Is the right time for next generation histopathological diagnostics?

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Pathology

What my friends think I do.

What my mom thinks I do.

What society thinks I do.

What my boss thinks I do.

What I think I do.

What I actually do.
Anatomical pathology (Commonwealth) or Anatomic pathology (U.S.) is a medical specialty that is concerned with the diagnosis of disease based on the macroscopic, microscopic, biochemical, immunologic and molecular examination of organs and tissues.
Prato della Valle: Pietro Danieletti’s sculpture with the Morgagni’s bust
Fig. 27. Schematische Darstellungen von Leberzellen. A Einfache physiologische Anordnung derselben. B Hypertrophie, a einfache, b mit Fettaufnahme (fettige Degeneration, Fettleber) C Hyperplasie (numerische oder adjungische Hypertrophie) a Zelle mit Kern und geteiltem Kernkörperchen. b geteilte Kerne. c, e geteilte Zellen.
Histology after fixation: the kidney paradigm

Acetic acid  Bouin  Formaldehyde  Glutaraldehyde

Mercuric chloride  Potassium dichromate  Zenker
autostainer

2019 - Padua
~55,000 histology reports
~30,000 cytology reports
~2,000 molecular path reports
470,500 FFPE blocks
Reference Unit for IOV (Padua)
Breast surgery – Gastroenterology – Radiology - Oncology
Type of samples to be processed

Surgical

Biopsies

Biopsy specimens: organ of origin

- Skin
- Gastrointestinal = Bone marrow
- Urogenital
- Breast
- Gynecological
- Lung
- Other

Type of samples to be processed

- Biopsy specimens: organ of origin

- Surgical

- Biopsies

- Amyloid
  - Congo Red Cat # 24614
  - Alcian Blue PAS Cat # 25008
  - PAS Cat # 24200
  - Rapid Mucus Cat # 24208
  - Cresyl Violet Cat # 21083
  - Balsamow Cat # 25964
  - Luxol fast Blue Cat # 24111

- Carbohydrates
  - Oil Red O Cat # 25992
  - Reticulin Cat # 25004
  - Jones PAS-M Cat # 25091

- Neuronal Tissue
  - Biopsies

- Triglycerides & Lipids

- Reticulin Fibers

- Connective Muscle Tissue
  - Biopsies

- Pigment, Minerals & Granules

- Microorganisms

- Special Stains
The multistep model of scientific paradigms

1761
De sedibus et causis morborum per anatomiam inquisitionis

1858
Die Cellularpathologie in ihrer Begründung auf physiologische und pathologische Gewebenlehre

1953
Molecular structure of nucleic acids; a structure for deoxyribose nucleic acid

Diagnostic
Biomarkers
Prognostic

Predictive

= Affected individuals

No response
Response

Low risk
Good outcome

High risk
Poor outcome
From the molecular alteration to the targeted therapy

**HER2 amplification**
20-30% Breast ductal k

**EGFR mutation**
15-20% Lung adenocarcinomas

**BRAF V600E mutation**
50-60% Melanoma

Trastuzumab plus adjuvant chemotherapy for operable HER2-positive breast cancer. Romond et al. - NEJM 2005

Gefitinib or carboplatin-paclitaxel in pulmonary adenocarcinoma. Mok et al. - NEJM 2009

Improved survival with vemurafenib in melanoma with BRAF V600E mutation. Chapman et al. - NEJM 2011

The international journal of science / 6 February 2020

CANCER CATALOGUED
Whole genome sequences for 38 types of tumour
“Cancer is driven by genetic change, and the advent of massively parallel sequencing has enabled systematic documentation of this variation at the whole-genome scale.”

“On average, cancer genomes contained 4-5 driver mutations when combining coding and non-coding genomic elements; however, in around 5% of cases no drivers were identified, suggesting that cancer driver discovery is not yet complete.”
Whole genomes redefine the mutational landscape of PDAC

- **Stable** (<50 events): 20%
- **Focal** (50-200, 50% on 1 Chr): 30%
- **Scattered** (50 – 200 widespread): 36%
- **Unstable** (>200 widespread): 14%


Pan-cancer analysis of whole genomes

The ICGC/TCGA Pan-Cancer Analysis of Whole Genomes Consortium

Nature 578, 82–93(2020) | Cite this article

91% of tumors had at least one identified driver mutation
Tissue and molecular diagnostics

- Choice of the right diagnostic approach for the available tissue sample
- Tumor is a tissue and the patologist’s evaluation matter!
- Next generation sequencing in old generation laboratories

Tissue and molecular diagnostics

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- Tumor is a tissue and the patologist’s evaluation matter!
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The molecular diagnostics’ recipe

The ingredients
(i.e. the samples)

The kitchen accessories
(i.e. the molecular methods)

The molecular diagnostics’ recipe

The ingredients
(i.e. the samples)

The kitchen accessories
(i.e. the molecular methods)
### The heterogeneous landscape of diagnostic kits for targeted mutational assessment

<table>
<thead>
<tr>
<th>Method</th>
<th>Limit of Detection</th>
<th>Reference Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sanger Sequencing</td>
<td>10 – 20%</td>
<td>All the mutations present in the analyzed gene regions</td>
</tr>
<tr>
<td>Real Time PCR</td>
<td>1 – 5%</td>
<td>Only «hot spot» Mutations (probe based)</td>
</tr>
<tr>
<td>Digital PCR</td>
<td>0.1 – 1%</td>
<td>Only «hot spot» Mutations (probe based)</td>
</tr>
<tr>
<td>Next Generation Sequencing</td>
<td>0.01 – 5%</td>
<td>All the mutations present in the analyzed gene regions</td>
</tr>
</tbody>
</table>

**IHC, FISH, RT-PCR, Pyrosequencing, Sanger, Real Time-PCR, ddPCR, NGS are methods, not tests!**
We can apply different filters, but she still is Marilyn Monroe!

We can apply different methods to perform a test (and get an adequate result; ALK fusion)

- IHC
- FISH
- NGS
- Real time

The heterogeneous landscape of diagnostic kits for targeted mutational assessment

We have to chose the most adequate method for the molecular lesion we have to analyze!
Situation 1
We have to test 1 gene with a known alteration (mutation/translocation/amplification/deletion)

- The best option is a «hot spot» single gene method such as Real time, FISH, IHC depending on the alteration we are looking for.
- NGS is not the best option in this case.

The diagnostics’ duel

- Real Time PCR: BRAF p.V600E
- NGS: BRAF wt
The diagnostics’ duel

Real Time PCR
*BRADF* p.V600E

NGS
*BRADF* wt

NGS technical problems

<table>
<thead>
<tr>
<th>Read depth</th>
<th>Gene length</th>
<th>% gene coverage</th>
</tr>
</thead>
<tbody>
<tr>
<td>7x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5x</td>
<td></td>
<td></td>
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<tr>
<td>4x</td>
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<tr>
<td>3x</td>
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<tr>
<td>2x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1x</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

10% not covered
NGS technical problems

Situation 2
We have to test 1 gene with different known and unknown alterations (mutation/translocation/amplification/deletion)

- Forget the «hot spot» option! It requires a large amount of material, is time consuming and has a relatively higher cost.
- NGS is the best option. Need to consider the best NGS approach (RNA- or/and DNA-based)

BRCA1 - Lots of mutations, lots of dilemmas
Collins FS – NEJM 1996
A combination of encorafenib (anti BRAF), cetuximab, and binimetinib (anti MEK) resulted in significantly longer overall survival and a higher response rate than standard therapy in patients with metastatic colorectal cancer with the BRAF V600E mutation.

The BRAF diagnostic scenario

<table>
<thead>
<tr>
<th>SANGER SEQUENCING</th>
<th>REAL-TIME PCR</th>
<th>MASS SPECTROMETRY</th>
<th>NEXT-GENERATION SEQUENCING</th>
<th>IMMUNOHISTOCHEMISTRY (VE1 clone)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10-20% AF</td>
<td>1-5% AF</td>
<td>1-5% AF</td>
<td>0.001-5% AF</td>
<td>0.001-5% AF</td>
</tr>
<tr>
<td>Low cost</td>
<td>Low cost</td>
<td>Low cost</td>
<td>High cost</td>
<td>Low cost</td>
</tr>
<tr>
<td>High TAT</td>
<td>Low TAT</td>
<td>Only hotspot BRAF mutations</td>
<td>High TAT</td>
<td>Low TAT</td>
</tr>
<tr>
<td>All BRAF mutations</td>
<td>Only hotspot BRAF mutations</td>
<td>All BRAF mutations</td>
<td>Only wild-type BRAF mutations</td>
<td></td>
</tr>
</tbody>
</table>

Angerilli V, et al. - Crit Rev Oncol Hematol 2022
BRAF p.V600E-specific immunohistochemical assessment in colorectal cancer endoscopy biopsies is consistent with the mutational profiling.

Class 1: codon 600
Class 2: codons 601 and 597
Class 3: codons 594 and 596

Poor prognosis
Similar to BRAF wt

Situation 3
We have to test multiple genes with different known and unknown alterations (mutation/translocation/amplification/deletion)

- Forget the «hot spot» option! It requires a large amount of material, is time consuming and has a higher cost.
- Comprehensive genomic profiling NGS is the best option. RNA- and DNA-based kits are usually required.
**More is better?**

Is better to use a comprehensive (=larger) or a more sensitive diagnostic NGS panel?

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**Targeted NGS**

Limited number of genes with a high diagnostic performance

I know the targetable alteration and I need reliable diagnostic results

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**CGP NGS (>50 genes)**

Large number of genes, higher risk of false negative results

I'm looking for unknown targetable alterations and I can miss something
Interpretation and definitions of NGS data!
- missense variants
- nonsense variants
- frameshift deletions/insertions
- splicing variants
- in-frame deletions
- VAF
- pathogenic/likely pathogenic
- uncertain significance variants
- benign/likely benign variants

The molecular diagnostics’ recipe

The ingredients
(i.e. the samples)

The kitchen accessories
(i.e. the molecular methods)
What a cancer is?

The clinical request for molecular testing:
MSI, MMR, BRAF, FGFR2, TP53, DAXX/ATRX, TMB, CGP, Methylation, RAS, ALK, ROS1, BAP1, chromatin remodeling, MGMT, NTRK

- High quantity and good quality of DNA/RNA/tissue sections.
- Most of the methods and diagnostic approaches are applicable.
Biopsy

- Low quantity of DNA/RNA/tissue sections (usually of high quality).
- Need for tests’ prioritization.
- Inadequate sampling/material.

FFPE tissue blocks may be inadequate for molecular analysis due to scarcity of material following previous sectioning for diagnostic purposes. Keep in mind that a tertiary centre receives different types of FFPE tissue specimens obtained with different workflows and processes.

The example of gastroesophageal adenocarcinomas

2.6 mm is estimated to be the average diameter of endoscopic biopsies (in reality, it’s much lower); a 27G (23G) needle gives a biopsy of 0.42 (0.6) mm of diameter

**DIAGNOSIS**
- 1 × 4 µm H&E
- 1 × 4 µm Giemsa
- 1 × 4 µm possible IHC (CK)
  + wastage 10–20 µm
Total = around 20–30 µm

**PREDICTIVE BIOMARKERS**
- 1 × 4 µm HER2 (plus further 2 sections if 2+)
- 1 × 4 µm PD-L1
- 4 × 4 µm MMR
- 1 × 4 µm EBER
  + wastage 10–20 µm
Total = around 30–50 µm
Not all biopsies are adequate for molecular testing!

I do not have enough material to perform all my tests:

- Need for tests’ prioritization
- NGS: it is possible (quantity/quality DNA/RNA)?
- Liquid biopsy approaches (!!liquid biopsy is not the solution for all our requests!!)?
The heterogeneous landscape of diagnostic kits for targeted mutational assessment

**Personalized molecular diagnostics** is the ground of personalized medicine

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</tr>
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Limit of Detection

- Low
- High

Tissue and molecular diagnostics

- Choice of the right diagnostic approach for the available tissue sample
- Tumor is a tissue and the patologist’s evaluation matter!
- Next generation sequencing in old generation laboratories
Tumor is a tissue!

High grade CRC
BRAF V600E
MSS

Medullary CRC
BRAF V600E
MSI
Enrichment for cancer cells
(diagnostic sensitivity of molecular testing)

The selected area was highly variable, and the average difference between the highest and lowest estimation ranged between 51% and 78%.

The number of overestimations was alarmingly high in samples containing <30% tumor cells.

Of concern is that 33 of 105 laboratories reported a wildtype result in a sample without tumor.
Despite primary and metastatic ileal NETs show a similar molecular landscape, tumor grading and mTOR signaling pathway may diverge in the metastatic setting.

Borga C, et al. – Endocr Relat Cancer 2021
Core signaling pathways in human cancer revealed by global genomic analysis

There appears to be only a **limited number of cellular signaling** pathways through which a growth advantage can be incurred.

Drugs should target the effect of the altered pathways (i.e. **downstream mediators or key nodal points**) rather than a single gene component!

Limited evolution of the actionable metastatic cancer genome under therapeutic pressure

van de Haar J, et al. – Nat Med 2021

For standard of care genomic biomarkers, we observed full concordance between the first and the second biopsy in 99% of pairs. Of the 219 biomarkers for clinical trial enrollment that were identified in the first biopsies, we recovered 94% in the follow-up biopsies. Furthermore, a second WGS analysis did not identify additional biomarkers for clinical trial enrollment in 91% of patients.

EGFR and MET amplifications determine response to HER2 inhibition in ERBB2 - amplified esophagogastric cancer

Sanchez-Vega F, et al. – Cancer Discov 2019
Tissue and molecular diagnostics

- Choice of the right diagnostic approach for the available tissue sample
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The performance of molecular testing relies not only on the quality of the method itself, but also, profoundly, on the quality of the biospecimen analyzed. Suboptimal material implies suboptimal results in molecular profiling.
The importance of being FFPE-extracted DNA

Sample A
iCCA (year: 2012)
Random G→A or C→T
Changes <1%

Sample B
iCCA (year: 2004)
Random G→A or C→T
Changes >10%

Mafficini A, et al. – Plos ONE 2014
Deamination Misspriming

NGS is a good technology to analyze FFPE samples

Similar coverage of targeted regions analyzed in 5 matched fresh-frozen (F) and FFPE (P) samples of solid pseudopapillary tumor (SPT).

DNA qualification may impact MSI testing results in mucinous colorectal adenocarcinoma...

"we demonstrated that preanalytical parameters as neoplastic cellularity and DIN may influence analytical performance for MSI testing. In particular, a minimum input of 50% of neoplastic cells is fundamental to correct perform molecular analysis by using Idylla™ system. DIN < 4 significantly affected TapeStation 4200 results."

- FFPE tissue, cytology, plasma
- 1-40 ng DNA/RNA
- 1->500 genes
- Timing/Clinical setting for CGP

DNA-based
- Simpler than RNA analysis
- Limited loss of analyses for low sample’s qualification
- May miss translocations/fusions

RNA-based
- 20-25% of samples cannot be analyzed
- Gold standard for translocations/fusions analysis
The liquid biopsy era...

Circulating tumor DNA (ctDNA) analysis through liquid biopsy has proven to be a robust method to tailor personalized treatments for CRC patient care.

Malla M. et al. – JCO 2022
A real-world application of liquid biopsy in metastatic colorectal cancer: the Poseidon study

Procaccio L, et al. – Cancers 2021

n= 33 mCRC (from spoke centers to hub center)

83% = 7 days

22 days → 17 days

"...the absence of harmonized procedures corresponds to an unmet clinical need, ultimately affecting the rapid implementation in clinical practice."
Limitations of tissue biopsy

- Tissue biopsy may be infeasible in 9–18% of patients.
- Tissue biopsy procedural failure rates vary by technique and can range from 4–42%.
- Samples are inadequate for testing in 8–26% of patients.
- Access to testing varies by country and region and depends on infrastructure and reimbursement.

The overall tissue biopsy failure rate may be up to 43%.

* e.g., tissue sample successfully extracted from target lesion; † Molecular diagnosis and/or histological diagnosis. aNSCLC, advanced NSCLC.
The prognostic impact of patient-specific liquid biopsy

<table>
<thead>
<tr>
<th>ctDNA</th>
<th>Negative (N = 48)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive (N = 51)</td>
<td>4.59 (2.512 - 8.4)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Gender</th>
<th>Liver (N = 48)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distant Nodes (N = 6)</td>
<td>0.31 (0.073 - 1.3)</td>
<td></td>
</tr>
<tr>
<td>Lung (N = 21)</td>
<td>0.63 (0.306 - 1.3)</td>
<td></td>
</tr>
<tr>
<td>Peritoneum (N = 14)</td>
<td>0.53 (0.243 - 1.1)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Margins</th>
<th>Liver (N = 99)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.49 (1.072 - 2.1)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

HR: 22; 95% CI: 3.0-166.0; P=0.002

Loupakis F, et al. – JCO Prec Oncol 2021

Comprehensive Genomic Profiling (CGP)-informed personalized molecular residual disease (mrd) detection: an exploratory analysis from the predator study of mCRC patients undergoing surgical resection

Tissue CGP identified potentially actionable alterations in 54% (37/69) of patients. MRD-positivity was significantly associated with lower disease-free survival (DFS) (HR: 4.97, 95% CI: 2.67–9.24, p < 0.0001) and overall survival (OS) (HR: 27.05, 95% CI: 3.60–203.46, p < 0.0001).

Lonardi S, et al. – JSM 2022
Take home messages

The introduction of TCGA/ICGC data into clinical practice

The clinical impact of the pathological report (educational programs)

The diagnostic performance of the different technologies in the therapeutic management of the neoplastic patient

The need of molecular test prioritization (TMB’s role)

Is the right time for next generation histopathological diagnostics?
Veneto Institute of Oncology – IOV
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The Institute of Cancer Research,
Sutton, UK
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Chiara Braconi
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UniPD – Department of Biology
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Padua University Hospital
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Marco Agostini
The Ohio State University
Carlo M. Croce
University of Verona
Aldo Scarpa
Claudio Luchini
Institute of Oncology Research (IOR) - Bellinzona
Luciano Cascione
Cancer Research UK – Manchester Institute
Michela Garofalo
Semmelweis University Budapest
Andras Kiss