CURRENT PRACTICE OF PATHOLOGY IN THE MOLECULAR ERA

Abstract

Molecular Pathology 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. The use of antibody integrated specific immunohistochemical stains marks the dawn of the Molecular era of pathology. This chapter discusses briefly the various modern biology tools used in diagnostics and the role of molecular pathologist in understanding diseases and malignancies in particular as a part of the multidisciplinary team consisting of pathologist, radiologist, clinico-oncologist, surgeon and therapist. The commonly used modern biology tools in diagnostics are Immunohistochemistry, Pyrosequencing, Sanger sequencing, Reverse transcriptase (RT)-PCR, q RT-PCR, FISH, Next-generation sequencing (NGS) targeted panels etc. Newer tools like Liquid biopsy, digital pathology, tissue biobanking, patients derived organoids are also briefly mentioned. The importance of integration of morphological pathology and molecular diagnostics and the need for trained Molecular Pathologist is emphasized here. Molecular pathology is currently of great importance, and is becoming increasingly significant as it enables more precise diagnosis and treatment selection. 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. Establishment of a multidisciplinary cancer institute with a single laboratory equipped with both diagnostics and research facilities is the need of the hour.

Keywords: Molecular Pathology, modern biology tools, malignancy, multidisciplinary team.

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I. 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 the case based autopsic reports. The introduction of the first compound microscope by Zacharias Janssen in the 16th century and a later insight by Antoni van Leeuwenhoek in the 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 was 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, the introduction of cytochemical stains and later, immunohistochemistry (IHC) has 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.

II. MOLECULAR PATHOLOGY

Molecular Pathology (MP) is defined by the Association of Clinical Pathologists as "the study of molecules in a disease state, 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 after establishment of the MP laboratory. MP is at-present substantial in providing a precise diagnosis and treatment.

Precision Oncology is defined as "the use of therapeutics to confer benefit to patients with cancers displaying specific molecular or cellular features". Thus, molecular revolution has embarked on cancer diagnosis and treatment, thereby escalating the pathologist's role in therapeutic decisions. Some of the currently available techniques in molecular diagnostics are listed in table 1 [3].

Table 1: Majorly used molecular diagnostic techniques [3].

Diagnostic modality	Uses	Limitations
Sanger sequencing	Inexpensive equipment Widely used in various molecular laboratories	High turn-around-time compared to next-generation-sequencing (NGS) Little data on the molecular patterns of tumors Low sensitivity
Reverse transcriptase (RT)-PCR	High sensitivity for detection of genetic fusions Decreased turn-around-time Widely used in various other molecular laboratories	Only RNA-based Alteration-specific primers
qRT-PCR	Higher sensitivity Decreased turn-around-time Widely used in various other molecular laboratories Feasibility for liquid biopsy	Alteration-specific primers
Pyrosequencing	Inexpensive equipment Widely used in various other molecular laboratories Suitable for methylation studies	Little data on the molecular patterns of tumors
Immunohistochemistry	Decreased turn-around-time Widely used in various other molecular laboratories	Pre-analytical artifacts
Next-generation sequencing (NGS) targeted panels	Higher sensitivity Multiple targets can be analyzed simultaneously Rapid turn-around-time Inexpensive	Analytical complexity
Whole-genome sequencing (WGS)	Picks up useful mutations outside exons Picks up GC rich sequences	Interpretational complexity High cost
Whole-exome sequencing (WES)	Lower cost than WGS Only important genomic alterations picked Validation of rare diseases	Limited role in identifying genes responsible for regulation of transcription and splicing

MP has become the foundation of the upcoming diagnostic techniques and translational research methodologies. In the future, modern medicine will plausibly be determined by molecular medicine. Therapeutic pathology and personalized medicine will pave the way for generation of newer and more specific drugs targeting molecular pathways and specific genes, which along with various specific biomarkers will help predict the response and efficacy of the given drugs. Bridging the gap between diagnosis and therapy will be possible with advancement in translating the biomarker applications, thereby

strengthening the field of molecular pathology [4]. Apart from diagnostics, MP can also be utilized in the prevention and treatment of various diseases like testing of serum viral RNAs for viral infections, specific mutation analysis of tumour cells in diagnosing gene-specific neoplasms, screening of heritable genetic risk factors such as adenomatous polyposis coli (APC) gene in colorectal cancer (CRC), various biomarkers such as Bcr-Abl for assessment of leukemic recurrence and targeted therapy for specific cancers (personalized medicine) [1, 2].

Genomic medicine has come in the forefront, attributing to the introduction of a wide range of parallel sequencing as well as NGS technologies. Cancer-related gene mutation studies, gene rearrangements, copy number variations (CNVs), and RNA expression signatures are being routinely tested in various molecular technology equipped institutes. The idiosyncrasy of genetic mutations compels identification of mutations which initiate tumorigenesis, so called the ‘driver mutations’ which can be targets of specific therapies thereby serving as the anlage of personalized or precision medicine [5]. The multi-omics approach, comprising of genomic, epigenomic, transcriptomic, proteomic and metabolomic data will help unravel the complexity of mechanisms involved in the development as well as progression of cancers. This brings a paradigm shift from the conventional “one-gene, one-drug” and “multi-gene, multi-drug” strategy to a more pronounced and improvised “multi-molecular, multi-drug” regime. Nonetheless, many pre-analytical obstacles lie ahead including expensive equipment, high turn-around-time and unavailability of optimal tissue biopsy material. There is also a need for simultaneous innovation in computational methods and bioinformatics [3, 6].

III. MOLECULAR DIAGNOSTICS IN SPECIFIC TUMORS

1. **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].

2. **Ovarian cancer:** Germline mutations of BRCA1/2 are associated with high grade serous carcinoma of the 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 now considered the main treatment of these BRCA mutated high grade tumors in combination with newly approved PARP [poly (ADP-ribose) polymerase] inhibitor Olaparib for maintenance therapy [9].

3. **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 a new paradigm shift in classifying CRCs based on their comprehensive gene expression profiles and correlates with tumor behavior. There are 5 subtypes in this classification: CMS1 (MSI-immune), CMS2 (Canonical), CMS3 (Metabolic), CMS3 (Mesenchymal) and Mixed. CMS1 has shown to associate more commonly with elderly females with a higher histological grade at presentation in proximal colon and has a worse survival after relapse. CMS4 commonly present at an advanced stage and has worse relapse-free overall survival. In contrast, CMS2 generally involve the dorsal colo-rectum and has better prognosis [10]. Additionally, patients with no mutations in RAS gene benefit from epidermal growth factor receptor (EGFR) targeting antibodies [11,12].

4. **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]. ROS1inhibitors and MET inhibitors are now implicated in adenocarcinoma patients as testing for ROS1 and MET alterations using FISH has now been standardized. Furthermore, RET, HER2, BRAF mutations can be detected using FISH and PCR.

5. **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 checkpoint receptors on 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, soft tissue and Hodgkin's lymphoma [14].

IV. NEWER TOOLS

1. **Liquid biopsy:** A wide range of tumor-associated components circulating in peripheral blood and body 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]. The assessment of these biomarkers is rapid and requires minimal sampling. Currently, liquid biopsy can be used to identify genomic alterations for targeted therapy, to predict the burden of a 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.

2. **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. A variety of machine

learning approaches have claimed to improve diagnostic accuracy in the setting of IHC quantification and scoring, assessing inter-tumor heterogeneity, differentiating benign from malignant lesions and 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 [4]. Some of the uses of digital pathology are listed in table 2.

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

3. **Tissue bio-banking:** Tissue bio-banking 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. Samples for tissue bio-banking 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].

4. **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].

Table 3: Recent techniques – advantages v/s disadvantages [3]		
Techniques	**Uses**	**Limitations**
Liquid biopsy	Noninvasive procedure Rapid turn-around-time	Low specificity
Digital pathology	Improves quality for data analysis Low costs Histological slides easily circulated	Costly and time consuming Require skill training for software analysis and use
Patients' derived organoids (PDO)	Effective for testing in vitro effect of drugs	Consumes time and resource Non-availability of few cellular and molecular data of tissue

V. 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. **Pathologist's role:** The role of a pathologist in such a scenario becomes indispensable. A pathologist has to integrate histomorphology with molecular behavior, prognostic and predictive factorial information. This integrated information can then be adequately relayed to the clinician concerned. Conventional morphological analysis and other cyto-chemistry cannot be replaced by MP. Both are complementary to each other as are genotype and phenotype. Therefore, high-quality microscopy is essential for high-quality molecular diagnoses [1,5]. Morphologic assessment is indispensable to understand the molecular profiles which will be provided by MP [1]. There is also a need for 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].

Table 4: Pathology-focused activities in research [4]
Tissue analysis ahead of molecular analysis
Integration of validated biomarkers into routine diagnostics
Molecular diagnostics in the context of clinical trials
Biomarker validation
Digital pathology
Data management
Pathology informatics
Tissue biobanking

2. **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.

3. **Challenges:** Although in 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.

4. **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. The multi-disciplinary cancer institute also needs to have a single laboratory equipped with both diagnostic and research facilities in order

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 gateway to the 'City of Gold'.

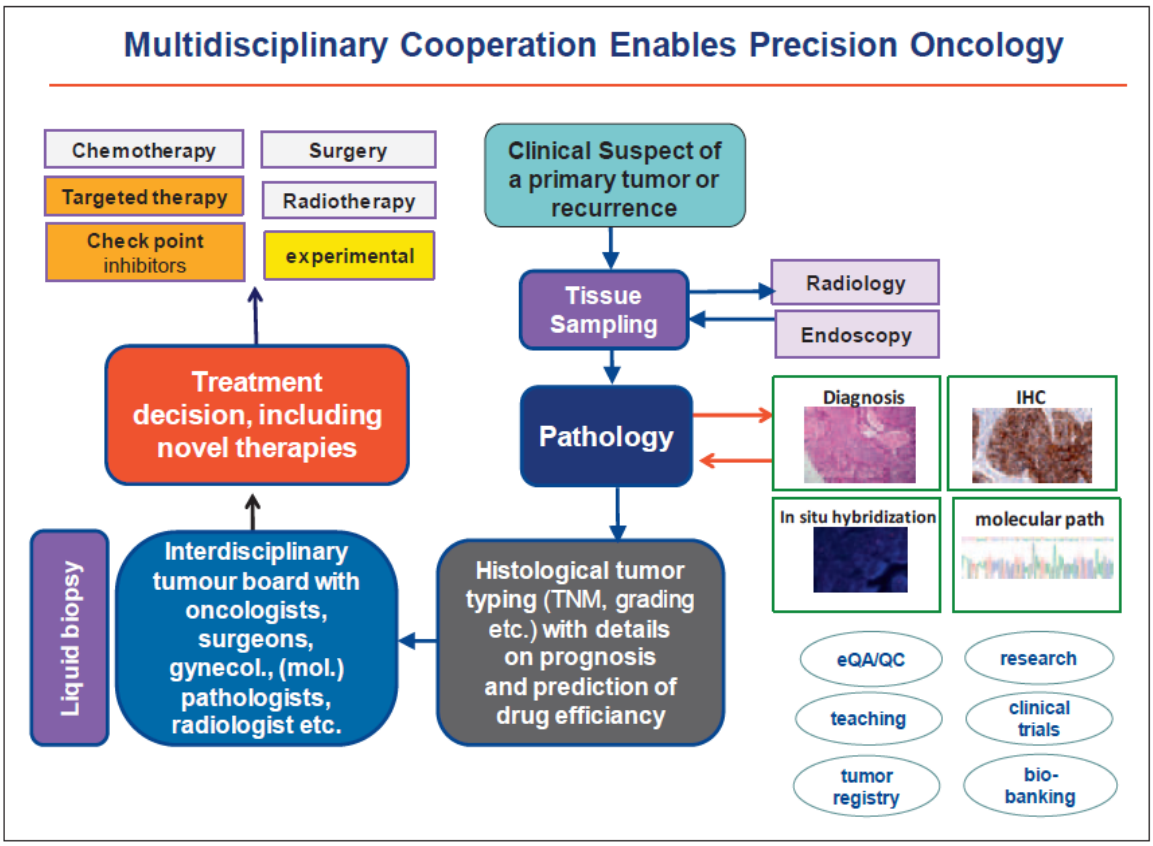

Figure 1: Multidisciplinary approach to precision oncology [1].

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