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Review

Fluorescence *in situ* hybridization (FISH)-based companion diagnostics in oncology

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ABSTRACT

Companion diagnostics are integral to the application and success of personalized medicine. In oncology, many companion diagnostics are molecular-based, including a number of fluorescence *in situ* hybridization (FISH) based companion diagnostics. Here, we review the currently approved FISH based companion diagnostics and evaluate several new FISH assays as potential companion diagnostics.

KEYWORDS: companion diagnostic, personalized medicine, fluorescence *in situ* hybridization, FISH, oncology, research

INTRODUCTION

Personalized medicine is the customization of healthcare − with medical decisions, practices, and/or products being tailored to the individual based on a patient's unique clinical, genetic, genomic, and environmental information. This practice results in significant improvements in outcomes with a subsequent long term reduction in health care costs. Perhaps, the most impressive applications of personalized medicine have been in the treatment of cancer, where the last few decades have seen significant advances in personalized treatment resulting from the sophistication of genomic analysis and the development of "targeted" therapeutics. First, advances in genomic analysis have shown that common tumors such as breast cancer are, in fact, a heterogeneous mixture of molecular genotypes. Second, "targeted" therapeutics that inhibit specific genetic pathways have become more prevalent [1-3]. The combination of these two scientific developments has allowed physicians to take a new pharmacogenomic approach, in which the evaluation of genetic variations of each patient may predict how they will likely respond to a particular therapy [4]. Overall, this approach has had a significant impact on patient stratification, determination of prognosis, and selection of treatment [5]. In general, most cancer treatments benefit only a minority of patients to whom they are administered [6]. Therefore, using genetic analysis to stratify likely responding patients for treatment before it is initiated targets those patients at the outset. This can also reduce the number of adverse effects experienced by those trialed on other, potentially ineffective treatments. Kongkaew *et al.* [5] estimated that more than 5% of hospital admissions are associated with adverse reactions to prescribed drugs. Many of these are due to individual genetic differences that render one hypersensitive to the drug, or unable to metabolize it properly [7].

Integral to personalized medicine is the concept of a companion diagnostic, or the development of diagnostic tests to deliver the essential genetic information that identifies those patients for which a specific diagnosis or treatment may be appropriate. One use of companion diagnostics is to identify patients who will (or will not) respond to a particular drug prior to its administration, or identify those who should not be treated with the drug because of a high risk for adverse events [8].

Companion diagnostics in the oncology sector are primarily molecular-based, with several recent

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approvals being fluorescence *in situ* hybridization (FISH) assays [9], Figure 1. FISH is a more reliable, reproducible, sensitive, and accurate procedure which is less affected by tissue fixation and analytical variables compared to immunohistochemistry, for example. It also offers the benefit of simultaneous evaluation of morphology and gene amplification. This review will specifically discuss the therapeutic products and their FISH-based companion diagnostics currently required by the FDA, as well as prospective FISH-based assays that could, or are currently being co-developed with promising cancer drugs (Table 1).

Relevant FISH-based companion diagnostics in oncology

Breast cancer

The human epidermal growth factor receptor 2 gene (*HER2*) is localized to chromosome 17q and encodes

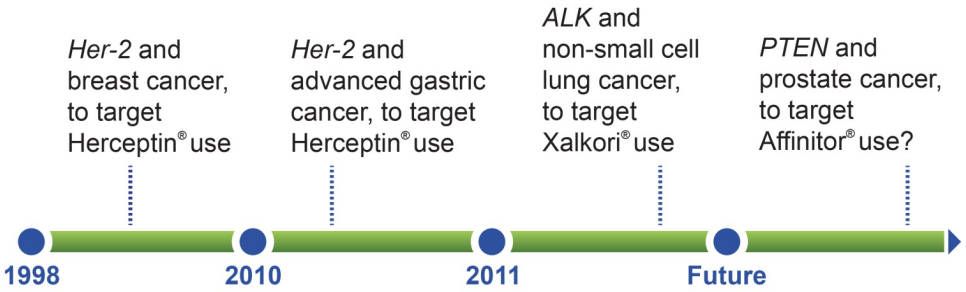

Figure 1. Timeline of FDA approvals of key FISH-based companion diagnostics in oncology.

Table 1. Examples of FISH-based companion diagnostics required by the US FDA and future prospects for companion diagnostic and drug therapy co-development.

Cancer therapy	Indication(s)	FISH companion diagnostic	US FDA status
Herceptin® (trastuzumab) Tykerb® (lapatinib)	Overexpression of *HER2* in metastatic breast tumor cells	Single probe Roche Ventana Inform™ Dual probe Abbott Pathvysion™ Dako Cytomation Her2 PharmDx™	Required
Herceptin® (trastuzumab) Tykerb® (lapatinib)	Overexpression of *HER2* in metastatic gastric cancer tumor cells	Single probe Roche Ventana Inform™ Dual probe Abbott Pathvysion™ Dako Cytomation Her2 PharmDx™	Required
Xalkori® (crizotinib)	*ELM4-ALK* translocation-positive advanced or metastatic non-small-cell lung cancer	Abbott Vysis *ALK* Break-Apart FISH Probe™ Kit	Required
Affinitor® (everolimus) ridaforolimus, bicatulamide	*PTEN* deletion in prostate cancer	Examples are: Abbott Vysis *PTEN*/CEP10 FISH Probe™ Kit, CymogenDx PTEN-del-TECT™ Four Color Panel	Unapproved
EPZ-5676	11q23 rearrangements in lymphoid/myeloid leukemias	Abbott Vysis LSI MLL Dual Color Break-Apart™ Probe kit	Unapproved
TBD	3q26 amplification in cervical cancer	Ikonysis oncoFISH™ Cervical Test	Unapproved

a transmembrane tyrosine kinase receptor. Amplification and/or overexpression occur in approximately 15-20% of invasive breast carcinomas, and are associated with earlier recurrence, shortened disease free survival, and poor prognosis [10-13]. Thus, analysis of the status of HER2 has emerged as a critical prognostic and predictive factor, and has become the standard of care for patients presenting with breast cancer. Trastuzumab (Herceptin®), a "humanized" monoclonal antibody, targets the extracellular domain of HER2 and is widely used in the management of HER2-positive breast cancers [14-16]. It has been shown to confer a significant survival benefit in the treatment of women with advanced or metastatic HER2+ breast cancer [12, 13]. Herceptin® was FDA-approved in 1998 and today is a standard of care in both the adjuvant and metastatic settings [17].

Because accurate assessment of HER2 is critical in the management of breast cancer, methods such as FISH have regularly been used in *HER2* assays, particularly in the setting of equivocal immunohistochemistry results. Although FISH analysis is more expensive and time consuming than IHC, numerous studies have concluded that this cost is justified by the increased accuracy and more precise use of anti-HER2 targeted therapies [18]. Three versions of the FISH assay are FDA approved. The single probe Ventana Inform™ test (Ventana Medical Systems Inc., Tuscon, AZ, USA) that measures only *HER2* gene copies is approved as a prognostic test. The two dual probe (*HER2* gene probe plus the chromosome 17 centromere probe [CEP 17]) kits, the Pathvysion™ (Abbott Molecular, Downer's Grove, IL, USA) test and the Dako Cytomation Her2 PharmDx™ test (Dako North America Inc., Carpinteria, CA, USA) are approved for the selection of patients for trastuzumab-based therapies. In 2007, the American Society of Clinical Oncology (ASCO) and the College of American Pathologists (CAP) created a set of joint guidelines for the laboratory evaluation of HER2 status [19]. They recommend either using IHC assays for initial evaluation of HER2 status followed by reflex testing by FISH for some IHC categories (i.e. 2+) or utilization of FISH in initial testing. Based on studies showing that FISH is the most accurate commercially available assay method, rather than IHC [20, 21], a number of laboratories, including the Breast Cancer International Research Group (BCIRG) and the Cleveland Clinic have selected FISH as the primary method for assessing *HER2* gene status of breast cancer patients being screened prospectively for adjuvant and metastatic Herceptin-based therapy [18, 22, 23].

Recently, bright-field *in situ* hybridization techniques such as chromogenic *in situ* hybridization (CISH) and the automated silver-enhanced *in situ* hybridization (SISH) have been introduced for the determination of *HER2* gene status. These new techniques combine features of immunohistochemical analysis and FISH as they use a peroxidase enzyme-labeled probe with chromogenic detection, instead of a fluorescent-labeled probe, allowing results to be visualized by standard bright-field microscopy. Studies have reported a high rate of concordance (> 85%) between FISH and CISH [24-26]. Some advantages of the brightfield ISH methods are that the signals do not decay, so slides may be stored for a long period at room temperature, and they do not require the use of costly equipment such as a fluorescence microscope. However, brightfield ISH techniques are relatively new and have not been broadly established in pathology laboratories. Thus, careful validation, preferably against FISH as the standard, is required when establishing immunohistochemical analysis as the routine test for *HER2* gene status [12].

Gastric cancer

Patients with advanced or metastatic gastric or gastroesophageal junction (GEJ) cancer have few available treatment options and generally poor survival rates. Most patients with gastric cancer present with advanced or metastatic disease with poor 5-year survival rates of approximately 5% to 20% [27, 28]. In most cases treatment is palliative.

Similar to breast cancer, *HER2* gene amplification and protein overexpression is present in 6-35% of gastric cancer or GEJ adenocarcinoma [29, 30]. Therefore, there is continued interest in developing anti-HER2 targeted therapy for this well-known, difficult to treat disease. Unlike breast cancer, however, HER2 overexpression in gastric cancer is often classified as heterogeneous since it was shown that, in up to 30% of patients, there is focal rather than generalized HER2 amplification as assessed by immunohistochemical (IHC) staining of tumor cells [31]. Thus, the prognostic significance

of HER2 status in stomach and GEJ cancers had varied significantly and, in contrast with breast cancer [32], a consensus as to whether gene amplification or protein overexpression was a validated adverse prognostic factor in upper gastrointestinal adenocarcinomas has been difficult to reach [33-35]. In 2010, findings from the Trastuzumab for Gastric Cancer (ToGA) study, a Phase III international randomized controlled trial involving 122 centers in 24 countries, showed that trastuzumab in combination with chemotherapy significantly improved survival of patients with HER2 overexpression or amplification. Thus, trastuzumab was considered as a new standard option for patients with HER2-positive advanced gastric or GEJ cancer [36].

The European Medicines Agency (EMA) label for trastuzumab recommends that IHC be used as the initial testing method for gastric or GEJ cancers. Current National Comprehensive Cancer Network guidelines recommend that 8 to 10 biopsies be taken to allow adequate histologic interpretation of gastric cancers because of the high tumor heterogeneity of HER2 gastric cancer (some biopsy specimens may be HER2-positive, whereas others from the same tumor may not). On the basis of ToGA, patients with tumors scoring IHC 2+ and FISH-positive gained a survival benefit; thus the EMA recommends that samples scoring IHC 2+ should be retested by FISH. If positive (*HER2*:chromosome 17 ratio ≥ 2), patients are eligible for trastuzumab therapy [29]. After overexpression in tumor cells is confirmed, both trastuzumab and chemotherapy are used as first-line treatment, specifically for advanced gastric cancer [30]. The United States Food and Drug Administration (FDA) approval states that patients with HER2-overexpressing tumors are eligible for trastuzumab therapy, according to the definition of HER2-positivity in individual test assays [29]. Accurate and high-quality HER2 testing, using reliable methods such as IHC and FISH analyses, is vital to ensure that patients receive the best possible treatment for their HER2-positive disease with respect to survival benefit and quality of life. To date, HER2 is the only validated therapeutic target in gastric cancer.

Lung cancer

Lung cancer, including non-small cell lung cancers (NSCLC), is the most common invasive cancer and the leading cause of cancer death in the world [37]. Currently more than 70% of lung cancers are advanced or metastatic at diagnosis and have poor prognosis, with a median survival of approximately 12 months using chemotherapy [38]. In 2004, the success of targeted therapy using epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitor (TKI), such as gefitinib and erlotinib, for treatment of EGRF-mutant lung adenocarcinoma has made targeted therapy the most common modality for major human cancers [39-42]. Recently, in search of new and effective targets other than EGFR in lung cancer treatment, Soda, *et al.* [43] identified the echinoderm microtubule- associated protein-like-4 and the anaplastic lymphoma kinase (*EML4-ALK*) fusion gene with transforming ability in NSCLC patients. *ALK* is now recognized as the second most frequent oncogenic driver in NSCLC after the *EGFR* mutations [44, 45].

The *ALK* gene is located on chromosome 2p and encodes an acid receptor tyrosine kinase in the insulin receptor superfamily. Chromosomal rearrangements involving the *ALK* gene are found in 3-5% of non-small cell lung cancer cells [43, 46]. Oncogenic activation of *ALK* occurs in a variety of human malignancies. In most cases it is aberrantly activated due to chromosomal rearrangement, either an intrachromosomal inversion or an interchromosomal translocation [43, 46]. Crizotinib (Xalkori®) is a selective oral inhibitor of ALK. The efficacy of crizotinib in patients with *ALK*-positive advanced NSCLC has been demonstrated in two multi center, multinational, single-arm studies of crizotinib and a recently completed open-label, randomized, multicenter, multinational, phase III study of crizotinib vs. standard-of-care chemotherapy. These studies demonstrated that in patients previously treated with first-line chemotherapy, crizotinib significantly prolonged progression-free survival compared with standard, single-agent chemotherapy [47, 48]. In light of these results, the detection of *ALK* rearrangements is a critical companion diagnostic for the treatment of lung cancer. ALK status can be determined in many ways, including FISH, IHC, and reverse transcriptase PCR (RT-PCR). However, FISH analysis for *ALK* rearrangements, using a break apart probe set is seen as the "gold-standard" for *ALK* testing. In the US, approval of crizotinib for *ALK*-positive NSCLC depends on the result of FISH analysis [45]. Because *ALK* and its most common fusion partner,

EML4, are located close together on chromosome 2, *ALK* FISH is technically challenging. FISH amplification is defined by the separation between the 5′ and 3′ signals of more than 2 signal diameters and at least 50 cells to be counted to ensure 100% sensitivity and specificity [49, 50]. Those found to have *ALK* rearrangements are then selected for treatment by crizotinib (Xalkori®). Noteworthy is the short time line from the original identification of *ALK* gene rearrangements in NSCLC (2007) to the FDA approval of crizotinib for this indication (2011) [51].

Future possibilities

The chromosome 3q26 region and cervical cancer

Uterine cervical cancer is the second most common gynecological malignancy in the world in both incidence and mortality [52]. Even though effective screening programs have lowered the rates of morbidity and mortality in developed nations, a major problem of the cervical screening program is the inability of current, commonly used screening tests (cytology, high-risk HPV panels, and HPV genotyping) to distinguish which low-grade lesions of the cervix are destined to either regress or progress to high-grade lesions or malignancies [52]. Thus, continuous effort has focused on discovering molecular markers that would enhance the efficiency of the screening process. Progression from a precancerous lesion to invasive cervical cancer has been associated independently with three different genetic events: integration of high-risk papillomavirus into the cellular genome [53, 54], accumulation of numerical chromosome aberrations [55-58] and development of genomic instability with a consistent gain of chromosome arm 3q [59-62]. Infection with oncogenic human papillomaviruses (HPVs) is generally considered an initiating factor in the carcinogenesis of the uterine cervix [63, 64]. Although 95% of patients with precancerous lesions harbor oncogenic HPV, only a small fraction of these eventually progresses to invasive carcinoma (invCA) [65]. Therefore, HPV infection alone is not sufficient for malignant conversion, but integration of HPV in the genome is considered the driving factor for invCA. Following integration of HPV into the host genome causing genomic instability, the most recurrent structural chromosomal aberration in cervical cancer is 3q amplification. The smallest consensus region of 3q amplification in cervical cancer was mapped to chromosomal bands 3q26–27, in which the human telomerase RNA gene (*hTERC*) is located. hTERC is the RNA subunit of telomerase that provides telomere stability and regulates telomere length [60]. Additionally, other genes residing in the 3q26 region such as *PIK3CA*, which encodes a catalytic subunit of phosphatydilinositol 3-kinase and is associated with a number of cancer-related functions including apoptosis and cellular growth, have the potential of acting as cervical oncogenes [66]. It was shown that the expression of the *TERC* gene and viral oncogenes increases significantly with histopathological severity of the lesion and was found to mark the transition from high-grade premalignant lesions to invCA. Amplification of 3q, as determined by FISH, increases in copy number as the severity of the cytologic specimen approaches cervical cancer [67]. There are relatively infrequent 3q gains in women with low-grade squamous intraepithelial lesion (LSIL) screening results, but up to 70% of women with high-grade squamous intraepithelial lesions (HSILs) and 100% of women with cervical cancer express the 3q gain [52, 59, 60, 68]. Heselmeyer-Haddad *et al.* [59, 60] reported that targeting the *TERC* gene on chromosome 3q and establishing its copy number in routinely prepared cytological material by means of FISH could serve as a test to determine the progressive potential of individual CIN 2/3 lesions. Women with LSIL cytologic findings have undergone assessment for the 3q gain to predict which will have cervical intraepithelial neoplasia (CIN) 2/3 lesions [61, 69]. Absence of this biomarker can also indicate which women with LSIL cytologic findings are less likely to progress to CIN 2/3 or cervical cancer, and can then be followed more conservatively [67]. Using cervicovaginal liquid-based preparations, a FISH assay to assess for gain of 3q could be used as an adjunct to cytology, particularly for high-risk women, without the use of invasive procedures. The use of 3q26 as persistence-progression indicator has previously been shown [70]. This may not only aid in avoiding unnecessary colposcopies and biopsies, particularly in women who do not present with obvious high-grade dysplasia [71], but could also be used to develop a novel therapy using this genomic status as a companion diagnostic.

The *PTEN* gene and prostate cancer

Prostate cancer is one of the leading causes of cancer mortality in men in the Western world. In the United States it is the most commonly diagnosed cancer in men and second only to lung cancer in the number of male cancer deaths [72]. Prostate cancers display a variable range of clinical behaviors, from slow-growing tumors of little clinical significance to aggressively metastatic and lethal diseases. Current prognostic tools, such as pre-operative prostate specific antigen (PSA) levels, histological Gleason grading, clinical tumor, node, and metastasis (TNM) staging are used to place men in low-, intermediate-, and high-risk prostate cancer risk groupings. However, these prognostic tools often fail to accurately stratify individual patients at early stages of the disease. Given the wide range of clinical outcomes and associated treatments, the main challenge for physicians remains to distinguish indolent from clinically significant tumors. With the goal of improving clinical management of the disease, current efforts are focusing on identifying the genes and understanding the pathways involved in mediating disease progression and treatment resistance.

The primary negative regulator of the phosphatidylinositide 3-kinases (PI3K) pathway is the tumor suppressor phosphatase and tensin homolog gene (*PTEN*). The *PTEN* tumor suppressor gene maps to human chromosome 10q23.3, and this region is known to exhibit high rates of loss of heterozygosity in a variety of human malignancies [73]. However in recent years, it has become evident that relatively large deletions and genomic rearrangements affecting *PTEN* are most prevalent in prostate cancer [74, 75]. FISH analyses have provided a robust evaluation of the genomic status of *PTEN* in prostate cancer. In early studies by Yoshimoto *et al.* [76] analyzing 35 radical prostatectomy specimens showed no *PTEN* deletion in benign glandular epithelium or low-grade Prostatic Intra-epithelial Neoplasia (PIN), while *PTEN* deletions were found in 23% of High-Grade Prostatic Intra-epithelial Neoplasia (HGPIN), a pre-malignant stage of prostate carcinoma, and 68% of overt prostate cancer. The authors concluded that acquisition of a *PTEN* deletion is an important step toward prostatic tumorigenesis [76]. Recently, the development of a novel four-color FISH assay, in which a *PTEN* probe is flanked by *BMPR1A* and *FAS* probes, has significantly improved the specificity and sensitivity of the detection of *PTEN* genomic status compared to the traditional two-color FISH assay [77, 78]. With the improved design of the four-color FISH assay, *PTEN* genomic status can be used as a reliable diagnostic tool and potential companion diagnostic for emerging anticancer drugs such as everolimus, ridaforolimus, or bicatulamide, which are all potent inhibitors of the PI3K signaling pathway [79-83]. Overall, it is clear that the status of *PTEN* is a powerful biomarker that promise effective diagnosis and improved patient stratification and management.

The *MLL* gene and acute lymphoid/myeloid leukemia

Aggressive leukemias arise in both children and adults as a result of rearrangements to the Mixed Lineage Leukemia (*MLL*) gene located on chromosome 11q23. The *MLL* gene encodes a large histone methyltransferase that regulates Hox gene expression through direct promoter binding and histone modification [84, 85]. *MLL* rearrangements occur in 5% to 10% of acute leukemia, and include chromosomal translocations, partial tandem duplication and amplifications, all of which result in hematopoietic malignancies due to sustained HOX expression and stalled hematopoetic differentiation [84, 86]. The vast majority of translocations result in oncogenic fusion proteins in which the native methyltransferase domain is replaced by fusion partner sequences that interact with DOT1L, a histone methyltransferase, in complexes that promote transcriptional elongation [87, 88]. As a result, *MLL*-fusion proteins gain the ability to recruit DOT1L to MLL target genes where the resulting hypermethylation leads to aberrant expression of a characteristic set of genes, including *HOXA9* that drive leukemogenesis [89]. *MLL* gene rearrangements associated with both acute myeloid leukemia (AML) and acute lymphoid leukemia (ALL) are usually associated with a relatively poor prognosis despite improved treatment options like allogeneic hematopoietic stem cell transplantation underscoring the need for new treatment regimen. The drug EPZ-5676 (Epizyme, Inc. Cambridge, MA, USA), a potent inhibitor targeting DOT1L [89], is currently under Phase I clinical trial to conduct a preliminary assessment of the anti-leukemia activity of EPZ-5676 in patients with acute leukemias bearing rearrangements of the *MLL* gene (http://clinicaltrials.gov/show/NCT01684150).

An *MLL* break-apart FISH probe is also currently being co-developed by Abbott Diagnostics to assist in identifying eligible patients for its DOT1L inhibitor. According to pre-clinical data and the initial findings from its Phase I clinical study, EPZ-5676 and its FISH-based companion diagnostic show some promising results in the treatment of these aggressive tumors.

CONCLUSIONS

In the past decade, the clearest advances in personalized medicine have been within the field of oncology, and there is substantial expectation that cancer genomics and personalized oncology will continue to increasingly impact cancer care and patient outcome. FISH-based biomarkers currently provide important approved companion diagnostics, and additional FISH assays will likely also receive FDA approval. With ongoing effort focused on the discovery of biomarkers, therapeutic drugs and the development of their companion diagnostics, the treatment of cancer will progressively move from a reactive to a proactive discipline; a discipline that will be more prognostic, predictive and preventive.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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