TITLE: CURRENT PRACTICE OF PATHOLOGY IN THE MOLECULAR ERA

Authors:

1. Yengkhom Daniel Singh, SR, Department of Pathology, RIMS, Lamphelpat, Imphal, Manipur -795004, India. Email: danielyengkhom2297@gmail.com
2. Deeparani Thingujam, SR, Department of Pathology, RIMS, Lamphelpat, Imphal, Manipur -795004, India. Email: drdeeparanithingujam@gmail.com
3. Babina Sarangthem, Associate Professor, Department of Pathology, RIMS, Lamphelpat, Imphal, Manipur -795004, India. Email: [babeesrang789@gmail.com](mailto:babeesrang789@gmail.com)
4. INTRODUCTION

It was only until the 15th century the field of Pathology was historically described for the first time by an Italian physician Antonio Benivieni in his article on case based autopsic reports. The introduction of the first compound microscope by Zacharias Janssen in 16th century and a later insight by Antoni van Leeuwenhoek in 17th century shoved the path for examining the solid tissue and body fluids. The cornerstone of pathology however was in the period of Rudolf Virchow, regarded as the “Father of Modern Pathology”, who revolutionized disease classification and rightfully has been credited by his colleagues with the title “The Pope of Medicine”. With the advent of time, the practice of Pathology slowly shifted towards histologic examination of fixed tissue biopsy or resected specimens, cytologic examination of aspirates, body fluids, exfoliated cells, squash preparations and scraps. Furthermore, introduction of cytochemical stains and later, immunohistochemistry (IHC) have boosted the utility of tissue sections and cytological smears. Most recently, the understanding of proteomics has led to antibody integrated specific immunohistochemical stains that allow us explore well beneath the scope of morphologic appearances of lesions which not only help in understanding the disease process but also the expected behavior of cells and disease. This marks the dawn of the Molecular era of pathology.

1. MOLECULAR PATHOLOGY

Molecular Pathology (MP) is defined by the Association of Clinical Pathologists as “the study of molecules in a disease state”. by using the tools of molecular biology to better understand the aetiology, pathogenesis, diagnosis and prognosis of diseases. It is an integrated approach combining molecular medicine with technological advances and more so, necessitates a coordinated teamwork among the pathologist, radiologist, clinico-oncologist, surgeon and therapist [1]. Molecular pathology and the so-called personalized medicine are the mainstay of many new targeted therapeutic regimes, disease pathways and biomarkers to predict response. It is a multidisciplinary approach in neo-medicine which uses advanced high-throughput molecular technologies to fill the gap between diagnostic and therapeutic interface, but keeping in place the traditional morphology-based diagnosis at the backbone. Formalin fixed paraffin embedded (FFPE) specimens, cytologic preparations and even blood can be used in molecular analyses [2]. Emphasis over carefully planned pre-analytic steps, SOP’s and training of the bio-scientists are vital prior to establishing testing facilities and regular assessment of external quality controls post establishment. MP is currently of great importance, and is becoming increasingly significant as it enables more precise diagnosis and treatment selection. Precision Oncology is defined as “the use of therapeutics that are expected to confer benefit to a subset of patients whose cancer displays specific molecular or cellular features (most commonly genomic changes and changes in gene or protein expression patterns)” . Hence, the “molecular revolution” has deeply transformed cancer care, re-evaluating the role of the pathologic diagnoses as the backbone of the therapeutic decision-making process. Some of the currently available techniques in molecular diagnostics are listed in table 1 [3].

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| **Table 1: Majorly used molecular diagnostic techniques [3].** | | |
| **Techniques** | **Advantages** | **Disadvantages** |
| Sanger sequencing | - Low-cost machinery  - Widespread on a large scale in all molecular biology laboratories | - Higher turn-around-time in  comparison to NGS technologies  - Low sensitivity  - Limited information on tumor molecular landscape |
| Reverse transcriptase (RT)-PCR | - Great sensitivity in detecting fusion genes  - Low turn-around-time  - Widespread on a large scale in all molecular biology laboratories | - Alteration-specific primers  - RNA-based |
| qRT-PCR | - Great diagnostic sensitivity  - Low turn-around-time  - Widespread on a large scale in all molecular biology laboratories  - Reliable for liquid biopsy analysis | - Alteration-specific primers |
| Pyrosequencing | - Low-cost machinery  - Widespread on a large scale in all molecular biology laboratories  - Best performances in studies on methylation | - Limited information on tumor  molecular landscape |
| Immunohistochemistry | - Low turnaround-time  - Widespread on a large scale in all molecular biology laboratories | - More affected by preanalytical  artifacts than molecular pathology diagnostics |
| Next-generation sequencing (NGS) targeted panels | - Greater sensitivity  - Allows lot of targeted fragments to be sequenced in a single run  - Faster turnaround time  - Lower cost than comprehensive profiling. | - Biostatistical analysis of the results |
| Whole-genome sequencing (WGS) | - Identifies meaningful mutations even they occur outside of exons  - GC-rich gene sequences appear more accurately captured | - Difficult interpretation of genomic data  - Relatively expensive |
| Whole-exome sequencing (WES) | Cost-effective alternative to WGS  - Focuses on the most relevant portion of the  genome and facilitates the discovery and  validation of common and rare variants | - Unable to interrogate many variants  that may be important for controlling  gene transcriptional regulation  or splicing |

MP is the backbone of modern diagnostics and translational research. In future, modern medicine is likely to be determined by molecular medicine and technical advances which marks the beginning of personalised medicine and therapeutic pathology, i.e., the development of a new generation of drugs targeting specific genes and pathways, coupled with biomarkers that predict the individual patient’s response to those drugs. novel biomarkers have become indispensible in the field of medical advancement. Advancement in the translation of biomarker discovery into diagnostic and therapeutic application acts as the connecting link between basic research and diagnostics which should be strengthened in field of molecular pathology [4].Currently, MP mainly involves molecular diagnostics. There are also several ways that MP can be used specifically in the diagnosis, prevention and/or treatment of disease: serum viral RNAs used to diagnose viral infections; a mutation in tumour cell genes can be used to diagnose specific neoplasms; genetics can be used to screen individuals for preventable inheritable conditions (e.g. APC testing in colorectal cancer patients); treatment and response can be monitored using biomarkers (e.g. Bcr-Abl testing for relapse in leukaemia); personalised medicine can be applied to cancer patients by tailoring drugs to certain specific cancers [1, 2].

MP, when incorporated with recent advances in genomics can change the future of diagnosis and treatment of genetic, infectious and neoplastic diseases. As a result, Personalized medicine has come in the forefront.Genomic medicine has been advancing rapidly since the introduction of massive parallel sequencing/ NGS technologies and fundamentally altered management of cancer patients. Many institutions have been offering extensive molecular testing for cancer care in a clinical setting, including cancer-related gene mutation analysis, copy number variation (CNV), gene rearrangement analysis, and RNA expression signatures. Each patient’s malignancy is unique. Identification of a driver gene mutation can lead to specific targeted therapies, resulting in personalized / precision medicine [5].The field of precision oncology is currently moving towards a multi-omics approach. Only a comprehensive and integrated analysis of genomic, transcriptomic, epigenomic, metabolomic and proteomic data will be able to unravel the complex mechanisms guiding cancer development and progression. Hence, biomarker research is currently shifting from a “one-gene, one-drug” and “multi-gene, multi-drug” paradigm to a “multi-molecular, multi-drug” perspective. However, generating multi-omics data is expensive, time-consuming and relies on the availability of suitable biopsy material, raising many preanalytical issues. Moreover, advances in computational approaches and bioinformatic pipelines are needed to allow a better stratification of patients’ cohorts into subpopulations able to respond to a given therapy [3, 6].

1. MOLECULAR DIAGNOSTICS IN SPECIFIC TUMORS:
2. *Breast cancer*

Most recently, the molecular classification of breast cancer is being integrated with the morphology-based classification based on the immunohistochemistry (IHC) panel of markers comprising of estrogen receptor (ER), progesterone receptor (PR), HER2neu and Ki67 (MIB-1) thereby classifying them into 5 molecular subtypes: luminal A, luminal B (HER2 negative), luminal B (HER2 positive), HER2 positive/enriched (non-luminal) and triple negative breast cancer (basal) [7]. It is now well established that luminal A type has excellent response to endocrine therapy. Further adding to its glory is the advent of newer methods like OncotypeDX assay, Mammaprint, Endopredict assay and PAM50/Prosigna test which are based on mRNA detection using FFPE tissue to classify breast cancers into high or low risk categories, wherein the latter would benefit from chemotherapy and the former not so much [8].

1. *Ovarian cancer*

Germline mutations of BRCA1/2 are associated with high grade serous carcinoma of ovary which is the most common type, and leads to a homologous recombination deficiency (HRD) entailing reduced capacity to repair dsDNA breaks. Platinum-based chemotherapy is the mainstay of treatment of these BRCA mutated high grade tumors in combination with newly approved PARP [poly (ADP-ribose) polymerase] inhibitor Olaparib for maintenance therapy [9].

1. *Colorectal cancer*

Approximately 10-15% of CRC’s (colorectal cancers) have microsatellite instability (MSI) due to defective DNA mismatch repair. Lynch syndrome/Hereditary non-polyposis colorectal cancer syndrome has characteristically high MSI (MSI-H). Genetic counselling for CRC patients with MSI-H is necessary after evaluating MSI status with immunohistochemical analysis of MLH1, MSH2, MSH6, PMS2 and EPCAM. The consensus molecular classification (CMS) of CRC also added new paradigm shift in classifying CRCs based on their comprehensive gene expression profiles and correlates with the tumor behavior. There are 5 subtypes in this classification: 1. CMS1 (MSI-immune) 2. CMS2 (Canonical) 3. CMS3 (Metabolic) 4. CMS3 (Mesenchymal) 5. Mixed. CMS1 has shown to associate more commonly with elderly females with a higher histologic grade at presentation in proximal colon and has a worse survival after relapse. CMS4 commonly present at an advanced stage and has a worse relapse free overall survival. CMS2 in contrast involve the dorsal colorectum and has superior survival [10]. Additionally epidermal growth factor receptor (EGFR), a target for therapy of advanced CRCs is well highlighted by patients with no mutations in RAS gene who benefit from EGFR-targeting antibodies [11,12].

D. *Non-small cell lung cancer*

Advanced-stage adenocarcinomas usually accompany mutations in EGFR and anaplastic lymphoma kinase (ALK) gene and the common testing is done using NGS and FISH respectively [13]. Testing for ROS1 and MET alterations using FISH is now standardized with their role fastened by use of ROS1inhibitors and MET inhibitors being implicated in adenocarcinomas. Furthermore, RET, HER2, BRAF mutations can be detected using FISH and PCR.

*F. Immune-oncology*

The evasion of immune surveillance by the tumor cells is now well understood. After extensive research, it is now clear that tumor cells express certain checkpoint receptors at their surfaces, e.g. CTLA4 and PD1/PD-L1, making them invisible to the immune defense mechanism. The method of detection of PD1/PD-L1 is immunohistochemistry (IHC). Certain drugs (monoclonal antibodies) directed against PD1/PD-L1 are now available as a therapeutic approach to cancers of stomach, lung, breast, sarcomas and Hodgkin’s lymphoma [14].

1. NEWER TOOLS:

*A. Liquid biopsy*

A wide variety of tumor associated components present in circulating blood and bodily fluids are available for detection which include circulating tumor cells (CTCs), cell free circulating tumor DNA (ctDNA), circulating free RNA (cfRNA), miRNA, and other cell components [15]. Assessment of these biomarkers are rapid and require minimal sampling. Currently, liquid biopsy can be used to identify genomic alterations for targeted therapy, to predict burden of tumor, to predict response to treatment as well as identification and characterization of acquired drug resistance. Liquid biopsy also aids in early detection and screening of patients to identify risks of cancer in populations of high-risk.

*B.Digital pathology*

With advancing technology and artificial intelligence (AI), the morphology based histopathological diagnosis needs to be supplemented with techniques such as high-resolution whole-slide imaging with or without AI. Various machine learning approaches have claimed to improve workflow and diagnostic accuracy in the setting of immunohistochemical quantification and scoring, in assessing inter-tumor heterogeneity, in differentiating benign from malignant lesions and in some part, tissue grading of severity [16]. However, such approaches should be used in caution, considering the possible technical errors, computational errors and shortcomings of machine learning. Skill development of the pathologists and an inter-communication among pathologists and engineers are constantly and inevitably required to harness the advantage of such tools. Some of the uses of digital pathology are listed in table 2 [4].

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| **Table 2: Uses of Digital Pathology [4]** |
| Automated digitalization of images for storage and multi-site discussion  Automated scoring of IHC  Automated counting of hybridization signals  Automated identification of tumor in sections for subsequent microdissection |

*C. Tissue biobanking*

Tissue biobanking is a part of translational science that gives access to a number of bio samples which are collected and stored in optimal conditions for research purposes. The approach to such methodologies is a combination of molecular data, pathologic taxonomy, response to therapy and clinical outcomes. The samples for tissue biobanking can be either fresh frozen collections or FFPE specimens which allow prospective and retrospective analysis respectively. It should be kept in mind about the ethical considerations in utilizing pathologic tissues and patient derived xenografts for translational cancer research [17].

*D. Patients’ derived organoids (PDO)*

PDOs are patient derived 3-dimensional tissue structures grown from the adult stem cells in vitro. They are a resemblance of the parent organ of origin and is subsequently bio-banked [18]. These models allow an opportunity to test for new drugs and their effects in an effort to recapitulate the patient’s pattern of response or resistance observed clinically [19]. Also notably, PDOs allow an opportunity for translational research thus paving the way for precision oncology by integrating histomorphology, immunohistochemistry (IHC) and molecular profiling of tumors [3].

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| **Table 3: Newer available techniques – advantages and disadvantages [3]** | | |
| **Techniques** | **Advantages** | **Disadvantages** |
| Liquid biopsy | - Noninvasive test  - Quick result turnarounds | Low specificity, especially as the list of biomarkers expands with better understanding of the underlying biology of cancers |
| Digital pathology | - Improves data and analysis quality  - Low storage costs  - Easier sharing of histological slides | - Adds time and cost to the typical surgical pathology clinical workflow  - Expensive machinery  - Need for analysis software and skills for their use |
| Patients’ derived organoids (PDO) | - Effective biological model to test the in vitro effect of drugs | - Time- and resource-consuming  - Lack of some cellular and molecular background of the natural tissue |

1. CONCLUSION:

In the evolving era of targeted therapy and ever-increasing translational studies on newer drugs, it needs to be emphasized that there is a need for universal standardization of methodologies and bridge the gap between diagnostics and therapeutics. .

1. *A Pathologist’s role:*

The role of a pathologist in such a scenario becomes indispensable for integrating histomorphology with molecular behavior, prognostic and predictive factorial information which are to be adequately relayed to the clinician concerned. The traditional morphological analysis and other chemical techniques used in conventional pathology laboratories cannot be replaced by MP. Both are complementary to each other as are genotype and phenotype. So, high-quality microscopy is a condition sine qua non for high-quality molecular diagnostics [1,5]. Morphologic assessment is indispensible to provide understanding and context to the molecular profiles that are subsequently provided via MP [1].There is also a need for the improvisation of laboratories to infuse newer technologies in the armory of already available tests. Optimal robust training of the current and future pathologists supported by an amenable financial backbone is an absolute need. A list of pathology centered activities is shown in table 4 [4].

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| **Table 4: Pathology-centered activities in the research endeavor [4].** |
| Molecular diagnostics in the context of clinical trials  Analysis of tissues ahead of molecular analyses  Tissue biobanking  Digital pathology  Pathology informatics  Data manager  Biomarker validation  Integration of validated biomarkers into routine diagnostics |

1. *The multidisciplinary team:*

There is an ever-increasing role of molecular pathology in multi-disciplinary approach for care of cancer patients. Appropriate education and training are required across all healthcare workforce involved in patient care. Experts in informatics, molecular genetics and communications thereby play an ever-increasing role. Incorporation of molecular profiles of tumors with morphologic types are thereby becoming the basis for many recent WHO classification systems of tumors.

1. *Challenges*

Although in the recent decades we saw a rapid development of scientific knowledge and a relative reduction in the cost of these advanced testing/diagnostic platforms, major challenges still lie ahead: a) reluctance of traditional pathologists to adapt to these innovative techniques b) lack of molecular scientific knowledge c) relatively confined proven molecular targets for therapy d) a notwithstanding cost.

1. *Future perspectives:*

Molecular pathologists will play a pivotal role in the near future in addressing the shortcomings of current practice in the diagnostics and treatment of many cancers. Pathology residents should also be trained in a manner to adapt skills in genomic technologies above the already acquired histopathological knowledge and be encouraged to take part in translational researches. Multi-disciplinary cancer institute also need to have single laboratory with both diagnostic and research capabilities to bridge this gap. All being desirable noteworthy facets of the gem of oncology, pathologists should not forget the value of morphology as there lies the gate to the city of gold.

“The task of science is to stake out the limits of the knowable, and to center consciousness within them.”  
― **Rudolf Virchow**

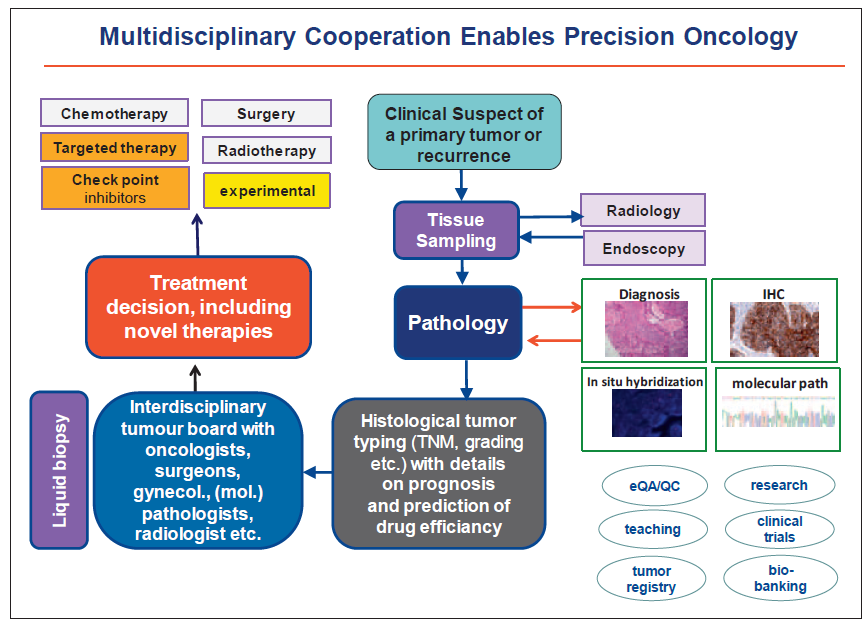


Figure 1: Multidisciplinary approach to precision oncology [1].

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