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From Cell Biology to Tissue Engineering

Review

Immunohisto(cyto)chemistry: an old time classic tool driving modern oncological therapies

Tomer Cooks[1]*, Sofia D.P. Theodorou[2]*, Eleni Paparouna[2]*, Sophia V. Rizou[2], Vassilios Myrianthopoulos[2,3,4], Vassilis G. Gorgoulis[2,5,6,7] and Ioannis S. Pateras[2]

[1]The Shraga Segal Department of Microbiology, Immunology and Genetics, Faculty of Health Sciences, Ben Gurion University of the Negev, Beer Sheva, Israel, [2]Molecular Carcinogenesis Group, Department of Histology and Embryology, School of Medicine, National and Kapodistrian University of Athens, [3]Division of Pharmaceutical Chemistry, School of Pharmacy, National and Kapodistrian University of Athens, [4]PharmaInformatics Unit, Athena Research Center, Athens, [5]Center for New Biotechnologies and Precision Medicine, Medical School, National and Kapodistrian University of Athens, [6]Biomedical Research Foundatin of the Academy of Athens, Athens, Greece and [7]Faculty of Biology, Medicine and Health, University of Manchester, Manchester Academic Health Science Centre, Wilmslow Road, Manchester, UK.

*: equally contributing authors

Summary. In the era of precision medicine immunohistochemistry (IHC) and immunocytochemistry (ICC) share some of the highlights in personalized treatment. Survival data obtained from clinical trials shape the cut-offs and IHC scoring that serve as recommendations for patient selection both for targeted and conventional therapies. Assessment of Estrogen and Progesterone Receptors along with HER2 status has been among the first approved immunostaining assays revolutionizing breast cancer treatment. Similarly, ALK positivity predicts the efficacy of ALK inhibitors in patients with non-small cell lung cancer (NSCLC). In recent years, Programmed Death Ligand 1 (PD-L1) IHC assays have been approved as companion or complimentary diagnostic tools predicting the response to checkpoint inhibitors. Anti-PD-L1 and anti-PD-1 monoclonal antibodies have inaugurated a new period in the treatment of advanced cancers, but the path to approval of these biomarkers is filled with immunohistochemical challenges. The latter brings to the fore the significance of molecular pathology as a hub between basic and clinical research. Besides, novel markers are translated into routine practice, suggesting that we are at the beginning of a new exciting period. Unraveling the molecular mechanisms involved in cellular homeostasis unfolds biomarkers with greater specificity and sensitivity. The introduction of GL13 (SenTraGor®) for the detection of senescent cells in archival material, the implementation of key players of stress response pathways and the development of compounds detecting common mutant P53 isoforms in dictating oncological treatments are paradigms for precision oncology.

Key words: Biomarkers, Immunohistochemistry, Immunocytochemistry, Oncology, Therapy

Offprint requests to: Ioannis S. Pateras, Molecular Carcinogenesis Group, Department of Histology and Embryology, School of Medicine, National and Kapodistrian University of Athens, 75 Mikras Asias St. GR-11-527, Athens, Greece. e-mail: ispasath2004@yahoo.com or ipateras@med.uoa.gr

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Introduction

Advances in technology have revolutionized the analysis of tumor samples rendering feasible the development of personalized treatments. Yet conventional assays play a major impact on decision making in routine practice. Immunohistochemistry (IHC) and immunocytochemistry (ICC) are old-fashioned still powerful techniques for visualization of cellular and extracellular components (Coons et al., 1942; Nakane and Pierce, 1966; Sternberger et al., 1970). The basis of IHC and ICC exploits a fundamental immunologic principle which lies in the specificity of antigen-antibody reaction. According to Brandtzaeg (1998) IHC is a "way of talking to cells; the result can

identify the cells and may indicate what they are doing *in vivo* if one asks the right questions by means of the right antibodies" (Brandtzaeg, 1998). Our understanding of the functions of cells and tissues is tightly related with the development of the appropriate antibodies that react with the target antigens. ICC refers to the immunostaining assay that is employed for the detection of antigens in cells (from the greek "cyto-"), whereas IHC refers to antibody-based assessment of tissue antigens (Maity et al., 2013). When the antibody is conjugated to a fluorophore, the immunostaining assay is called immunofluorescence (IF). Hence samples are viewed in pathology practice mainly in light and to a much lesser extent in electron microscopy or alternatively in fluorescence microscopy (Sethi et al., 2016).

Nowadays, thousands of antibodies exist, and the list is still growing, enabling the localization of a wide range of single as well as multiple antigens on the same cell or tissue section, correlating morphological with functional parameters. The latter renders immunostaining assays attractive tools for research, diagnostic and therapeutic purposes (Matos et al., 2010; Inamura, 2018). In the current review we demonstrate the significance of these classical *in situ* assays in dictating modern oncological treatments.

ER, PR and Her2/Neu

A milestone in breast cancer management is estrogen receptor (ER), progesterone receptor (PR) and tyrosine protein kinase Her2/Neu testing through *in situ* staining (Leong and Zhuang, 2011). Although IHC is considered as the gold standard, a strong concordance of these markers between ICC and IHC has been repeatedly demonstrated (Shabaik et al., 2011; Vohra et al., 2016).

ER exists in two isoforms i.e. ERα and ERβ, which share a similar primary structure and are encoded by two identical genes residing in 6q25.1 and 14q22-q24 respectively, though only ERα has a clinical utility (Heldring et al., 2007). ERα is expressed in nearly 70% of invasive breast carcinomas and its status is directly related with the response to hormonal therapy. Given that patients with even low ERα positive staining exhibit a significant benefit from anti-ER therapy versus matched ERα negative cases, a 1% cut-off employing IHC/ICC has been validated as the definition of ER+ cases (Allred, 2010; Hammond et al., 2010; Hicks et al., 2017) (Table 1). PR also exists in two isoforms encoded by the same gene mapped in 11q22.1 by employing alternate transcriptional start site (Daniel et al., 2012). Available antibodies for PR detection bind to the N-terminus which is identical for both isoforms (Leong and Zhuang, 2011). Since ERα positively regulates PR expression; PR is rarely observed in ER negative cases (Leong and Zhuang, 2011; Daniel et al., 2012). PR expression predicts response in endocrine therapy in a similar manner to ERα (Table 1).

ERBB2 resides in 17q12 encoding Her2/Neu/ErbB2. It is a member of the ErbB family of tyrosine kinase receptors (Roskoski, 2014). Her2/Neu is amplified in nearly 20% of invasive breast carcinomas which results in its overexpression (Allred, 2010). This has been exploited therapeutically revolutionizing the prognosis of Her2/Neu positive patients. Specifically, breast cancer patients exhibiting a strong complete membrane

Abbreviations and Notes. Cancer*, referring to the types of cancer where already approved drug is administered upon examination of the particular marker; Criteria*: IHC scoring systems employed prior approval for the treatment of the corresponding drug; 28-8 pharmDx (DAKO-Agilent)*: the (%) in each type of cancer refer to the percentage of positive PD-L1 expression of cancer cells; SP142 (Ventana-Roche) NSCLC (≥50% TC or ≥10% IC)*: TC refers to percentage of PD-L1 expressing cancer cells of any intensity and IC refers to the percentage of PD-L1 expressing tumor-infiltrating immune cells of any intensity; SP142 (Ventana-Roche) Urothelial carcinoma (≥5% IC)**: tumor infiltrating immune cells, which refers to the percentage of lymphocytes and macrophages exhibiting discernible positive PD-L1 immunostaining of any intensity in ≥5% of tumor area; SP263 (Ventana-Roche)*: the (%) refers to the percentage of viable cancer cells of any intensity above background staining; 73-10 (DAKO-Agilent) (≥1%)*: the (%) refers to percentage of viable cancer cells; ADC: antibody-drug conjugate; Aromatase Is: aromatase inhibitors; ALCL: anaplastic large cell lymphoma; ALK: anaplastic lymphoma kinase; ALK inhibitors: a class of drugs targeting ALK tyrosine kinase activity; anti-EGFR therapeutic mAbs: anti-EGFR therapeutic monoclonal antibodies belong to a class of drugs binding to EGFR resulting in inhibition of its activity; AR: androgen receptor; BrCa: Breast carcinoma; ATRX: Alpha thalassemia/mental retardation syndrome X-linked (ATRX); CDK4/6: Cyclin D Dependent Kinase 4/6 inhibitor 4/6, a class of drugs that abrogate the activity of CDK4/6; cHL: classical Hodgkin lymphoma; CLC: small- cell lung cancer; CPS: Combine Positive Score, it refers to the number of PD-L1 stained cells including cancer cells, lymphocytes and macrophages divided by the total number of viable cancer cells; CRPC: castration-resistant prostate cancer; DLL3: Delta Like Canonical Notch Ligand 3; anti-DLL3-ADC: anti-DLL3 antibody drug conjugate; EGF: epidermal growth factor; EGFR: epidermal growth factor receptor; EGFR H-score (ranging from 0-300) = 1 × (percentage of weakly stained cells, 1+) + 2 × (percentage of moderately stained cells staining, 2+) + 3 × (percentage of strongly stained cells, 3+); EGFR TKIs: EGFR tyrosine kinase inhibitors: a class of drugs including first-, second- and third-generation recognizing mutant and wild type EGFR; ER: estrogen receptor; 5-FU: Fluorouracil; GCa: Gastric carcinomas; GBM: glioblastoma multiforme; GEJCa: gastroesophageal junction carcinoma; GIST: gastrointestinal stromal tumors; HNSCC: head and neck squamous cell cancer; IHC: Immunohistochemistry; IMT: inflammatory myofibroblastic tumor; LCNEC: large cell neuroendocrine carcinoma; mAbs: monoclonal antibodies; MGMT: O6-methylguanine-DNA methyltransferase; mTOR inhibitors: mammalial target of rapamycin inhibitors, a class of drugs (including rapamycin and its analogues) inhibiting mTOR signaling pathway; NETs: neuroendocrine tumors; NTRK: neurotrophic tyrosine receptor kinase; MMR: mismatch repair; MSI: microsatellite instability; MSI-H: microsatellite instability high; NB: neuroblastoma; NSCLC: non-small cell lung cancer; PCa: prostate cancer; PD-1: programmed death protein 1; PD-L1: programmed death ligand 1; PR: progesterone receptor; RCC: renal cell carcinoma; ROS1: c-ROS oncogene 1; SCCHN: squamous head carcinoma of head and neck; SCLC: small cell lung cancer; SERDs: selective estrogen receptor degraders (also known as selective estrogen receptor downregulator), a class of drugs that bind to ER, leading in turn to ER degradation; SERMs: selective estrogen receptor modulators, a class of drugs modulating ER activity in a tissue specific manner; TKIs: tyrosine kinase inhibitors; TPS: Tumor Proportion Score, it refers to the percentage of viable cancer cells.

Table 1. Biomarkers, function, type of cancer, type of treatment and criteria.

Biomarker	Function	Cancer*	Type of Treatment	Criteria*	References
ER/PR	Growth; Differentiation	Breast cancer	SERMs; SERDs; Aromatase Is, CDK4/6 inhibitors, mTOR inhibitors	≥1% of tumor cells express nuclear immunoreactivity (positive immunostaining)	Hammond et al., 2010; Asano et al., 2017; Kneubil et al., 2017; Tong et al., 2018
Her2 (Neu)	Growth; Apoptosis	Breast cancer, Gastric and GEJ adenocarcinoma	anti-HER-2 mAbs, Tyrosine kinase inhibitors, anthracycline and taxane-based chemotherapies	i) BrCa, HER2-positive (3+): ≥10% of tumor cells showing homogeneous membrane staining; ii) GCa/GEJCa, HER2-positive (3+): ≥10% of tumor cells showing strong complete, basolateral or lateral membranous reactivity	Hofmann et al., 2008; Wolff et al., 2013; Abrahao-Machado and Scapulatempo-Neto, 2016; Nitta et al., 2016; Asano et al., 2017; Kneubil et al., 2017; Dominguez et al., 2018
EGFR	Growth; Differentiation; Survival	NSCLC, colorectal cancer, breast cancer	EGFR TKIs, anti-EGFR therapeutic mAbs.	EGFR H score ≥200 (high EGFR expression) - although IHC exhibits several limitations	Goldstein and Armin, 2001; Spano et al., 2005; Pirker et al., 2012; Abdelrahman et al., 2017; Avilés-Salas et al., 2017; Hitij et al., 2017; Wei et al., 2017
ALK	Growth; Differentiation; Angiogenesis	NSCLC, IMT, ALCL	ALK inhibitors (TKIs)	Any percentage of tumor cells exhibiting strong granular cytoplasmic immunoreactivity [VENTANA ALK (D5F3 clone) CDx Assay]	Pulford et al., 2004; Mino-Kenudson et al., 2010; Travis et al., 2011; Sugawara et al., 2012; Wynes et al., 2014; Antonescu et al., 2015; O'Malley et al., 2015; Marchetti et al., 2016; Gallant and Lovly, 2018; Janoueix-Lerosey et al., 2018; Ricciuti et al., 2018; Trigg and Turner, 2018
ROS1	Growth; Differentiation	NSCLC	ROS1 inhibitor	Lack of standarized scoring system	Rimkunas et al., 2012; Sholl et al., 2013; Yoshida et al., 2014; Boyle et al., 2015; Cao et al., 2015; Bubendorf et al., 2016
DLL3	Differentiation - Development	SCLC	anti-DLL3 ADC	Lack of standarized scoring system	Saunders et al., 2015; Sharp et al., 2016; Lambert and Morris, 2017
IDH1	Metabolism	Gliomas	Guides therapy-decision making	Any percentage of cancer cells with intense cytoplasmic immunoreactivity for IDH1-R132H	Capper et al., 2010; Weller et al., 2017
ATRX	Chromatin remodeller	Gliomas	Guides therapy-decision making	ATRX loss: lack of nuclear immunoreactivity	Capper et al., 2010; Weller et al., 2017
MGMT	DNA repair	Gliomas	Guides alkylating agent therapy: lack of MGMT expression increases chemosensitivity	IHC is not an accepted method to study MGMT status	Brell et al., 2005; Rodriguez et al., 2007; Capper et al., 2008; Sharma et al., 2009; Weller et al., 2017; Wick et al., 2018
MLH-1, MSH-2, MSH-6, PMS-1, PMS-2	DNA repair	Colorectal cancers	Guides therapy-decision making: MSI-H/MMR deficient benefit from Immunotherapy whereas they lack benefit for 5-FUbased cytotoxic chemotherapy	Positive signal: unequivocal nuclear staining in cancer cells	Boland et al., 1998; Casorelli et al., 2008; Shia, 2008; Bertagnolli et al, 2009; Shia et al., 2009; Amira et al., 2014; Kawakami et al., 2015; Birendra et al., 2017
PD-1, PD-L1	Immune checkpoint	Melanoma, NSCLC, Urothelial cancer, HNSCC, cHL, RCC	PD-1 and PD-L1 inhibitors	a) 22C3 pharmDx(DAKO-Agilent): NSCLC (TPS≥1%: PD-L1 expression for 2nd line; TPS ≥50%: high PD-L1 expression for 1st line); Gastric or GEJ Adenocarcinoma (CPS≥1) of at least moderate intensity; Urothelial Carcinoma (CPS≥10%: PD-L1 expression); b) 28-8 pharmDx (DAKOAgilent)*: non-squamous NSCLC (≥1%, ≥5%, ≥10 %); SCCHN (≥1%); Melanoma (<5%; ≥5%); Urothelial carcinoma (≥1%); c) SP142 (Ventana-Roche) NSCLC (≥50% TC or ≥10% IC)*; Urothelial carcinoma (≥5% IC)**; d) SP263 (Ventana-Roche)* NSCLC (≥25%); e) 73-10 (DAKO-Agilent) (≥1%)*	Keir et al., 2008; Brahmer et al., 2012; Herbst et al., 2014; Tumeh et al., 2014; Wang et al., 2014; Bellmunt et al., 2015; Geng et al., 2015; Patel and Kurzrock, 2015; Phillips et al., 2015; Ferris et al., 2016; Papaioannou et al., 2016; Reck et al., 2016; Roach et al., 2016; Chae et al., 2017; Contratto and Wu, 2017; Diggs and Hsueh, 2017; Feng et al., 2017; Kang et al., 2017; Mino-Kenudson, 2017; Nishino et al., 2017; Paulsen et al., 2017; Sharma et al., 2017; Gong et al., 2018; Inamura, 2018

Her2/Neu staining in >10% of tumor cells are eligible to receive anti-Her2 therapy (Table 1) (Nitta et al., 2016). On the other hand a weak to moderate complete membrane staining in >10% or intense immunostaining in ≤10% of cancer cells is borderline and warrants further assessment with *in situ* hybridization. Interestingly, an inverse relationship between Her2/Neu and ER or PR status is typically evident (Nicolini et al., 2018). Of note, Her2/Neu is also overexpressed in approximately 20% of carcinomas arising from gastroesophageal junction (GEJ) and gastric carcinomas basically of intestinal rather than diffuse type (Gunturu et al., 2013). Administration of anti-Her2 therapy in patients with metastatic gastric cancer or carcinomas arising from GEJ exhibiting score 3+ (Table 1) significantly improves their overall survival (Gunturu et al., 2013). A major issue when assessing Her2/Neu status is intratumoral heterogeneity, which is frequently observed in gastric carcinomas (Abrahao-Machado and Scapulatempo-Neto, 2016). Therefore it is recommended to examine more than one tissue sample from each patient to avoid potential discrepancies.

EGFR

Epidermal Growth Factor Receptor (EGFR/ErbB1/HER1) belongs to the ErbB family that also comprises Her2/Neu as we previously mentioned. *EGFR* gene is mapped to 7p11.2 encoding a 170-kDa transmembrane receptor (Herbst and Shin, 2002). Expression of EGFR in normal cells ranges from 40,000 to 100,000 molecules per cell (Carpenter and Cohen, 1979) whereas in several cancer types EGFR is significantly overexpressed exceeding 10^6 receptors per cell (Gullick et al., 1986). Elevated EGFR expression in cancer is attributed to *EGFR* gene amplification and transcriptional up-regulation (Roskoski, 2014). Additionally, mutations in the *EGFR* locus lead to constitutive activation of EGFR (Herbst and Shin, 2002; Suzuki et al., 2005; Pinter et al., 2008; Liang et al., 2010; Douillard et al., 2014), although there are studies that do not confirm it (Cappuzzo et al., 2005; Tsao et al., 2006). Of note, the association of EGFR mutations at least in exon 19 with gene amplification occurs in a subset of patients (Bethune et al., 2010). Moreover, these genetic lesions are often accompanied by increased EGFR ligand production that further boosts EGFR signaling pathway (Sigismund et al., 2018). Hence, EGFR status has been exploited therapeutically by developing EGFR-directed therapies that are currently employed for the treatment of several types of cancer including non-small cell lung cancer (NSCLC), colorectal carcinomas and breast carcinomas (Table 1).

Long ago it was demonstrated that up-regulation of EGFR expression in NSCLC is related with intense and abundant EGFR immunostaining (Gorgoulis et al., 1993). However, unlike Her2/Neu there are several limitations in translating EGFR results obtained from IHC into clinical practice. Although a significant percentage of patients with strong EGFR immunostaining have a survival benefit from EGFR targeted therapies, there are certain cases with EGFR negative status that exhibit a complete response to anti-EGFR therapy rendering the utility of IHC/ICC rather controversial (Bethune et al., 2010; Pirker, 2012; Hutchinson et al., 2015). The latter can be attributed: (i) to the fact that conventional antibodies do not recognize all mutant EGFRs and (ii) to the heterogeneity of EGFR overexpression in the tumor. Moreover, the presence of activating mutations in downstream effectors like KRAS, BRAF and NRAS allow autonomous signaling independent of EGFR activation, which in turn negatively predicts efficient response to anti-EGFR therapy (Hsu et al., 2016; Mondaca and Yaeger, 2018). Additionally, an interesting understudied issue relates to the effect of subcellular localization of EGFR (membranous versus cytoplasmic) as a potential prognostic biomarker for anti-EGFR therapy (Petersen et al., 2017). Although it is widely reported for the membranous staining, cytoplasmic localization of EGFR seems to be rather prominent in predicting poor outcome in pancreatic and thyroid cancer (Hutchinson et al., 2015), suggesting that exploiting *in situ* assays to assess EGFR subcellular staining pattern may have a predictive utility.

ALK

In 1994 the characterization of the chromosomal translocation t(2;5) (p23;q35) in an anaplastic lymphoma cell line, revealed a unique rearrangement leading to the fusion of the amino terminus of nucleophosmin (NPM) to the catalytic domain of a previous unidentified protein called Anaplastic Lymphoma Kinase (ALK) (Morris et al., 1994). ALK is a type I transmembrane tyrosine kinase receptor belonging to the insulin receptor superfamily. It is normally expressed in several tissues, most abundant in neurons, whereas it is physiologically silent in lymphoid cells. ALK signaling is activated in several cancers driving malignant progression through three principle mechanisms: a) ALK rearrangements triggering the tyrosine kinase domain as evidenced by NPM-ALK and EML4 (echinoderm microtubule-associated protein-like 4)-ALK, b) gene amplifications, and c) activating point mutations including three hot-spot mutations at residues F1174, F1245 and R1275 (Bayliss et al., 2016). Since 1994 several ALK rearrangements activating the tyrosine kinase domain have been described in a variety of tumors apart from anaplastic large cell lymphoma (ALCL), including NSCLC, neuroblastoma and inflammatory myofibroblastic tumor (IMT) (Holla et al., 2017). ALK fusions account for approximately 5% of patients with NSCLC, mainly identified in young, never- or light ex-smokers with adenocarcinoma-type mainly with acinar, signet-ring, papillary and to a lesser extent solid pattern, where EML4-ALK is the most prevalent (Shaw et al., 2009; Inamura, 2018; Kometani et al., 2018). The

presence of EML4-ALK positive tumors are often mutually exclusive for EGFR, KRAS and TP53 mutations suggesting that ALK positive cases form a distinct entity (Inamura et al., 2009). On the other hand more than 50% of cases with ALCL exhibit ALK rearrangements. Interestingly, the various ALK fusion proteins exhibit different oncogenic potential *per se* (Hallberg and Palmer, 2016).

The high benefit cost ratio of IHC over fluorescent *in situ* hybridization (FISH) renders the former the method for choice for the detection of ALK expression (Conklin et al., 2013; To et al., 2013; von Laffert et al., 2014) (Table 1). Within this context given the practical value in routine practice of cytological specimens, a high accuracy of ICC for the detection of ALK status in NSCLCs has been demonstrated (Savic et al., 2013; Zito Marino et al., 2017). Of note, the ALK staining pattern is related with the ALK fusion partner. Accumulating evidence demonstrates that different fusion proteins are related with distinct subcellular localization, which in turn may affect the downstream signaling pathway and finally the clinical outcome. In ALCL, NPM-ALK fusion proteins exhibit nuclear and cytoplasmic immunostaining, whereas TPM3 (Tropomyosin 3)-ALK fusion products have cytoplasmic and membranous staining (Zeng and Feldman, 2016). In a cohort of patients with ALK positive Large B Cell Lymphoma (LBCL), a rare subtype of lymphomas, granular cytoplasmic staining was related with a superior overall survival versus non-granular staining (Sakamoto et al., 2016). The US Food and Drug Administration (FDA) has approved an IHC assay employing the D5F3 clone antibody as a companion diagnostic kit for crizotinib in the treatment of ALK-rearranged NSCLC patients (Mino-Kenudson, 2017) (Table 1). Within this frame intense granular cytoplasmic ALK staining is considered positive (To et al., 2013; Zwaenepoel et al., 2014). Notably a small proportion of neurodoendocrine lung carcinomas may exhibit ALK positivity despite being absent of ALK rearrangements (Nakamura et al., 2013). On the other hand, NSCLC patients harboring KIF5 (Kinesin-related protein 5)-ALK fusion, exhibit ALK perinuclear halo immunostaining pattern, which should also be taken into consideration during ALK evaluation (Takeuchi et al., 2009). Of note, multiple reports have demonstrated the efficacy of ALK inhibitors in IMT and ALCL (Butrynski et al., 2010; Richly et al., 2015; Lin et al., 2017).

ROS1

ROS1 (c-ROS oncogene 1) is a tyrosine kinase receptor with high homology to ALK encoded by 6q22.1 (Bubendorf et al., 2016; Uguen and De Braekeleer, 2016). ROS1 is aberrantly expressed in a subset of cancers. Initially, it was reported the presence of ROS1 rearrangement in glioblastoma multiforme leading to a constitutive active kinase activity (Birchmeier et al., 1987; Charest et al., 2003). Since then ROS fusions have been detected in NSCLC, cholangiocarcinoma, gastric cancer, ovarian cancer, colorectal cancer and angiosarcoma (Rikova et al., 2007; Gu et al., 2011; Bergethon et al., 2012). ROS1 rearrangements are found in 1-2% of patients with lung adenocarcinoma, dictating a subset of NSCLC patients who will benefit from treatment with the FDA-approved protein kinase inhibitor called crizotinib. Similar to ALK fusions, ROS1 rearrangements are frequently observed in young never or light smokers (Subramanian and Govindan, 2013). Interestingly, most adenocarcinomas with ROS1 rearrangements exhibit solid, micropapillary, cribriform and signet ring morphology (Viola et al., 2016). The frequency of coexisting ALK and ROS1 rearrangements is very rare (Song et al., 2017), suggesting that ALK and ROS1 positive cases represent unique clinical entities.

The subcellular localization of ROS1 is highly dependent on the fusion partner of ROS1. Lung adenocarcinomas with CD47-ROS1 fusions exhibit granular cytoplasmic immunostaining, while membranous immunoreactivity has been found in cases with EZR-ROS1 fusion (Yoshida et al., 2014). Within this context, several studies have clearly demonstrated that IHC can become an initial screening assay for the detection of ROS1-rearranged in NSCLC patients (Liu et al., 1995; Liang et al., 2010; Boyle et al., 2015; Viola et al., 2016; Selinger et al., 2017) (Table 1). Given the rarity of ROS1 positive cases, and the fact that IHC is less expensive and time-consuming than FISH or next Generation sequencing (NGS), implementing IHC should be considered a starting point followed by FISH confirmation. Besides, given that the diagnosis of NSCLC is often based on cytological specimens, assessment of ROS1 status employing ICC has also been implemented in NSCLC cases demonstrating high concordance with IHC (Bubendorf et al., 2016; Pisapia et al., 2017).

DLL3

Delta-like protein 3 (DLL3) is an inhibitory ligand for Notch receptors that is overexpressed in more than 80% of small cell lung cancer cancers (SCLC) (Sabari et al., 2017). DDL3 appears to act downstream of achaete-scute homologue 1 (ASH-1) (Karachaliou et al., 2016). ASH-1 plays an important role in pulmonary neuroendocrine differentiation and there is strong evidence supporting its capacity to driving tumor initiation in SCLC. Hence, DLL3 could also contribute to SCLC oncogenesis. Indeed, preclinical studies in SCLC settings (Saunders et al., 2015) and phase 1 clinical studies in recurrent SCLC (Rudin et al., 2017) show anti-tumor activity of rovalpituzumab tesirine (Rova-T). Rova-T is a novel first-in class DLL3 monoclonal antibody conjugated to a DNA-damaging pyrrolobenzodiazepine (PBD) dimer toxin that binds to DLL3 expressed on the cell surface enabling targeted therapy for SCLC (Baize et al., 2017). The status of DLL3 in the first-in-human, first-in-class phase 1 trial

was assessed by IHC, classifying patients as DLL3-high and DLL3-low when tumor cells expressed cytoplasmic or membranous staining in at least 50% and fewer that 50% respectively (Rudin et al., 2017). A significant disease control for DLL3-high versus DLL3-low patients was observed. However since several DLL3-low patients exhibited a favorable outcome, future employment of a lower companion diagnostic cut-off below 50% may expand the number of patients having a clinical benefit.

IDH1, ATRX, MGMT

The impact of isocitrate dehydrogenase 1 (IDH1), alpha thalassemia/mental retardation syndrome X-linked (ATRX) and O6-methylguanine-DNA methyltransferase (MGMT) status in guiding current therapeutic approaches in gliomas is highly appreciated (Salles et al., 2011; Weller et al., 2017).

Wild type IDH1 is encoded by *IDH1* gene mapped to 2q33.3 and catalyzes in the cytosol and peroxisomes the oxidative decarboxylation of isocitrate leading to α-ketoglutarate, NAD(P)H and CO_2. Advances in cancer genetics revealed the identification of mutations in *IDH1*, most commonly missense mutations leading to substitution of the Arginine residue at position 132 including IDH1-R132H in gliomas and IDH-R132C in acute myeloid leukemia (AML) which are by far the most common alterations (Ichimura et al., 2009; Mardis et al., 2009). Mutations of Arginine 132 residue reside in the active site of IDH1 resulting in neomorphic activity that promotes in turn the conversion of α-ketoglutarate to D-2-hydroxyglutarate (D-2HG) along with the oxidation of NADPH. Accumulation of D-2HG leads to epigenetic deregulation due to aberrant histone and DNA methylation patterns favoring hypermethylation. Besides, disruption of [NAD(P)+/NAD(P)H] levels has functional consequences on cellular homeostasis. Notably, patients with glioma harboring IDH1 mutations have a more favorable prognosis in comparison with those with wild type IDH1 (Miller et al., 2017).

ATRX is an X-linked gene encoded by *ATRX* gene located at Xq21.1. It is part of the SNF2 (SW1/SNF2) family of DNA helicases involved in chromatin remodeling and telomere maintenance. It interacts with the transcription cofactor Death Associated Protein 6 (DAXX), forming a complex that possesses an ATP-dependent translocase activity mediating the deposition of H3.3 at telomeres and DNA pericentric repeats (Nandakumar et al., 2017). ATRX is frequently inactivated in gliomas (86%), due to mutations and to a lesser extent to deletions and fusions (Cancer Genome Atlas Research Network et al., 2015). Among IDH mutated gliomas with no loss of 1p/19q, absence of ATRX is related with improved clinical outcome (Karsy et al., 2017).

MGMT is a DNA repair protein that removes alkyl adducts from the O6 position of guanine in DNA restoring guanine to its normal state (Pegg, 2000), thereby blunting alkylating agents' efficacy (Wick et al., 2014). MGMT becomes an important drug resistant factor (Liu and Gerson, 2006). The promoter of MGMT is methylated in 40-50% of patients with glioblastoma leading to decreased transcriptional and protein expression (Shah et al., 2011). The latter has a favorable predictive value, since down-regulation of MGMT is related with a striking benefit in patients with glioblastoma treated with the methylating agent temozolamide (Hegi et al., 2005; Stupp et al., 2005).

Given that IDH1, ATRX and MGMT play a significant role in treatment decision-making of patients with glioma (Karsy et al., 2017), it is necessary to assess their profile. IDH-R132H, referring to the most frequent mutation, and ATRX status are evaluated by performing immunohistochemistry (Weller et al., 2017). A significant concordance occurs between the presence of R132H mutation (c.395G>A) and immunopositivity employing a mouse monoclonal antibody recognizing the R132H mutated form (Capper et al., 2010; Mellai et al., 2011). Immunoreaction for IDH1-R132H scores positive when tumor cells exhibit strong cytoplasmic immunoreactivity (Weller et al., 2017) (Table 1). The mitochondrial IDH2 isophorm is also examined, although mutations in *IDH2* are much less common than in IDH1 (Miller et al., 2017). As for ATRX, nuclear immunostaining in cancer cells denotes intact ATRX status. On the other hand, loss of ATRX presents with lack of nuclear immunoreactivity in tumor cells while the surrounding stroma stains positive for ATRX (Reuss et al., 2015) (Table 1). Regarding the assessment of MGMT, IHC has been extensively employed, considering uniform nuclear immunostaining as positive. Although, MGMT immunoreactivity should reflect the methylation status of MGMT promoter, discordance between IHC results with the methylation status prevents its implementation in routine practice (Mason and McDonald, 2012; Tanboon et al., 2016). The lack of correlation of MGMT immunostaining with the methylation analysis can be attributed to the following parameters: a) the contamination from non-neoplastic cells during assessment of the methylation status can affect the analysis leading to false-positive results due to the expression of MGMT by normal glia and infiltrating lymphocytes, b) even with a standardized cut-off point for MGMT immunopositivity there is often significant intra-observer variability in MGMT evaluation, c) the differences among the MGMT clones employed for IHC analysis suggests the necessity for a systematic approach on this issue, d) the expression of MGMT may be determined by additional epigenetic factors. Therefore, IHC is not acceptable for the examination of MGMT status in clinical practice (Wick et al., 2018) (Table 1).

MLH1, MSH2, MSH6, PMS1, PMS2

MutL Homolog 1 (MLH1), MutS Homolog 2 (MSH2), MutS Homolog 6, PMS1 Homolog 1 (PMS1) and PMS1 Homolog 2 (PMS2) encoded by 3p22.2, 2p21, 2p16.3, 2q32.2 and 7p22.1 locus respectively are

DNA mismatch repair (MMR) proteins (Arzimanoglou et al., 1998). Somatic or germline mutations, along with epigenetic silencing in MMR genes increase the spontaneous rate of mismatch mutations (Liu et al., 1995; Wheeler, 2005). Repetitive DNA sequences and particularly microsatellite sequences are highly susceptible to MMR defects during DNA replication leading to microsatellite instability (MIN). Inherited mutations in *MLH1* and *MSH2*, followed by *MSH6* and *PMS2* is a hallmark of Lynch syndrome alternatively called hereditary nonpolyposis colorectal cancer (HNPCC), to emphasize the lack of multiple colonic polyps. HNPCC is characterized by increased susceptibility to the development of MIN positive malignancies including colorectal, endometrial and ovarian carcinomas (Sehgal et al., 2014). Besides, Muir-Torre syndrome and Turcot's syndrome are two less common inherited conditions than HNPCC that are also associated with germline mutations in MMR genes and increased incidence of MIN positive cancers.

Approximately 15% of sporadic colorectal carcinomas exhibit MIN that is mainly attributed to promoter hypermethylation of MLH1 and to a lesser extent to somatic mutations in MMR genes (Wheeler, 2005). While epigenetic inactivation of MLH1 is responsible for MIN in sporadic colorectal cancers, the majority of MIN positive colorectal cancers associated with HNPCC follow a different route exhibiting allelic loss and mutations in MMR genes (Wheeler, 2000). Colorectal carcinomas associated with MMR defects are usually right-sided; they have a mucinous component or exhibit signet ring cell phenotype, accompanied by Crohn's-like peritumoral lymphoid reaction and prominent intratumoral lymphocytic infiltrate (Alexander et al., 2001). The latter implies that MIN positive cancers elicit an immunological response rendering them good candidate targets for immunotherapy (analyzed below). Of note, survival data from patients with colorectal carcinoma suggests that MIN high profile (exhibiting instability in more than 30-40% of the microsatellite loci tested) is an independent factor of favorable prognosis (Lawes et al., 2003; Pawlik et al., 2004).

Analysis of MMR status employing immunohistochemistry is widely performed in routine practice (Kawakami et al., 2015) (Table 1). Positive staining is characterized by unequivocal nuclear immunopositivity of cancer cells. A major advantage of IHC analysis is that the absence of staining of a particular MMR component can direct the genetic analysis. To this end, absence of MMR immunostaining exhibits a high concordance with genetic-based MSI analysis (Kawakami et al., 2015). Of course the analysis of MMR proteins employing IHC along DNA testing for MSI are complimentary (Poulogiannis et al., 2010). Within this context, several findings support that MMR deficient or MSI positive cases with advanced colorectal cancer benefit from anti-PD-1 immunotherapy (Le et al., 2017; Overman et al., 2017) (Table 1). Besides, stage II colorectal cancer patients with MMR deficiency do not benefit from Fluorouracil (5-FU)-based adjuvant therapy (Kawakami et al., 2015) (Table 1). Hence, awareness of the MMR status may help us to substitute toxic for more targeted therapies.

PD-1 and PD-L1

Blocking selectively Programmed Death Ligand 1 (PD-L1) - PD-1 axis has reformed systemic cancer therapy (Postow et al., 2015). PD-1 (CD279) is a 288 amino acid protein encoded by 2q37.3 and expressed on activated T cells, naïve and activated B lymphocytes, NK cells, monocytes, dendritic cells and on a significant proportion of tumor infiltrating lymphocytes (TILs) (Keir et al., 2008). The two ligands for PD-1 are PD-L1 (CD274; B7-H1) and PD-L2. PD-L2 has a more restricted expression pattern, whereas the former is widely expressed. PD-L1 is encoded on 9q24.1 and is found on a wide range of hematopoietic cells including T, B lymphocytes, macrophages, dendritic cells and non-hematopoietic cells, such as endothelial cells, keratinocytes, astrocytes, corneal cells and placenta synciotrophoblast (Pardoll, 2012). Under physiological conditions the PD-L1/PD-1 axis plays a key role on the maintenance of self-tolerance by restraining autoimmunity and therefore preserving immune homeostasis. The latter translates into an efficient immune escape mechanism within the cancer microenvironment. Within this frame, PD-L1 is up-regulated on the cell surface of cancer cells of solid tumors and hematologic malignancies. Besides, PD-1 is expressed in a significant proportion of Tumor Infiltrating Lymphocytes (TILs). Hence, the elevated expression of both PD-L1 and PD-1 favors in turn tumor escape from host immunosurveillance (Pardoll, 2012). As expected, blockade of PD-L1/PD-1 axis can induce durable remissions. Indeed a number of immune checkpoint inhibitors targeting PD-L1 or PD-1 are in clinical development in various phases and during the past five years several of them have entered into routine practice (Kotsakis and Georgoulias, 2017; Malhotra et al., 2017; Martin-Liberal et al., 2017; Rijnders et al., 2017). PD-1 and PD-L1 checkpoint inhibitors are being evaluated in ongoing clinical trials and have been clinically approved for the first (1^{rst}) and second (2^{nd}) line treatment of several cancers including: melanoma, NSCLC, head and neck squamous cell carcinoma (HNSCC), urothelial carcinoma, Renal cell carcinoma (RCC), Hodgkin's Lymphoma, Merkel cell carcinoma, cervical carcinoma, gastric and GEJ adenocarcinoma, hepatocellular cancer along with microsatellite instable (MSI-H) or MMR deficient (dMMR) solid tumors including MSI-H/dMMR colorectal carcinoma (Gong et al., 2018; Sun et al., 2018). For clinical practice two anti-PD-1 inhibitors, namely pembrolizumab and nivolumab and three anti-PD-L1 inhibitors, specifically atezolizumab, durvalumab and avelumab have been approved (Gong et al., 2018).

The expression of PD-L1 status on cancer cells as well on tumor infiltrating immune cells employing IHC is currently the most effective biomarker to examine the response to anti-PD-L1/PD-1 therapy (Table 1) (Herbst et al., 2014; Sun et al., 2018). Although it is still a matter of controversy, patients with PD-L1 positive tumors have a higher response rate to PD-L1/PD-1 blockage and overall survival compared to PD-L1 negative tumors (Sun et al., 2018). Collectively the FDA-approved PD-L1 companion/complementary diagnostic IHC assays are the following: a) 22C3 pharmDx (Dako-Agilent), b) 28-8 pharmDx (Dako-Agilent), c) SP142 (Ventana-Roche), d) SP263 (Ventana-Roche), e) 73-10 (Dako-Agilent) (Gong et al., 2018) (Table 1). For PD-L1 evaluation partial or complete linear membrane immunostaining on cancer cells along with cytoplasmic and or membrane staining for lymphocytes and macrophages is considered positive PD-L1 staining and is scored. An assay-specific scoring is presented in Table 1, even though in certain settings there is still a lack of approved evaluating system. Specifically, the IHC testing for both SP263 and 73-10 based assay is not approved yet, although certain cut-offs are provided based on the available literature data (Scheel and Schäfer, 2018). Within this frame, subgrouping according to PD-L1 IHC status predicts response rate. Hence, higher clinical PD-L1 cut-offs refer to a superior overall response rate to a particular agent versus lower one. Of note, inter-assay variability when scoring cancer cells for NSCLC can be minimal, whereas there is poor concordance when scoring immune cells (Hirsch et al., 2017; Rimm et al., 2017). However, based on the existing data a thorough examination is pertinent before utilizing different assays interchangeably, at least in NSCLC setting (Marchetti et al., 2017; Mino-Kenudson, 2017; Munari et al. 2018). Besides, there are various cut-offs according to the IHC assay which should be taken into consideration before drawing firm conclusions. Notably PD-L1 assessment in cytological specimens is not recommended yet, even though high concordance has been demonstrated between cytological specimens and matched histological samples (Mino-Kenudson, 2017).

Future perspectives

As we move into the era of personalized treatment, the oncology community is witnessing major advances in translating basic research into routine practice. Of multiple testing methods, *in situ* assays including IHC and ICC are extensively used providing meaningful data in a rapid and cost effective way. Survival data from clinical trials are shaping IHC cut-offs and scoring, establishing the recommendations and guidelines for selection of cancer patients for treatment with targeted therapies. In such a rapidly evolving era we expect that novel biomarkers monitoring the different hallmarks of cancer along with the identical stress phenotypes (Luo et al., 2009; Negrini et al., 2010; Hanahan and Weinberg, 2011) will be introduced into clinical practice. Within this background, below we give emphasis to novel biomarkers that can be assessed with traditional *in situ* platforms and translated into major clinical advances in oncology.

TP53 (encoding for the p53 protein) is considered the most commonly mutated gene in human cancer (Yue et al., 2017). Since the presence of p53 mutations in various histotypes has implications on prognosis and treatment (Moreira et al., 2015; Robles et al., 2016) - detecting such mutations became of great clinical importance. While using sequencing methods may provide an accurate mutational spectrum, IHC-based approaches are still commonly used as a surrogate for mutational analysis (Bellizzi, 2013). Importantly, the wild-type form of the p53 protein is maintained in low cellular levels and considered relatively unstable and therefore typically undetectable using IHC. In contrast, most mutant p53 proteins have a much longer half-life, resulting in nuclear accumulation suitable for detection using IHC (Schon et al., 2002; Alsner et al., 2008). The mutation status of p53 may be used clinically to predict outcome across several tumors as well as within a given histotype (Salinas-Sánchez et al., 2007; Bellizzi, 2013; Köbel et al., 2019). In Endometrial carcinomas, for example, diffuse staining of more than 80% of tumor cells will have indications regarding prognosis but also be affiliated with high grade serous carcinoma, where p53 mutations are a founder event (Chen et al., 2017). The fact that mutations in p53 are so predominant makes them a desirable target for therapy. Several identified small molecules reactivating specific "hot-spot" missense mutant p53 have been found to restore the wild-type conformation and induce tumor cell death. Two of such compounds are currently being tested in clinical trials (Bykov et al., 2018; Schulz-Heddergott and Moll, 2018), suggesting that positive results will affect future clinical decision making based on p53 status. While current p53 IHC is limited to the detection of nuclear accumulation and cannot differentiate between specific mutants, recent novel antibodies have been developed to target epitopes uniquely found in three gain-of-function mutants of the gene (Hwang et al., 2018). Since the R175H, R248Q and R273H positions are amongst the most prevalent spots of mutations in TP53 and also proven to gain oncogenic functions (Sabapathy and Lane, 2018), uniquely detecting these mutant proteins using IHC could be integrated into clinical diagnosis (Fig. 1).

An interesting aspect of traditional histochemical techniques is their capacity to sustain the development of more advanced analytical or diagnostic methodologies that are based on the same principles. Small molecule reagents with high affinity for specific molecular targets have been extensively used in the past for staining tissue or cellular samples, thus facilitating the establishment of diagnostic protocols with particular success and broad limits of applicability (Veuthey, 2014). In most instances, such molecules are excessively hydrophobic

as a result of their structure, which is characterized by extended aromatic or conjugated heterocyclic systems that usually contain nitrogens. Such an electronic arrangement gives rise to easily excitable chromophores that are used as a readout for the histochemical analysis. Molecules that belong to these classes offer excellent opportunities for developing sophisticated chemical tools that are more compatible with current state-of-the-art instrumentation and can be integrated into present day labs toward the establishment of analytical methods that are more sensitive, more accurate and more high-throughput with respect to the original methodologies they evolved from. A great example of utilizing an old-fashioned dye for developing a cutting-edge immunohistochemical method is the SenTraGor® reagent (Evangelou et al., 2017). In this case, Sudan Black-B (SBB), a stain developed more than 80 years ago has been successfully used as a lead for designing a bivalent molecule that can identify aged cells and quantify cellular senescence with high accuracy. The reagent was developed by coupling the SBB scaffold with biotin through a flexible linker. This modification enables detection of lipofuscin, the main by-product of cellular senescence through the use of a secondary streptavidin-based antibody. The use of SenTraGor® permits the establishment of simple and efficient assays based on immunochemistry for monitoring a cellular response of high clinical but also biological importance for which, notably, detection was particularly challenging until SenTraGor® discovery. Given that lipofuscin is considered as a biomarker of cellular senescence, this novel reagent may drive treatment with a new class of drugs triggering the death of senescent cells, called senolytics (Myrianthopoulos et al., 2018). SenTraGor® can be employed first to predict therapeutic response and second to monitor the efficacy of senolytics thus allowing for a personalized therapeutic approach (Fig. 1). It is reasonable to anticipate other analogous chemical tools based on already known reagents to emerge in the future expecting to open new routes to a field of research where disciplines such as histology, chemical biology and synthetic chemistry integrate toward a common endeavor.

So far it is clear that *in situ* techniques play a major role in dictating modern oncological treatments, however it is obvious that the potential of these assays is just beginning to unfold and new biomarkers will develop based on the following: a) the rewiring of DNA repair processes that takes place during cancer development favoring error prone at the expense of error free mechanisms (Galanos et al., 2018); assessment of RAD52 versus RAD51 employing quantitative immunofluorescence analysis (QIBC) will open a new dimension in the visualization of tumor dynamics (Fig. 1) b) given that components of the DNA damage response and repair (DDR/R) machinery predict the

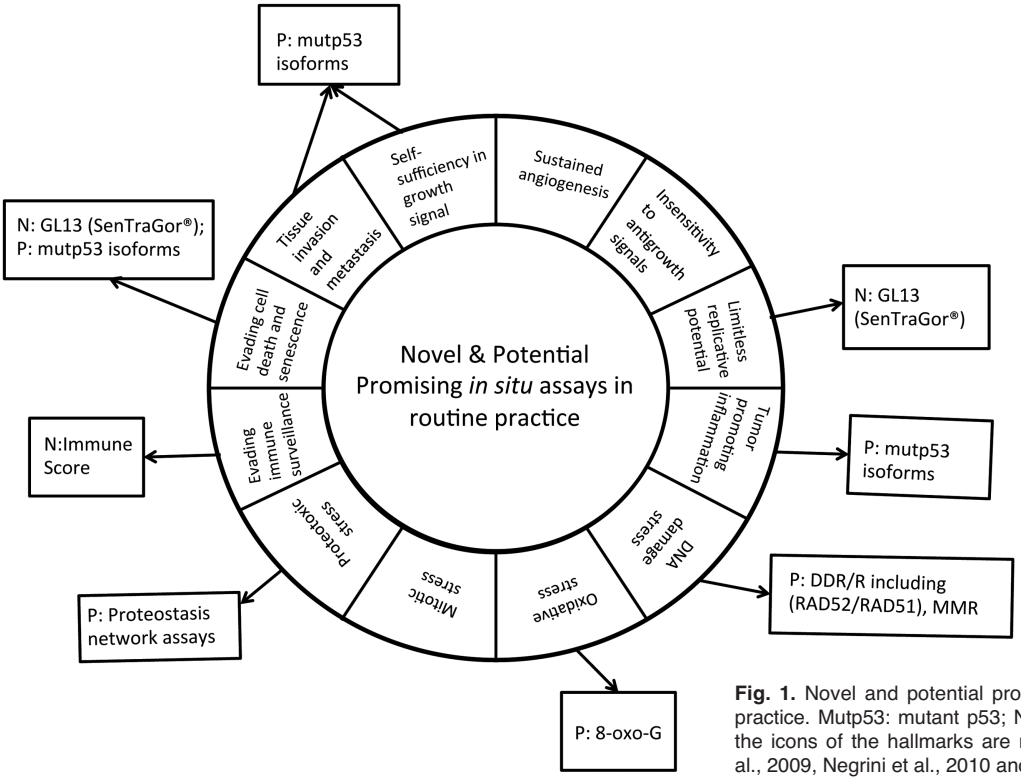

Fig. 1. Novel and potential promising *in situ* assays in routine practice. Mutp53: mutant p53; N: Novel; P: Potential promising; the icons of the hallmarks are modified icons based on Luo et al., 2009, Negrini et al., 2010 and Hanahan and Weinberg, 2011.

response to PD-L1 blockade (Wang et al., 2018) apart from MMR, additional proteins involved in homologous recombination and base excision repair will complement PD-L1 assessment (Fig. 1), c) the reprogramming of key molecular/metabolic pathways that results in an altered cellular homeostatic state often characterized by elevated stress levels (Luo et al., 2009; Gorgoulis et al., 2018); evaluating stress overload directly or indirectly reveals potential ways to sensitize cancer cells, i.e. the examination of 8-oxo-G immunostaining status may predict the response to MutT Homolog 1 (MTH1) inhibitors (Warpman Berglund et al., 2016) or the assessment of proteotoxic stress can be a marker for the efficacy of proteostasis network inhibitors (Sklirou et al., 2018) (Fig. 1) d) the crosstalk between cancer cells and the surrounding microenvironment (Chatzinikolaou et al., 2014; Pateras et al., 2015; Nakad and Schumacher, 2016); the immune score based on the evaluation of cytotoxic T lymphocytes (Pagès et al., 2018) can supplement along with DDR/R factors PD-L1 testing (Fig. 1).

Collectively, the impact of molecular pathology in oncology is ongoing and will increase in the near future, by further dictating the assays of biomarkers and encouraging the precision medicine approach in cancer.

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