Upgrading capacity for molecular diagnosis in hospital laboratories in Nigeria: The imperatives

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Molecular biology is the study of macromolecules such as proteins, the nucleic acids including DNA, the storehouse of genetic information; the RNAs (mRNA and tRNA) and enzymes that are essential to life processes. Essentially, molecular diagnostics deal with identification, isolation and manipulation of molecular components in cells and organisms in the diagnosis of benign conditions such as viral infections, malignant or any genetic disease (a disease in which there is an abnormality in or a deficiency of a particular molecule, such as haemoglobin in sickle cell anaemia, or a clonal Philadelphia (Ph) chromosome abnormality in chronic myelocytic leukaemia (CML).

It was in 1938 that the term "molecular biology" was coined by Warren Weaver (1894-1978) a mathematician and one time Director of the Natural Sciences Division of the Rockefeller Foundation in California, USA[1]. Warren created the term to describe the use of techniques from the physical sciences such as X-rays, radioisotopes, ultracentrifuges, mathematics, and others to study living matter. It was in the same institution that Linus Pauling and his students established *Sickle Cell Anemia as a Molecular Disease in 1949*[2]. The seminal paper introduced the concept of molecular medicine; and it was also established that genes control not just the presence or absence of enzymes but also the specific structure of protein molecules.

Pathologists in Nigeria are very much aware of the poor state of molecular diagnostics in most public health institutions in the country. Readers need be reminded that at the launching of this Journal, Annals of Tropical Pathology (ATP) at the 14th Faculty of Pathology *All Fellows Congress* on Saturday 6th November 2010, the Faculty reported on its request for the *Development of Molecular Pathology Programmes* in the memorandum of understanding (*MOU)* it signed with Dr. Dhiren Govender, the African representative on the International Committee of the Royal College of Pathologists (UK).

In the past two decades or so, progress in molecular biology has resulted in the emergence of molecular or targeted therapies, especially for cancers, the focus of this discuss. Molecular therapies comprise of a group of highly selective medications that directly interrupt molecular abnormalities within the cancer cells that induce tumorigenesis. The development of targeted cancer medications has been greatly enhanced with the elucidation of the major steps essential for development of cancers in that each of the basic tumorigenic steps serves as a potential target for molecular therapy[3, 4]:

- Acquisition of autonomous growth signalling (oncogene addiction, tumour growth dependent on continued activation of specific mutated oncogenes such as MYC, RAS, BCR-ABL);

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- insensitivity to growth inhibiting signals (loss of tumour suppressors);
- evasion of apoptosis (anti-apoptosis);
- limitless growth potential (aberrant cell cycle);
- sustenance of angiogenesis (formation of new blood vessels/ blood and nutrients to cancer cells)
- potential for metastasis to distant organs; and
- failure of immune system to eradicate established cancer (immune evasion).

Our patients can only benefit from modern therapies if we manage to effectively combine the traditional morphologic pathology with reliable molecular diagnoses. A situation where a very large majority of pathology and haematology laboratories in the country rely largely on the highly subjective morphologic techniques such as the *French-American-British (FAB)*[5, 6] and the *Working Formulation* [7] for diagnosis of leukaemias and lymphomas cannot ensure best practices in cancer therapy. It is doubly regrettable that diagnostic adjuncts such as histochemistry and immunophenotyping are also not readily available! The adoption of the current WHO diagnostic criteria by Nigerian pathologists would be the ultimate goal in this era (Swerdlow, *et al;* 2008)[8]. The WHO criteria fulfil all the expected requirements for molecular diagnosis including morphology, cytogenetics, molecular genetics and immunophenotyping.

The Imperatives for Ensuring Accurate Diagnosis of Cancers in the Era of Molecular Medicine

In addition to cost effective use of molecular therapies, other advantages include:

(i.) Upgrading of laboratories to molecular pathology grade;

(ii.) Prognostication of diseases, in particular malignancies;

(iii.) More objective diagnosis, thus allowing application of appropriate therapy and better approach to counselling patients and/or carers;

(iv.) Rational use of targeted therapy;

(v.) Accurate diagnosis of lymphomas for good treatment outcome; molecular diagnostics should complement immunophenotyping into B and T-cells. For example, the highly aggressive but potentially curable Burkitt lymphoma is easily distinguishable from the heterogynous diffuse large B-cell lymphoma (DLBCL); various subtypes of DLBCL and Hodgkin lymphoma are identifiable[9, 10] (Table 1);

(vi.) Identification of the more aggressive and poor prognostic chronic lymphocytic leukaemia (CLL) variant expressing the marker for unmutated IgH, ZAP70, from the rather indolent variants of the disease facilitates early commencement of appropriate chemotherapy and chances of good treatment outcome in such patients;

(vii.) Molecular diagnosis facilitates easy identification of chronic myeloid leukaemia (CML) patients expressing t(9;22) (q34;q11) and or BCR-ABL1 chimeric gene that will benefit from novel tyrosine kinase inhibitors (TKI), such as imatinib that is available free in Nigeria;

(viii.) Accurate diagnosis of the non-leukaemic myeloproliferative neoplasms (myelofibrosis, polycythaemia vera and essential thrombocythaemia) expressing JAK2 V617F mutation, that may benefit from ruxolitinib (Jakafi) a Janus-associated kinase (JAK) enzymes inhibitor;

(ix.) Prognostic stratification of acute leukaemias such as acute myeloblastic leukaemias with t(8;21)(q22;q22) and t(15;17)(q22;q12 or q21) translocations often associated with good prognosis, and ALL with very poor prognostic

Table 1: Chromosomal Translocations in Lymphoid Neoplasms and the Affected Genes

Translocations	Non-Hodgkin Lymphoma	Genes Involved
t(8;14)(q24;q32)	Burkitt lymphoma	c-myc and IgH
t(2;8)(p12;q24)	Burkitt lymphoma	Igk and c-myc
t(8;22)(q24;q11)	Burkitt lymphoma	c-myc and Igλ
t(9;14)(p13;q32)	Lymphoplasmacytoid lymphoma	pax-5 and IgH
t(11;14)(q13;q32)	Mantle cell lymphoma	CCND-1 (bcl-1) and IgH
t(14;18)(q32;q21)	Follicular lymphoma	IgH and bcl-2
t(14;19)(q32;q13)	B-cell chronic lymphocytic leukemia	IgH and bcl-3
t(3;var)(q27;var)*	Diffuse large B-cell lymphoma	bcl-6 and IgH*
t(14;15)(q32;q11-13)	Diffuse large B-cell lymphoma	IgH and bcl-8
t(1;14)(q21;q32)	Pre ± B acute lymphoblastic leukemia, mantle cell lymphoma[2]	bcl-9 and IgH
t(11;18)(q21;q21)	Low-grade B-cell MALT[3] lymphoma	api2 and mlt
t(1;14)(p22;q32)	Low-grade B-cell MALT lymphoma	bcl-10 and IgH
t(2;5)(p23;q35)	T/null-cell anaplastic large cell lymphoma	alk and npm
t(11;14)(p15;q11)	Pre ± T acute lymphoblastic leukemia, lymphoblastic lymphoma	LMO1 and TCRd
t(4;14)(p16;q32)	Plasma cell myeloma	mmset and IgH
t(14;16)(q32;q23)	Plasma cell myeloma	IgH and c-maf
t(16;22)(q23;q11)	Plasma cell myeloma	c-maf and IgH

* 14q32 (IgH) is most commonly involved in translocations involving 3q27 (bcl-6), but in less than 50% of cases; other sites that can be involved in bcl-6 translocations include 2p12, 22p11, 8q24, 11q13, and 5q31.
[2] Very few cases of mantle cell lymphoma have been assessed for bcl-9 rearrangements.
[3] MALT = mucosa-associated lymphoid tissue.

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marker, t(9;22)(q34;q11) compared to the ALL patients expressing hyperdiploidy (> 50 chromosomes) that is generally associated with better survival;

(x.) Detection of minimal residual disease (MRD; up to 1 abnormal cell in 10^4 to 10^5);

(xi.) Rational evaluation of treatment outcome in terms of survival;

(xii.) Prevention and reduction of drug toxicity through appropriate use of medication; etc.

Table 2: Major recurrent translocations in acute and chronic myeloid leukaemias

Disease	Genes Involved
t(8;21)(q22;q22);	RUNX1/RUNX1T1;
inv(16)(p13.1q22 or (16;16)(p13.1;q22);	CBFB/MYH11;
t(15;17)(q22;q12) (acute promyelocytic leukaemia-APM);	RARA/PML;
t(9;11)(p22;q23);	MLLT3/MLL;
t(6;9)(p23;q34;	DEK/NUP214;
t(1;22)(p13;q13) (acute megakaryoblastic leukaemia);	RBM15/MKL1
t(9;22)(q34;q11) (CML and Philadelphia chromosome)	BCR/ABL1

Our Options

Which of the numerous molecular diagnostics should we go after, considering the financial implications and manpower resources? I should be comfortable to have the following in all our teaching and specialist hospitals across the nation. It would be more cost effective to share the facilities rather than duplicating the facilities in all centres.

Conventional cytogenetic facilities and polymerase chain reaction (PCR) techniques must be available in the designated molecular diagnostic centres across the country. Although not really a molecular diagnostic test, facilities for immunophenotyping should also be available for objective definition of the characteristics on the surfaces of the gene products, the protein molecules:

(i.) ***Conventional cytogenetic*** facilities for direct evaluation of genetic abnormalities in benign and malignant tissues through karyotyping must be available (see Tables 1 and 2);

(ii.) ***Polymerase chain reaction (PCR)*** is mandatory for all nucleic acid based molecular tests, including bacterial and viral diseases, cancers and other diseases with genetic bases. The versatility of PCR techniques is based on the fact that the technique can be used for analysis of fresh tissues, frozen tissue and even archival paraffin-embedded tissues. The major variants of PCR are the conventional PCR for the amplification of small quantities of target DNA obtained from the test specimen; the highly sensitive reverse transcriptase (RT) PCR and real-time quantitative RT-PCR (qRT-PCR) designed for quantitation of a specific mRNA. In fact, RT-PCR and qRT-PCR are the foundation of molecular biology, and therefore a must in all designated molecular diagnostic centres. Reverse transcription can be described as the synthesis of a complimentary DNA sequence from an mRNA template with the aid of the enzyme, reverse transcriptase, an RNA-dependent DNA polymerase[12]. The qRT-PCR is the most sensitive and accurate technique for the quantification of specific mRNAs such as the bcr-abl1 chimeric gene of chronic myelogenous leukaemia (CML) and the HIV-1 viral capsids. It is most reliable for detection and monitoring of MRD (as low as 1 abnormal cell in over 10^5 normal cells) in CML and HIV-AIDS patients on tyrosine kinase and antiretroviral therapies, respectively[13].

(iii.) ***Gel electrophoresis and blotting*** **techniques** are used for separation of DNA, RNA, and proteins based on their size with application of an electric field as the nucleic runs through agarose gel (less commonly polyacrylamide gel). Blotting techniques are run along with PCR procedures[14]. The techniques involve initial separation of nucleic acid molecules and proteins by electrophoresis (e.g., agarose electrophoresis), followed by transfer onto a synthetic membrane such as nylon and nitrocellulose. The materials are covalently linked (in a permanent form) to the membrane by UV irradiation for further analysis. A labelled copy of the material of interest (e.g., DNA) is allowed to hybridise with the membrane; the specific DNA, if present on the membrane will couple with the labelled copy and can be detected by autoradiography. Blotting techniques include Southern blot for separation of specific DNA, Northern blot for detection of specific RNA and Western blot to separate specific proteins from a mixed sample.

(iv.) ***Immunophenotyping*** for determination of cellular lineage and disease clonality (eg, a monoclonal B-cell malignancy will express χ or λ light chains, while polyclonal infective disorder has both light chains) using immuno-histochemistry or better still, flow cytometry. The limitations of immunophenotyping are that it assesses only the gene expression (the proteins), rather than the more diagnostic direct assessment of the genes, which the molecular genetic techniques will test; and also it may not be conclusive. Highly versatile Flow cytometry machines for detection of minimal residual disease in leukaemias are now available.[11]

(v.) ***Fluorescence in situ hybridization*** (FISH) is the use of non-isotopic labelling of nucleic acid for detection of specific DNA sequence in both metaphase chromosomes and in non-dividing (interphase) cells; it can be used to assess archival tissues. The technique bypasses the culture stage of conventional chromosomal analysis, since all cells can be used rather than the dividing cells. FISH allows detection of MRD undetectable by the conventional cytogenetic technique (e.g. t(8;21) in AML, t(9;22) in CML, etc). Using FISH, MRD detection level is generally lower than that of PCR, about 1 in 10^3cells.

(vi.) ***The DNA microarray*** is a collection of very small (100 micrometer diameter) DNA spots attached to a solid support such as a microscope slide. Each spot has a DNA fragment molecule that is complementary to a single DNA sequence. The array enables simultaneous identification of multiple, differentially expressed genes from normal and malignant cells in a single sample of tissue. The known and unknown probes on the slide hybridize with cDNAs generated from RNAs from the cells or tissue under investigation. The analysis of the hybridisation results helps to identify genes that are upregulated, downregulated, or unaltered in the transformation process.[12]

The Way Forward

The Faculty of Pathology and the Postgraduate Medical College of Nigeria should, as a matter of urgency revisit the MOU with the Royal College of Pathologists of the UK for the development of a Molecular Pathology curriculum. Resident Pathologists should be better educated on the importance of molecular

diagnostics as mandatory for achieving best practices in the management of benign and malignant conditions alike.

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