Cancer Biomarkers and Molecular Theranostics

ESK Ma, CLP Wong
Division of Molecular Pathology, Department of Pathology, Hong Kong Sanatorium and Hospital, 2 Village Road, Happy Valley, Hong Kong

ABSTRACT
Cancer biomarkers have evolved from assays based on proteins, hormones and enzymes to molecular assays based on DNA or RNA. These molecular cancer biomarkers have broad clinical applications in disease screening, diagnosis, classification, offering a prognosis, risk stratification, treatment selection and monitoring. The prevailing trend in oncology is to harness molecular biomarkers to predict treatment efficacy or toxicity in the individual patient, and thereby guide the choice of treatment. The term theranostics was recently coined to indicate this marriage between an individual’s specific diagnosis and tailored therapy. Before therapeutics can be directly linked to diagnostics, the analytical validity, clinical validity, and clinical utility of the test in question should be carefully considered, not to mention the consequential ethical and financial implications. Recourse to predictive cancer markers with a view to targeted molecular therapy may have positive and negative connotations. The best known examples of positive predictors are the EGFR gene mutation in lung cancer and the presence of HER2 gene amplification in breast cancer, whilst the KRAS gene mutation is a negative predictor in metastatic colorectal cancer. Development of companion diagnostics is now double fuelled by the ever-expanding repertoire of agents used for targeting; detection of EML4-ALK gene fusion for consideration of ALK inhibitor therapy in lung cancer being a recent example of this phenomenon. Apart from such predictive markers, pharmacogenomics is another important facet of cancer theranostics. For instance, thymidylate synthase expression or genotype is related to the response to 5-fluorouracil and related compounds. It is envisaged that in the future, emerging diagnostic tools — including the next generation of sequencing technology and array-based comparative genomic hybridisation applied to the cancer genome — will further advance personalised oncology.

Key Words: Breast neoplasms; Colorectal neoplasms, hereditary nonpolyposis; Lung neoplasms; Mutation/genetics; Tumor markers, biological
INTRODUCTION

Molecular cancer biomarker assays based on DNA and RNA testing are widely utilised in clinical oncology. These markers aid in disease diagnosis both in terms of confirming histological findings and offering a diagnostic label, even when conventional methods are inconclusive or inadequate. Notable examples include the molecular diagnosis of acute promyelocytic leukaemia, which is an oncological emergency. This involves reverse transcription–polymerase chain reaction (RT-PCR) or fluorescence in-situ hybridisation (FISH), whenever the morphology is atypical, and the differentiation of essential thrombocythaemia from reactive thrombocytosis through detection of the clonality marker JAK2 V617F mutation in the former condition. Besides diagnosis, disease classification may be based on molecular markers, the first example of which is found in the World Health Organization classification of cytogenetically normal acute myeloid leukaemia (AML) based on the mutational status of \textit{NPM1} and \textit{CEBPA}. These gene mutations, together with the gene fusions resulting from chromosomal translocations, are powerful prognostic markers in AML. Risk stratification of patients can be achieved through a combination of several prognostic markers. For example, the combined use of interphase FISH markers and immunoglobulin heavy chain gene mutation status can identify subgroups at risk of chronic lymphocytic leukaemia. What is most relevant to the practising oncologist is to directly use the molecular cancer biomarkers to guide therapy, and will be further elaborated on in the article. After treatment is administered, monitoring of the clinical response can be undertaken through the utilisation of molecular markers, which often allows greater analytical sensitivity or depth than is possible by conventional means. The most successful example of molecular monitoring prevails in chronic myeloid leukaemia, where international guidelines are available to define the milestones of optimal treatment response and standardisation of the testing method. Finally, use of molecular markers in cancer screening is beginning to emerge, e.g. plasma EBV DNA is used to screen for nasopharyngeal cancer in our locality.

Theranostics is a newly coined term that refers to the utilisation of diagnostic markers to direct therapeutic decisions, with the objective of individualising patient therapy. These diagnostic markers are often molecular in nature and also termed predictive markers, because they predict either clinical outcome (prognosis) or treatment response. The latter predictive markers can be further categorised into positive or negative therapeutic predictors. Thus, epidermal growth factor receptor (\textit{EGFR}) gene mutation in non–small-cell lung cancer (NSCLC) and human epidermal growth factor receptor-2 (\textit{HER2}) gene amplification in breast cancer are positive. Whereas, the Kirsten rat sarcoma viral oncogene homolog (\textit{KRAS}) gene mutation in metastatic colorectal cancer (CRC) is classified as negative. Notably, before the predictive markers are put into routine diagnostic use, they have to be validated for both analytical performance (in terms of measurement accuracy) and clinical performance (in terms of distinguishing normal from the diseased). Even more desirable is the documentation of their clinical utility (i.e. evidence of survival benefit), but this may not always be available when the marker is first launched. Also to be considered are issues related to the patent for the diagnostic tests and the method of reimbursement, especially with respect to constraints due to tight health care budgets. Since the literature on the subject is expanding at a phenomenal pace and is widely disseminated over the internet, it is important for the relevant patient population be properly educated and counselled on the predictive markers to improve their uptake and avoid undue anxiety over test results.

PHARMACOGENOMICS

Apart from the predictive markers (related to tumour factors or drug targets), another important facet of personalised oncology is pharmacogenomics (host factors). There has been increasing utilisation of genetic polymorphisms, especially with respect to drug-metabolising enzymes that could predict treatment
The uridine diphosphate glucuronosyltransferase (UGT) superfamily of endoplasmic reticulum–bound enzymes is responsible for conjugating a glucuronic acid moiety to a variety of compounds to allow their elimination, whilst UGT1A1 catalyses the glucuronidation of bilirubin for excretion in the bile. As irinotecan therapy for advanced CRC became more widely used, it was observed that patients with Gilbert’s syndrome suffered from severe toxicity. An intermediary metabolite SN-38 of irinotecan shares the same glucuronidation pathway with bilirubin and its reduced clearance leads to more pronounced toxicity. The most common polymorphism associated with low activity of UGT1A1 is the *28 variant with 7 (rather than 6) TA repeats at the gene promoter, in which case irinotecan dose reduction is recommended in patients homozygous for that allele.\(^{12}\)

Frequently, 5-fluorouracil (5-FU) is used together with irinotecan in advanced CRC and acts by inhibition of thymidylate synthase (TS). This leads to depletion of the thymidine in the cell, which shuts off DNA synthesis and triggers apoptosis and other cell death processes. A substantial body of evidence has accumulated over the past few years, showing that TS expression varies considerably among tumours and the clinical response to 5-FU and related compounds is inversely related to TS expression. Low TS expression is associated with significantly higher response rates and a superior median survival as compared to high TS expression tumours. Polymorphisms within the TYMS gene regulate TS expression and have even been found to exert an effect on the response to 5-FU–based chemotherapy.\(^{13}\)

The significant polymorphisms include either double (2R) or triple (3R) tandem repeats of 28-bp sequences at the 5′-UTR, a G→C substitution at the second repeat of 3R allele, and a 6-bp deletion at the 3′-UTR of the gene. The 2R, 3RC and -6 alleles are associated with low levels of TS expression. Therefore the high expression genotypes are 2R/3RG, 3RC/3RG, 3RG/3RG plus +6/+6 or +6/-6, whereas the low expression genotypes are 2R/2R, 2R/3RC, 3RC/3RC plus -6/-6. Such TYMS genotyping may obviate the methodological drawbacks of intra-tumoural TS mRNA and protein expression studies.\(^{14}\) However, germline polymorphism is only a predictor of expression without taking into account acquired changes in the tumour and the interaction of different polymorphisms may be more complicated than expected.

Thiopurine S-methyltransferase (TPMT) is a cytosolic enzyme that inactivates thiopurine drugs such as 6-mercaptopurine and azathioprine through methylation, before their conversion into 6-thioguanine nucleotides, the cytotoxic metabolites incorporated into DNA. Variability of TPMT enzyme activity is attributable to polymorphisms of the TPMT gene. The most common alleles (TPMT*2, TPMT*3A and TPMT*3C), account for 95% of TPMT deficiency. Genotyping is therefore a convenient method for predicting TPMT enzyme activity before treatment with thiopurines.\(^{15}\) Low TPMT activity puts the patient at risk of developing toxicity, whereas high activity indicates a need to use a higher dose.

In addition to pharmacogenomic markers, protein and mRNA expression levels of ERCC1 and RRM1 appear to influence the response to cisplatin and gemcitabine respectively in NSCLC, and are therefore determinants of survival after surgical treatment of early-stage disease.\(^{16}\)

**HER2 AMPLIFICATION IN BREAST CANCER**

The HER2 gene is amplified in approximately 18 to 20% of breast cancers and is the primary mechanism of HER2 protein over-expression on the tumour cells. The HER2 status is associated with clinical outcome in breast cancer. HER2 positivity predicts an inferior prognosis (i.e. higher recurrence rate and mortality) in newly diagnosed breast cancer patients not in receipt of adjuvant systemic therapy. More importantly, the monoclonal antibody trastuzumab that targets the receptor is highly effective in HER2-positive breast cancer patients, in both the metastatic and adjuvant setting. Recently the small molecule tyrosine kinase inhibitor (TKI) lapatinib was reported to improve clinical outcome in patients with advanced breast cancer when added to capecitabine.\(^{17}\) Overall these results
show the usefulness of HER2 testing in therapeutic decision making for breast cancer patients.

Detection of HER2 status in breast cancer is routinely performed by immunohistochemistry (IHC) for protein over-expression and FISH for gene amplification. Recommendations for the testing algorithm, definition of positivity and quality assurance issues were recently issued jointly by the American Society of Clinical Oncology and College of American Pathologists. A positive HER2 result was defined as IHC 3+ (i.e. uniform, intense membrane staining of >30% of invasive tumour cells) or FISH+ (i.e. >6 average HER2 gene copies per nucleus or a ratio of HER2 gene signals to chromosome 17 signals of >2.2). An equivocal HER is said to occur in face of IHC 2+, there are between 4 and 6 HER2 gene copies per nucleus or the FISH ratio is 1.8 to 2.2. A negative HER2 result is inferred from an IHC of 0 or 1+, a FISH result of <4 copies or FISH ratio of <1.8. Studies have shown that only patients with IHC 3+ staining and/or HER2 gene amplification by FISH benefit from trastuzumab, and that only 20 to 25% of the IHC 2+ tumours show gene amplification when tested by FISH. Therefore the algorithm for IHC is to reflect to FISH for 2+ cases only, noting that when an equivocal result is encountered, patients with a HER2/CEP17 ratio of ≥2 are eligible for trastuzumab. The algorithm for FISH in the event of an equivocal result involves counting additional cells, repeat testing, or performing the IHC testing (or reviewing the IHC result if already available).

The advantage of using an internal control for the copy number of chromosome 17 is to distinguish between HER2 gene amplification and polysomy 17. The presence of polysomy 17 may complicate the interpretation of HER2 testing and is a major cause of equivocal results (Figure 1). Polysomy 17 per se does not significantly increase mRNA levels but may increase HER2 protein expression (used in the IHC 2+ range). In contrast to HER2 gene amplification, polysomy 17 is not associated with high-grade tumour, hormone receptor negativity and reduced disease-free survival, and therefore biologically it resembles HER2 negativity. In the rare event of polysomy 17 found in association with IHC 3+, the ploidy level is usually high and the indication for trastuzumab is established by the IHC result.

HER2 over-expression is also detectable in gastric cancer, and an early study showed an incidence of 12.2% in gastric and 24% in gastroesophageal adenocarcinoma. The recently completed ToGA trial reported that trastuzumab in combination with chemotherapy may be considered a new standard option for patients with HER2-positive advanced gastric cancer. The HER2 testing in gastric cancer differs from that in breast cancer in 2 aspects. First, owing to their structure, the basolateral membranous immunoreactivity of the glandular cells may result in incomplete membranous staining (in contrast to complete and uniform staining in breast cancer). Second, compared to breast cancer, in gastric lesions a higher rate of heterogeneity may necessitate the examination of more tumour areas.

Emerging technologies such as silver in-situ hybridisation (SISH) are being developed for the purpose of HER2 testing and show excellent concordance with FISH in breast cancer. The interpretation of SISH results is most reproducible using the HER2/CEP17 ratio. However separate slides are needed for detection of HER2 gene copy number and chromosome 17 signals. To circumvent the issue, a dual-colour dual-hapten in-situ hybridisation (dual ISH) system was recently developed in which the HER2 gene signal was detected by SISH whilst the chromosome 17 copy number was detected by chromogenic red ISH on the same slide. The advantages of these new ISH systems include automated testing, facilitation of reflex testing after IHC on the same machine, direct identification of the tumour cells under bright-field microscopy, and amenability for later review.

A related gene locus which is adjacent to and slightly telomeric to the HER2 gene is the topoisomerase II-a (TOP2A) gene, the amplification or deletion of which is often linked with HER2 gene amplification and increased responsiveness to anthracycline-containing chemotherapy regimens. The TOP2A status can be detected on its own, and paired with chromosome 17 copy number controls by dual-colour FISH, or together with HER2 status (by triple-colour FISH).

**EGFR GENE MUTATION IN NON–SMALL-CELL LUNG CANCER**

Activating mutations of the EGFR gene are highly correlated with treatment responsiveness to gefitinib and erlotinib, both of which are small molecule TKIs targeting EGFR. These mutations occur at the tyrosine kinase domain of the EGFR gene and preferentially involve a subset of lung cancers characterised by
female sex, non-smoker, adenocarcinoma histology, and East Asian ethnicity. EGFR mutations involve the adenosine triphosphate–binding pocket of the tyrosine kinase receptor domain, clustering at exons 18 to 21 of the EGFR gene, and in the form of in-frame deletions, in-frame insertions/duplications and point mutations. Two hotspots namely deletion at exon 19 and the point mutation L858R at exon 21 constitute the majority of mutations. These mutations increase the kinase activity of EGFR, leading to the heightened activation of downstream pathways that favour cell survival on the one hand, but also opens up an avenue for inhibition through TKI therapy on the other. Patients with NSCLC having exon 19 deletions seem to respond better to gefitinib and erlotinib than those with L858R mutations. A few mutations — notably D761Y, S768I, T790M, and insertions at exon 20 — are resistant to TKI therapy. Two recent randomised clinical trials IPASS and SATURN have confirmed that the EGFR mutation status is a strong predictive biomarker of clinical benefit from TKI therapy, but the same cannot be said for EGFR gene copy number by FISH or EGFR protein expression by IHC.

Besides serving as a cancer biomarker to predict TKI response in NSCLC, the detection of different EGFR gene mutations in multiple lung cancers enables the distinction between synchronous primaries from intrapulmonary metastases, and metachronous primaries from disease recurrence.

The detection of EGFR mutations was performed...
at Hong Kong Sanatorium and Hospital by PCR, followed by Sanger sequencing on DNA extracted on micro-dissected paraffin tissue to enrich tumour cells.35 A review of 481 consecutive samples accrued from September 2005 to April 2009 at our laboratory showed detectable EGFR mutations in 210 samples (44%) overall. Among the positive samples, exon 19 deletions accounted for 86 cases (41%) in which ELREA deletion accounted for 62 cases (30%) and exon 21 L858R mutations accounted for 82 cases (39%). These two mutation hotspots accounted for 80% of cases, which was in accordance with international literature. Rare insertion mutants were detected at exon 20 and although some were unreported, they were most probably associated with primary or de-novo drug resistance, especially those at position D770. These should be distinguished from secondary or acquired resistance conferred by T790M that was usually detected in association with a hotspot drug sensitive mutation, as seen in one of our cases. Double mutations were detected in 22 cases (10%) and usually involved at least one TKI-sensitive mutant. Unexpectedly, 3 cases showed a combination of a TKI-sensitive hotspot mutation and a known TKI resistance exon 20 S768I mutation, in whom documentation of the TKI response would be of interest. Finally, although EGFR and KRAS mutations should be mutually exclusive,36 one sample showed concurrent presence of both, which might reflect tumour heterogeneity.

Reflex KRAS mutation detection was performed only in samples tested negative for EGFR gene mutation, unless a specific request was made. Among the 271 (56%) EGFR mutation–negative cases, KRAS mutations were detected in 27; all were hotspot mutations at exon 2 (Gly12Phe = 1, Gly12Cys = 7, Gly12Asp = 1, Gly12Ala = 4, Gly12Val = 12, and Gly13Asp = 2). KRAS mutations accounted for 10% of all EGFR negatives and 5.6% of all cases. Overall 244 cases (51%) of samples were negative for both EGFR and KRAS gene mutations.

**EML4-ALK GENE FUSION IN NON–SMALL-CELL LUNG CANCER**

First reported in 2007, this new molecular aberration in NSCLC occurs as a result of a small inversion within chromosome 2p that leads to the formation of a fusion gene that comprises portions of the echinoderm microtubule-associated protein-like 4 (EML4) upstream and the anaplastic lymphoma kinase (ALK) downstream.37 The fusion gene, initially reported at a frequency of 3 to 5% in NSCLCs, was oncogenic and represented a novel molecular target. The patients tended to be younger, more likely to be male, never or light smokers, and tested negative for EGFR and KRAS gene mutations.38 EML4-ALK fusion may be detected by a dual-colour split-apart FISH probe that targets the ALK gene (Abbott Molecular, Abbott Park, IL).38 While the typical FISH signal pattern in a positive cell emits one fusion yellow signal, one orange signal and one green signal, atypical signal pattern in the form of one fusion yellow signal and one orange is also recognised as a result of deletion of the 5’ portion of the rearranged ALK gene (Figure 2). A dual-colour dual fusion probe system was also developed but since both

![Figure 2. Detection of ALK gene rearrangement by dual-colour interphase fluorescence in-situ hybridisation.](image-url)

(a) ALK dual-colour split apart probe (Abbott Molecular) showing one yellow fusion signal of wild-type allele, and split orange signal and green signal indicating ALK translocation or rearrangement (arrow). (b) ALK dual-colour split apart probe (Abbott Molecular), showing atypical signal pattern with one yellow fusion signal of wild-type allele and one residual orange signal. The green probe targeting the 5'-portion of the rearranged ALK allele is deleted. (c) Dual colour dual fusion EML4-ALK probe (GSP, Japan) showing two fusion yellow signals, one green signal targeting the wild-type EML4 gene and one red signal targeting the wild-type ALK gene. Since both genes that are involved in the fusion are located on the same chromosome arm, the distinction between wild type and fusion signals may be more challenging than inter-chromosomal translocations.
genes involved in the fusion were originally located on the same chromosome arm, the distinction between wild type and fusion signals may be more challenging than for inter-chromosomal translocations. The fusion transcript can be detected by RT-PCR, and a multiplex method is reported that allows the characterisation of various isoforms of the fusion transcript.\(^{39}\) Finally, the utilisation of IHC for cytoplasmic expression of ALK protein in the detection of EML4-ALK fusion is also under investigation. Patients who harbour this gene fusion are not expected to derive clinical benefit from EGFR TKI and are candidates for trials of ALK-targeted therapy (e.g. crizotinib).

**KRAS GENE MUTATION IN COLORECTAL CANCER**

The benefit of anti-EGFR monoclonal antibody cetuximab in advanced CRC is limited to patients with wild-type KRAS tumors but not in those harbouring mutations in codons 12 or 13 of the KRAS gene.\(^{40}\) There is, however, a lack of validated testing methods and standardised protocols for the detection of KRAS gene mutations. Few studies have systematically compared the sensitivity, specificity, and concordance between different diagnostic methods. Similar to EGFR mutation testing, the methodologies currently in use can be categorised as allele-specific PCR assays that cover specific mutations and direct nucleotide sequencing that covers all variations that may be present. This has to be balanced against the higher analytical sensitivity of mutation detection for the former assays (\textit{vide infra}). For example, while the TheraScreen (DxS, Manchester, UK) allows the detection of a KRAS mutant clone size down to 1%, only 7 codon 12 and 13 mutations are targeted by the TheraScreen assay; rarer ones are not covered. Moreover, mutations at exon 3 and other positions of exon 2 (apart from codons 12 and 13) are also not detected. The higher detection sensitivity of allele-specific PCR assays obviates the need for microdissection and is simpler to perform when compared with direct sequencing, but are considerably more expensive.

At Hong Kong Sanatorium and Hospital, we retrospectively analysed 210 consecutive samples from patients with CRC submitted for KRAS mutation detection from May 2008 to April 2009 (Table 1). DNA was extracted from formalin-fixed paraffin-embedded tissue after micro-dissection to select areas with at least 30% tumour cells by a histopathologist. Detection of KRAS mutations at exons 2 and 3 was performed by PCR sequencing. Bidirectional sequencing was performed. Abnormal results were repeated for confirmation to exclude PCR artefacts. KRAS gene mutations at codons 12 and 13 were identified in 76 samples (36%). Double mutations were seen in 3 patients. Of note, 5 (7%) of 76 samples harbouring rare mutations at codons 12 and 13, namely Gly12Phe (n = 1), Gly13Arg (n = 2) and Gly13Cys (n = 2) respectively, would have escaped detection by TheraScreen. Finally, patients who harbour this gene fusion are not expected to derive clinical benefit from EGFR TKI and are candidates for trials of ALK-targeted therapy (e.g. crizotinib).

**Table 1.** The results of KRAS gene mutation detection in 210 patients.

<table>
<thead>
<tr>
<th>Codon</th>
<th>Nucleotide change</th>
<th>Amino acid change</th>
<th>No. of cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>c.34_35delinsTT</td>
<td>Gly12Phe*</td>
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<td>c.34G→A</td>
<td>Gly12Ser</td>
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<tr>
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<td>Gly12Cys</td>
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<td>Gly13Cys* &amp; Gly60Asp</td>
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No KRAS mutation detected in exons 2 & 3 127

Total No. of cases 210

* KRAS gene mutations at codons 12 and 13 not detected by TheraScreen.
The era of molecular theranostics in cancer paves the way for a paradigm shift in pathology, from purely providing a diagnosis and investigating disease mechanisms, to directly ensuring appropriate treatment. With an increasing role to play, the pathologist should keep abreast of recent developments in companion diagnostics of targeted therapy, since clinical practice is expected to move ahead rapidly and new cancer biomarkers once validated will replace existing ones. With respect to the cancer genome, it is envisaged that emerging, next-generation, diagnostic sequencing technology to detect mutations as well as array-based comparative genomic hybridisation (to interrogate copy number variations) will further advance personalised oncology.43

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