caused by the release of hydrogen ions during DNA extension is measured [132]. While each platform and associated sequencing method has its own advantages and disadvantages, it has been repeatedly demonstrated that performance is relatively comparable for similar applications [133,134], and they uniformly allow for high throughput and multiplexing, low sequencing costs, and low error rates [130]. When comparing these two NGS platforms against each other, the main differences are the turnaround time and upfront cost; the elimination of optics in Ion semiconductor sequencing has allowed for a faster runtime and lower upfront cost than Illumina, but Illumina ultimately offers a lower cost per base pair [133,134]. The primary disadvantage of NGS, independent of the platform, is the shorter read lengths, averaging 150bp, compared to the 900bp attainable on ThermoFisher's Applied Biosystems 3730XL first-generation sequencing platform [135]. Sanger sequencing is often used for validating variants found using NGS, but it has been recently suggested that this should no longer be the standard of practice, as NGS is showing itself to be as reliable as – and in some cases, more reliable than – Sanger sequencing [136].

4.3 Third Generation Sequencing

Continually advancing technologies are pushing us across the border from NGS to third generation sequencing (TGS). While NGS, now also frequently referred to as second

generation sequencing, made impressive strides towards reaching the impossible trifecta of good, fast, and cheap, TGS promises to bring us even closer. The available TGS platforms, namely Pacific Biosciences' RSII and Sequel single-molecule real time (SMRT) sequencers, and the portable Oxford Nanopore MinION (soon to be followed by PromethION, a benchtop sequencer), focus on single molecule sequencing with real-time results and very long read lengths. Pacific Biosciences' original RS SMRT platform was the first commercially available means to single molecule sequencing. This platform and its successors, the RSII and the Sequel, use a sequencing-by-synthesis approach in which fluorescently-tagged nucleotides are read in real-time during synthesis [137]. MinION is the first commercially available sequencer to use nanopore technology; as the molecules pass through the nanopore, changes in electrical conductivity are measured and the event-level data is generated [48]. These technologies allow for direct sequencing of both DNA and RNA without prior amplification, and in the case of RNA, without prior reverse transcription to cDNA [131,137,138]. In addition, and of considerable advantage to those studying the epigenome, these platforms also offer the ability to directly identify cytosine methylation during sequencing [137,139,140]. Both of these technologies are more error-prone than current NGS systems, but workarounds are possible with a hybrid sequencing effort, using high-quality NGS short read information for error correction in the longer TGS reads [141]. Furthermore, despite these error rates, it has been demonstrated that even at extremely low coverage (58,000 reads compared to Illumina or Ion's millions of reads typically generated), MinION has potential clinical applications in prenatal testing, accurately assessing aneuploidy in 2–4 hours using a short read sequencing library preparation [142].

Each platform has shown its muscle: in conjunction with high-fidelity PCR, SMRT sequencing was used in 2013 to resolve a gap in the human genome of the complex and highly repetitive central exon of the Mucin5AC gene. With the SMRT technology's ability to generate long reads, a complete sequence of the region was generated; following this *de novo* sequencing of the region, SMRT sequencing further identified genetic variation in the troublesome exon in four individuals [143]. Also of considerable interest is the use of the MinION system in 2015 for surveillance during the West Africa Ebola virus outbreak [144]. The device proved to be nearly ideal for the situation, given its portable nature (the MinION is approximately the size of a flash drive), quick turnaround, and ability to be powered off a connection to a laptop computer. These factors, taken with its low upfront capital investment [145], point toward future use in clinical applications.

4.4 Single-Cell Sequencing

The advent of NGS technologies – and looking forward, TGS technologies – has advanced our ability to interrogate the genetic content of a single cell through single-cell sequencing. Even cells of the same type may have variances in their genomes, transcriptomes, and epigenomes [146], as well as copy number variations [147] and whole chromosome aneuploidy [148]. This advancement in single-cell sequencing capabilities has made it possible to better understand the effects a single rare cell can impart, particularly in cancer, where a single cell resistant to chemotherapy can give rise to an entire mass of resistant cells through clonal evolution [149]. Furthermore, single-cell sequencing has special implications in the study of CTCs, allowing them to be used as a means of monitoring disease

progression and response to treatment [23,149]. Our ability to analyze the effects of single cells is currently hindered by the requisite amplification of the cell's genetic material prior to sequencing. Depending on the amplification method used, to varying degrees, amplification bias and a lack of fidelity are inherent, resulting in uneven genome coverage and inaccuracies in detecting single nucleotide and copy number variations. Through ongoing efforts to optimize amplification methodology and the employment of computational algorithms these challenges are being overcome; upcoming TGS technologies should offer further respite from these difficulties [146].

5. Expert Commentary and Five-Year View

Given the rapid growth of personalized therapies, with the hope that this approach may change the future of cancer treatment, and the growing usefulness of liquid biopsies to facilitate personalized treatments, we expect significant growth in these fields over the next five years. Rapid advances in CTC isolation and sequencing will position CTCs as pivotal players in determining patient care. Since CTCs can be isolated without employing invasive methods and hold the potential to contain much of the same clinically relevant information as traditional biopsies, their application in individualized treatments and monitoring therapy response is invaluable. Gain of knowledge will also direct the requirements for technical validation and help decide the most suitable companion diagnostic for CTCs. For example, it has been suggested that molecular characterization of CTCs is much needed in order to take advantage of the full potential of this biological material for biomarker purposes [9]. Cost effectiveness remains a limiting factor, however, as sequencing technologies evolve, so will CTC analysis.

As testing of circulating DNA is in its infancy, we will likely see growth of cfDNA's use as a diagnostic tool. Based on the success of commercially available cfDNA tests for prenatal diagnosis, we anticipate the application of cfDNA as cancer biomarker to follow in those footsteps. As they develop, cfDNA analysis for cancer and in prenatal care will face different issues and future prospects. While detecting cancer biomarkers still confronts technical issues, prenatal diagnostics challenges lie in ethical issues. Given the complexity of the ethics surrounding diagnosis of potential genetic disorders in utero, debate will likely continue as cfDNA prenatal testing becomes more accepted. When solving technical issues as they relate to cfDNA as a biomarker, the path is less challenging. A PubMed search reveals the number of reports exploring this route have grown exponentially in the past years with 2016 expected to double last year's publications. Combined with current clinical testing, we anticipate the use of cfDNA as a cancer biomarker to be implemented in the clinic in the near future.

In terms of biological therapy advancement, based on the patterns of double-digit growth in the past few years, we can expect to see this continue as more therapies become available and established therapies become more widely prescribed [150]. We foresee a large increase in the array of personalized antibody treatments made available, especially for cancer treatment. As we learn more about tumor heterogeneity, translational research investigators will be able to provide clinicians with valuable information for the selection of antibodies to target each patient's particular tumor. This will likely center on developing polyclonal

antibodies, which overcome the limits imposed by single epitope targeting and the possibility of off-target effect. CART will likely expand its growth and broaden the cancer types eligible for treatment.

EV's have the unique benefit of potential use for both diagnostic purposes and also for treatment. We will likely see forward progress using EVs synergistically with other upcoming therapeutics, e.g., miRNAs, for disease treatment. More accessible and cost effective NGS, paired with advancement in overcoming current limitations in vesicular fraction differentiation, will likely lead to greater use of EVs as biomarkers to monitor disease progression and treatment response. Improved methodology for vesicle isolation will enhance capabilities in selection of these bodies for delivery of therapeutics.

In the long run, we anticipate CRISPR landing in many clinical trials. Several biotech companies have been founded based on the concept of CRISPR therapies such as Editas Medicine, Intellia Therapeutics, and CRISPR Therapeutics. The CEO of Editas Medicine has announced the company plans to begin human clinical trials using CRISPR for the treatment of Leber congenital amaurosis, a disease causing retinal defects due to several possible inherited gene mutations. While preliminary CRISPR work has been successful, it is still a new technology and may fall short of expectations. In the near future we will begin to understand how feasible it is to translate CRISPR technology from the lab into the clinic. Optimistically, CRISPR could emerge as a curative option for previously incurable genetic disorders, but the possibility remains it will prove ineffective or cause unanticipated side effects. In addition to clinical trials in individuals, we may see more embryonic work emerging in attempts to correct genetic conditions before birth. This will likely be dependent on the political climate as it enters more of an ethical quagmire that could take many years to traverse.

Lastly, on the sequencing front, from a clinical perspective, we will continue to see an emphasis on targeted gene panels for genetic testing of patients, mainly because of fast turnaround and existing knowledge of actionable targets. Though current generation sequencing is more economical than Sanger sequencing, genetic testing is still fiscally out of reach for many patients due to high out-of-pocket costs or poor insurance coverage [151]. Clinics face limitations due to upfront costs and space requirements for the larger Ion and Illumina platforms, as well as the personnel needed to run them. The vast quantity of data generated require bioinformatics support that is likely unattainable by most clinics. Though open access analytical pipelines are available, accessing them can still present a roadblock for clinics, and they bring potential concerns over patient privacy. As accessibility of these pipelines increases, we will gain more information regarding the clinical utility of genetic variants, which will cause an increase in patient interest [151] and the likelihood of insurance-covered testing. This, paired with the growing market of direct-to-consumer genetic testing (Color) and DNA sequencing (23 and Me) also generating patient interest, will produce an upward trend in the use of these platforms for clinical purposes. These tests will likely remain contracted to outside providers, as the ability to multiplex sequencing runs plays a key role in the cost efficiency and may not be possible in smaller clinic settings. As TGS platforms move mainstream, we will see an upswing in the use of these platforms and we may see their more frequent use in a clinical setting, given their low capital investment

and minimal space requirements. A continued challenge in the clinical use of WGS or WES is the reporting of incidental findings; the American College of Medical Genetics and Genomics has issued a policy statement on how and whether these variants should be reported, but this could be an ongoing ethical issue [152].

From a translational research perspective, as current sequencing technologies continue to evolve, we will continue to see advancements in the areas of genetic profiling and a greater understanding of the importance of genetic variation. This will occur on both a smaller scale, through single-cell sequencing, and on more encompassing scale, through greater analysis of the non-coding regions of the genome made possible by more affordable WGS. As we are able to mine more data from the human genome, it will be important to employ a broader analytical approach to understand the interplay between the genome, epigenome, and transcriptome. Future advancements in sequencing technology, specifically improvements in TGS, will make simultaneous sequencing of the genome, transcriptome, and epigenome of even a single cell a reality. Taken together, all these technologies will allow us to obtain a more global overview of a patient's health status and to better understand the interplay of all these factors, ultimately guiding us to a more educated and precise treatment choice.

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6. Key Issues

- Technological advances have been fundamental for the progress of precision medicine, and to support the development of new therapeutics.
- Liquid biopsy holds the most exciting potential in cancer treatments and early diagnosis.
- Circulating tumor cells (CTCs) are currently used in clinical settings to support prediction of outcome in cancer patients.
- Analysis of cell free DNA (cfDNA) has been adopted as a tool in prenatal diagnostic.
- The use of extracellular vesicles (EVs), and microRNA as diagnostic tools remains experimental.
- Immunotherapies are at the forefront of cancer treatment.

Figure 1.

Summary of Current State and Speculative Use of Liquid Biopsies in Clinical Settings through Advancement of Sequencing Technology. Circles represent specified use indicated by color coordination. It is assumed that use in each timeframe will continue into subsequent periods.

* Indicates current use in only prenatal diagnostics.