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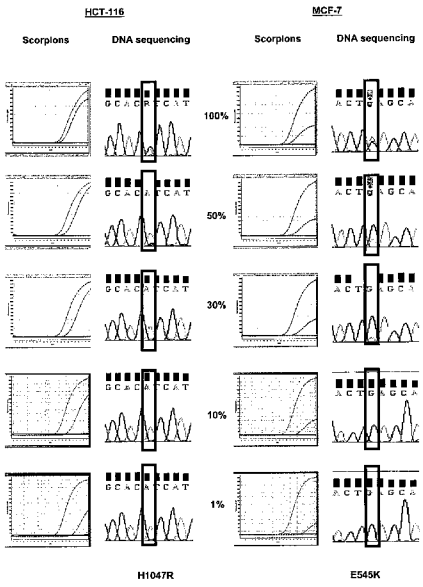

Figure 1

(57) **Abstract:** A polynucleotide comprising at least the final six nucleotides of one of the following primer sequences, or a sequence complementary thereto: SEQ. ID NOS. 3 to 16, 18, 20 to 33, 35 or 37 to 39.

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POLYNUCLEOTIDE PRIMERS

Technical Field

The present invention relates to a polynucleotide, a kit comprising a polynucleotide and a method for detecting the presence or absence of mutations in a gene.

Background Art

Phosphatidylinositol 3-Kinases (PI3K) are a large family of lipid kinases involved in cell signalling. The PI3K-AKT pathway is activated in a number of tumour types, resulting in abnormalities of cell growth, proliferation and survival (add ref of 1 recent review). Recently, mutations in the catalytic subunit of the class 1A PI3K (PIK3CA) have been identified in human cancers[1]. The precise role of these mutations in carcinogenesis is still to be clearly defined but with ongoing development of a number of targeted PI3K inhibitors, detection of mutations will become increasingly important for patient selection. Technical challenges in the detection of such mutations result from the limitations of tumour biopsies that may only contain small quantities of the mutated sequences. Furthermore, DNA extracted from paraffin embedded tissue is often degraded and of poor quality. The minimum level of mutant DNA required for detection by sequencing is 15-25% and so there is a pressing need for development of sensitive assays able to detect small amounts of mutated alleles in a heterogenous sample and the products necessary for carrying out the assays.

The present invention seeks to address this need.

Disclosure of the Invention

The present invention provides sensitive and robust tests for tumour-borne PIK3CA mutations.

According to one aspect of the present invention, there is provided a polynucleotide comprising at least the final six nucleotides of one of the following primer sequences, or a sequence complementary thereto: SEQ. ID NOS. 3 to 16, 18, 20 to 33, 35 or 37 to 39. That is, the polynucleotide comprises at least the six nucleotides at the 3' end of one of the following primer sequences, or a sequence complementary thereto: SEQ. ID NOS. 3 to 16, 18, 20 to 33, 35 or 37 to 39.

Preferably, the polynucleotide comprises at least 75% of the 8, 10, 12, 14, 16, 17, 18 or 20 nucleotides at the 3' end, or the entirety, of one of the following primer sequences, a sequence complementary thereto, or a sequence having 80%, 90%, 95% or 99% sequence identity thereto: SEQ. ID NOS. 3 to 16, 18, 20 to 33, 35 or 37 to 39.

In some embodiments of the present invention there is provided a polynucleotide comprising at least 75% of the ten nucleotides at the 3' end of one of the following primer sequences, or a sequence complementary thereto: SEQ. ID NOS. 3 to 16, 18, 20 to 33, 35 or 37 to 39.

Conveniently, the polynucleotide is less than 100 nucleotides long, preferably less than 80 nucleotides long, more preferably less than 60 nucleotides long, more preferably less than 40 nucleotides, more preferably less than 30 nucleotides long.

Advantageously, the polynucleotide further comprises a quencher group and a fluorophore group.

Conveniently, the quencher group and the fluorophore group are separated by a nucleotide tail sequence comprising first and second regions, the nucleotides of the first region being complementary to but in reverse order from the nucleotides of the second region, such that hybridisation of the first region to the second group results in the quencher group to be sufficiently close to the fluorophore group to quench the fluorophore group.

Preferably the tail sequence further comprises a third region having a sequence complementary to a region of the PIK3CA gene.

Advantageously, the polynucleotide comprises at least the six nucleotides at the 3' end of SEQ. ID NO. 18 and the tail sequence comprises SEQ. ID NO. 17.

Alternatively, the polynucleotide comprises at least the final nucleotides at the 3' end of SEQ. ID NO. 35 and the tail sequence comprises SEQ. ID NO. 34.

Alternatively, the polynucleotide comprises at least the final nucleotides at the 3' end of SEQ. ID NO. 39 and the tail sequence comprises SEQ. ID NO. 38.

Conveniently, the quencher group comprises Dabcyl.

Preferably the fluorophore comprises Hex, Fam or Rox.

According to another aspect of the present invention, there is provided a kit comprising at least two of the polynucleotides of the invention.

Advantageously, the kit comprises a polynucleotide comprising SEQ ID NO. 18 and a polynucleotide comprising any one of SEQ ID NOS. 3 to 16; or a polynucleotide comprising SEQ ID NO. 35 and a polynucleotide comprising any one of SEQ ID NOS. 20 to 33; or a polynucleotide comprising SEQ ID NO. 39 and a polynucleotide comprising SEQ ID NO. 37.

Conveniently, the kit further comprises nucleotide triphosphates, a polymerisation enzyme and/or a buffer solution.

According to a further aspect of the present invention, there is provided the use of a polynucleotide or a kit of the invention; or a polynucleotide comprising four or five of the six nucleotides at the 3' end of SEQ. ID NOS. 3 to 16, 18, 20 to 33 or 35 or sequences complementary thereto for detecting a mutation in a nucleic acid sample containing at least a fragment of the PIK3CA gene.

Advantageously, the fragment of the PIK3CA gene in the nucleic acid sample is at least 10 nucleotides long, preferably 20 nucleotides long, more preferably 30 nucleotides long and more preferably 40 nucleotides long.

According to another aspect of the present invention, there is provided a method of detecting the presence or absence of a mutation in the PIK3CA gene comprising the steps of:

a) mixing a nucleic acid sample comprising at least a fragment of the PIK3CA gene with a polynucleotide comprising at least the six nucleotides at the 3' end of one of the following primer sequences, or a sequence complementary thereto: SEQ ID NOS. 3 to 16 or 20 to 33 ; and

b) detecting hybridisation of the polynucleotide to the nucleic acid sample wherein hybridisation indicates the presence of a mutation.

Conveniently, the polynucleotide comprises one of the following primer sequences: SEQ ID NOS. 3 to 16 or 20 to 33.

Preferably, the method further comprises the step of, prior to step a), amplifying the number of copies of the fragment of the PIK3CA gene using thermal cycling nucleic acid amplification, preferably PCR.

Advantageously, step b) comprises carrying out DNA polymerisation using the polynucleotide as a first primer and detecting the extension product of polymerisation.

Conveniently, step b) comprises the step of mixing the nucleic acid sample and the polynucleotide with a second primer which corresponds to a region of the fragment of the PIK3CA sequence downstream of the region to which the polynucleotide is complementary and carrying out PCR on the mixture.

Preferably, the second primer comprises: SEQ. ID NO. 18 and the polynucleotide comprises at least four or five of the six nucleotides at the 3' end of SEQ. ID NOS. 3 to 16; or the second primer comprises SEQ. ID NO. 35 and the polynucleotide comprises at least four or five of the six nucleotides at the 3' end of SEQ. ID NOS. 20 to 33.

Alternatively, the method further comprises the step of carrying out PCR on the sample using control primers and comparing the amplification of the PIK3CA gene with amplification using the polynucleotide and the second primer.

Advantageously, the control primers comprise SEQ ID NOS. 37 and 39.

Conveniently, the polynucleotide comprise a quencher group and a fluorophore group and wherein step b) comprises exposing the mixture to light of a wavelength to which the fluorophore is responsive in the absence of the quencher group and detecting light at the wavelength emitted by the fluorophore group in the absence of the quencher group.

It is preferred that the PIK3CA gene is the sequence available as GenBank accession no. NM_006218 version no. NM_006218.2 GI:54792081, which is incorporated herein by reference.

Where reference is made in the specification to "at least four or five of the six nucleotides at the 3' end" of a reference sequence, this means that, of the six nucleotides in the reference sequence, either one or two of the nucleotides may be missing or replaced with a different nucleotide. Of course, in some embodiments, the sequence comprises all six of the nucleotides of the reference sequence.

In this specification, "ARMS" is the amplification refractory mutation system disclosed in, for example, EP-A-0332435.

Where reference in this specification is made to a percentage of a polynucleotide compared with a reference polynucleotide, this can be determined by algorithms known in the art.

For example the percentage identity between two sequences can be determined using the BLASTP algorithm version 2.2.2 (Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schäffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997), "Gapped BLAST and PSI-BLAST: a new generation of

protein database search programs", Nucleic Acids Res. 25:3389-3402) using default parameters.

Brief Description of Drawings

Figure 1 shows the results of carrying out Scorpions detection and sequencing on samples containing mutant PIK3CA gene.

Detailed Description

Embodiments of the present invention provide polynucleotide primers that can be used in assays for the detection of mutations of the PIK3CA gene in a sample containing nucleic acids.

In specific embodiments, the polynucleotide primers are forward and reverse primers that hybridise with the PIK3CA gene to enable a PCR amplification reaction to take place. Thus the forward primer hybridises upstream of and to the opposite strand from the reverse primer and the forward and reverse primers together define an amplicon sequence which is amplified during PCR. The sequence of the forward primer is selected such that it is not complementary to the wild type sequence but is capable of hybridising with a mutant PIK3CA sequence.

In order to detect the presence the mutant PIK3CA gene in the sample, the primers are mixed with the sample. The necessary agents for PCR (appropriate nucleotide triphosphates, DNA polymerase enzyme and a buffer solution) are then added to the sample and PCR is carried out. If the sample contains the mutant sequence to which the forward primer is able to hybridise then the amplicon is amplified during PCR and the presence of the mutant sequence in the sample is thus indicated. If the sample does not contain the mutant sequence then the forward primer binds to the PIK3CA sequence with low efficiency and so there is little or no amplification of the amplicon sequence.

In order to detect the mutation E542K, the forward primer sequence may be one of SEQ ID NOS. 3 to 9, preferably SEQ ID NO. 5. In order to detect the mutation E545K, the forward primer sequence may be one of SEQ ID NOS. 10 to 16, preferably SEQ ID NO. 14. In order to detect the mutation H1047R, the forward primer sequence may be one of SEQ ID NOS. 20 to 26, preferably 21. In order to detect the mutation H1047L, the forward primer sequence may be one of SEQ ID NOS. 27 to 33, preferably 28. However, it is to be appreciated that the precise sequence of the forward primer need not be identical to these sequences, provided that the forward primer hybridises to the mutant sequence more readily than to the wild type sequence. In the sequences set out above, it is the final six nucleotides (i.e. the nucleotides at the 3' end) of the primers that provide the binding specificity so these nucleotides must be identical to the given sequence.

In order to detect the presence of the amplicons formed in the sample, the reverse primer is a so called "Scorpions" primer in embodiments of the present invention. Details of Scorpions primers are provided in WO-A-99/066071 which is incorporated herein by reference. A Scorpions primer comprises a primer sequence complementary to a first target sequence of a gene (in this invention PIK3CA) and a tail sequence comprises a probe sequence flanked by two mutually complementary sequences. A DNA polymerase blocking moiety (such as a hexethylene glycol (HEG) monomer) is provided between the primer sequence and the tail sequence. A fluorophore group is provided at one end of the tail sequence and a quencher group is provided at the other end of the tail sequence. In use, the primer sequence of the Scorpions primer acts as a reverse primer during PCR in the normal way and thus the entire Scorpions primer, including the tail sequence, becomes incorporated into each amplicon. The DNA polymerase blocking moiety prevents duplication of the tail sequence. Thus the mutually complementary sequences in the tail sequence have the tendency to hybridise with each other, bringing the fluorophore group and the quencher group into proximity and preventing emission from the fluorophore group. However, if the amplicon contains a second target sequence complementary to the probe sequence, the probe sequence preferentially binds to the second target sequence, separating the mutually complementary sequences. This results in the fluorophore group and the quencher group being spatially distanced such that the

fluorophore group emits light of one wavelength in response to incident light of another wavelength. Accordingly, the Scorpions primer enables easy detection of amplicons and moreover, avoids false positive results (caused by primer dimers, for example) because a signal is only generated when the amplicon contains the second target sequence.

The fluorophore group may be Hex (4,7,2',4',5',7'-hexachloro-(3',6'-dipivaloylfluoresceinyl)-6-carboxamidohexyl]-1-O-(2-cyanoethyl)-(N,N-diisopropyl)-phosphoramidite), Fam ([(3',6'-dipivaloylfluoresceinyl)-6-carboxamidohexyl]-1-O-(2-cyanoethyl)-(N,N-diisopropyl)-phosphoramidite) or Rox (5,6,-Carboxy-X-Rhodamine). The quencher group may be Dabcyl (5'-Dimethoxytrityloxy-5-[(N-4'-carboxy-4-(dimethylamino)-azobenzene)-aminohexyl-3-acrylimido]-2'-deoxyUridine-3'-[(2-cyanoethyl)-(N,N-diisopropyl)]-phosphoramidite).

In embodiments of the present invention, a Scorpions primer is provided for detection of the E542K and E545K mutations wherein the primer sequence is SEQ ID NO. 18 and the probe sequence is SEQ ID NO. 17. A Scorpions primer is provided for detection of the H1047R and the H1047L mutations wherein the primer sequence is SEQ ID NO. 35 and the probe sequence is SEQ ID NO. 34.

It is to be appreciated, however, that the use of Scorpions primers is not essential to the invention and other methods of detecting the synthesis of amplicons may be employed such as TaqMan™ product detection, as described in patent numbers US-A-5487972 and US-A-5210015

In some embodiments, a control assay is also carried out to detect the overall concentration of the PIK3CA gene in the sample. This is achieved by carrying out a separate PCR reaction with control forward and reverse primers which define an amplicon in another region of the PIK3CA gene. It is preferred that the forward primer is SEQ ID NO. 37 and the reverse primer is a Scorpions primer wherein the primer sequence is SEQ ID NO. 39 and the probe sequence is SEQ ID NO. 38. The number of PCR cycles required to generate a threshold number of control amplicons is then compared with the number of PCR cycles required to generate the threshold number of amplicons containing the mutant sequence in order to assess the proportion of

mutant copies of the PIK3CA gene in the sample. Such control assays are generally carried out separately from the test assays.

The PCR assays are preferably carried out as multiplexed real time PCR assays.

The test sample of nucleic acid is conveniently a sample of blood, faeces, sputum, colonic lavage, bronchial lavage or other body fluid, or tissue obtained from an individual. The individual is conveniently human, preferably Homo sapiens. It will be appreciated that the test sample may equally be a nucleic acid sequence corresponding to the sequence in the test sample. That is to say that all or a part of the region in the sample nucleic acid may firstly be amplified using any convenient technique such as thermal cycling nucleic acid amplification, in particular PCR, or whole genome amplification (WGA) before use in the method of the invention.

Any convenient enzyme for polymerisation may be used provided that it does not affect the ability of the DNA polymerase to discriminate between normal and mutant template sequences to any significant extent. Examples of convenient enzymes include thermostable enzymes which have no significant 3'-5' exonuclease activity, for example Taq DNA polymerase, particularly "Ampli Taq Gold™ DNA polymerase (PE Applied Biosystems), Stoffel fragment, or other appropriately N-terminal deleted modifications of Taq or Tth (Thermus thermophilus) DNA polymerases.

In further embodiments of the present invention, there are provided kits comprising one or more polynucleotides of the invention and the nucleotide triphosphates, DNA polymerase enzyme and buffer solution required to carry out a PCR reaction. Preferred kits comprise forward and reverse primers for detection of a specific mutation and forward and reverse control primers.

EXAMPLES

Materials and Methods

Primers were designed against the 4 most common mutations in the PIK3CA gene (Accession Number: NM_006218). ARMS primers were designed to detect 2

mutations in exon 20: H1047R and H1047L; and 2 mutations in exon 9: E452K and E454K. A control primer was designed to cDNA position 2450 in the PIK3CA gene.

Scorpions were also designed. To allow multiplexing of a number of assays in each reaction the three scorpion primers were labelled with different fluorophores.

Primer designs

A number of ARMS primers were designed specific for each target region. The target region for the E542K and E545K mutations are shown below as SEQ ID NOS. 1 and 2 respectively (the mutant bases are shown in brackets with the normal variant first). The forward primers to the mutations are also shown below (SEQ ID NOS. 3 to 16). To enhance the specificity of these reactions, additional primer mismatches close to the 3'-terminus were used (shown underlined in the primer sequences). The optimal primers (E542K-2 and E545K-4) were used for the experiments described. The Scorpions primer usable with the primer sequences is shown as SEQ ID NOS. 17 and 18. Regions of correspondence between the Scorpions primer and the target regions are shown in identical highlighting or underlining.

Exon 9 Region

AACAGAGAATCTCCATTTTAGCACTTACCTGTGACTCCATAGAAAATCTTTCTCC
TGCTCAGTGATTT(C/T)AGAGAGAGGATCTCGTGTAGAAATTGCTTTGAGCTGTT
CTTTGTCATTTTCCCTTAATTCATTGTCTCTAGCTAGTCTGTTACTCTGTAAAATA
AAATAATATCTTATATA (SEQ ID NO. 1)

AACAGAGAATCTCCATTTTAGCACTTACCTGTGACTCCATAGAAAATCTTTCTCC
TGCT(C/T)AGTGATTTCAGAGAGAGGATCTCGTGTAGAAATTGCTTTGAGCTGTT
CTTTGTCATTTTCCCTTAATTCATTGTCTCTAGCTAGTCTGTTACTCTGTAAAATA
AAATAATATCTTATATA (SEQ ID NO. 2)

Mutation	Primer Sequence	SEQ ID NO.
E542K-0	5'-CTTTCTCCTGCTCAGTGATTTT-3'	3
E542K-1	5'-CTTTCTCCTGCTCAGTGATTAT-3'	4
E542K-2	5'-CTTTCTCCTGCTCAGTGATTCT-3'	5
E542K-3	5'-CTTTCTCCTGCTCAGTGATTGT-3'	6
E542K-4	5'-CTTTCTCCTGCTCAGTGATATT-3'	7
E542K-5	5'-CTTTCTCCTGCTCAGTGATCTT-3'	8
E542K-6	5'-CTTTCTCCTGCTCAGTGATGTT-3'	9
E545K-0	5'-ACTCCATAGAAAATCTTTCTCCTGCTT-3'	10
E545K-1	5'-ACTCCATAGAAAATCTTTCTCCTGCAT-3'	11
E545K-2	5'-ACTCCATAGAAAATCTTTCTCCTGCCT-3'	12
E545K-3	5'-ACTCCATAGAAAATCTTTCTCCTGCGT-3'	13
E545K-4	5'-ACTCCATAGAAAATCTTTCTCCTGATT-3'	14
E545K-5	5'-ACTCCATAGAAAATCTTTCTCCTGGTT-3'	15
E545K-6	5'-ACTCCATAGAAAATCTTTCTCCTGTTT-3'	16
Exon 9 scorpion	Hex-CGCGCTCGTGTAGAAATTGCTTTGAGCGCG-que-heg-CAATGAATTAAGGGAAAATGACA	17 and 18

Exon 20 region

The target region for the H1047R and H1047L mutations are shown below as SEQ ID NO. 19 (the mutant bases are shown in brackets with the normal variant first). The forward primers to the mutations are also shown below (SEQ ID NOS. 20 to 33). To enhance the specificity of these reactions, additional primer mismatches close to the 3'-terminus were used (shown underlined in the primer sequences). The optimal primers (H1047R-1 and H1047L-1) were used for the experiments described. The Scorpions primer usable with the primer sequences is shown as SEQ ID NOS. 34 and 35. Regions of correspondence between the Scorpions primer and the target regions are shown in identical highlighting or underlining.

AGTGCAGTGTGGAATCCAGAGTGAGCTTTCATTTTCTCAGTTATCTTTTCAGTTC
AATGCATGCTGTTTAATTGTGTGGAAGATCCAATCCATTTTTGTTGTCCAGCCAC
CATGA(T/C/A)GTGCATCATTCATTTGTTTCATGAAATACTCCAAAGCCTCTTGCTC
AGTTTTATCTAAGGCTAGGGTCTTTCGAATGTATGCAATGTCATCAAAAGATTGT
AGTTCTGGCATTCCAGAGCCAAGCATCATTGAGAAAAGATTTATGAAGAGATTG
GCATGCTGTCGAATAGCTAGATAAGCCTT (SEQ ID NO. 19)

Mutation	Primer Sequence	SEQ ID NO.
H1047R-0	5'-TGTTGTCCAGCCACCATGAC-3'	20
H1047R-1	5'-TGTTGTCCAGCCACCATGCC-3'	21
H1047R-2	5'-TGTTGTCCAGCCACCATGGC-3'	22
H1047R-3	5'-TGTTGTCCAGCCACCATGTC-3'	23
H1047R-4	5'-TGTTGTCCAGCCACCATAAC-3'	24
H1047R-5	5'-TGTTGTCCAGCCACCATCAC-3'	25
H1047R-6	5'-TGTTGTCCAGCCACCATTAC-3'	26
H1047L-0	5'-TGTTGTCCAGCCACCATGAA-3'	27
H1047L-1	5'-TGTTGTCCAGCCACCATGCA-3'	28
H1047L-2	5'-TGTTGTCCAGCCACCATGGA-3'	29
H1047L-3	5'-TGTTGTCCAGCCACCATGTA-3'	30
H1047L-4	5'-TGTTGTCCAGCCACCATAAA-3'	31
H1047L-5	5'-TGTTGTCCAGCCACCATCAA-3'	32
H1047L-6	5'-TGTTGTCCAGCCACCATTAA-3'	33
Exon 20 scorpion	Fam-CGCGGCATGAAATACTCCAAAGCCGCG-que-heg-CCCTAGGCTTAGATAAAACTGAGCAA	34 and 35

Control Primers

The control primers are shown below. Regions of correspondence between the Scorpions primer and the target regions are shown in identical highlighting or underlining.

AGGCTTGAAGAGTGTCGAATTATGTCCTCTGCAAAAAGGCCACTGTGGTTGAAT TGGGAGAACCCAGACATCATGTCAGAGTTACTGTTTCAGAACAATGAGATCATC TTTAAAAATGGGGATGG (SEQ ID NO. 36)

Mutation	Primer Sequence	SEQ ID NO.
Control primer	5'-AGATGATCTCATTGTTCTGAAACAG-3'	37
Control scorpion	Rox-CCGGCCAATTCAACCACAGTGGCCGG-que-heg-GGCTTGAAGAGTGTCGAATTA	38 and 39

All primers were synthesised and supplied by Invitrogen. PCR Buffer, Taq and Magnesium were supplied by Eurogentec and dNTPS were purchased from Abgene Ltd. Scorpions were synthesised and supplied by ATDBio.

Assays were multiplexed into 2 reactions containing a control assay and 2 ARMS assays (1 x exon 9 and 1 x exon 20). Assays were performed in 25ul reaction volume containing 1x PCR Buffer, 4.0mM $MgCl_2$, 200uM dNTP mix, 0.25uM of each primer (control primer and 2 ARMS primers) and 0.25uM of each scorpion (control scorpion (SEQ ID NOS. 38 and 39), exon 20 scorpion (SEQ ID NOS. 34 and 35) and exon 9 scorpion (SEQ ID NOS. 17 and 18)). 2.5ul of DNA template was added to each reaction. The H1047R and E542K primers were multiplexed with 2.5 unit Taq polymerase per reaction. The H1047L and E545K primers were multiplexed with 3.0 unit Taq polymerase per reaction. The E542K primer used was E542K-2 (SEQ ID NO. 5). The E545K primer used was E545K-4 (SEQ ID NO. 14). The H1047R primer used was H1047R-1 (SEQ ID NO. 21). The H1047L primer used was H1047L-1 (SEQ ID NO. 28).

In all cases the reactions were amplified on a Stratagene Mx3000P under the following conditions: 95°C for 10minutes, followed by 45 cycles of 90°C for 30 seconds and 60°C for 1 minute.

DNA cassettes harbouring point mutations to use as positive controls were constructed based on a method described by Higuchi et al.[2]. In brief, corresponding outer and mutamer primers were used to generate half cassettes with complementary ends, each half cassette containing a mutant base. These PCR products were mixed and amplified with inner nested primers. Self priming of the complementary half cassettes and subsequent amplification created a final product with a mutated base. Products were sequenced to ensure the correct sequence had been created. This process was repeated for each mutation of interest. The DNA cassette was mixed with an equal amount of genomic DNA to create a 100% positive control.

Example 1

To determine the specificity of the reactions and the primers each assay was performed with 5-50ng of genomic DNA per reaction to assess breakthrough signal caused by extension of mismatched primer. For each reaction a ΔCt value (control Ct- mutation Ct) was defined (Ct = threshold cycle). The reactions were performed six times for each DNA concentration and repeated in triplicate on separate occasions to define a cut off ΔCt value below which any amplification can be said to be due to the presence of mutant sequence and not due to breakthrough signal. The cut off ΔCt value was determined to be 1 Ct below the lowest ΔCt value seen in all reactions for each assay. For H1047R and H1047L assays the cut off ΔCt was defined as 12, for the E542K assay the cut off ΔCt was 9 and for the E545K assay the cut off ΔCt was 8.

Example 2

To assess the sensitivity of the assay, 5 copies of mutant DNA were diluted in varying concentrations of genomic DNA to give final concentrations of 5, 2, 1, 0.5 and 0.1% mutant DNA to wild type. Table 1 illustrates the sensitivity of the 4 ARMS assays. The table shows the ΔCt values for reducing concentrations of mutant DNA within a background of wild type DNA. The predefined cut off ΔCts are illustrated in the final column. The exon 20 assays were able to detect 5 copies of mutant DNA when this comprised only 0.1% of the total DNA (within the previously defined cut off ΔCt). The exon 9 assays were able to detect 5 copies of DNA at 1% concentration with a ΔCt within the predefined cut off values (Table 1).

Table 1:

WT DNA /reaction (copies)	MUT DNA /reaction (copies)	Relative % of MUT alleles	ΔCT					
			H1047L	H1047R	E542K	E545K	CUT OFF ΔCt	
100	5	5%	5.9	4.3	5.1	5.8		
250	5	2%	7.2	6.7	7.2	6.4	H1047L	12
500	5	1%	8.3	7.6	8.4	7.0	H1047R	12
1000	5	0.5%	9.3	9.0	10.2	8.1	E542K	9
5000	5	0.1%	11.5	10.5	12.1	10.1	E545K	8

Example 3

Admixtures of cell lines containing mutation H1047R (HCT-116) and E545K (MCF-7) were used to compare the relative sensitivities of the ARMS assays compared with sequencing. Both cell lines were heterozygous for the mutation. Sequencing was performed using primers and PCR cycling conditions as described by Samuels et al[1]. ARMS assays and sequencing were carried out at concentrations of the mutant gene of 100%, 50%, 30%, 10% and 1% of the total mixture. The results are shown in Figure 1 in which the results under the heading "Scorpions" show the increase in amplicon copy number after successive rounds of PCR (results using control primers and mutant primers are shown as separate lines). Under the heading "DNA Sequencing" is shown the results of sequencing the reverse strand of the gene in the mixture. Sequencing was not able to detect the presence of H1047R mutant when present at less than 50% of the total mixture and was unable to detect the presence of E545K mutant when present at less then 30% of the total mixture. Assays using

the primers of the invention, by contrast, were able to detect the presence of mutants at 1% concentration.

Example 4

This assay was applied to DNA extracted from fresh frozen tissue from a variety of tumour types that were assessed for the presence of PIK3CA mutations using the ARMS/Scorpion assay. In total DNA was available from 279 tumour samples. The assay reported mutations in 5 of 49 (10.2%) colorectal cancer samples, 19 of 49 (38.7%) breast cancer samples, 1 of 51 (1.9%) lung cancer samples, and 1 of 34 (2.9%) melanoma samples. No mutations were detected in 50 prostate or 46 ovarian cancer samples. Of the colorectal samples positive for PIK3CA mutations 3 were H1047R, 1 was H1047L and 1 was E542K; of the breast cancer samples positive for PIK3CA, 15 were H1047R, 1 was H1047L and 3 were E545K; both the mutations in the lung cancer sample and melanoma sample positive for PIK3CA mutations were H1047R. Sequencing identified only 14 of the total 26 (53%) mutations detected. Sequencing detected a mutation in one breast cancer specimen which the ARMS assay was not designed to detect (c.1634 A>G; p E545G). This is not a novel mutation and has been previously described in breast and colorectal cancers[3-5].

The incidence of PIK3CA mutations in the samples analysed was consistent with previous studies with the exception of ovarian cancer [1, 3-9]. PIK3CA mutations have been previously described in ovarian cancers but it has been suggested that there may be an associated with endometriod and clear cell cancers [8, 10]. All the ovarian cancers tested in this study were serous adenocarcinomas which may explain the absence of any PIK3CA mutations.

The ARMS assay identified significantly more mutations in the clinical samples than seen by direct sequencing. The cell line admixtures confirm that this assay is more sensitive than sequencing in detecting the PI3KCA mutations of interest. It is likely that the heterogeneity of clinical samples that will contain both tumour and normal tissue will mean that in some instances the incidence of mutation will be below that detectable by sequencing methods and as such the ARMS assay is more suitable for

clinical application. The drawback is that only certain ARMS specific mutations will be detected. However in this series of 279 samples only a single mutation in exon 9 or 20 of the PI3KCA gene was detected that the ARMS assay was not designed to detect.

In summary, the examples show that the present invention provides a sensitive, high throughput assay for the detection of the 4 most common mutations in the PIK3CA gene. This assay may be applied to small amounts of DNA and can detect low levels of mutant PIK3CA within a sample.

Sequence Listing Free text

<210> 1
<223> E542K target region

<210> 2
<223> E545K target region

<210> 3
<223> E542K-0 fwd primer

<210> 4
<223> E542K-1 fwd primer

<210> 5
<223> E542K-2 fwd primer

<210> 6
<223> E542K-3 fwd primer

<210> 7
<223> E542K-4 fwd primer

<210> 8
<223> E542K-5 fwd primer

<210> 9
<223> E542K-6 fwd primer

<210> 10
<223> E545K-0 fwd primer

<210> 11
<223> E545K-1 fwd primer

<210> 12
<223> E545K-2 fwd primer

<210> 13
<223> E545K-3 fwd primer

<210> 14
<223> E454K-4 fwd primer

<210> 15
<223> E545K-5 fwd primer

<210> 16
<223> E545K-6 fwd primer

<210> 17
<223> Exon 9 Scorpion

<210> 18
<213> Exon 9 Scorpion 2

<210> 19
<223> H1047R and H1047L target regions

<210> 20
<223> H1047R-0 fwd primer

<210> 21
<223> H1047R-1 fwd primer

<210> 22
<223> H1047R-2 fwd primer

<210> 23
<223> H1047R-3 fwd primer

<210> 24
<223> H1047R-4 fwd primer

<210> 25
<223> H1047R-5 fwd primer

<210> 26
<223> H1047R-6 fwd primer

<210> 27
<223> H1047L-0 fwd primer

<210> 28
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<210> 29
<223> H1047L-3 fwd primer

<210> 30
<223> H1047-3 fwd primer

<210> 31
<223> H1047L-4 fwd primer

<210> 32
<223> H1047L-5 fwd primer

<210> 33
<223> H1047L-6 fwd primer

<210> 34
<223> Exon 20 Scorpion

<210> 35
<223> Exon 20 Scorpion 2

<210> 36
<223> Control target region

<210> 37
<223> Control primer

<210> 38
<223> Control Scorpion

<210> 39
<223> Control Scorpion 2

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CLAIMS

1. A polynucleotide comprising at least 75% of the ten nucleotides at the 3' end of one of the following primer sequences, or a sequence complementary thereto: SEQ. ID NOS. 3 to 16, 18, 20 to 33, 35 or 37 to 39.

2. A polynucleotide according to claim 1 wherein the polynucleotide is less than 100 nucleotides long, preferably less than 80 nucleotides long, more preferably less than 60 nucleotides long, more preferably less than 40 nucleotides, more preferably less than 30 nucleotides long.

3. A polynucleotide according to claim 1 or 2 comprising at least 75% of the 12, 14, 16, 17, 18 or 20 nucleotides at the 3' end, or the entirety, of one of the following primer sequences, or a sequence complementary thereto: SEQ. ID NOS. 3 to 16, 18, 20 to 33, 35 or 37 to 39.

4. A polynucleotide according to any one of the preceding claims further comprising a quencher group and a fluorophore group.

5. A polynucleotide according to claim 4 wherein the quencher group and the fluorophore group are separated by a nucleotide tail sequence comprising first and second regions, the nucleotides of the first region being complementary to but in reverse order from the nucleotides of the second region, such that hybridisation of the first region to the second group results in the quencher group to be sufficiently close to the fluorophore group to quench the fluorophore group.

6. A polynucleotide according to claim 5 wherein the tail sequence further comprises a third region having a sequence complementary to a region of the PIK3CA gene.

7. A polynucleotide according to claim 6 comprising at least the six nucleotides at the 3' end of SEQ. ID NO. 18 and the tail sequence comprises SEQ. ID NO. 17.

8. A polynucleotide according to claim 6 comprising at least the six nucleotides at the 3' end of SEQ. ID NO. 35 and the tail sequence comprises SEQ. ID NO. 34.

9. A polynucleotide according to claim 6 comprising at least the final nucleotides at the 3' end of SEQ. ID NO. 39 and the tail sequence comprises SEQ. ID NO. 38.

10. A polynucleotide according to any one of claims 4 to 9 wherein the quencher group comprises Dabcyl.

11. A polynucleotide according to any one of claims 4 to 10 wherein the fluorophore comprises Hex, Fam or Rox.

12. A kit comprising at least two of the polynucleotides defined in one of the preceding claims.

13. A kit according to claim 12 comprising a polynucleotide comprising SEQ ID NO. 18 and a polynucleotide comprising any one of SEQ ID NOS. 3 to 16; or a polynucleotide comprising SEQ ID NO. 35 and a polynucleotide comprising any one of SEQ ID NOS. 20 to 33; or a polynucleotide comprising SEQ ID NO. 39 and a polynucleotide comprising SEQ ID NO. 37.

14. A kit according to claim 12 of 13 further comprising nucleotide triphosphates, a polymerisation enzyme and/or a buffer solution.

15. Use of a polynucleotide according to any one of claims 1 to 11 or a kit according to any one of claims 12 to 14; or a polynucleotide comprising four or five of the six nucleotides at the 3' end of SEQ. ID NOS. 3 to 16, 18, 20 to 33 or 35 or sequences complementary thereto for detecting a mutation in a nucleic acid sample containing at least a fragment of the PIK3CA gene.

16. Use according to claim 15 wherein the fragment of the PIK3CA gene in the nucleic acid sample is at least 10 nucleotides long, preferably 20 nucleotides long, more preferably 30 nucleotides long and more preferably 40 nucleotides long.

17. A method of detecting the presence or absence of a mutation in the PIK3CA gene comprising the steps of:

a) mixing a nucleic acid sample comprising at least a fragment of the PIK3CA gene with a polynucleotide comprising at least the six nucleotides at the 3'

end of one of the following primer sequences, or a sequence complementary thereto: SEQ ID NOS. 3 to 16 or 20 to 33 ; and

b) detecting hybridisation of the polynucleotide to the nucleic acid sample wherein hybridisation indicates the presence of a mutation.

18. A method according to claim 17 wherein the polynucleotide comprises one of the following primer sequences: SEQ ID NOS. 3 to 16 or 20 to 33.

19. A method according to claim 17 or 18 further comprising the step of, prior to step a), amplifying the number of copies of the fragment of the PIK3CA gene using thermal cycling nucleic acid amplification, preferably PCR.

20. A method according to any one of claims 17 to 19, wherein step b) comprises carrying out DNA polymerisation using the polynucleotide as a first primer and detecting the extension product of polymerisation.

21. A method according to claim 19 or 20 wherein step b) comprises the step of mixing the nucleic acid sample and the polynucleotide with a second primer which corresponds to a region of the fragment of the PIK3CA sequence downstream of the region to which the polynucleotide is complementary and carrying out PCR on the mixture.

22. A method according to claim 21 wherein the second primer comprises: SEQ. ID NO. 18 and the polynucleotide comprises at least four or five of the six nucleotides at the 3' end of SEQ. ID NOS. 3 to 16; or the second primer comprises SEQ. ID NO. 35 and the polynucleotide comprises at least four or five of the final six nucleotides of SEQ. ID NOS. 20 to 33.

23. A method according to claim 20 further comprising the step of carrying out PCR on the sample using control primers and comparing the amplification of the PIK3CA gene with amplification using the polynucleotide and the second primer.

24. A method according to claim 23 wherein the control primers comprise SEQ ID NOS. 37 and 39.

25. A method according to any one of claims 17 to 24 wherein the polynucleotide comprise a quencher group and a fluorophore group and wherein step b) comprises exposing the mixture to light of a wavelength to which the fluorophore is responsive in the absence of the quencher group and detecting light at the wavelength emitted by the fluorophore group in the absence of the quencher group.

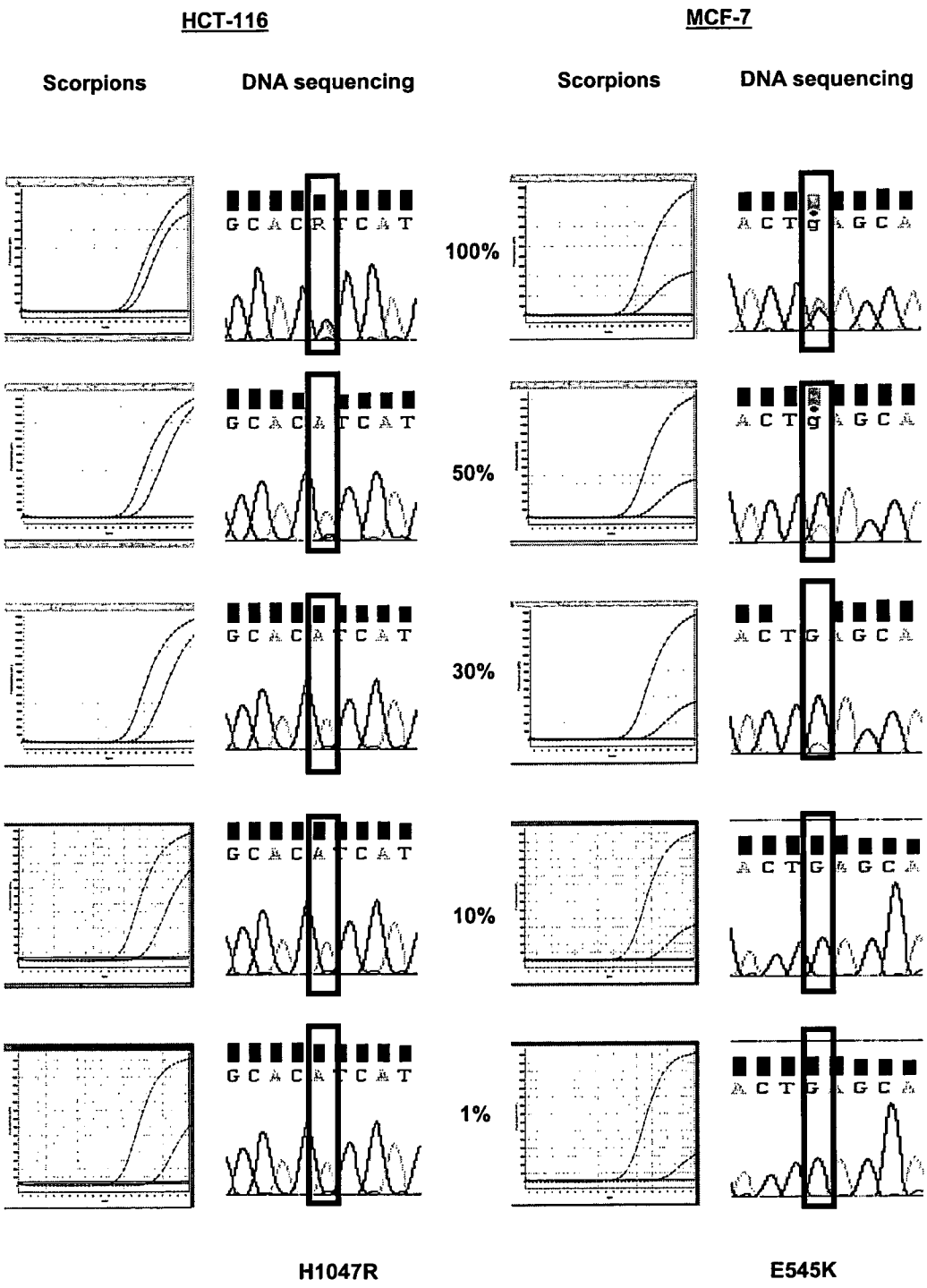
HCT-116
MCF-7
Scorpions
DNA sequencing
Scorpions
DNA sequencing
100%
50%
30%
10%
1%
H1047R
E545K

Figure 1