Molecular pathology platform in a university hospital: Hassan II University Hospital of Fez experience

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Abstract— Molecular pathology plays an important role in tumor pathology after introducing targeted therapy. Therefore, molecular classification of tumors is currently required to predict the response to treatment. According to molecular biology results. These targeted cancer therapies have changed the practice of laboratories in surgical pathology and has led to establish and organize several hospital molecular genetic platforms around the world. In this context, it is necessary to adjust the organization of work and to perform molecular biology assessments in pathology laboratories of hospitals. This would cover growing demand of oncologists in the field of targeted therapies. Our aim is to describe the different steps of regulation of molecular genetic platform in University Hospital of Fez and to report the experience of this laboratory.

Index Terms—Molecular pathology, Molecular biology, Targeted therapy, Genomic alterations

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INTRODUCTION

The identification of molecular alterations in cancer cells allowed to identify new therapeutic targets and/or to develop targeted therapies. The molecular assessment level would allow tumor targeted therapy, thus tailored to each patient and promote "personalized" medicine. Nowadays, the main developments and applications are involving therapies targeting specific molecules, particularly EGF receptor, KRAS, HER2, c-Kit.

Molecular assessments are possible using deparaffinized tissue sections and cytological material by fluorescence in situ hybridization (FISH). These assessments are done also using nucleic acids (DNA) extracted from paraffin embedded tissue and frozen tissue [1-2]. Furthermore, other molecules, RNA, certainly promising targets for the diagnosis, prognosis and therapeutic strategy against cancer [3-4].

During the last decade, the molecular characterization is a critical factor in the choice of the treatment strategy, beyond choices based only on the type and stage of disease. Specific molecular alterations identified in tumor cells are useful to guide and clarify the diagnosis of disease, and might provide prognostic information of patient's treatment. Hence, it is required to develop molecular tests for care of patients.

The integration of molecular biology in pathology yielded increasing number of publications, this reflects the need to develop this field among activities of our laboratories [5-11].

The purpose of this article is to describe the "molecular pathology" platform development of University Hospital of Fez despite constraints and requirements related to this development. We report more particularly development of assessed pathologies in our laboratory, the investigated molecular alterations and the used methods for each molecular abnormality.

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MATERIAL AND METHODS

This is a retrospective study was achieved over 3 years

Developed assessments of pathologies

The most assessed pathologies in the laboratory of Molecular Pathology of the University Hospital of Fez are colon cancer, breast cancer, lung cancer, melanoma, glioma, sarcoma and gastrointestinal stromal tumor (GIST).

The assessed samples were obtained for tissue fragments from surgical pieces or biopsies. The tissues were fixed and embedded in paraffin. The samples were obtained from patients hospitalized in the University Hospital of Fez, while most patients underwent their treatments in private Hospitals.

The pre-assessment stage

The pre-assessment stage is definitely the most difficult to control and also the most concerned by samples nonconformities. It is the one that drives a sample from the patient to molecular biology (MB) investigations. It is therefore crucial to be completely controlled by researchers in the laboratory of pathology who are working closely with clinical departments.

The samples are treated in laboratory of pathology by fixing it in formalin solution at 10%. The benefits of formalin fixation are maintaining excellent morphology, low cost, rapid tissue penetration, reproducibility of obtained results, and standardized immunohistochemistry already for most antibodies [12]. The duration of fixation is certainly the most limiting step and the most difficult. This period can vary for the same type of sample and organ, depending on the day and even the time of arrival of the surgical piece to the laboratory. This time setting would impact the quality of MB examinations passed downstream by modifying the structure of nucleic acids (RNA, DNA) and proteins [13]. However, the results of immunohistochemical methods, FISH, PCR, and Real Time PCR depend basically on this fixing time [14, 15].

DNA extraction from fixed material and embed in paraffin

The DNA is extracted from fixed material and embedded in paraffin using the QIAamp DNA FFPE Tissue Kit (no. 56404), it is the first step in the molecular assessment of tumors according to the protocol described in the kit. It consists to isolate DNA from tumor tissue cells with sufficient quantity and quality for best assessment. A minimum percentage of tumor cells of 20% is accepted in the used method. Paraffin embedded samples are incubated in xylene at elevated temperature to remove the paraffin from tissue. They are then washed in alcohol solutions to remove traces of xylene. The deparaffinized samples are then subjected to digestion of proteins step bound to the DNA and RNA by the action of a protease. Finally, the nucleic acids are purified by capture on a filter then washed and eluted.

After extracting DNA from tumor tissue, a quality test is performed. This quality test is used to quantify the DNA, and to determine its purity. The assay is done with a spectrophotometer by measuring OD at 260 nm that is the maximum absorbance area of the nucleic acids. DNA quantification is important for downstream applications.

Molecular alterations assessment

The laboratory is mostly involved in *KRAS*/*NRAS* mutations including exon 2, codon 12 and 13, and exon 3 and 4; *EGFR* mutations in codons 18,19,20,21, V600E mutations (melanoma, colon cancer, *KIT* gene)., exons 9, 11 and in exon 18 of the *PDGFRA* gene, *IDH1* and *IDH2* in gliomas, and in *SS18* and *EWSR1* genes in sarcomas.

FISH is used for the determination of several rearrangements (*SS18*, *EWSR1* in sarcomas), amplifications (*MDM2* in sarcomas and *HER2* in breast cancer).

FISH is used to detect molecular rearrangements, following recommendations from the developers of kit. Real Time PCR using the probe Taq man is realized for glioblastoma to explore the amplification of *MDM2* gene.

The set of genomic alterations investigated and corresponding pathologies are summarized in Table 1.

Inter-laboratory controls

The inter-laboratory controls were carried out either using the same mutation detection technique of extracted DNA, or from the primary sample after further extraction and results checking. Different techniques are used to detect mutations.

The list of external laboratories involved in these inter-laboratory controls included:

- Institute for Brain and spinal cord (France, Paris)
- Bergonié Institute (France, Bordeaux)
- Institute of Pathology (Germany, Munich)
- University Hospital of Clermont-Ferrand (France, Clermont-Ferrand)

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Analysis of concordances was performed for the results of inter-laboratory controls.

RESULTS

454 cases have been studied including 159 colorectal cancers, 13 lung cancers, 54 stromal tumors, 5 melanomas, 99 gliomas, 45 Breast cancer and 79 sarcomas.

The findings are summarized as follows:

Table 1: Main genomic alterations determined at the platform of molecular biology and corresponding tumor pathology

Gene	Colon	Lung	Melanoma	GIST	Glioma	Breast cancer	Sarcoma	Technique
KRAS	Exons 2/3/4							PCR Sequencing
NRAS	Exons 2/3/4							PCR Sequencing
EGFR		Exons 18/19/20/21						Real Time PCR
BRAF	Exon 15 (V600E)		Exon 15 (V600E)					PCR Sequencing AS-PCR
KIT				Exon 9 Exon 11				PCR Sequencing
PDGFRA				Exon 18				PCR Sequencing
$IDH_{1/2}$					Exon 4 (IDH_1 R132H) (IDH_2 R172K)			PCR Sequencing
HER2						Chr 17 (amplification)		FISH
SS18							Chr 18 (chromosomal rearrangements)	FISH
EWSR1							Chr 22 (chromosomal rearrangements)	FISH
MDM2					Chr 12 (amplification)		Chr 12 (amplification)	FISH Real Time PCR

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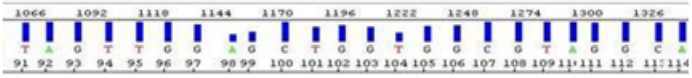
- The colorectal cancers represented 30% of detected mutations on exon 2 of *KRAS* gene, particularly on codons 12 and 13 dominated by 35G>A (Fig. 1), followed by a percentage that ranges between 4 and 5% of mutations found on exon 3 of *KRAS* gene, exon 2 and 3 of *NRAS* gene. However, no mutation was found on exon 4 of *KRAS*, *NRAS*, and *BRAF* genes.
- The melanoma represented 40% of V600E mutations on *BRAF* gene.
- The lung cancer showed 16% of cases with mutations on exon 18, 19, 20 and 21 of *EGFR* gene
- GIST represented 37% and 0% of mutations respectively on exons 11, 9 of *KIT* gene, there was also 38% of mutation found on exon 18 of *PDGFRA* gene.
- The soft sarcomas tissue, *SS18* gene rearrangement were observed in 65% of synovial sarcomas (Fig. 2A), while the rearrangement of *EWSR1* gene was recorded in 71% of PNET/Ewing tumors (Fig. 2B). 7 cases among 14 cases of liposarcoma showed amplification of *MDM2* gene.
- Breast cancer demonstrated *HER2* gene amplification in 29% of cases.



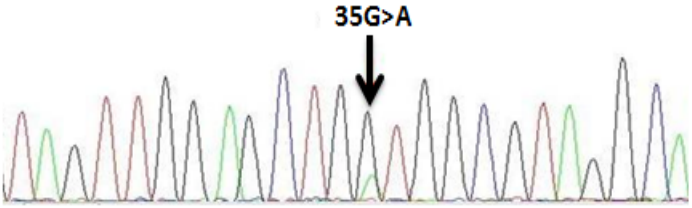


Fig. 1. Electropherogram showing the mutation c. 35G>A detected in forward strand in *exon* 2 of *KRAS gene*

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- In gliomas, 43.43% of cases showed a mutation on exon 4 of *IDH1* gene (Fig. 2C; while *IDH2* gene mutation was also observed on exon 4, this corresponded to anaplastic oligodendroglioma grade III (Fig. 2D). 15 cases of glioblastoma were studied for the detecting *MDM2* gene amplification using Real Time PCR technique. We found *MDM2* amplification in 7 cases representing 46.67%, while 2 cases were inconclusive.

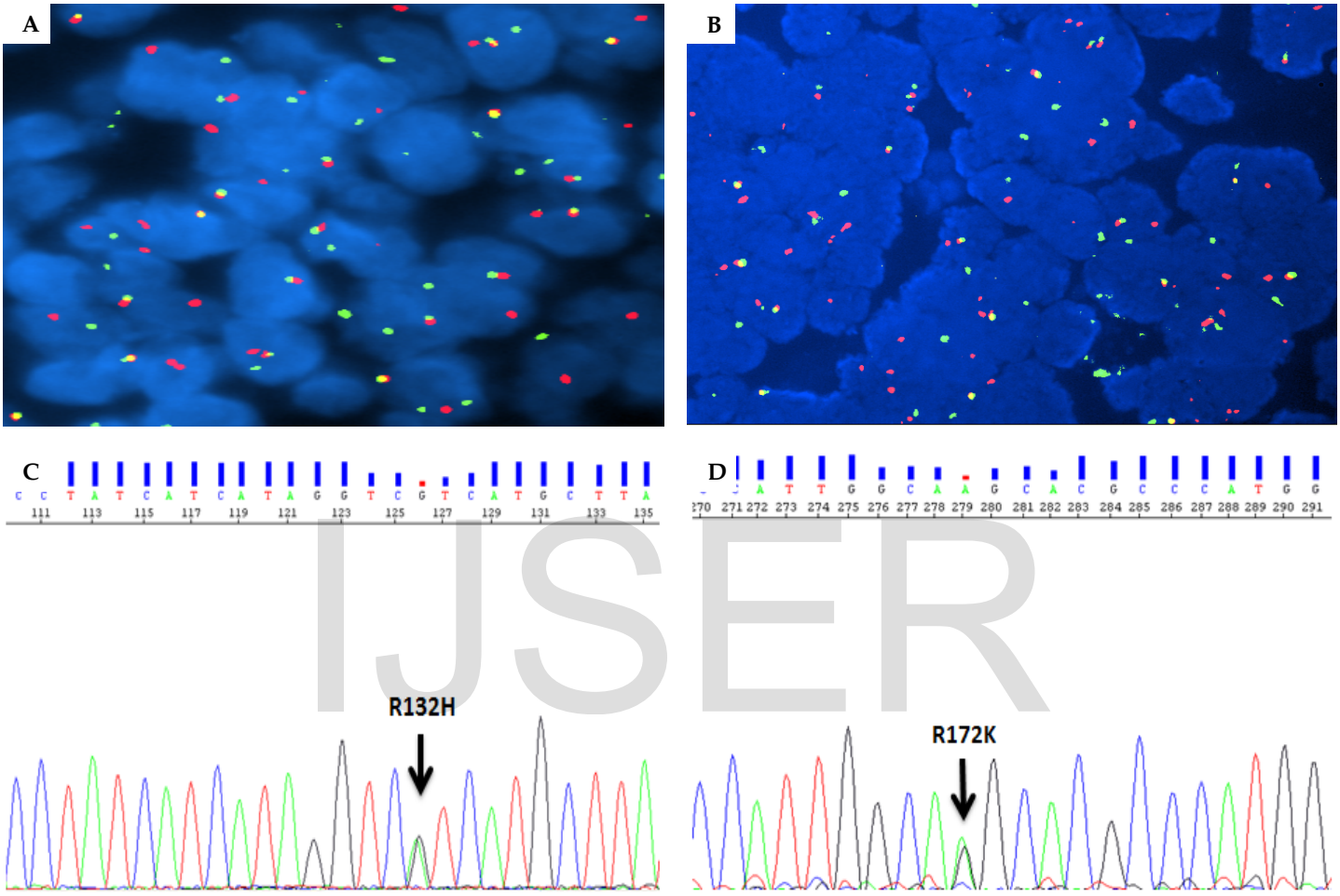

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Fig. 2. A. Observation of *SS18* gene rearrangement realized with probe Vysis Break Apart (x 1000). B. Observation of *EWSR1* gene rearrangement realized with probe Vysis Break Apart (x 1000). C. Electropherogram showing the mutation R132H detected in forward strand in exon 4 of *IDH1* gene. D. Electropherogram showing the mutation R172K detected in forward strand in exon 4 of *IDH2* gene

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Discussion

The innovation of treatments targeting the genetical alterations in oncology allowed to pathology genetic laboratories to be opened to other field and implement molecular pathology platforms.

In 2012, a molecular diagnostic's activity was developed within our pathology laboratory, this was established according to European standards while inspired from French Model (Laboratory of Molecular Pathology, Bergonié Institute). This was followed by focusing on tumor pathologies whose morphological diagnosis was established.

It is important to perform quality control within intra- and interlaboratory framework. These controls must concern each gene and mutation of interest, according to pathology of investigated organ. Interlaboratory tests are performed using or not the same analytical techniques; This can be achieved to compare the sensitivity of used methods, including the percentage of tumor cells present in the sample. Quality control should be done in all stages of the process including pre-assessment, assessment, and post-assessment. It is required since the initial management of tissue such as hot and cold ischemia allows determining genomic alterations. External quality control is benefiting from national and European tests developed. This process would enhance the laboratory potential toward standardized certification [16].

The constant evolution of new targeted therapies in oncology, their possible combination with multiple simultaneous mutations in the same tumor, let to consider transferring in short-term to industry new offers of care extraction technologies of nucleic acids and also the assessment of genomic alterations.

Molecular complexity characteristics play key role in mutation's identifications on small-size tumors, and allows minimal invasive approach while the morphological diagnosis are more difficult [17]. Indeed, it is required to optimize the technique of genomic alteration's detection.

And new sequencing techniques including next sequencing generation or NGS, should allow multiple mutations detections from DNA of several patients simultaneously from small samples [18,19]. These techniques should also allow reducing assessment time to obtain results faster. However, it is difficult to maintain the quality of DNA after formalin fixation and paraffin embedding.

This methodological complexity requires a rapid emergence of new skills in hospital laboratories, including bioinformatics expertise. Besides, complexity of tumors showing higher heterogeneity of mutations according to the mapping done in the tumor sample [20]. Thus, the concept of personalized medicine must also incorporate the underlined difficulties to identify with certainty the mutational profile of the entire tumor [20]. The future of a molecular pathology sector in a pathological laboratory should also take account any new developed antibodies used in immunohistochemistry, this might indicate mutations and genomic rearrangements [21-23]. However, the assessment with molecular biology approach remains expected and will become necessary to compare results [21-23]. The synergy of pathologists and molecular biologists with required expertise will be the best guarantee of optimal use of these antibodies and a better interpretation of results.

The detection of specific molecular abnormalities is technically possible and useful in routine, even essential in terms of diagnosis and prognosis. Therefore, many pathologists and clinicians are increasingly indicating systematically this complementary technique. Indeed, molecular biology is becoming a gold standard and is offering several advantages: the finding of a molecular abnormality allows a precise and reproducible diagnosis especially on biopsy; there are genetical disorders that constitute inclusion criteria for targeted therapies. The systematic identification of genetic abnormalities allows to better classify different tumors and improve patient's selection for a given treatment (e.g. sarcoma). The confrontation of histology and molecular biology results would increase the skills of pathologists. However, some drawbacks such as the cost, the small number of assessing laboratories and the absence of quality assurance has so far limited their larger use.

Beyond the contribution within the framework of offering patient care, we cannot ignore that the integration of molecular pathology assessment service anatomicopathological should allow to new generations of pathologists, to acquire also new culture in biological assessments, and new trusts of knowledge in the pathophysiology of cancer for better service patients [24-26]. Finally, establishing a strong link with a biobank ideally within the same laboratory would also strengthen the optimization of molecular pathology and allows to to the concept of "integrative pathology" to emerge in hospitals [25,27,28]. This

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concept of "integrative pathology" or Pathobiology would combine molecular data, the morphology in clinical pathology and management of different biological collections of data.

Conflict of interests

The authors declare that they have no conflicts of interest related to this article.

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