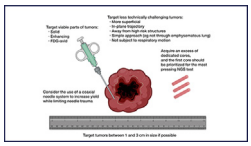
NARRATIVE REVIEW



Next-Generation Sequencing and Image-Guided Tissue Sampling: A Primer for Interventional Radiologists

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ABSTRACT

The discovery of increasing numbers of actionable molecular and gene targets for cancer treatment has driven the demand for tissue sampling for next-generation sequencing (NGS). Requirements for sequencing can be very specific, and inadequate sampling leads to delays in management and decision making. It is important that interventional radiologists are aware of NGS technologies and their common applications and be cognizant of the factors that contribute to successful sample sequencing. This review summarizes the fundamentals of cancer tissue collection and processing for NGS. It elaborates on sequencing technologies and their applications with the aim of providing readers with a working understanding that can enhance their clinical practice. It then describes imaging, tumor, biopsy, and sample collection factors that improve the chances of NGS success. Finally, it discusses future practice, highlighting the problem of undersampling in both clinical and research settings and the opportunities within interventional radiology to address this.

ABBREVIATIONS

cDNA = complementary DNA, CNB = core needle biopsy, ddNTP = dideoxynucleotide triphosphate, DNA-seq = DNA sequencing, FDA = U.S. Food and Drug Administration, FF = fresh-frozen, FFPE = formalin-fixed and paraffin-embedded, FNA = fine needle aspiration, GC = guanine-cytosine, HCC = hepatocellular carcinoma, LRS = long-read sequencing, mRNA = messenger RNA, NGS = next-generation sequencing, PCR = polymerase chain reaction, RNA-seq = RNA sequencing, SRS = short-read sequencing, WES = whole exome sequencing, WGS = whole genome sequencing

Image-guided percutaneous needle tissue sampling has become the diagnostic cornerstone of modern clinical oncology. It allows histopathological diagnosis in primary cancer and confirmation and staging of secondary disease. Growing understanding of the molecular basis of cancer and advances in the processing of big data have heralded an era of precision oncology, where genomic and transcriptional tumor features in the individual patient can guide treatment and prognostication. In the last 2 decades, multiple actionable gene alterations with a corresponding specific "targeted" therapy have been identified. A 2019 review by Colomer et al (1) described 24 targetable molecular alterations and 64 different specific anticancer therapies. Targeted therapies are increasingly being integrated into routine clinical management, and molecular traits have become important determinates of treatment in many cancers (examples from the United Kingdom and the United States are summarized in **Table 1**).

The development of next-generation sequencing (NGS) technologies has allowed rapid, cost-effective, accurate, and high-throughput detection of novel and rare somatic mutations in clinical and research practice (2). However, the utility of NGS is not limited to the positive identification of molecular targets because even wild-type (ie, normal) genes can affect treatment strategy, for example, panitumumab is approved for use in patients with colorectal cancer with wild-type rat sarcoma virus genes in England.

Early investigation of targeted therapies focused on specific rare and metastatic cancers for which treatment options were limited. Emerging clinical trial data now demonstrate improvement in the response rate and progression-free survival (3–5) with a tumor-agnostic approach—in which treatment is based on genetic and molecular features without regard to the cancer type or tissue of origin. There are now 8 U.S. Food and Drug Administration (FDA)-approved tumor-

Appendix A contains a glossary of biological terms which may be helpful for the reader and this can be found by accessing the online version of this article on *www.jvir.org* and selecting the Supplemental Material tab.

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Table 1. Detected Genetic/Molecular Alterations and Their Associated Targeted Therapies for Tumor-Agnostic Treatment (Based on Genetic and Molecular Features only Regardless of Site of Origin) and the 5 Most Common Cancers Worldwide (Breast, Colorectal, Lung, Melanoma, and Prostate Cancers)

Gene or molecular alteration	Cancer type	Therapeutic agents
NTRK gene fusion*	Solid tumors anywhere* NSCLC†	Entrectinib,* larotrectinib* Atezolizumab,† larotrectinib,† nintedanib,† nivolumab,† pembrolizumab†
TMB-H	Solid tumors anywhere	Pembrolizumab
dMMR*	Solid tumors anywhere Colorectal cancer*	Dostarlimab Ipilimumab,* nivolumab,* pembrolizumab*
MSI-H*	Solid tumors anywhere Colorectal cancer*	Ipilimumab, pembrolizumab Ipilimumab,* nivolumab,* pembrolizumab*
BRAF mutation*	Solid tumors anywhere Colorectal cancer,* melanoma,* NSCLC	Dabrafenib, trametinib Atezolizumab, binimetinib,* cextuximab,† cobimetinib, dabrafenib,* encorafenib,* trametinib,* vemurafenib*
RET fusion gene*	Solid tumors anywhere NSCLC*	Selpercatinib Selpercatinib,* pralsetinib, atezolizumab,† nivolumab,† bevacizumab,† nintedanib,† pembrolizumab†
EGFR mutation*	Colorectal cancer,* NSCLC*	Afatinib,* amivantamab, atezolizumab,* cetuximab, dacomitinib,* erlotinib,* gefitinib,* mobocertinib, nivolumab,* nintedanib,† osimertinib,* pembrolizumab,* ramucirumab
PD-L1 protein*	Breast cancer,* NSCLC*	Atezolizumab,* cemiplimab-rwlc, ipilimumab, nivolumab, nintedanib,† pembrolizumab*
ALK mutation*	NSCLC*	Alectinib,* atezolizumab,* brigatinib,* ceritinib,* lorlatinib,* nivolumab,* pembrolizumab,* nintedanib,† ramucirumab
MET mutation*	NSCLC*	Atezolizumab,† bevacizumab,† capmatinib, nivolumab,† nintedanib,† pembrolizumab,† tepotinib*
ROS1 mutation*	NSCLC*	Crizotinib,* entrectinib,* atezolizumab,† nivolumab,† nintedanib,† pembrolizumab†
HER2 (mutation, amplification, or expression)*	Breast cancer,* NSCLC	Fam-trastuzumab deruxtecan-nxki, lapatinib,* margetuximab-cmkb, neratinib,* pertuzumab,* pertuzumab, trastuzumab, and hyaluronidase-zzxf, trastuzumab,* tucatinib*
PIK3CA mutation*	Breast cancer*	Alpelisib*
KRAS mutation*	NSCLC*	Atezolizumab,† bevacizumab,† nivolumab† Nintedanib,† pembrolizumab,† sotorasib*
BRCA1/BRCA2 mutation	Breast cancer, prostate cancer	Olaparib, rucaparib, talazoparib
HHR pathway gene mutations	Prostate cancer	Olaparib
PSMA protein	Prostate cancer	Lutetium Lu 177 vipivotide tetraxetan
Estrogen receptor expression*	Breast cancer*	Abemaciclib,* ado-trastuzumab emtansine, alpelisib, anastrozole,* everolimus, exemestane,* fulvestrant,* letrozole,* palbociclib,* pertuzumab, ribociclib,* tamoxifen,* toremifene

Note–The therapeutic agents that are in normal text are those that are approved by the U.S. Food and Drug Administration (FDA).
ALK = anaplastic lymphoma kinase; BRAF = v-Raf murine sarcoma viral oncogene homolog B; BRCA1 = breast cancer gene 1; BRCA2 = breast cancer gene 2; dMMR = mismatch repair deficiency; EGFR = epidermal growth factor receptor; HER2 = human epidermal growth factor receptor 2; HHR = homologous recombination repair; KRAS = Kirsten rat sarcoma virus; MET = mesenchymal epithelial transition; MSI-H = high microsatellite instability; NSCLC = non–small cell lung cancer; NTRK = neurotrophic tyrosine receptor kinase; PD-L1 = programmed death-ligand 1; PIK3CA = phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; PSMA = prostate-specific membrane antigen; ROS1 = c-ros oncogene 1; RET = rearranged during transfection; TMB-H = high tumor mutational burden.
*Approved by both FDA and National Institute for Health and Care Excellence.
†Approved by the National Institute for Health and Care Excellence (England and Wales).

agnostic therapies for 6 targetable molecular alterations for solid tumors anywhere in the body (Table 1).

The benefits of individualizing therapy (3–5), together with the progressive improvement in the efficiency and cost of NGS, will raise demand for tumor sampling—a technical and resource challenge that radiologists and radiology departments must consider. However, current interventional radiology pathways prioritize core biopsy for histopathological analysis and may not adequately consider the specific requirements of NGS.

This review aims to be a short introductory resource on NGS. It expands on existing reviews (6,7) by elaborating on sequencing technologies with clinically relevant applications to provide readers with a working knowledge in this space. It also discusses factors that affect sequencing success and the future of radiological sampling in personalized oncology.

WHAT IS NGS?

Sanger Sequencing

The founding breakthrough in DNA sequencing was developed in 1977 by Frederick Sanger and colleagues with their chain-termination method—Sanger sequencing.

Sample DNA is first amplified using polymerase chain reaction (PCR). Amplification is necessary to produce enough DNA copies to generate a sufficient signal for detection. In PCR, sample DNA is heat-denatured into single strands, and DNA primer, DNA polymerase, and

nucleotides are added. DNA primers are short single-stranded sequences of DNA that are specific and complementary to the target DNA portion to be sequenced. They are necessary to guide DNA polymerase to the site where chain extension begins. PCR results in multiple copies of the DNA sequence of interest.

These DNA strands then undergo Sanger sequencing. To determine the sequence, radioactive or fluorescently labeled modified nucleotides (dideoxynucleotide triphosphate [ddNTP]) are introduced during PCR. These modified ddNTPs cannot form a phosphodiester bond with a subsequent nucleotide, and hence, the random incorporation of ddNTPs results in the termination of DNA extension. Chain-termination PCR results in millions/billions of copies of the DNA sequence of interest that terminate at random lengths.

DNA fragments are then separated by size through electrophoresis. By arranging the fragments in order of size and then identifying the terminal ddNTP (eg, by fluorescence detection), the DNA sequence is determined.

Second-Generation Sequencing or NGS

Despite its success, the relatively high cost and low throughput of Sanger sequencing prevent its large-scale use. The first commercially available second-generation (NGS) platform was introduced in 2005 (8). The key difference with Sanger sequencing is that sample DNA must be made into libraries prior to sequencing. These libraries are pools of DNA fragments with adaptors introduced. Adaptors bind to the ends of DNA fragments and allow them to interact with their specific sequencing platform where they are clonally amplified. Adaptors can also contain individual "barcode" sequences that allow multiplexing—the parallel readout of multiple different samples from a single run (9). A simplified schematic (**Fig 1**) of a commonly used clonal bridge amplification sequencing process (Illumina, San Diego, California) is provided to aid understanding of the general NGS process.

Bridge Amplification. Adaptors first bind fragmented DNA with complementary adaptors on a flow cell (a hollow glass slide with multiple channels coated with adaptors). Synthesis of a complementary DNA (cDNA) strand then occurs. cDNA forms a bridge by virtue of another adaptor at its free end, which binds to a complementary adaptor on the slide. The strand undergoes amplification, and this process is repeated multiple times. The strands on the slide are spaced such that clonal clusters comprising approximately 1,000 copies of each DNA fragment are generated, and each slide can support millions of parallel reactions.

Sequence Readout. After amplification, in a process similar to Sanger sequencing, the bases of each fragment are then identified serially by the detection of a transduced signal. This occurs by the incorporation of labeled modified nucleotides that terminate synthesis. After detection, the modified nucleotide is chemically unblocked, the next one in the series is added, and the process is repeated until the whole fragment is read. Transduced signals can differ according to NGS platform.

A bioinformatics software then aligns the millions of reads to a reference sequence. The number of unique reads that include a given nucleotide is known as the sequencing depth; greater depth increases sequencing accuracy. A patient's own germline DNA, which can be extracted from normal tissue, such as blood, will serve as a more accurate reference than a generic library. After alignment, any differences between the reference and newly sequenced reads (eg, mutations) are identified.

These NGS methods allow the processing of millions of reactions in parallel, resulting in high throughput, scalability, and speed. Genome sequencing projects that took years with Sanger sequencing can now be completed in hours. It can also detect a wider range of mutations and analyze sequences without requiring preexisting knowledge of gene locations, and it is more cost-effective (10).

Limitations of NGS. Second-generation sequencing technologies use short-read sequencing (SRS) methodology—DNA samples require fragmentation prior to sequencing. They are unable to sequence long stretches of DNA (11). Signal read comes from the extension of a template strand on every molecule within a clonal cluster. However, this extension does not occur perfectly in synchrony on every strand. This "dephasing" decreases the signal and limits the read length to between 75 and 400 base pairs.

The use of PCR is also an issue. Certain regions of DNA are more challenging to amplify with PCR, and preferential amplification of repetitive DNA segments can introduce bias. Guanine-cytosine (GC) base pairs are also more thermostable than adenine-thymine base pairs, and the amplification process results in underrepresentation of bases in areas of high or low GC content. Most housekeeping genes, tumor-suppressor genes, and approximately 40% of genes with tissue-specific regulation contain high-GC sequences in their promoter region (12)—reduced sensitivity to potentially important sequence changes here could impact biomarker discovery.

The inability of SRS to generate sufficient sequence overlap from DNA fragments is a major challenge for the detection of large sequence changes. A clinical example of such a change is the chromosomal translocations classical of some hematological malignancies (13). Short fragments of reads within the long translocated fragment will not be correctly aligned to its real position and are, therefore, not detected.

Long-Read Sequencing

Long-read sequencing (LRS) technologies—sometimes referred to as third-generation sequencing—are capable of sequencing single molecules without the need for DNA amplification. This avoids the associated biases and errors of SRS, allowing longer read lengths (5–30 kilobase pairs) and the exploration of genomic regions that were previously

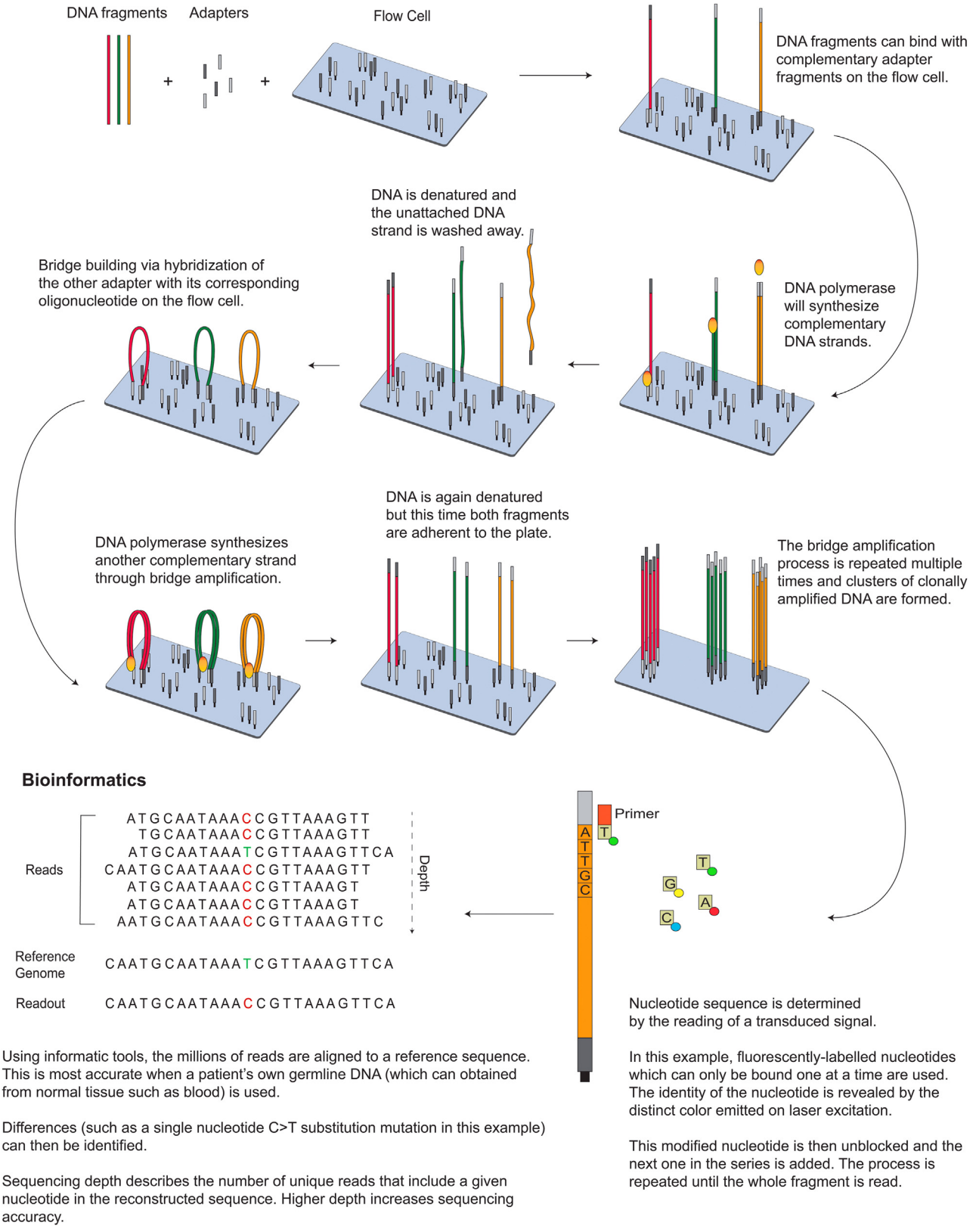

Figure 1. The principle of Illumina sequencing in brief. Amplification of nucleic acid material resulted in a detectable signal to identify the nucleotide sequence in sampled DNA fragments. By aligning with a reference sequence in silico, these fragments could then be reassembled to obtain the tumor sequence and identify any differences, such as mutations.

Table 2. Advantages and Disadvantages of Short-Read Long-Read Sequencing

	Advantages	Disadvantages
Short-read sequencing	• Low cost • High yield • High speed • Improved sequence fidelity • Ability to sequence fragmented DNA	• Underrepresentation of high–GC-content regions • Limited resolution of complex regions of the genome (eg, MHC), repetitive regions (where short reads will not map uniquely), large sequence changes (eg, structural variation), and paralogous regions of the genome Systematic errors during amplification
Long-read sequencing	• De novo assembly from long reads that span low-complexity and repetitive regions • Targeted sequencing of complex genomic and paralogous regions • Detection of structural variants • Single-molecule sequencing allows examination of clonal heterogeneity Allows full-length sequencing of isoforms and examination of splicing in transcriptomics	• Low yield • High error rate/lower per read accuracy • Bioinformatic challenges (eg, coverage biases, scalability, and limited availability of pipelines) Instruments were generally more expensive, but this is changing with the development of new devices, such as Minion

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GC = guanine-cytosine; MHC = major histocompatibility complex.

inaccessible to SRS platforms. Table 2 (14) outlines the advantages and disadvantages of SRS versus LRS.

A pioneering study by Nattestad et al (15) of the SKBR-3 breast cancer cell line demonstrated that human epidermal growth factor receptor 2 amplification appeared within complex rearrangements that could only be precisely identified by LRS. The group also sequenced SKBR-3 cells and patient-derived organoids from 2 patients with breast cancer and found that structural variation detection was much more accurate and sensitive in LRS and that hundreds of known variants within known cancer-related genes were only detectable with LRS (16).

The 2 current major producers of LRS technologies are Pacific Biosciences and Oxford Nanopore Technologies. The latter is briefly described to familiarize the reader with an example of LRS methodology.

Long DNA molecules can pass through protein nanopores—small-diameter holes within a membrane. A steady current is applied to the nanopores during a read—this alters depending on the size of the pore opening. Single strands of sample DNA are threaded through the pore. Each nucleotide differs in size and, therefore, has a unique electrical signature that allows identification. This technology has been commercialized by Oxford Nanopore Technologies (Oxford, UK), and there is excitement over its small, handheld-sized Universal Serial Bus device (Minion). This offers portability and LRS data at a fraction of the cost and time previously possible (9,17). A comparison of common SRS and LRS platforms is summarized in Table 3.

COMMON APPLICATIONS IN NGS

It is worth noting that the definition of NGS varies in the literature but usually refers to second-generation technologies (as in the previous section). Here, NGS refers to second-generation and beyond.

DNA Sequencing

The human DNA sequence dictates the developmental processes and influences susceptibility to diseases. The use of high-throughput DNA sequencing (DNA-seq) technologies allows the identification of genetic variations that cause or influence cancer, with implications on diagnosis and management. DNA analyses on tumor samples broadly fall into 2 categories: (*a*) targeted approaches investigating a limited number of clinically significant genes ("hotspot" panels) and (*b*) whole exome sequencing (WES) or whole genome sequencing (WGS), which are often used in exploratory research to elucidate new mechanisms of response or resistance (18).

Because each sequencing run can only read a limited number of sequence fragments, it is more cost-effective to sequence specific parts of the genome of interest. The use of assays with complementary oligonucleotides that capture targeted genes or exomes facilitates this. In contrast, WGS sequences the entire genome, including exons and introns—potentially providing crucial and previously unknown information (19).

High-throughput DNA-seq is now crucial in the management paradigm of numerous cancers, in which targeted therapy decisions are based on specific genetic alterations (Table 1). Its role will continue to develop—in advanced cancer, in which there may be several commonly identified molecular targets; in directing patients to clinical trials, and with individualized treatment for patients with rare cancers (1). An early use example is FoundationOne CDx [Foundation Medicine, Cambridge, Massachusetts] (20), an FDA-approved NGS-based diagnostic test validated for all solid tumors. This assay is designed to find clinically relevant variations in 324 genes as well as specific gene rearrangements and other alterations such as microsatellite instability that can guide targeted therapies.

RNA Sequencing

RNA provides information on gene expression. It turns the information stored in DNA into functional proteins—with messenger RNA (mRNA) carrying instructions from DNA in the nucleus to cytoplasmic ribosomes where protein is synthesized. Thus, mRNA provides information on the current state of cancer cells and how disease states or therapy affects gene expression (because some DNA sequences are not expressed or underexpressed/overexpressed).

Table 3. Comparison of the Illumina, Pacific Biosciences (PacBio, Menlo Park, California), and Oxford Nanopore Technologies (Nanopore) Sequencing Platforms

	Illumina	PacBio	Nanopore
Mean read length	2 × 150–250 bp	10–15 kb	15–20 kb
Maximum read length	2 × 250 bp	>60 kb	>800 kb
Accuracy per nucleotide	>99%	>85%	60%–85%
Error bias	Substitutions in high–/low–GC-content regions	Small indels (mostly insertions)	Small indels (mostly deletions)
Coverage bias	Low coverage of high–/low–GC-content regions Mapping issues with highly homologous regions	Homopolymers	Truncation of homopolymers and low-complexity regions
Accuracy after error correction	N/A	After CCS, 95%–99%	After $1D^2$, 97% After hybrid correction, >99%
Sample requirements	1–2 μg	10-μg HMW	0.4–1-μg HMW
Low-throughout sample requirements	100 ng	400–800 ng	10–100 ng

Source–Adapted from (34) under a Creative Commons Attribution 4.0 International License.
bp = base pair; CCS = circular consensus sequencing; GC = guanine-cytosine; HMW = high molecular weight; indels = insertion/deletion polymorphism; kb = kilobase; N/A = not applicable.

Compared with microarrays, RNA sequencing (RNA-seq) is more sensitive, provides absolute quantity levels, is not affected by on-chip sequence biases, and gives additional information on gene expression levels and splice junction variants.

RNA-seq is similar to DNA-seq except that RNA is first converted to a more stable cDNA through reverse transcription before sequencing. RNAase enzymes are ubiquitous and extremely stable, and without due care and preparation, contaminated samples can fragment, affecting RNA quality. RNA quality will determine whether a sample is suitable for RNA-seq and whether specific processing is required. For example, the selection of the polyadenylated tail of mRNA is the typical method to extract mRNA from a sample, but this would not be possible with fragmented mRNA. Another method, such as the selective amplification of nonribosomal RNA, would be necessary (19).

Tumor gene expression results, which are essentially the number of sequencing reads mapping to different transcripts, can be used to prognosticate, predict treatment efficacy, and guide therapy options. Oncotype DX [Exact Sciences, Madison, Wisconsin] (21) is one such diagnostic test licensed for use in breast cancer, specifically in early-stage estrogen receptor–positive and human epidermal growth factor receptor 2–positive invasive breast cancer with no lymphatic involvement. It measures the expression of 21 specific genes and can predict the likelihood of metastatic spread within 10 years of diagnosis and the likelihood that additional chemotherapy will be helpful.

Epigenomic Sequencing

Epigenetic modifications, such as DNA methylation or histone modification, can influence gene expression. These refer to phenotypic changes that do not involve alterations in the DNA sequence—for example, the addition of a methyl group to DNA can repress gene expression. Epigenetic studies are crucial for a better understanding of these cellular processes in cancer.

There are different methods of epigenomic sequencing depending on the epigenetic modifications of interest. In methylation sequencing, fragmented DNAs are separated into 2 volumes, with one being treated with bisulfite. Bisulfite changes cytosine to uracil but leaves methylated cytosine unchanged. After sequencing, data analysis compares the 2 volumes and identifies the regions that were methylated.

A number of cancer drugs targeting epigenetic regulators have been developed for the treatment of hematological malignancies (eg, DNA methyltransferase inhibitors in myelodysplastic syndrome and acute myeloid leukemia), and it shows potential for use in solid tumors in preclinical and clinical trials (22).

FACTORS THAT AFFECT NGS SUCCESS

The sequencing failure rates in published studies (3,23–27) involving DNA molecular profiling from core needle biopsy (CNB) samples range from 3% to 38%. Test failures fall into 3 categories: (*a*) insufficient tumor tissue, (*b*) insufficient nucleic acid extracted, and (*c*) failure of library generation. The first 2 account for most failures, with a study of 1,528 various types of biopsy samples sent for NGS testing by Al-Kateb et al (26) demonstrating that 94% of the failed samples (n = 343) were due to insufficient tumor tissue (64%) or insufficient DNA extraction (29%).

The relevant tissue sampling and processing factors that contribute to NGS success are discussed in the following section. **Figure 2** distills this evidence and summarizes the key points to help radiologists improve NGS success.

Tumor Selection and Targeting

Fundamental to successful tumor sequencing is accurate tissue sampling. In metastatic disease, target selection on image review is driven by technical feasibility and safety with a focus on the largest and/or most accessible lesion. The solid and perfused parts of the tumor as well as biologically active volumes (identified on functional imaging)

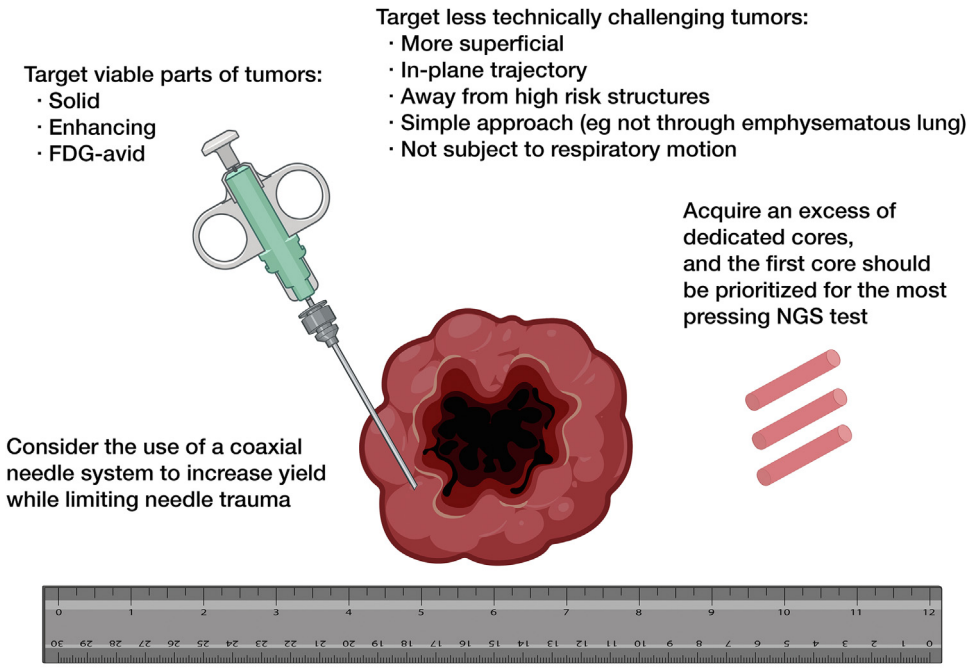

Figure 2. Factors that could improve the rates of successful next-generation sequencing (NGS) after core needle biopsy. FDG = fluorodeoxyglucose. Created with BioRender.com.

are targeted to optimize collection of viable cancer tissue. These are preferred over necrotic and apoptotic cells for molecular profiling because autolysis of cellular components within necrosis can render material unsuitable and apoptosis can result in nucleic acid fragmentation (28).

Sabir et al (29) investigated the characteristics of CNB samples that were adequate for NGS in 153 patients and devised a likelihood of adequacy score on the basis of problematic imaging characteristics, which included small size, proximity to high-risk structures, unfavorable surrounding tissue (eg, emphysematous lung or through the bone), highly angled approach (necessitating out-of-plane trajectories), location susceptible to motion (eg, lung bases), and presence of sclerosis or necrosis. The fewer factors present, the higher the likelihood of adequacy score. This score was significantly associated with NGS success, and its use is described in a number of publications (6,29).

Most criteria logically relate to increasing technical difficulty. Biopsies of necrotic and sclerotic areas reduce the amount of viable tumor sampled. Smaller lesions (<3 cm in diameter) had higher odds of NGS success, with the thought that larger lesions are more likely to have outgrown their blood supply and have necrotic areas (29). In a powerful study, Bhamidipati et al (27) analyzed 5,000 prospectively collected research biopsy cores and demonstrated that the mean malignant area per core increases substantially with lesion sizes of >1 cm (**Fig 3**). In combination, these data suggest that an optimal result could be achieved by targeting lesions between 1 and 3 cm in diameter.

A prospective study of 334 image-guided biopsy samples from the molecular-screening for cancer treatment optimisation-01 trial (30) showed no significant difference in tumor cellularity between targeting the central regions and targeting the peripheral regions of tumors in 242 patients who had matched biopsies (central < peripheral, 35%; central = peripheral, 28%; and central > peripheral, 37%).

A large retrospective study of 614 solid tumor specimens by Goswami et al (31) showed that biopsies from deep organs and the lung generally yielded extremely small specimens with low tumor cellularity—with low DNA yield and poor NGS success rates. This was attributed to reduced targeting accuracy with deeper sites and less aggressive sampling given relative increased proximity to major vessels. Goswami et al (31) also showed that the NGS success rate was significantly lower in the bone (63%, $P < .001$) than in other sites (eg, breast [96%], colorectal area [93%], liver [77%], lung [84%], and lymph nodes [78%]). The molecular-screening for cancer treatment optimisation-01 study (30) supports this, with cellularity within bone lesions (median, 0%) being significantly lower than that in other sites. The inherent low cellularity of bony disease and strong acids (which degrades DNA) used to decalcify the specimen prior to histological assessment result in poor NGS success.

Bhamidipati et al (27) corroborated these findings, showing that the malignant area per core was lower in deep locations, the thorax, bone, and thyroid (**Fig 3**b). In combination, these data underline that selecting solid organs and more superficial locations increases the chances of success. Where deep or bony lesions are targeted, increased sample collection via multiple passes and a coaxial technique (32) should be considered.

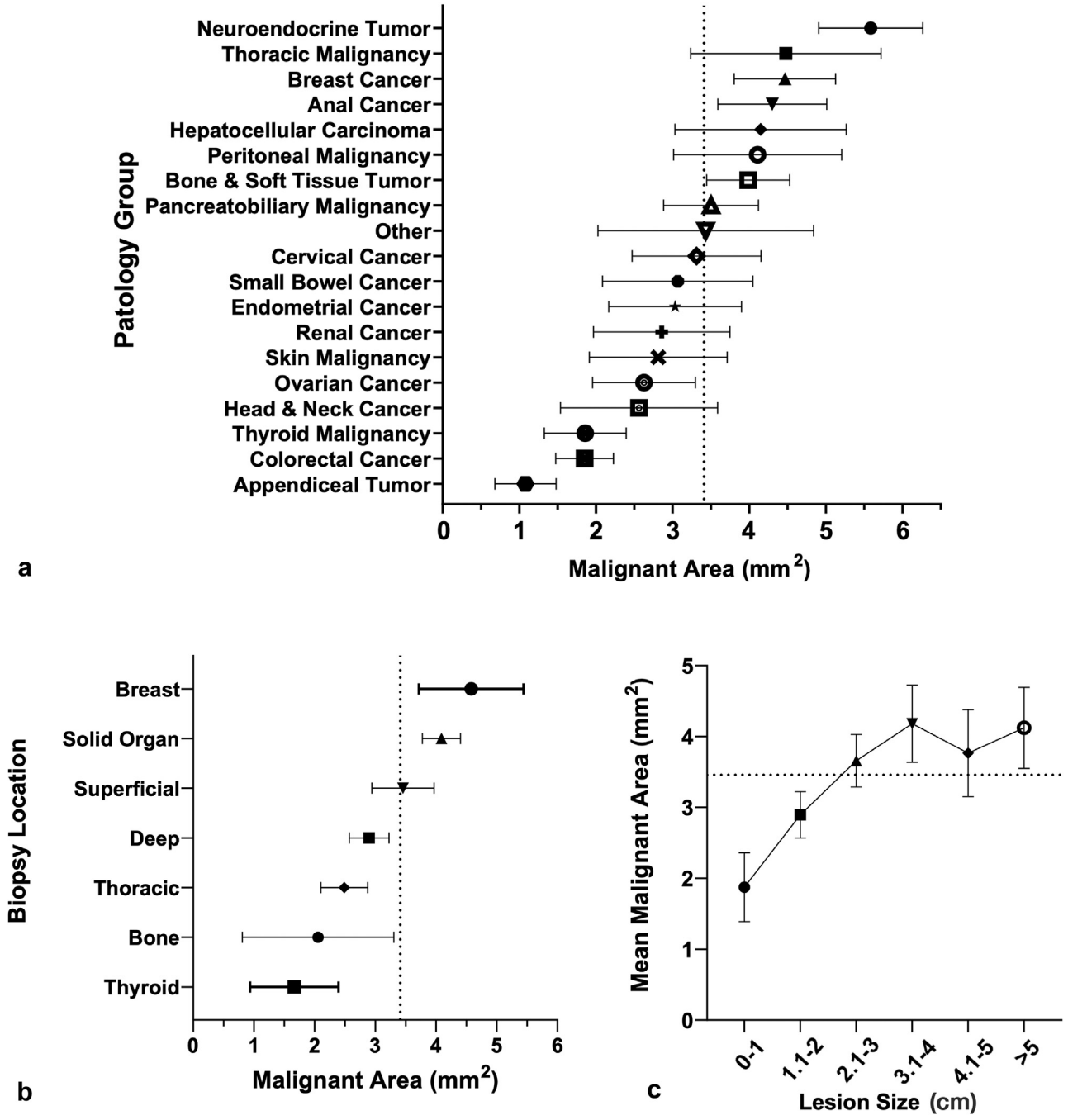

Figure 3. Malignant area by **(a)** tumor pathology, **(b)** biopsy location, and **(c)** lesion size. Each graph displayed the mean malignant area per core along with 95% CI estimates. The dotted line represented the mean malignant area per core for all biopsies. It should be noted that the majority of breast biopsies were performed using 14-gauge needles and the majority of thyroid and thoracic biopsies were performed using 20-gauge needles. Reproduced from (27) under a Creative Commons Attribution 4.0 International License.

Variation in sequencing success by tumor type has been suggested. Bhamidipati et al (27) found that the malignant area per core varied significantly between different tumor types (Fig 3a). However, Goswami et al (31) found no differences in NGS success rates between major tumor types, and other studies (29,30,33) have reported mixed results. It is likely that tumors with a greater propensity for necrosis (eg, colorectal adenocarcinoma and treated tumors) and mucinous neoplasms offer reduced tumor yield. In these contexts, extra care should be taken to target solid, enhancing or functionally active tumor regions.

A number of different NGS platforms exist (34), and the choice of platform and assay type (eg, WGS vs targeted panel) affects the amount of nucleic acid required. This can range from 10 to 3,000 ng (35)—approximately 2,000–500,000 cells (28). Tumor fraction refers to the proportion

Table 4. Range of number of tumour cells per idealised full cylindrical core for typical gauges and lengths of needle biopsy samples.

Gauge (G)	Nominal inner diameter (mm)	Length (mm)	Volume of core (mm^3)	Number of tumour cells*
14	1.600	10	20.1	603,187–1,206,374
14	1.600	20	40.2	1,206,374–2,412,749
14	1.600	30	60.3	1,809,562–3,619,123
16	1.194	10	11.2	335,908–671,817
16	1.194	20	22.4	671,817–1,343,633
16	1.194	30	33.6	1,007,725–2,015,450
18	0.838	10	5.5	165,463–330,925
18	0.838	20	11.0	330,925–661,851
18	0.838	30	16.5	496,388–992,776
20	0.603	10	2.9	85,674–171,347
20	0.603	20	5.7	171,347–342,694
20	0.603	30	8.6	257,021–514,041
22	0.413	10	1.3	40,189–80,379
22	0.413	20	2.7	80,379–160,758
22	0.413	30	4.0	120,568–241,137

*Assuming tumour fractions of 30%–60%.

of tumor nuclei over total nuclei within a specimen, and published literature (18,23,27,36) suggests that a typical CNB sample contains a median tumor fraction of approximately 30%–60%; **Table 4** summarizes the number of tumor cells per cylindrical core for different gauges and lengths of biopsy needle samples. This is an idealized estimate assuming full-length cylindrical cores and that a 1-mm^3 tumor contains approximately 10^5 cells (37). Depending on length, this suggests that a single core from smaller gauge needles (i.e. 18G and above) may not be sufficient for more demanding assays such as WGS or RNA-seq. **Table 4** serves as a guide, however given real-world issues of core fragmentation, variable tumour fraction, and notched needles that acquire horizontal cylindrical segments, additional tissue collection may guard against assay failure. It is also important to note that the throw lengths of biopsy devices do not correspond to the actual length of the sample. For instance, throw lengths of 13, 23, and 33 mm using the BioPince Full Core instrument (Argon Medical Devices, Plano, Texas) correspond with core lengths of 9, 19, and 29 mm, respectively (38).

Determining an optimal sampling system and technique is known to be challenging (6), with a wide range of views expressed in the literature. Two prospective studies on lung nodule biopsy for nucleic acid analysis presented conflicting conclusions, with one (39) suggesting 10 cores from a 20-gauge side-cut needle and the other (40) suggesting a single pass of an 18-gauge core as preferable. However, it is well established that increasing the number of cores increases pathological diagnostic success (41), and similarly, multiple cores should increase the likelihood of obtaining sufficient viable tumor.

Bhamidipati et al (27) demonstrated that the malignant area per core decreased with sequential core samples (median malignant areas in the first, second, third, fourth, and fifth cores were 2.40, 2.40, 2.10, 2.1, and 1.8 mm^2, respectively)—suggesting that the first sample core should be prioritized for the most pressing molecular test.

Tumor Fraction and Cellularity in the Sample

The size of the tumor-rich areas and tumor cellularity within the sample determine the nucleic acid yield. As discussed earlier, certain tumors (eg, treated or necrotic) can be hypocellular, and a greater volume of tissue is required. Higher tumor fractions are also required in hepatic tumors because tumor signal is diluted by the often tetraploid normal hepatocytes (which contain double the normal DNA) (42).

Minimum tumor fraction requirements are dependent on the analytical sensitivity of the specific NGS platform and are typically between 10% and 20% (28). Tumor fractions below the analytical sensitivity result in a reduced read depth of the tumor-derived sequence with an increased risk of false-negative results and difficulty in determining the validity of low-frequency variant mutations.

There exists a complex relationship between tumor size, tumor cellularity, and tumor fraction. A larger sample does not necessarily equate to better results. Increasing the tumor sample size may increase the total nucleic acid yield by collecting a greater number of cells; however, this could trade-off with decreasing tumor fraction if more nontumor components, such as stroma, are included. Limiting sampling to carefully selected areas of viable tumor is most likely to deliver a tumor-rich sample.

Tissue Processing and Storage

The standard fixative used in clinical practice is 10% formalin. It causes random breaks in nucleic acids, resulting in a high number of fragmentations and reduced quality. Fragmented nucleic acids are not suitable for LRS, which limits the use of formalin-fixed and paraffin-embedded (FFPE) material for specific analyses, such as in complex or repetitive regions of

the genome (Table 2). Fragmentation and poor quality can also introduce errors during analysis or render specimens unsuitable for SRS. In addition, the cross-linking of nucleic acid bases to other macromolecules can reduce the yield of extracted DNA/RNA (43).

Despite this, improvements in technology, particularly with DNA/RNA extraction kits, DNA repair kits, library preparation protocols, and bioinformatic removal of artifacts, make it possible to harness sufficient quality DNA/RNA from formalin-fixed tissue to allow sequencing. There are multiple studies comparing DNA-seq (44–50) and RNA-seq (51,52) NGS data from paired FFPE and fresh-frozen (FF) tissue specimens in various cancer types. The majority demonstrates high concordance [eg, DNA NGS studies (44–46,50) with concordance rates between 91% and 99%]—with the authors concluding that FFPE samples can be a reliable alternative substrate for clinical and research NGS use. One study was less promising—De Paoli-Iseppi et al. (48) reported a mean concordance rate of 43.2% in 27 genes of clinical relevance with 0/3 v-raf murine sarcoma viral oncogene homolog B1 (BRAF) FFPE calls in WES of 10 patients with melanoma. This suggests that FFPE tissue could fall short with more exploratory WES or WGS studies. Increasing sequencing depth could partly address this issue, and new commercially available NGS platforms (eg, MGI's DNBSEQ platforms [MGI Tech, Shenzhen, China) have significantly decreased costs enabling wider use.

Review of the aforementioned literature also highlighted that publications do not always report the number of FFPE samples that fail to meet the DNA quality criteria. Although FFPE and FF concordance is good when good-quality DNA is available, a greater proportion of failure in the FFPE samples is probable. For example, Hedegaard et al (53) reported a failure rate of 69.5% with 43 of 61 FFPE samples, which was thought to be due to poor amplification resulting from DNA modifications caused by fixation and storage.

FF samples have been widely held as the gold standard for NGS, but this method is relatively expensive and resource-intensive, limiting large-scale adoption. The aforementioned study data show that FFPE specimens can meet certain clinical and research NGS needs. Although FF samples provide higher-quality nucleic acids than those provided by FFPE samples, it should not be thought of as the default gold standard. Rather, the right sample is that which meets the specific requirements of the clinical or research question.

Preservative preparations, such as RNAlater (Thermo Fisher Scientific, Waltham, Massachusetts), stabilize and protect nucleic acids in specimens and, thus, eliminate the need for immediate processing or freezing (54). Biopsied tissue is simply placed into the solution in a collection vessel. RNAlater allows the sample to be stored for 1 week at room temperature without any significant degradation of nucleic acid quality. Once transferred to a −20 °C or −80 °C freezer, it can then be stored indefinitely. Although currently limited to research use, feasibility for use in hospital practice has been demonstrated in large-scale multicenter studies (55,56) of DNA and RNA NGS from RNAlater-held samples, and it may soon be validated for clinical use.

FUTURE OF RADIOLOGICAL BIOPSIES IN PERSONALIZED ONCOLOGY

Opportunities to Study Heterogeneity and Therapy-Induced Evolution

Current standard sampling protocols for tumor sequencing are reliant on a small volume of tissue usually from a single site. However, tumors are now acknowledged to be heterogeneous, with variation within a patient or even in a single tumor. Standard samples, which are a fraction of the total disease volume, are underrepresentative, reducing sensitivity in detecting key tumor biomarkers and limiting the understanding of cancer evolution (57). Published cancer sequencing studies to date largely focus on primary disease, in which tumors are frequently surgically resected and banked. Most patients with cancer die of metastatic disease, in which sampling and resection are limited. Expansion of sampling in advanced disease will improve understanding of the molecular transition to the metastatic state, enable further targeted therapy development, and provide crucial information on the resistance mechanisms that lead to therapy failure (58).

Most biopsies are performed under imaging guidance, and interventional radiology will play a central role in expanding efficient pathways that enable systematic sampling. Underutilized opportunities for sampling collection may already exist. For example, patients undergoing focal ablative therapy are usually not sampled during the procedure. With the right precautions (eg, the use of a coaxial needle to minimize the risk of tumor seeding), sampling could be performed here without significant additional risk.

Hepatocellular carcinoma (HCC) is unique in that definitive diagnosis and staging are frequently based on imaging without mandatory histopathological confirmation. The Liver Imaging Reporting and Data System is a classification system that reflects the probability of HCC in at-risk livers on the basis of computed tomography and magnetic resonance imaging findings, with advantages of reporting standardization and the reduction of "unnecessary" biopsies for LR-5 lesions (definite HCCs). A systematic review (59) of 18 studies highlighted an increased possibility of misdiagnosis in small, early-stage, and nontypical nodules. The emergence of immune therapies, such as checkpoint inhibitors, has shown promise in HCC; however, there is a need to better select patients likely to respond on the basis of individual tumor profile (60). Paired pretreatment and on-treatment sampling would enable this work; biopsies of LR-5 lesions would aid this and could also reduce the risk of imaging misdiagnosis.

Enabling Multiplatform Profiling of Metastases

Tumors are complex ecosystems of malignant cells and their microenvironment (including stroma, blood vessels, and

immune cells), and this complexity is not fully characterized by single profiling methods performed in isolation. Recent studies (61–63) have shown that integrative multiplatform analyses capture the complexity of the tumor ecosystem much more effectively and that data derived from DNA, RNA, and methylation sequencing as well as digital pathology and radiomics can be combined using machine learning to accurately predict clinically relevant endpoints. The integration of imaging data with multisite molecular profiling facilitated by systematic interventional sampling will help develop better biomarkers of response to therapy, and because the acquisition of these data is becoming increasingly more affordable, it is likely that such technologies will be routinely used in the clinic in the near future. Similarly, the emergence of federated learning (64) will allow these models to be built cooperatively among several cancer centers.

Reducing Sampling Error and Facilitating Multisite and Serial Sampling

Liquid biopsy has shown great promise as a less invasive method to enable NGS, but its clinical use at present is limited to the analysis of circulating tumor DNA and tumor cells (65). It provides no information on mRNA, protein, or the tumor microenvironment. Although certain genetic/epigenetic profiles or cell surface markers obtained from liquid biopsy may point to specific cancer types, it cannot definitively localize disease. Molecular profiles can also vary between disease sites, and this spatial information is lost. Multiple factors (eg, low tumor burden, tumor type, and tumor vascularity) affect the amount of tumor-derived material shed into the bloodstream, which can also limit assay sensitivity (66). In contrast, needle biopsies target specific locations, obtaining relatively higher yields of tumor, and provide samples suitable for the full range of assays described earlier.

There is a need for low morbidity needle sampling protocols that can reduce sampling error and facilitate multisite material collection. Although CNBs are the mainstay of solid organ lesion sampling, evidence suggests that fine needle aspiration (FNA) can perform as well as or better than CNB for NGS (23,36,67). For example, Roy-Chowdhuri et al (23) demonstrated that tumor fraction and cellularity were significantly higher in FNAs than in CNBs (ie, high tumor fraction in 60% of FNA samples vs 15% of CNB samples), with FNA samples also demonstrating superior sequencing metrics. The potential benefits of FNA include reduced trauma, rapid-onsite evaluation, tumor cell enrichment, reduced costs, and faster turnaround because FNA does not require prolonged formalin fixation or tissue processing.

CONCLUSION

The growth of personalized cancer care and targeted therapies will lead to an increase in demand for radiological sampling and NGS. It is important for radiologists to have a working understanding of NGS technologies and be cognizant of the factors that can influence NGS success. The growing understanding of tumor heterogeneity, the tumor ecosystem, and the sampling error associated with standard biopsy should be addressed in future sampling protocols.

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APPENDIX A. GLOSSARY

Chromosomal translocation: A genetic change in which a piece of 1 chromosome breaks off and attaches to another chromosome.

Epigenetics: The study of stable phenotypic changes that do not involve alterations in the DNA sequence.

Extron: An exon/extron is a region of the genome that ends up within a messenger RNA molecule. Some exons are coding, in that they contain information for making a protein, whereas others are noncoding.

Genome: The entire set of DNA instructions found within a cell.

Histone: A protein that provides structural support for a chromosome, acting as spools around which DNA winds to create structural units called nucleosomes. Epigenetic modifications can cause the loosening or tightening of DNA wound around the histone, which will affect the accessibility to specific regions of DNA and its expression.

Intron: An intron is a region that resides within a gene but does not remain in the final mature messenger RNA molecule after transcription of that gene and does not code for amino acids that make up the protein encoded by that gene.

Microarray: A laboratory tool with a collection of microscopic DNA spots attached to a solid surface that is used to detect the expression of thousands of genes at the same time.

Microsatellite instability: Microsatellites are short repeated sequences of DNA that reside adjacent to each other in the genome. They result from impaired DNA mismatch repair, and the presence of microsatellite instability is phenotypic evidence of this. It is associated with various cancers.

Next-Generation Sequencing library: A key step in the next-generation sequencing workflow is preparing the input for sequencing, known as creating a library. This is a collection of similarly sized DNA fragments with known adapter sequences added to the 5′ and 3′ ends, which allow interaction with a specific sequencing platform. Adaptors also contain indexing barcodes that allow individual sample identification.

Promoter: A region of DNA upstream of a gene where relevant proteins (such as RNA polymerase and transcription factors) bind to initiate transcription of that gene.

Oligonucleotide: Short strands of synthetic DNA or RNA molecules that serve as the starting point for many molecular biology applications.

Splice junction variants: A genetic alteration in the DNA sequence that occurs at the boundary of an exon and an intron (splice site). This change can disrupt RNA splicing, resulting in the loss of exons or the inclusion of introns and an altered protein-coding sequence.

Transcriptome: A set of all RNA transcripts (including coding and noncoding) in an individual.