Cancer Targeted Therapy Strategy: The Pathologist's Perspectives

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ARTICLE HISTORY

Accepted: June 28, 2016

Abstract: The effectiveness of new personalized treatment procedures in oncology is based on the fact that certain tumors exhibit specific molecular features.

More in detail, neoplastic tissues of patients should display a specific biomarker, most often a specific genetic alteration and/or under/overexpression of a definite protein, that could be the target of its respective drug. Immunohistochemical and molecular analyses, which usually include examination of nucleic acids from either tissues or fluids, are common tests to define the status of a tumor.

This review focuses on the pathologist's role in carefully controlling pre- analytic procedures and standard operating procedures that are a crucial prerequisite to reach reliable and reproducible results. Six paradigmatic applications of targeted therapy, for which pathological diagnosis plays a fundamental role, are summarized. Traditional and next-generation sequencing are also addressed from the pathologist's perspective as well as the importance pathologists have in this shift to more accurate definition of disease risk and prognostication of therapy response in the personalized medicine era.

Keywords: Target therapy, biomarker, pathologist's perspective, Immunohistochemistry, FFPE tissue, molecular analyses, traditional sequencing, next-generation sequencing.

1. THE PAST

Traditionally (and unfortunately), in the past decades, pathologists have not been influent in clinical/therapeutic decision-making process.

The choice of the appropriate laboratory tests and the interpretation of results were first started by clinical physicians, and pathologists were neither involved in the selection of the more suitable tests nor in therapeutic decisions following the tests' results.

However, the discipline of pathology had a relevant role in discovering, classifying and interpreting biomarkers at cellular and subcellular level, that could guide physicians in the management of patients [1]. There has always been a historical tradition of investigation in pathology, with an important contribution in scientific discoveries.

2. THE PRESENT: THE PATHOLOGIST'S PERSPECTIVES

Nowadays, the effectiveness of new personalized treatment procedures in oncology is based on the fact that certain tumors exhibit specific molecular features. More in detail, neoplastic tissues of patients should display a specific biomarker, most often a specific genetic alteration and/or under/overexpression of a definite protein, that could be the target of its respective drug [2].

Immunohistochemistry (IHC) and molecular analyses, which usually include the examination of nucleic acids from either tissues or fluids, are common tests to define the status of a tumor.

Such new and constantly evolving molecular tests have a deep impact on "classical pathology". On one side, malignant tumors are classified on the basis of histogenesis, histological type, grading, staging and other morphophenotypical features, according to the World Health Organization (WHO) so-called 'Blue Books' of pathology. However, tumors belonging to the same category are heterogeneous entities, encompassing different lesions with distinct biological properties. As morphology-based pathological parameters are only partly able to predict clinical behavior of tumors, further available analyses have to be added in the diagnostic procedure.

The principal aims of these ancillary tests are:

- to provide clinicians with predictive information on the behavior of each tumor, for example, drug response, prognosis and the risk of metastases;

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- to define specific characteristics of each tumor (*i.e.* expression of proteins, genetic alterations) relevant for the most useful drug selection for each patient;
- to prompt future research on human tissue that will enable the discovery of new targeted drugs.

On the other hand, it has to be stated that at present and in the next future, WHO-based surgical pathology will still have its prevailing role in daily diagnostic and patient management. Tissue samples will be a more easily available source for new diagnostic techniques, which require conventional histomorphology-based characterization. The new molecular analyses will provide 'additional information' that conventional morphology is not yet able to contribute.

2.1. Technical Considerations: The Pathologist's Role

Carefully controlled pre-analytic procedures and standard operating procedures are a crucial prerequisite to reach reliable and reproducible results.

2.1.1. Tissue Specimens Procurement and Examination

Tissue specimens may be analysed fresh (*e.g.* for the establishment of cell cultures) or may be rapidly frozen, set inside an optimal cutting temperature, used to obtain histologic frozen sections.

Formalin-fixed paraffin-embedded (FFPE) tissues are achieved for various uses including standard hematoxylin & eosin (H&E), IHC, in situ hybridization-ISH, other molecular analyses.

In order to ensure high quality of tissue and its adequacy for further tests, it is of critical importance to collect and process tissue timely.

Warm ischemia time is the range of time after tissue resection from the human body, during which it is kept at room temperature but prior to being stabilized. It also progressively occurs in the lesional tissue during the surgical resection itself. The cold ischemia time is defined as the period in which tissue is kept on ice or at 4 °C in a refrigerator after resection but before formalin fixation or freezing [3]. Time from surgical removal to stabilization affects protein preservation (and therefore immunohistochemical expression) in a variable manner.

When adequate refrigeration lacks, a decrease in progesterone receptor IHC staining is observed in breast cancer samples after 2 h [4]. Similarly, for Her2/neu testing, the American Society of Clinical Oncology/College of American Pathologist (ASCO/CAP) currently recommends a cold ischemia time of less than one hour for breast cancer [5]. During gross examination and sampling of fresh tissue for molecular analyses, the pathologist can assure that tissue is sampled from the right location (*i.e.*, including neoplasia and not healthy tissue), avoiding necrotic areas and that correct preservation is taken. Additionally, pathologists can also select the appropriate dimensions of neoplastic areas, to be fixed or frozen in an adequate time [6].

2.1.2. FFPE Tissue and Molecular Analyses

FFPE is still the most widely used technique to process tissue in the routine diagnostic setting. It does not require expensive tools, and it is easy-to-use and cost-effective. In the past, molecular analysis from FFPE tissue was difficult to perform.

To solve this problem, in the last years a large number of protocols for extraction of nucleic acids and/or proteins from FFPE tissue have been well-thought-out and the use of neutral buffered formalin for 24-48 h for fixation has become a standard procedure [7-9]. Nowadays, DNA/RNA of good quality for molecular analyses can easily be acquired if the tissue is quickly fixed in 10% neutral buffered formalin and if standard procedures are adopted in routine processing [10]. Important factors affecting final molecular results in fixed specimens include chemical parameters of the fixative (*e.g.*, concentration, zinc content, and pH), length of fixation, type (routine or microwaved) of tissue processing, conditions of the storage unit where paraffin blocks are kept [11, 12]. Despite being better suitable for the extraction of DNA, RNA as well as proteins, the use of frozen tissue for diagnostic assays is still limited to large and specialized hospitals. Nucleic acids from frozen specimens are generally high molecular - weight and without crosslinking, thus suitable for a wide variety of purposes. Frozen tissue yields DNA and RNA, ideal for current approaches such as whole genome amplification, whole genome sequencing, and cDNA microarray analyses [13, 14]. However, frozen storage for molecular analyses has also some drawbacks. RNA and DNA fragmentation has been proven in different studies after five years despite storage at −70 °C or −80 °C [15, 16]. Many medical centers do not have the trained personnel or infrastructure for frozen specimen procurement and storage. Biomolecules can degrade with increasing freeze–thaw cycles. Storage costs for frozen specimens are much higher than for room temperature specimens. Finally, loss of temperature control for technical problems can definitively damage irreplaceable specimens [3].

2.1.3. Pre-analytic Tumor Tissue Selection

Before the molecular assay, a pathologist under the microscope must identify the tumor containing parts of the tissue (so- called 'tumor cell enrichment' procedure), marking the area of the H&E slide section containing neoplasia for macrodissection or microdissection, if used (Fig. **1**). Especially within large tumors, it is fundamental to select different regions of the tumor for manual microdissection to account for heterogeneity of the tumor itself [17]. Instead of microdissection, 1 mm punches may be taken from the marked areas [18]. The pathologist should express an estimate of the percentage of neoplastic cells present in the sample selected in the final pathology report.

Considering that many tests have a lower limit of detection, the assessment of this measure could be very important in determining the results of each technique [19]. To prevent false negative results, the presence of necrosis should be best avoided in samples for molecular analysis, as well as widely dispersed tumour cells within a sample. The pathologist is clearly responsible for the evaluation of neoplastic cell content and for performing macrodissection or microdissection.

2.1.4. Traditional Sequencing

Currently, traditional sequencing remains the basis to discover cancer predisposition in the patient's germline

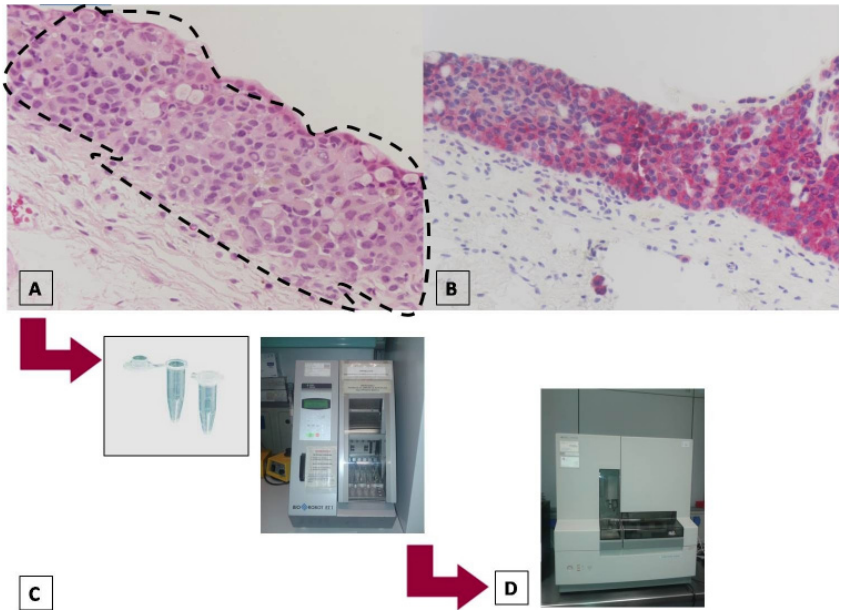

Fig. (1). Example of a workflow of mutation analyses in routine diagnostic. (**a**) Tumor area of a mucosal melanoma was marked on hematoxylin and eosin (H&E) slide and subsequently manually microdissected for the detection of a BRAF mutation. (**b**) Immunohistochemical stainining for S-100 in the same lesions as in (**a**) highlights malignant melanoma cells. **c**) DNA was extracted from the microdissected tissue fragments and Sanger (**d**) sequencing was performed.

[20, 21] and has also been used to determine polymorphisms associated with anticancer drug resistance and toxicity [22-24]. Moreover, in routine oncology practice, assessments that predict the efficacy of drugs already in the market or at various stages of clinical development are based on DNA sequence determination [25-27]. Sanger sequencing and other traditional platforms have been used for the following analyses [28]:

- BRAF mutation testing to predict anti-EGFR (epidermal growth factor receptors) antibody resistance in colon cancer;
- BRAF mutational analyses to select patients with metastatic thyroid cancers and melanoma that could benefit from treatment with the BRAF inhibitors;
- EGFR sequencing in non-small cell lung cancer (NSCLC) patients to those that could respond to treatment with EGFR tyrosine kinase inhibitors;
- KRAS mutational analyses for treating patients with advanced colorectal cancer with anti-EGFR therapies [29];
- sequence-based detection of the EML4-ALK translocation in NSCLC to select patients for treatment with crizotinib;
- CKIT mutations detection for the treatment of gastrointestinal stromal tumors and of a subset of malignant melanomas;

2.2. Six Paradigms: Breast Cancer, Gastric Cancer, Lung Cancer, Melanoma, Malignant Mesothelioma and Lymphoid Malignancies (Table 1)

2.2.1. HER-2 in Gastric and Breast Cancers

HER-2 belongs to the family of EGFR, and is a transmembrane protein showing a tyrosine kinase domain which is transphoshorylated by binding to other members of the same family, thus activating downstream signaling pathways. Both gene amplification leading to protein overexpression and gene mutation can lead to HER2 activation. Downstream pathways activated by HER receptors include Ras-Raf-MAPK, PI3K-AKT (Fig. **2**), and STAT, which promote cell migration, invasiveness and metastases through inhibition of apoptosis, and promotion of angiogenesis and proliferation [30]. For its characteristics, HER receptors represent ideal targets for personalized drugs. Among other tumors, amplification of HER-2 is also found in gastroesophageal adenocarcinomas. Overexpression of HER-2 is mandatory for the use of trastuzumab, which is a monoclonal, recombinant, humanized antibody directed against the extracellular domain interfering with HER-2 dimerization.

The detection of HER2 overexpression can be performed either by immunohistochemistry or by in situ hybridization. The most used technique for routine determination of HER2 status is immunohistochemistry, which evaluates the membranous expression according to the ASCO/CAP recommendations [5]. At the DNA-level, several in situ hybridization methods like fluorescence (FISH), chromogen (CISH) or Silver in situ hybridization (SISH) could be performed [31]. In addition to the monoclonal antibody trastuzumab, newer available targeted drugs such as lapatinib, pertuzumab and trastuzumab-emtansin are currently under evaluation in clinical studies [32].

Due to the challenges in the IHC assessment of HER-2 staining in gastric cancer (heterogeneity with focal and incomplete membrane staining), accurate and consistent testing can be achieved only following the guidelines stated by expert pathologists [33]. HER-2 positive immunostaining ranges widely (up to 53.4 %) [34]. When amplification is studied by in situ hybridization approaches, it ranges from 8.7% to 18.1% of cases in 8 studies enrolling 1,597 patients

Table 1. Overview of molecular tests with therapeutic implications recommended for routine testing in different tumors.

Tumor Type	Affected Gene(s)	Type of Alteration	Method for Detection	Related Treatment
Breast Cancer	HER2 PIKCA Gene expression assays (EndoPredict, OncotypeDX *etc.*)	Amplification SNV mRNA levels	IHC, ISH Sequencing qRT-PCR	Herceptin Reduced response to anti-HER2 treatment Prognostic assay
Serous ovarian cancer	BRCA1/BRCA2	SNV, MNV, indel	Sequencing	Olaparib
NSCLC	EGFR ALK	SNV, MNV, indel Translocation	Sequencing IHC; ISH, Sequencing	Gefitinib, Erlotinib, Afatinib Crizotinib, Ceritinib
Colorectal cancer	RAS (KRAS,NRAS)	SNV	Sequencing	Cetuximab / Panitumumab
GIST	KIT PDGFR	SNV SNV, indel	IHC; Sequencing IHC; Sequencing	Imatinib, Sunitinib Imatinib, Sunitinib
Malignant Melanoma	BRAF KIT	SNV Indel	IHC, Sequencing Sequencing	Vemurafenib, Dabrafenib Sunitinib, Imatinib
Malignant mesothelioma	BAP-1 NF2	SNV, indel SNV, indel	IHC, sequencing Sequencing	Vorinostat? FAK inhibitors
DLBCL	MYC BCL2	Translocation, amplification, overexpression	IHC, FISH	BET inhibitors, BH3 mimetics
Classical hairy cell leukemia	BRAF	V600E	Sequencing, IHC	Vemurafenib

SNV= single nucleotide variation; MNV=multiple nucleotide variation; IHC= immunohistochemistry; ISH=in situ hybridization; DLBCL=diffuse large B-cell lymphoma; indel=insertion or deletion.

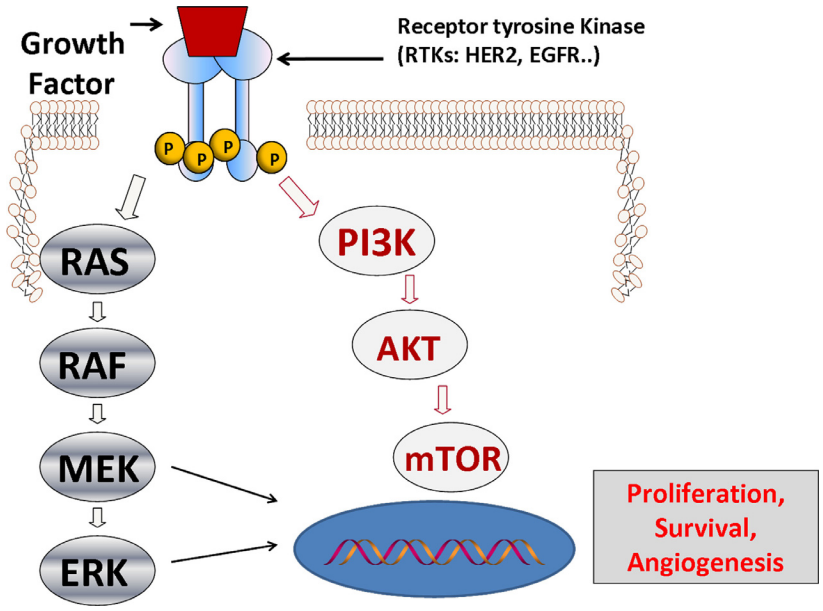

Fig. (2). Tyrosine kinase receptors and intracellular signaling networks. Stimulation of the tyrosine kinase receptors (HER2, EGFR…) by the growth factor results in activation of the RAS/RAF/MEK/MAPK and PI3K/AKT/mTOR intracellular signaling networks. These pathways promote cell proliferation, survival, and angiogenesis.

[34]. This wide range of expression could be partially due to technical issues and the proportion of esophageal and gastric neoplastic cells, according to different histotypes (tubular, papillary, mucinous, or poorly differentiated).

For cases with an IHC score of 3 +, the European Medicines Agency (EMA) has approved treatment with trastuzumab in combination with traditional chemotherapy. The samples with an IHC score of 2+ should be retested with in situ hybridization assay and only positive cases forwarded for treatment. Finally, an IHC score of 1 + is considered negative [33]. The ToGA trial, a randomized multicenter phase III study, has shown that trastuzumab in combination with chemotherapy is more effective in HER-2- positive advanced gastric cancer, with an increased overall survival, than the use of chemotherapy alone [35].

After the interesting results of trastuzumab in patients with HER2-positive gastric cancers, an increasing interest is evident in the development of selective drugs.

Different receptors and /or downstream pathways are known to be aberrantly activated in gastric cancer (*e.g.* HER2, MET and FGFR2) and therefore they may represent new treatment targets beyond HER2 inhibition. [36, 37]. Moreover, peculiar endocrine profiles in gastric cancer have been linked to an unfavorable outcome and may identify subsets of patients for tailored therapy [38]. Routinely, in breast cancer HER2 IHC is used commonly as a 1st step test, followed by HER2 ISH if the result is equivocal or discordant.

The most recent guidelines now define equivocal HER2 ISH as a HER2/CEP17 ratio <2.0 with a mean HER2 copy number between 4.0 and <6.0 [5]. Additional criteria for a HER2-positive result including any HER2/CEP17 ratio≥2.0, a HER2 copy number ≥6.0, where the HER2/CEP17 ratio is <2.0. In order to avoid false negative results and to determine the HER2 status of the tumour accurately, it is recommended that an equivocal ISH result must prompt reflex testing [5].

For the same aim, it could be useful to repeat HER2 testing performing HER2 IHC and/or ISH on a different tissue block or specimen, or ISH applying a different probe for CEP17. However, most laboratories that do not have access to alternative chromosome 17 probes, have validated a single ISH test and had performed IHC previously on the specimen. For such reasons, an equivocal ISH test is required at least to repeat ISH on a core biopsy or excisional specimen (the one that was not tested) or on another tumour block from the same specimen.

The rate of equivocal ISH results may have increased with routine application of the updated guidelines, whereas prior to the their application, equivocal HER2 ISH cases were not frequently encountered [39]. There has been consequently a rise in repeated tests with unknown benefits in identifying additional patients with HER2-positive breast cancer [40]. The issues ensuring reliable HER2 testing by IHC are preanalytic, analytic and postanalytic. Preanalytic issues mainly relate to time of and to fixation and the fixative used. Excessive delay to the initiation of formalin fixation (cold ischemia time) has been shown to adversely impact the analysis of hormone receptor assays and HER2 analysis [41, 42], as described by Khoury *et al.*, who suggested that delays in the start of fixation of 60 to 120 minutes may invalidate the accurate analysis of hormone receptors and HER2 FISH due to loss of signal intensity [42].

The comparison of IHC expression of ER, PR and HER2 on tissue fixed for a standard amount of time with tissue from the same samples that underwent prolonged fixation (72 to 96 hours) [43] showed that formalin fixation for up to 72 hours did not have any effect on ER, PgR and HER2 reactivity and therefore, is an acceptable upper limit of time in routine clinical practice. The immune-reactivity of breast prognostic biomarkers may be reduced by formalin fixation for an extended time.

Post-analytic issues involve interpretation criteria, reporting methods and quality assurance measures. Despite the fact that literature suggests 2+ score being the most problematic [44], 3+ scoring, which is often misinterpreted, has the most serious clinical consequences. The change in criteria is an attempt to reduce 3+ false positives, because a small percentage of cells may show intense staining owing to edge artifacts. Scoring such cases (11-30% of strongly staining cells as 2+ (which is defined equivocal according to updated guidelines) will result in additional confirmation by ISH if they are true positive. Image analysis systems could be used to achieve consistency in interpretation. It is also important that HER2 testing results are effectively communicated to the oncologist. In our practice, a standardized template stating the time taken to fix the tissue, the controls and antibody used and the HER2 score with description of staining, was used.

2.2.2. Lung Cancer

For decades, the dismal prognosis of lung cancer and the limited number of treatment options have narrowed the practical impact of pathologic diagnoses on the care of lung cancer patients. Lung cancer has been divided into 2 groups for diagnostic and treatment purposes: small cell lung carcinoma (SCLC) and the non-small cell lung cancer (NSCLC), the latter consisting of adenocarcinoma, squamous cell and large cell carcinoma. Traditionally, the primary role of the pathologist was to distinguish between SCLC and NSCLC on biopsy or cytology and, for the minority of NSCLCs that were potentially amenable to surgery, to examine stage resection specimens. Of the NSCLCs, about 20% to 25% are currently diagnosed as squamous cell carcinoma and 40% to 50% are diagnosed as adenocarcinoma. Because the great majority present in an advanced stage that is not potentially amenable to surgical resection, about 70% of lung cancers are diagnosed on small biopsies and /or cytology specimens and additional tissue are typically not obtained [45]. It can be very difficult to diagnose a specific cell type on some of these small samples, particularly based only on routine stains, because of the limited tissues available for examination, sampling of poorly differentiated areas, and crush and other artifacts [46]. In the recent past, because differentiation of SCLC from NSCLC had a potential impact on subsequent therapy, but differentiation of adenocarcinoma from squamous cell carcinoma was considered insignificant, from a practical perspective, diagnoses of tumor subtypes on small samples were retained not crucial. The introduction of new targeted molecular therapies has altered the traditional role of the pathologist: one widely publicized change is that a diagnosis of NSCLC, not otherwise specified, although unavoidable in some cases, is less satisfactory than in the past. Diagnosis of the specific cell type is now important for the selection of several of the new therapies by oncologists [45]. For example, only patients with nonsquamous NSCLC are reported to have improved survival when the new antifolate drug, pemetrexed, was included in their regimen [47]. In addition, the anti–VEGF monoclonal antibody bevacizumab was approved for patients with advanced nonsquamous NSCLC, as other histotypes may develop pulmonary hemorrhage, potentially life threatening [48, 49]. The use of immunostains is encouraged to reach a specific cell type diagnosis when doubts persist after H&E evaluation [45]. Many tumors previously called large cell carcinomas are now known to be specific cell types, mostly poorly differentiated adenocarcinomas. In addition, it may be difficult to differentiate a solid-pattern adenocarcinoma from a squamous cell carcinoma based on routine histology. Even with special studies, a few lung tumors cannot be classified as a specific cell type.

Moreover, adenosquamous carcinomas make up about 1% of lung cancers and have at least a 10% adenocarcinoma component and at least a 10% squamous cell carcinoma component.

EGFR is a transmembrane protein showing a tyrosine kinase domain which is transphoshorylated by binding to other members of the same family, thus activating downstream signaling pathways (Fig. **2**). In the previous years, the first generation of oral, selective, reversible EGFR tyrosine kinase inhibitors (TKIs), such as gefitinib and erlotinib, was investigated. The observation that activating EGFR mutations are a predictive biomarker for response to EGFR TKI therapy, introduced a new role for pathologists in precision medicine of lung cancer patients. Two mutations account for 90% of the activating EGFR mutations, short in-frame mutations in exon 19 and the L858R point mutation in exon 21, but a number of less frequent EGFR mutations are also clinically relevant. Multiplex testing allows simultaneous detection of multiple EGFR mutations, not just the major 2 [50]. While rare tumors such as adenosquamous carcinomas may have EGFR mutations and respond to EGFR TKI therapy, pulmonary adenocarcinomas that are initially responsive to the first- generation reversible EGFR TKIs, eventually developing acquired resistance due to one or more of several possible mechanisms. These second-generation drugs having higher-affinity, are irreversible EGFR tyrosine kinase blockers that also inhibit HER2 and sometimes HER4 and may have modest activity against T790M or other mutations that cause acquired resistance to the first-generation EGFR TKIs [51].

Cetuximab is an anti-EGFR immunoglobulin G1 monoclonal antibody that is currently undergoing clinical trials for lung cancer therapy [52]. Despite initial modest results for patients with advanced NSCLC treated with chemotherapy and cetuximab , subgroup analysis of the First Line Erbitux in Lung Cancer phase III trial found different outcomes associated with increased overall survival in patients with advanced NSCLC, receiving first-line chemotherapy plus cetuximab, with a high EGFR expression based on an immunohistochemistry score using the Dako pharmDx kit (Glostrup, Denmark) with an increased overall survival compared with patients treated with chemotherapy alone [53]. Therefore, although EGFR mutation testing is recommended as the best biomarker predicting response to EGFR TKIs, in the future, EGFR IHC and EGFR FISH may prove to be reliable alternative predictive biomarker tests for cetuximab therapy for lung cancer. FISH and, especially, IHC are conventional techniques familiar to surgical pathologists and are more likely to be available in pathology laboratories that lack their own molecular diagnostics laboratory [50].

Chromosomal alterations might also result in the overexpression of fusion proteins that could be targeted by newly developed drugs. One recent example is that of the EML-4 (echinoderm microtubule-associated protein like 4)-ALK inversion on chromosome 2, identifiable in a small subset of NSCLC [54], which involves the first exons of EML4 to the 3' part of ALK (from exon 20). Further fusion partners of ALK (which is a receptor tyrosine kinase belonging to the insulin receptor family) have been described [55]. However, the variability of EML4 breakpoint, along with its inversion on the same chromosome, has limitation to PCR-based assays and FISH-based tests. Due to these technical difficulties, the optimal method of detection of this specific genetic alteration still has to be established, although IHC determination has been considered effective. Early clinical trials of the first-generation ALK TKI crizotinib produced improved RR and PFS in ALK-positive NSCLC [56].

2.2.3. Melanoma

Most cutaneous melanomas show mutations in the genes of the RAS/RAF/MAPK pathway (Fig. **2**), which regulate the proliferation of melanocytes. Upstream, the stem cell factor (SCF) receptor (encoded by KIT), a receptor- linked tyrosine kinase, is activated by extracellular ligands triggering this signaling cascade. Cutaneous melanomas show alternatively BRAF or NRAS mutations resulting in downstream signaling, which account for 50-70% and 15-20% of all mutations, respectively [57]. Until now, more than 30 mutations within the BRAF gene have been identified; the most common of which is V600E, where thymine is replaced with adenine at nucleotide 1799. This causes a substitution of valine (V) by glutamate (E) at codon 600. The availability of targeted drugs (vemurafenib and dabrafenib) directed towards aberrant BRAF [57] has given clinical relevance to BRAF mutational status of metastatic melanomas. However, BRAF sequencing is not yet available in most pathology laboratories, as it is time consuming and expensive. On the contrary, IHC is widely performed, requires a limited amount of neoplastic tissue, and is a cost-effective familiar technique for all pathologists. Recently, a monoclonal antibody (clone VE1) against BRAF V600E protein has shown high sensitivity (90-100%) and specificity (97-100%), with good inter-observer reproducibility [58, 59]. Thus, V600E BRAF IHC could be used as a cheap and rapid test to select cases for molecular analysis. Despite the great efficacy of imatinib and other tyrosine kinase inhibitors in gastrointestinal stromal tumor (GIST) treatment, contrasting response rates have been reported in KIT mutated melanomas [54]. However, mutational screening is still critical to select cases for this targeted therapy, especially in mucosal melanomas, in which KIT mutations showed low prevalence [60].

2.2.4. Malignant Mesothelioma (MM)

Malignant mesothelioma is molecularly characterized mostly by the loss of tumor suppressor genes, rather than the gain of function mutations. Somatic inactivation of tumor suppressor gene located at 3p21.1, BRCA associated protein 1 (BAP1), has been described in MM. Germline BAP1 mutations have also been identified in cases of familiar mesotheliomas not caused by exposure to asbestos which usually show a longer survival [61]. Germline mutations of BAP1 seem also to predispose to other several different tumors such as ocular (uveal) and cutaneous melanomas and renal cell carcinoma, suggesting the existence of a BAP1 -related neoplastic syndrome, involving multiple organs.

In contrast with results of preclinical models, BAP 1 expression evaluated by immunohistochemistry (IHC) in 123 MM tissue samples was not related to asbestos exposure and inversely correlated with survival, suggesting that its role in the development of MM may be independent of the traditional asbestos-related effect [61]. It could be supposed that the identification of germline mutations could stratify indi-

viduals at high risk of MM. BAP1 has a role in DNA repair and control of gene expression through histone modification. *In vitro*, sensitivity of histone deacetylase inhibitor vorinostat on BAP1-knockdown MM cell lines did not seem significant.

This finding was confirmed by a recently completed Phase III trial (VANTAGE 014) of vorinostat in 660 pretreated advanced MM patients [62], which observed few (if any) significant responses, without improvement in overall survival and only a minor advantage in progression-free survival (PFS).

Neurofibromatosis type 2 (NF2) is a tumor suppressor gene located on chromosome 22q12 and encodes for the protein Merlin. Loss of NF2 function occurs in approximately 40% of patients with MM. Preclinical studies suggest that Merlin inactivation is a critical step in MM pathogenesis increasing its invasiveness through upregulation of focal adhesion kinase (FAK) expression [63]. A recently reported phase I study of GSK2256098 (an oral FAK inhibitor) proposed that Merlin loss patients might result in improved PFS in response to FAK inhibition [64].

2.2.5. Lymphoid Malignancies

Tumors of hematopoietic and lymphoid tissues frequently contain chromosomal translocations leading to the generation of novel fusion genes often exhibiting a tyrosine kinase activity. In addition, single gene mutations appear to be important in the pathogenesis of various leukemias and lymphomas. However, these discoveries have had only a superficial impact on traditional treatment. The only major biomarker routinely used is CD20, the expression of which (immunohistochemically evaluated) by B-cell malignancies is a prerequisite for Rituximab (an anti-CD20 monoclonal antibody) application.

Diffuse large B-cell lymphoma (DLBCL) represents the most common subtype of non-Hodgkin lymphoma accounting for 30–40% of all cases. It is an aggressive neoplasm that is fatal without treatment. Current immunochemotherapy employing rituximab and a combination of cyclophoshamide, doxorubicin, vincristine and prednisolone (R-CHOP) can reach good clinical results, but most patients who fail R-CHOP will ultimately die from their disease.

Important predictive information within DLBCLs of the activated B-cell (ABC) molecular subtype could be inferred by their cell of origin, as this group exhibits poorer outcome after usual treatment than DLBCLs of the germinal center cell (GCB) subtype [65, 66]. Unfortunately, the only standardized method to get this information is gene expression profiling, which is not routinely available. Conversely, several immunohistochemical staining protocols have been developed as substitution for gene expression profiling, even if they suffer from the relatively poor reproducibility of interpretation [67]. Other established biologic predictive factors that can influence the therapy of DLBCL are MYC and BCL2. The increased expression of MYC protein (identified in 10% of patients and is due to MYC gene rearrangement) is associated with increased proliferation of neoplastic cells and with a poorer outcome in R-CHOP-treated patients. So called "double hit" lymphomas harboring MYC and BCL2 translocation represent a small group (approximately 5% of DLBCL cases), which are usually refractory to treatment with a median survival of approximately 8 months. Overexpression of MYC protein due to an up-regulation by other mechanisms than gene translocation can be detected by IHC in up to 30% of cases. Interestingly, a negative prognostic impact of such MYC protein overexpression is observed only in patients who simultaneously overexpress BCL2 protein (nearly 25% of patients) [68]. DLBCL patients should be therefore assessed for translocations and protein overexpression of MYC and BCL2 in order to identify patients that could benefit from alternative therapies.

Another distinct biomarker expressed in more than half of the cases of the systemic anaplastic large cell lymphoma (ALCL), a peripheral T-cell lymphoma, is the nucleophosmin (NPM) – ALK fusion protein, which can be easily detected by immunohistochemistry. It results in constitutive activation of ALK tyrosine kinase leading to the activation of multiple downstream pathways. Crizotinib is an inhibitor of ALK tyrosine kinase and successful treatment of ALK + ALCL has been reported in pediatric patients, leading to ongoing trials in relapsed/refractory ALCL [69].

Mantle cell lymphoma (MCL) is a rare subtype of non-Hodgkin's (NHL) B-cell lymphoma defined by cyclin D1 overexpression or t(11;14). MCL often follows an aggressive clinical course with an overall poor prognosis. Chronic active signaling via the B-cell receptor (BCR) pathway has been implicated in the pathogenesis of many subtypes of B-cell malignancies, including MCL. Overexpression of an integral protein in the BCR pathway, Bruton's tyrosine kinase (BTK), has been observed in MCL. Activation of BTK and its downstream targets plays a vital role in normal and malignant B cells, including modulation of nuclear transcription, as well as regulation of B-cell proliferation, differentiation, survival and migration [70]. In MCL, phosphoproteomic analysis of cell lines indicated a prosurvival role of BCR signaling in this malignancy [71]. Ibrutinib is a potent, orally bioavailable inhibitor of BTK that binds irreversibly to a cysteine residue (C481) in the BTK active site. In preclinical studies in MCL cell lines, ibrutinib demonstrated the inhibition of downstream BCR signaling and cell apoptosis. Ibrutinib is a very promising agent for patients with MCL with high response rates. However, when used as a single agent, nearly 30% of patients relapse in the first 2 years of treatment [72].

3. THE FUTURE: THE PATHOLOGIST'S ROLE AND PERSPECTIVES

In October 2010, representatives from major national pathology organizations met in Cold Spring Harbor,NY [73], to discuss the challenges faced by pathology in the rapidly developing field of targeted therapy.

The main topics discussed were the advent of next-generation sequencing (NGS) and whole-genome analysis (WGA) in pathology practice and the role of pathologists in ensuring that the performance of genome-based tests and their interpretation would be overviewed by pathologists themselves and not by different professional figures. From the discussion, the new concept of "primary-care pathologist" emerged (Blue Dot Project 6- see below). A survey conducted by the CAP indicated that nearly half of patholo-

gists desire more direct interaction with patients in their clinical practice [74]. Pathologists must decide how to participate in this activity and how to partner with other health care professionals (such as geneticists) to develop direct interactions with patient and provide up-to-date interpretations of genomic information in the context of intercurrent health events and needs.

In order to achieve these goals, 7 "Blue Dot Projects" were recommended in the Branbury Conference [73]. The need to introduce NGS and WGT topics into medical student and pathology resident educational programmes, *i.e.* Project 1, was considered mandatory. Blue Dot Project 2 addressed the issue of listing all tests applied by pathologists in routine diagnostics, to decide which tests might be replaced by NGS or other high-throughput technologies. The establishment of a shared "repository" of genomic information, as stated in project 3, using appropriate data protection systems, should be controlled by pathologists. In particular, when cancer cells are sequenced, pathologists have to monitor critical issues. As an example, they have to assess the need for other test formats such as microRNA profiling, epigenomic status determination, and traditional in situ analyses (immunohistochemical analysis and fluorescence in situ hybridization) to personalize patients' care. Blue Dot Project 4 focused on identifying and validating operational models for WGA, by conducting 4 multi-institutional projects, involving analysis of 16 whole human genomes in major clinical areas such as cancer.

The purposes of these short-range projects were to test operational models, produce clinical variant database entries, and assess different whole-genome sequencing technologies and mapping analyses.

The accreditation and regulatory guidelines for WGS diagnostics (Blue Dot Project 5) were defined to have major input and control by pathologists and organized pathology depts. Due to Project 6, a new pathology towards direct clinical consultation was created. Irrespective of the specialist who has directly overseen the technical performance of the NGS "test" and regardless of whether it was done in-house or not, a surgical pathologist has to bring the information received to the clinical specialists (*e.g*, medical oncologists) in a workable format. That is, pathologists must be able to help "interpret" the report and emphasize the genetic alterations important for immediate personalization of the patient's care [73]. Finally, the last project, Project 7, referred to reimbursement issues.

3.1. Technical Considerations: NGS Sequencing Compared with Traditional Sequencing

Clinical oncologic applications demand high levels of accuracy, sensitivity and specificity for sequence-based tests detecting specific genetic alterations. Therefore, the expertise, especially in computational biology, required to perform clinical NGS testing for cancer patients is significantly higher for this NGS approach than for traditional Sanger sequencing. The traditional approach is less expensive than the cost for an NGS test incorporating hundreds or thousands of genes or a whole exome.

Regardless of the sequencing approach used, pathologists have to decide which is the best sample to test (*e.g.*, primary vs metastatic tumor tissue or tumor tissue *vs* circulating tumor cells).

Problems to be solved remain the following: small samples such as from fine-needle aspiration biopsies, extensive necrotic rather than viable tumor tissue, tumor heterogeneity for mutations and other genetic abnormalities, and samples that feature a very low percentage of tumor DNA [28].

NGS sequencing can confirm "expected" mutations in a given case but also discover "unexpected" sequence abnormalities, which may significantly alter treatment options. Unlike traditional sequencing, NGS sequencing can provide gene copy information such as homozygous and heterozygous deletions, gene amplifications and translocations, which also drive therapy selection, as in the case of the EML4-ALK translocation and the selection of crizotinib in NSCLC [28]. The longer turnaround time for NGS of a cancer genome sequence (eg,7-14 days) than for traditional sequencing will probably shorten as this technology continues to evolve.

Literature data also suggest that, in the near future, NGS will overcome traditional sequencing in sensitivity for mutation detection in samples in which a mutation is present in only a small percentage of the total DNA extracted. However, the modern tumor characterization is increasingly based on some forms of integrated laboratory report in which tumor cell DNA sequencing will be combined with traditional cancer cell diagnostics, including morphologic slide–based results (IHC studies and FISH), epigenetic testing such as methylation-specific RT-PCR profiling, full transcriptional analysis, and microRNA profiling. Moreover, the starting sample/s are going to change. Currently, surgical biopsy and resection specimens are the most widely analyzed samples but a transition to testing ever-smaller amounts of DNA is likely to be required.

NGS now enables non-invasive analyses such as early detection of relapse-determining mutations in blood or plasma samples from patients under treatment [75]. This process, today commonly addressed as liquid biopsy, has led to an improved understanding of the development of therapy resistance and opened the path for a future monitoring of cancer patients [76].

CONCLUSION

In their call for evolutionary pathology education and pathology practice leadership of the WGS and NGS technologies, Tonellato and colleagues [73] have clearly advised to place "Pathology's flag" in the field of newly developed molecular techniques, underlining the importance that pathologists have in this shift to more accurate definition of disease risk and prognostication of therapy response in the personalized medicine era.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

Declared none.

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