Multiplexed Assays for Detection of Mutations in PIK3CA

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BACKGROUND: Mutations in the PIK3CA gene (phosphoinositide-3-kinase, catalytic, alpha polypeptide) have recently been described in a number of cancers, and their detection is currently limited because of the low sensitivity of conventional sequencing techniques.

METHODS: We combined Amplification Refractory Mutation System (ARMS™; AstraZeneca) allele-specific PCR and Scorpions™ (DxS) to develop assays for tumor-borne PIK3CA mutations and used real-time PCR to develop high-throughput multiplexed assays for the most commonly reported PIK3CA mutants (H1047L, H1047R, E542K, E545K).

RESULTS: These assays were more sensitive than sequencing and could detect 5 copies of mutant DNA in proportions as low as 0.1% of the total DNA. We assayed DNA extracted from human tumors and detected PIK3CA mutation frequencies of 10.2% in colorectal cancer, 38.7% in breast cancer, 1.9% in lung cancer, and 2.9% in melanoma. In contrast, sequencing detected only 53% of the mutations detected by our assay.

CONCLUSIONS: Multiplexed assays, which can easily be applied to clinical samples, have been developed for the detection of PIK3CA mutations.

Phosphatidylinositol 3-kinases (PI3K) are a large family of lipid kinases involved in cell signaling. The activation of the PI3K-AKT pathway in a number of tumor types produces abnormalities in cell growth, proliferation, and survival (1). Recently, mutations in the gene for the catalytic subunit of the class 1A PI3K (PIK3CA, phosphoinositide-3-kinase, catalytic, alpha polypeptide) have been identified in human cancers (2). The presence of PIK3CA mutations in a tumor has been suggested as a positive prognostic factor (3) and may predict the response to treatment (4). The precise role of these mutations in carcinogenesis is still to be clearly defined, but with the ongoing development of a number of inhibitors for targeting PI3K, the detection of mutations will become increasingly important in patient selection. Technical challenges in the detection of such mutations arise from the limitations of tumor biopsies that may contain only small quantities of the mutated sequences. Furthermore, DNA extracted from paraffin-embedded tissues is often degraded and of poor quality. Traditional Sanger sequencing can detect 15%–25% mutant DNA in a background of wild-type DNA, so there is a pressing need to develop more sensitive assays capable of detecting smaller amounts of mutated alleles in a heterogeneous sample. We have combined 2 powerful techniques, Amplification Refractory Mutation System (ARMSd™; AstraZeneca) allele-specific PCR and Scorpions™ (DxS), a fluorescent signaling system that detects PCR products, to develop more sensitive assays for tumor-borne mutations in the PIK3CA gene.

ARMS is based on the principle that extension is efficient when the 3' terminal base of a primer matches its target, whereas extension is inefficient or nonexistent when the terminal base is mismatched. Primers are designed against the mutation of interest, and an amplicon is produced only if the mutation is present (5). Scorpion probes are bifunctional molecules that contain a PCR amplification primer attached to a probe sequence (6). During the PCR, the primer is extended to form an amplicon, and the probe, which contains an amplicon-specific sequence, binds to its target to produce a fluorescent signal. The amount of fluorescence will be proportional to the amount of amplicon generated.

Primers were designed against the 4 most common mutations in the PIK3CA gene (accession no. NM_006218) with Primer3 version 3.0 software (7). ARMS primers were designed to detect 2 mutations in exon 20 (encoding the H1047R and H1047L variants) and 2 mutations in exon 9 (E542K and E545K). A control primer was designed to cDNA position 2 450 in the PIK3CA gene.

All primers were synthesized and supplied by Invitrogen. PCR buffer, Taq, and magnesium were supplied by Eurogentec, and deoxynucleoside triphosphates were purchased from Abgene. Scorpions were synthesized and supplied by ATDBio.

Assays were multiplexed into 2 reactions containing a control assay and 2 ARMS assays (1 for exon 9 and 1 for exon 20). Assays were performed in a 25-μL reaction volume containing 1 × PCR buffer [Hot Gold Star Buffer (150 mM tris-HCl [pH 8.0 at 25 °C], 500 mM...
KCl, 0.1% Tween 20), 4.0 mmol MgCl2, 200 μmol of each deoxynucleoside triphosphate, 0.25 μmol of each primer (control primer and 2 ARMS primers), and 0.25 μmol of each Scorpion (control Scorpion, exon 20 Scorpion, and exon 9 Scorpion). DNA template (2.5 μL) was added to each reaction. The H1047R and E542K primers were multiplexed with 2.5 U Taq polymerase per reaction. The H1047L and E545K primers were multiplexed with 3.0 U Taq polymerase per reaction.

We used the following ARMS primers: control primer, 5′-AGATGATCTCATGGTTCTGAAACAG-3′; H1047R, 5′-TGTTGTCCAGCCACCATGCC-3′; H1047L, 5′-TGTTGTCCAGCCACCATGCA-3′; E542K, 5′-CTTCTTCTGCTCAGTGATTCT-3′; and E545K, 5′-ACTCATAGAAAATCTTTCTCCTGATT-3′. We designed Scorpions with Visual OMP 6.0 (DNA Software) and labeled the 3 Scorpion primers with different fluorophores as follows to allow multiplexing of a number of assays in each reaction: control Scorpion, Rox-CCGGCCAATTCAACCACAGTGGC CGG-que-heg-GGCTTGAAGAGTGTCGAATTA; exon 20 Scorpion, Fam-CGCGGCATGAAATACTCCAAA GCCGG-que-heg-CCCTAGCCTTAGATAAAACTGA GCAA; and exon 9 Scorpion, Hex-CGGGCTCGTGTA GAAATTGCTTTGAGCGCG-que-heg-CAATGAATTA AGGGAAAATGACA. (Rox is 5-carboxy-X-rhodamine; que, “quencher,” which in this case is dabcyldT; heg, hexaethylene glycol; Fam, 6 carboxyfluorescein; Hex, hexachlorofluorescein.) The reactions were amplified in all cases on a Stratagene Mx3000P instrument under the following conditions: 95 °C for 10 min followed by 45 cycles of 90 °C for 30 s and 60 °C for 1 min.

We constructed DNA cassettes harboring point mutations according to a method described by Higuchi et al. (8) for use as positive controls. 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 that 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.

To determine the specificity of the reactions, we performed each assay with 5–50 ng of genomic DNA per reaction to assess the breakthrough signal caused by extension of the mismatched primer. A value for the change in the threshold cycle (ΔCt) (mutation Ct – control Ct) was defined for each reaction. The reactions were performed 6 times for each DNA concentration and were repeated in triplicate on separate occasions to define a cutoff 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 cutoff ΔCt value was determined to be 1 Ct below the lowest ΔCt value observed in all reactions for each assay. The cutoff ΔCt was defined as 12 for the H1047R and H1047L assays, 9 for the E542K assay, and 8 for the E545K assay.

To assess the sensitivity of the assay, we diluted 5 copies of mutant DNA in various concentrations of genomic DNA to give final percentages of mutant DNA relative to wild-type DNA of 5%, 2%, 1%, 0.5%, and 0.1%. The exon 20 assays were able to detect 5 copies of mutant DNA when this mutant consisted of only 0.1% of the total DNA (within the previously defined cutoff ΔCt). The exon 9 assays were able to detect 5 copies of DNA at 1% of the total DNA with a ΔCt within the predefined cutoff values (Table 1).

Admixtures of cell lines containing mutation H1047R (HCT-116) and E542K (MCF-7) were used to compare the relative sensitivities of the ARMS assays with sequencing (Fig. 1). Both cell lines were heterozygous for the mutation. Sequencing was carried out with

| Table 1. Sensitivity of the 4 ARMS assays. a |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| WT DNA/ reaction, copies | MUT DNA/ reaction, copies | Relative percent MUT alleles | ΔCt | Cutoff ΔCt |
| 100 | 5 | 5% | 5.9 | 4.3 | 5.1 | 5.8 | H1047L | 12 |
| 250 | 5 | 2% | 7.2 | 6.7 | 7.2 | 6.4 | H1047R | 12 |
| 500 | 5 | 1% | 8.3 | 7.6 | 8.4 | 7.0 | H1047L | 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 |

a Table shows the ΔCt values for reducing concentrations of mutant (MUT) DNA within a background of wild-type (WT) DNA. The predefined cut off ΔCt values are illustrated in the final column.
primers and PCR cycling conditions as described by Samuels et al. (2). Sequencing was not able to detect the presence of the H1047R mutant when present at <50% of the total mixture and was unable to detect the presence of the E542K mutant when present at <30% of the total mixture. The ARMS/Scorpion assays, by contrast, were able to detect the presence of a mutant at 1% of the total DNA.

We assayed DNA extracted from fresh frozen tumor tissue to determine the presence of PIK3CA mutations. DNA was available from 279 tumor samples. The assay reported mutations in 5 (10.2%) of 49 colorectal cancers, 19 (38.7%) of 49 breast cancers, 1 (1.9%) of 51 lung cancers, and 1 (2.9%) of 34 melanomas. No mutations were detected in 50 prostate or 46 ovarian cancer samples. Of the colorectal samples positive for PIK3CA mutations, 3 were H1047R mutants, 1 was H1047L, and 1 was E542K. Of the breast cancer samples positive for PIK3CA mutations, 15 were H1047R mutants, 1 was H1047L, and 3 were E545K. Both of the mutations in the lung cancer and melanoma samples positive for PIK3CA mutations were H1047R. Sequencing identified only 14 (53%) of the 26 mutations detected and did not identify any false negatives. Sequencing detected a mutation in 1 breast cancer sample that the ARMS assay was not designed to detect (c.1634 A>G; p.E545G). This mutation has previously been described (9–11).

The incidence of PIK3CA mutations in the samples was consistent with previous studies, with the
exception of ovarian cancer (2, 9–15). PIK3CA mutations have previously been described in ovarian cancers, but they may also be associated with endometrioid and clear cell cancers (14, 16). All of the ovarian cancers tested in this study were serous adenocarcinomas, a result that may explain the absence of any PIK3CA mutations.

The ARMS assay identified more mutations in the clinical samples than direct sequencing. The cell line admixtures confirm that this assay is more sensitive than sequencing for detecting the PIK3CA mutations of interest. The heterogeneous nature of clinical samples, which will contain both tumor and normal tissue, means that the mutation incidence may be below that detectable by sequencing methods in some instances, and the ARMS assay will be more suitable for clinical application. The drawback is that only certain ARMS-specific mutations will be detected. In this series of 279 samples, however, we detected only a single mutation in PIK3CA exon 9 or 20 that the ARMS assay was not designed to detect.

In summary, we have developed a sensitive assay for detecting the 4 most common mutations in the PIK3CA gene. This assay may be applied to small amounts of DNA and can detect low concentrations of mutant PIK3CA within a sample. Future work with this assay will include detection of PIK3CA mutations in DNA extracted from paraffin-embedded tissue and DNA in the circulation.

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References


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