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Research paper

Diagnostic *RAS* mutation analysis by polymerase chain reaction (PCR)

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ABSTRACT

RAS mutation analysis is an important companion diagnostic test. Treatment of colorectal cancer with anti-Epidermal Growth Factor Receptor (EGFR) therapy requires demonstration of *RAS* mutation status (both *KRAS* and *NRAS*), and it is good practice to include *BRAF*. In Non-Small Cell Lung Cancer (NSCLC) and melanoma, assessment of *RAS* mutation status can be helpful in triaging patient samples for more extensive testing. This mini-review will discuss the role of PCR methods in providing rapid diagnostic information for cancer patients.

1. Introduction

The rat sarcoma viral oncogene homolog (RAS) family of membrane associated GTPase signalling molecules are involved in pathways that mediate cell growth. Many of these pathways interact, and different cell types use them differently, so the effects of activation by growth factors or mutation in key genes differ between cell and cancer types. The human RAS family consists of three genes Harvey RAS (*HRAS*), Kirsten RAS (*KRAS*), and Neuroblastoma RAS (*NRAS*) [1,2].

The clinical need for *KRAS* mutation testing is largely related to the use of anti-EGFR antibody therapy for patients with advanced colorectal cancer [3]. Virtually all colorectal cancers express EGFR, but few respond to treatment directed against the receptor because they have downstream activating mutations in signalling molecules including KRAS. It has now been shown that *NRAS* mutations in patients with colorectal cancer have the same effect. There is also increasing evidence from systematic reviews that B-Raf proto-oncogene (*BRAF*) mutations, which confer a worse prognosis, also confer a degree of resistance [4], and strong suspicion that *PIK3CA* mutations are also important [4]. This backs up reports from series of patients treated with anti-EGFR molecules [5], and in vitro data using cell lines, in which activation of downstream signalling leads to resistance to anti-EGFR molecules [6]. It also has recently become apparent that resistance may occur during treatment due to new mutations in *EGFR*, *KRAS*, *NRAS*, *BRAF*, and *PIK3CA* genes [7]. While the pharmaceutical licenses for anti-EGFR antibody therapeutics (e.g. cetuximab, panitumumab) granted by the Federal Drug Administration, and in the Europe by the European Medicines Agency (EMA), require the use of *KRAS* and *NRAS* mutation testing to exclude mutations before their use, this is not yet a requirement for *BRAF* or *PIK3CA* [8,9].

KRAS is of importance in other tumour types, and knowledge of *KRAS* mutational status can be helpful to guide further investigations. For instance, if a lung cancer has a *KRAS* mutation, there is little point in sending off biopsy material for Anaplastic lymphoma kinase (*ALK*) fusion gene testing, as the *KRAS* mutation will be the driver mutation and *ALK* will almost certainly be wild-type [10]. As yet, there are few therapeutic options for patients with *KRAS* mutated tumours, but this is likely to change, and knowledge of the *KRAS* mutational status of many tumours will then be of greater significance [8].

The activation of molecules such as *KRAS* by mutation requires conformational changes at the protein level, so not all mutations in *KRAS* are activating and able to drive carcinogenesis [1,9]. There are therefore 'hotspots' within *KRAS* that allow testing to be done without sequencing the entire gene. The American Society of Clinical Oncology (ASCO) have recently published guidance recommending testing of codons 12 and 13 of exon 2; 59 and 61 of exon 3; and 117 and 146 of exon 4 (known as "expanded" or "extended" RAS mutation testing, Table 1) [9]. This list is now widely used, but not all commercially available tests cover these codons. The corresponding mutations covered in guidance for testing laborato-

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Table 1
Mutations in *KRAS* and *NRAS* for which testing should be performed in patients with colorectal cancer. The exons and codons listed are common to both *KRAS* and *NRAS*.

Exon	Codon	Example Mutations
2	12	G12C (c.34G > T)
		G12R (c.34G > C)
		G12S (c.34G > A)
		G12A (c.35G > C)
		G12D (c.35G > A)
		G12V (c.35G > T)
	13	G13D (c.38G > A)
3	59	A59E (c.176C > A)
		A59G (c.176C > G)
		A59T (c.175G > A)
	61	Q61K (c.181C > A)
		Q61L (c.182A > T)
		Q61R (c.182A > G)
		Q61H (c.183A > C)
		Q61H (c.183A > T)
4	117	K117N (c.351A > C)
	146	A146P (c.436G > C)
		A146T (c.436G > A)
		A146V (c.437C > T)

ries are listed in Table 1, with example mutations. Most external quality assurance (EQA) schemes (e.g. UKNEQAS, Edinburgh, UK and European Society of Pathology EQA scheme) require reports to be submitted to their molecular pathology schemes, based on drug licence information (see http://www.ukneqas-molgen.org.uk/molecular-pathology and http://kras.eqascheme.org) [11,12].

2. PCR tests for *KRAS* mutation

Tests for *KRAS* mutations usually employ polymerase chain reaction (PCR). Modern, particularly automated, PCR methods are relatively simple to perform and provide rapid diagnosis at good sensitivity. It is perfectly possible to go from formalin-fixed paraffin-embedded (FFPE) tumour sample to result in a few hours, rather than a few days. There are a large number of methods available, suited to small and large laboratories. Most manufacturers of PCR machines used clinically have options for RAS analysis. The commonest commercial options are well validated, widely used, and as shown in Table 2, in three cases, approved by the US Federal Drug Administration (FDA). The Therascreen (Qiagen) offering is based on the amplification refractory mutation system (ARMS) technology [13,14], and is widely used. The Cobas (Roche) assay uses a CE-IVD marked TaqMelt PCR assay designed to detect the presence of 19 *KRAS* mutations in codons 12, 13, and 61 from just 100 ng of DNA extracted from FFPE samples [15]. Comparison the two assays have shown excellent concordance [16], but it should be noted that the cobas assay has a more extensive coverage. The most recently approved assay, the Idylla system from Biocartis, integrates DNA extraction and multiplex PCR in a simple-to-use cassette, and also has wide coverage (www.biocartis.com). There are presently few publications using this method, but its first offering for BRAF mutation has performed well [17–19].

The limit of detection (LoD) is given by most manufacturers as the lowest percentage mutant DNA detectable against background wild-type DNA. The best assays achieve 1%, but most manufacturers quote <5% in their literature. This means that the lower the percentage of neoplastic cells present, the higher the effective limit of detection. Manufacturers and users generally quote 10% neoplastic cells as a threshold below which it is not worth testing, as the effective limit of detection for mutations in such clinical material will be between 10% and 50% at that point [11]. It is important that this is taken into account when reporting the results of such tests, and close cooperation between histopathology and molecular pathology is essential [11].

Laboratory-developed tests (also known as in-house assays) for RAS mutation analysis (Table 3) are widely used in Europe and by clinical laboratories operating under the The Clinical Laboratory Improvement Amendments of 1988 (CLIA) regulations in the USA, as well as in research. The onus is on the laboratory to validate the test to ensure that the results are reliable [11]. Many of reagents are sold as research use only (RUO). The results from external quality assurance schemes (e.g. UKNEQAS, ESP) suggest that in well-run, suitably accredited laboratories, such tests are as safe and effective as those with approval for clinical use [11].

One advantage of developing tests locally is that there are a large number of different methods to choose from and it is possible to design tests to meet local requirements. Most methods use some of PCR enrichment to ensure low levels of detection of mutant DNA against a background of wild-type DNA. The castPCR (ThermoFisher) method can be used in both 96 well plates and TaqMan Arrays and shows good concordance with both Therascreen (Qiagen) and IonTorrent NGS [20,21]. PCR clamping incorporates peptide nucleic acids (PNAs) or locked nucleic acids (LNAs) to reduce the amplification of mutant DNA [22–25], while high resolution melt (HRM) methods use close control of annealing temperatures to favour amplification of mutant DNA[26,27]. HRM can also be used as a prior to sequencing to increase sensitivity [28,29].

3. Factors affecting assay choice

Guidance for molecular pathology [11] covers most of the requirements for test implementation and should be consulted before starting. One of the key considerations for assay choice is the number of samples needing testing. There is a trade-off between the cost per assay and the number performed. Efficiencies of scale mean that for many laboratories, it is better to send away rarely required tests, and to concentrate on those that are commonly requested. Turnaround time for patients, from biopsy to action on the result of a test, tends to be slower for those samples sent away, and if the result is required quickly, this may mean that a laboratory has to take on testing it would otherwise prefer to send away. Timeliness of diagnostic reporting is an essential component of the decision, and should be considered by multidisciplinary teams (tumour boards) considering setting up such services.

Laboratory facilities are a further consideration. Space is rarely an issue as PCR machines are all of bench-top type, but most molecular pathology methods require considerable expertise both to extract DNA from samples, to perform the tests, and to interpret the results.

Both NGS and PCR have advantages and disadvantages. While sequencing can look at entire genes, most PCR methods and targeted sequencing methods employ primers and probes that look for defined mutations. This makes them less comprehensive, but makes interpretation easier. Many next targeted generation sequencing (NGS) depend on PCR for library preparation and some can be thought of as a post-PCR methods (e.g. IonTorrent Ampliseq, ThermoFisher, Paisley, UK) [30,31]. Targeted NGS uses the application of highly parallel sequencing methods to analyse vast numbers of overlapping PCR products to cover whole exons or even genes. This allows even rare mutations to be detected, but at a cost in terms of time, complexity of analysis and interpretation, and economics [11].

Whatever the analytical method used, DNA needs to be extracted from formalin-fixed, paraffin-embedded (FFPE) tissue for assay. It is important to ensure that tissue is fixed promptly, but not overfixed (i.e. <72 h), and that neutral buffered formalin is used [11]. Tissue processing should not use high temperatures (i.e. <65 °C) to optimise DNA recovery [32]. DNA extraction can be manual, using one of several systems from companies such as