Nucleic Acid-Based Diagnostic Techniques in Malignancy

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Disclosures

There are no relevant financial interests to disclose for myself or my spouse/partner within the last 12 months

I will **not** be discussing off label use of medications

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Objectives

- Explain the differences between Sanger Sequencing and Next generation sequencing (NGS)
- Differentiate between the different applications of NGS
- Evaluate the pros and cons of utilizing circulating tumor DNA in solid tumors

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Abbreviations

DNA = Deoxyribose nucleic acid
PCR = Polymerase chain reaction
NGS = Next generation sequencing
dNTPs = Deoxyribose nucleotide triphosphates
SNV = Single nucleotide variant
JAK2 = Janus kinase 2
EGFR = Epidermal growth factor receptor
PIK3CA = Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha
PML-RARA = Promyelocytic leukemia-retinoic acid receptor alpha
FLT3-TKD = Fms-like tyrosine kinase 3 tyrosine kinase domain
FLT3-ITD = Fms-like tyrosine kinase 3 internal tandem duplication
CR = Complete remission
CRi = Complete remission with incomplete hematologic recover
CEBPa = CCAAT enhancer binding protein alpha
CalR = Calreticulin
IDH1/2 = Isocitrate dehydrogenase 1/2
NPM1 = Nucleophosmin 1
PMF = Primary myelofibrosis
MRD = Measurable residual disease
AML = Acute myeloid leukemia
CLL = Chronic lymphocytic leukemia
MM = Multiple myeloma
CML = Chronic myeloid leukemia
APL = Acute promyelocytic leukemia

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Background

- On October 1, 1990, the Human Genome project (HGP) launched
  - International effort to sequence and map all the human genes (~20,000-25,000)
  - Amazing feat was finished in 2003
- In 2006 National Cancer Institute and the National Human Genome Research Institute initiated the landmark Cancer Genome Atlas Program (TCGA)
  - Objective was to molecularly characterize thousands of primary cancers with matched normal samples of 33 cancer types
  - Completed in 2017

Cancer Science. 2019;110:6–15.
J Exp Clin Cancer Res. 2018;37(1):47.

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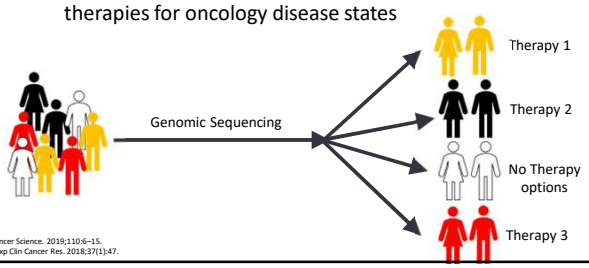
Background

- In 2015, President Barack Obama proposed the "Precision Medicine Initiative"
  - Initiative primarily focusing precision & targeted therapies for oncology disease states



Cancer Science. 2019;110:6–15.
J Exp Clin Cancer Res. 2018;37(1):47.

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Nucleic Acid Biochemistry

- Building blocks for deoxyribose nucleic acid (DNA) and ribose nucleic acids (RNA)
- Consist of four different bases
  - Sugar
  - Phosphate
  - Nitrogen-containing base
- Written as 5' → 3' (phosphate on 5' and hydroxyl on 3')
- Require purine-pyrimidine base pairing (A:T & G:C)

DNA	RNA	
Adenine	Adenine	Purines
Guanine	Guanine	
Cytosine	Cytosine	Pyrimidines
Thymine	Uracil	

5' – TCGATATATC – 3'
3' – AGCTATATAG – 5'
} Complementary Sequences

Greenspan, Daniel. (2003). From Genes to Genomes: Concepts and Applications of DNA Technology.
Tan, D., & Lynch, H. T. (2013). Principles of molecular diagnostics and personalized cancer medicine.

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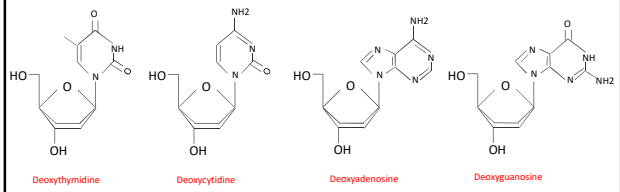
Nucleic Acid Structures



- Base pairings lead to double helix structure (primarily through differential hydrogen bonding)
- When unwound by DNA helicase → transcription

Greenspan, Daniel. (2003). From Genes to Genomes: Concepts and Applications of DNA Technology.
Tan, D., & Lynch, H. T. (2013). Principles of molecular diagnostics and personalized cancer medicine.

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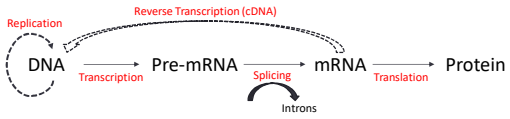
Nucleic Acid Information Flow



- Central dogma of molecular biology → flow of genetic information
- Complex processes requiring a myriad of proteins (e.g. scaffolding proteins, enzymes, etc.)
- Final product is protein

Greenspan, Daniel. (2003). From Genes to Genomes: Concepts and Applications of DNA Technology.
Tan, D., & Lynch, H. T. (2013). Principles of molecular diagnostics and personalized cancer medicine.

mRNA: messenger RNA
cDNA: complementary DNA

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Polymerase Chain Reaction (PCR)

- Molecular technique that amplifies a targeted nucleic acid region for sequencing or quantification
  - Sequencing
    - Sanger sequencing
    - Next generation sequencing (NGS) → Does not require PCR for sequencing
  - Quantification
    - Real time PCR
- DNA can be directly amplified
- RNA is converted back to cDNA using reverse transcriptase (transcriptosome)
  - Exons only

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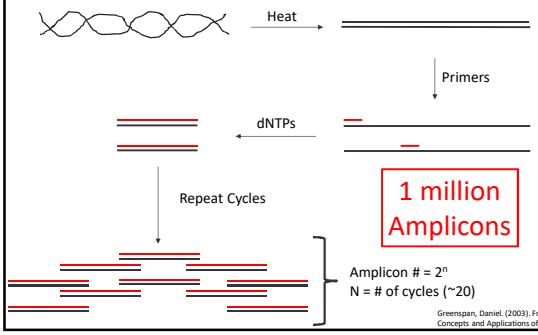

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DNA/Amplicon Sequencing

- Sequencing DNA is a way to determine nucleic acid base order
  - Sanger Sequencing
    - Target sequencing of one gene (eg FLT3-TKD)
    - Sequence length of 500-800 base pairs
    - Most pathology labs can run

    } Gives a targeted answer to a specific question
  - NGS
    - High-throughput sequencing/Deep sequencing
    - Sequence hundreds of genes simultaneously
    - Sequence length ~200-500 base pairs
    - Usually send out (takes 1-3 weeks)

    } Gives a complete picture of disease clonality

Greenspan, Daniel. (2003). From Genes to Genomes: Concepts and Applications of DNA Technology.
Tan, D., & Lynch, H. T. (2013). Principles of molecular diagnostics and personalized cancer medicine.

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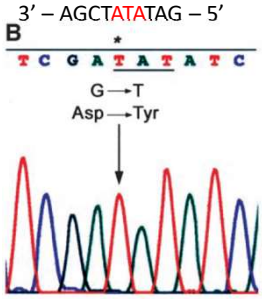
DNA/Amplicon Sequencing

- Gene/domain: FLT3-TKD
- Mutation: SNV missense
- NA sequence: GAT → TAT
- AA sequence: Asp835Tyr
- Therapy: FLT3 inhibitor

5' – TCGATATATC – 3'
3' – AGCTATATAG – 5'



FLT3-TKD: FLT3 tyrosine kinase domain
SNV: single nucleotide variant
NA: nucleic acid
AA: amino acid

Clin Cancer Res. 2004;10(4):1326-32.

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DNA Quantification

- Real-time PCR
  - Quantifies PCR product in "real time"
- If real-time PCR is used for RNA detection, it is called real-time RT-PCR
- Uses fluorescently labeled probes (e.g. SYBR green)
- The amount of amplicon is correlated with fluorescence intensity and calculated relative to standard control

Tan, D., & Lynch, H. T. (2013). Principles of molecular diagnostics and personalized cancer medicine.

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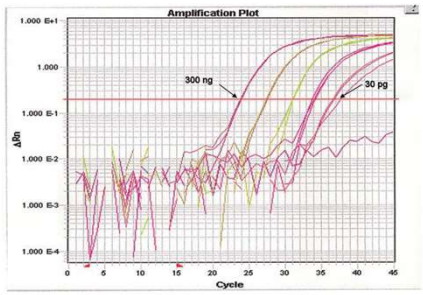
DNA Quantification



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Audience Response Question #1

JH is a 67-year-old with newly diagnosed early-stage B-cell lymphoproliferative disorder. Recent advances in this rare malignancy have led to 6 new therapeutics based on single nucleotide variant missense mutations all on different genes. Which of the following assays would be optimal and is correctly associated with its draw back

A. NGS; usually long turnaround time
B. Sanger sequencing; can only amplify short DNA regions
C. NGS; can only run one gene per panel
D. Pyrosequencing; requires radiolabeled nucleotides

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Applications of Nucleic Acid-Based Technology

- Sequencing
  - **Elucidating actionable and/or prognostic mutations**
    - Translocation – BCR-ABL1 (imatinib), RET-KIF5B (pralsetinib), PML-RARa (tretinoin), NTRK (larotrectinib)
    - SNV – FLT3-TKD (gilteritinib), cKIT (midostaurin), IDH1/2 (ivosidenib/enasidenib), Jak2 (ruxolitinib), BRAF (dabrafenib), EGFR (osimertinib), PIK3CA (alpelisib)
    - Insertion/deletion – FLT3-ITD (midostaurin), CalR (prognostic), CEBPa (prognostic)
  - **Identifying resistance mutations**
    - BCR-ABL1 tyrosine kinase domain
    - C481S missense mutation (confers resistance to BTK inhibitors)
    - cKit mutation in exon 17 (D816V confers resistance to imatinib but susceptibility to dasatinib)

SNV: single nucleotide variant
BTK: Bruton Tyrosine Kinase

NCCN. Myeloproliferative Neoplasms (v1.2020). | NCCN. Chronic Myeloid Leukemia (v2.2021). | NCCN. Non-Small Cell Lung Cancer (v8.2020). | NCCN. Cutaneous Melanoma (v4.2020). | NCCN. Acute Myeloid Leukemia (v4.2020). | NCCN. Breast Cancer (v5.2020). | NCCN. Systemic Mastocytosis (v1.2020). | N Engl J Med 2014; 370:2286-2294

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Applications of Nucleic Acid-Based Technology

- Sequencing
  - Elucidating actionable and/or prognostic mutations
    - Translocation – BCR-ABL1 (imatinib)
    - SNV – FLT3-TKD (gilteritinib), cKIT (midostaurin)
    - Insertion/deletion – FLT3-ITD (midostaurin)

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Applications of Nucleic Acid-Based Technology

The NEW ENGLAND JOURNAL of MEDICINE

ORIGINAL ARTICLE

Imatinib Compared with Interferon and Low-Dose Cytarabine for Newly Diagnosed Chronic-Phase Chronic Myeloid Leukemia

IRIS Trial

- Complete hematologic response
  - Imatinib: 95.3%
  - Cytarabine + Interferon: 55.5%

Landmark trial leading to paradigm shift in CML treatment to BCR-ABL1 tyrosine kinase inhibitors

N Engl J Med. 2003;348(11):994-1004.

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Applications of Nucleic Acid-Based Technology

The NEW ENGLAND JOURNAL of MEDICINE

ORIGINAL ARTICLE

A Double-Blind, Placebo-Controlled Trial of Ruxolitinib for Myelofibrosis

COMFORT I Trial

- ≥35% reduction in spleen size at week 24
  - Ruxolitinib: 41.9%
  - Placebo: 0.7%
- Symptom improvement
  - Ruxolitinib: 45.9%
  - Placebo: 5.3%

Landmark trial leading to standard of care treatment for myelofibrosis

N Engl J Med. 2012;366(9):799-807.

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Applications of Nucleic Acid-Based Technology

The NEW ENGLAND JOURNAL of MEDICINE

ORIGINAL ARTICLE

Efficacy and Safety of Midostaurin in Advanced Systemic Mastocytosis

Systemic Mastocytosis

- Overall response rate: 60%
- Mast-cell leukemia response rate: 50%

Landmark trial leading to only FDA approved therapy for mast-cell leukemia

N Engl J Med. 2016;374(26):2530-2541.

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Applications of Nucleic Acid-Based Technology

The NEW ENGLAND JOURNAL of MEDICINE

ORIGINAL ARTICLE

Midostaurin plus Chemotherapy for Acute Myeloid Leukemia with a *FLT3* Mutation

RATIFY Trial (CALGB 10603)

- Median overall survival
  - Chemotherapy + Midostaurin: 74.7 months
  - Chemotherapy + Placebo: 25.6 months

Landmark trial leading to first FDA approved therapy for FLT3+ AML

N Engl J Med. 2017;377(5):454-464.

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Applications of Nucleic Acid-Based Technology

The NEW ENGLAND JOURNAL of MEDICINE

ORIGINAL ARTICLE

Gilteritinib or Chemotherapy for Relapsed or Refractory *FLT3*-Mutated AML

ADMIRAL Trial

- Overall survival (months)
  - Gilteritinib : 9.3
  - Salvage chemotherapy: 5.6
- CR + CRi
  - Gilteritinib: 34.0%
  - Salvage chemotherapy: 15.3%

Landmark trial leading to first FDA approved therapy for relapse/refractory FLT3+ AML

N Engl J Med. 2019;381(18):1728-1740.

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Applications of Nucleic Acid-Based Technology

- Quantitative
  - Monitoring efficacy
    - CML = p210 → standard of care
    - APL = PML-RARa → standard of care
    - PMF = JAK2V617F → controversial
  - Measurable residual disease (MRD) monitoring
    - ALL, MM & CLL = ClonoSEQ® → rarely used
    - AML = NPM1 → ongoing area of research
  - Chimerism (allogeneic stem cell transplant)
    - CD3/CD33 chimerisms

NCCN. Myeloproliferative Neoplasms (v1.2020). | NCCN. Chronic Myeloid Leukemia (v2.2021). | NCCN. Acute Myeloid Leukemia (v4.2020). | ClonoSEQ Assay [package insert].

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Audience Response Question #2

Which of the following is NOT an appropriate application of NGS?

A. Running an assay to determine MRD status of a CLL patient after completion of chemotherapy to determine appropriateness of maintenance therapy
B. Running an assay of a 37 myeloid gene panel to determine the genetic aberrations in a newly diagnosed AML patient
C. Running an assay of a single 800 bp gene to determine prognosis of a newly diagnosed AML patient
D. Running an assay to determine if a patient harbors any resistance-associated mutations to a BTK inhibitor

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Liquid Biopsies

- Terminology
  - Liquid biopsy: Methods that can derive the same diagnostic information from a blood sample that is typically derived from a tissue biopsy sample.
  - Circulating cell-free DNA (cfDNA): DNA circulating in the bloodstream that is not associated with cells
  - Circulating tumor DNA (ctDNA): Tumor-derived, cell-free DNA that is thought to be representative of the entire tumor genome
  - Circulating cell-free RNA (cfRNA): Circulating gene transcripts (mRNA and non-coding RNAs) that are partly protected from degradation by their packaging into exosomes.

Nat Rev Genet. 2019;20(2):71-88.
Trends Mol Med. 2010;16(9):398-406.

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  - Circulating cell-free RNA (cfRNA): Circulating gene transcripts (mRNA and non-coding RNAs) that are partly protected from degradation by their packaging into exosomes.

Allows us to do a blood/plasma-based way of performing NGS panels to sequence hundreds of genes without tissue biopsy

Nat Rev Genet. 2019;20(2):71-88.
Trends Mol Med. 2010;16(9):398-406.

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Liquid Biopsies

- Gold standard for tumor-derived genetic information is tissue-based biopsy
  - Formalin-fixed paraffin embedded (FFPE) samples
  - Bone marrow biopsy
- In 2013, the College of American Pathologists in Lung Cancer did not include the use of cfDNA for identification of mutations (FFPE specimen or fresh/frozen/fixed specimen)
- In 2018, an updated guideline was published that provided settings when cfDNA would be indicated
  - EGFR mutational analysis with limited tissue sample
  - EGFR T790M mutations (progressive or clinical resistance)

J Mol Diagn. 2018;20(2):129-159.
J Thorac Oncol. 2013;8(7):823-859.

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Liquid Biopsies

- Prospective data
  - NILE study
    - Noninferiority of cfDNA to standard-of-care tissue genotyping in newly diagnosed metastatic non-small cell lung cancer patient (Guardant360)
    - The concordance rate between tissue and liquid results when available was > 98.2% with a 100% positive predictive value for cfDNA (*EGFR, ALK, ROS1, BRAF*)
- Several additional prospective studies currently ongoing
- Approved liquid biopsy (cfDNA) applications
  - *EGFR* in non- small-cell lung cancer (cobas®) and *SEPT9* in colorectal cancer (Epi proColon®)

Clin Cancer Res. 2019;25(15):4691-4700.
Nat Rev Genet. 2019;20(2):71-88.

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Advantages & Challenges of Liquid Biopsies

- Advantages
  - Less invasive (does not require invasive intervention)
  - Quicker turn around time
  - Possibility of early disease detection
- Challenges
  - Concentration of cfDNA varies considerably
    - ctDNA represents a small proportion of total cfDNA (0.1% to >10%)
  - Lack of standardization and standard operating procedures for ctDNA analysis (e.g. clinical validity)
  - Highly fragmented DNA (sensitivity ~70-80%)
  - Intratumor heterogeneity

Nat Rev Genet. 2019;20(2):71-88. | Trends Mol Med. 2010;16(9):398-406. | Nat Med 2008; 14(9): 985–990. | Ann Oncol. 2019;30(10):1580-1590.

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Audience Response Question #3

Which of the following is a challenge to implementing liquid biopsies into oncology practice?

A. Turnaround time is too long to be clinically useful
B. Inability to detect missense mutations such as single nucleotide variants (e.g. *EGFR)*
C. Lack of standard operating procedures such as blood collection and processing to avoid white blood cell lysis
D. Lack of available NGS panels on which to run specimen

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Conclusions

- DNA sequencing has allowed treatment of malignancies to shift towards targeted therapy with a focus on precision medicine
- NGS has created significant improvements in elucidating mutational profiles in various malignancies and has increased the detection level of MRD
- Liquid biopsies are an upcoming technology that adds an additional diagnostic technique to current armamentarium but still has many challenges before global implementation

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