Does Phaster Mean Better?

The accelerated pace of discovery of disease-causing genes has made efficient mutation detection a priority. Identification of DNA sequence variants allows human geneticists to determine, among other things, whether a candidate gene contributes to disease susceptibility, to identify new alleles at known loci, and to develop molecular diagnostic tests. Once a susceptibility gene has been identified, the investigator interested in molecular diagnostics or genotype–phenotype correlation must decide how to perform rapid, sensitive detection of mutations.

Grompe [1] divided these techniques into those that are most useful for identifying known mutations and those that are better for detecting novel mutations. However, he also allowed that no one method would be appropriate for all situations. Clearly, the choices investigators make will be based, in part, on what they are trying to accomplish (e.g., diagnostics vs population studies of allele frequencies); the goal will also dictate the level of sensitivity required and, conversely, the proportion of false positives or negatives that can be tolerated. Other factors such as knowledge of genomic size, sequence, and structure; availability of RNA/cDNA; and access to equipment and technical expertise will also influence the decision.

Since its identification in 1993 and 1994 as the (or, at least, a) major gene responsible for multiple endocrine neoplasia types 2A and 2B (MEN 2A, MEN 2B) and Hirschsprung disease (HSCR), respectively, the RET receptor tyrosine kinase [3] has provided a real-time case study of some of the more vexing aspects of both the detection and interpretation of mutations [2–7]. The latter problem, namely, correlating genotype with phenotype, is a fascinating one and has been considered elsewhere [8–10].

The former issue, determining the best approach to detecting RET mutations for a given set of patients, is easier to deal with, in that it is primarily a technical question. In this issue, Siegelman et al. [11] present an optimized single-strand conformation polymorphism (SSCP) assay performed with the automated Phast System™ (Pharmacia Biotech, Piscataway, NJ) for three RET exons. As the authors point out, the ability to detect these mutations has freed clinicians from the more invasive, less reliable pentagastrin test (which increases serum calcitonin concentrations) as a predictor for this set of late-onset diseases, mainly medullary thyroid carcinoma (MTC). The following bears emphasizing: Increased calcitonin concentrations are apparently present in only about half of MTC/MEN2 gene carriers. Moreover, biochemical and clinical evidence of MTC is age-dependent.

Thus, given that >92% of MEN2 families have been found to carry a RET mutation [10], identification of gene carriers by direct means offers a more reliable, age-independent alternative to biochemical screening. Although the cost-effectiveness of this may be difficult to quantify [10], the clinical implications are profound: Early diagnosis allows for prophylactic surgery (thyroidectomy and lymph node clearance) with a very high cure rate [12–15].

Given the size of RET (21 exons coding for a protein with two major isoforms of ~1100 amino acids each) and the difficulty in obtaining RET RNA from germline (non-tumor) tissue, scanning genomic DNA (in this case, PCR product) is clearly the way to go. If it were necessary to scan all exons for mutations (as is the case in HSCR), this would be too daunting, even for a small number of patients. However, because the neuroendocrine tumor-associated RET mutations are clustered among the cysteine residues in exons 10 and 11 (and, in the case of MEN 2B, a single methionine codon in exon 16), a simple and rapid assay should be possible.

What are the options for developing such an assay? Unfortunately, because of the variety of nucleotide changes in exons 10 and 11 that alter a small number of cysteine residues, restriction enzyme analysis becomes impractical. Sequencing is certainly sensitive, but relatively labor-intensive and expensive. Denaturing gradient gel electrophoresis (DGGE) is also highly sensitive, but requires a substantial initial investment in electrophoretic apparatus, primers containing long GC clamps, and determination of melting curves for each PCR product. For the laboratory with DGGE already up and running, or one that is prepared to make a long-term commitment to mutation detection, this approach may be the most fruitful, and reliable conditions for RET exons have been developed [16, 17].

What Phast System-based SSCP offers is, in a word, speed. This comes from the system’s use of precast gels, buffer strips precast in agarose, short run times (gels are only ~5 cm long), temperature control, and an automated silver-staining chamber. Thus, unlike in conventional SSCP, heteroduplex analysis, DGGE, and other sequencing gel-based detection methods, it is not necessary to pour gels, make buffer, run gels in the cold room, or wrestle with unwieldy staining apparatus—where many a gel has been lost. Moreover, the small scale of the Phast System requires only picogram quantities of PCR product for efficient detection. As for expense, beyond the initial $8000–$9000 for the apparatus, our laboratory has estimated that each RET genotype is obtained at a cost of no more than a few dollars, including DNA isolation and PCR. Clearly, this cost can vary widely, based on the availability and price of reagents and competent technical help. The system is also ideally suited to both denaturing and nondenaturing protein gels. A more compelling case can be made for purchasing the Phast System if a labora-

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1 I am biased, but I prefer this designation to the more popular “RET protooncogene” because we now know that RET is much more than the cellular version of a cancer susceptibility gene; it has critical roles in kidney and gut development. “Receptor tyrosine kinase” is a neutral term describing what the protein is (a receptor) and its generic cellular function (to phosphorylate tyrosine residues), without making reference to disease phenotype.
tory has a need for both DNA and protein applications. However, the present discussion is limited to the merits of Phast-based SSCP.

As stated, although the common MEN 2B mutation in exon 16 (M918T) removes a Fok I site and could therefore be easily restricted by resolution digest, exons 10 and 11 harbor a variety of mutations in multiple codons in MEN 2A and MTC patients (see Table 1 in reference 11). The Phast approach is particularly appropriate for this type of situation, i.e., where a large number of mutations are clustered among a small number of exons. Indeed, as Siegelman et al. point out, the system is a simple, rapid, and relatively inexpensive alternative to DGGE and conventional isotopic methods. Given the important decisions that must be made in light of a diagnosis of MEN2, this type of rapid mutation detection will undoubtedly continue to have great clinical utility.

Certainly, no mutation detection system is perfect, and the Phast System is no exception. One general problem with SSCP is that the assay provides no information as to the exact nature of the sequence variation. Clearly, for diagnostic purposes and genotype–phenotype correlations, sequencing is still an essential final step. Another limitation is sensitivity which, in our hands, fell well short of the 95% found by Siegelman et al. [11]. Subsequent to our Phast-based mutation screen of the entire length of \textit{RET} among HSCR patients [2], which detected 8 mutations, an additional 4 sequence variants were found by DGGE and (or) sequencing (R.M.W. Hofstra, S. Bolk, M. Angrist, C.H.C.M. Buys, and A. Chakravarti, unpublished data). This implies a detection rate of \( <70\% \). We are not a clinical laboratory and do not demand 100% detection, but still, for a large family with a linked haplotype, it is very frustrating not to be able to find a mutation, short of sequencing every exon. Also, false negatives make determination of genotype–phenotype correlations much slower. Like Siegelman et al. [11], we also tried two temperature conditions [2]. Some investigators will use as many as four conditions to increase sensitivity (R.G.H. Cotton, personal communication). Of course, this adds to the cost and defeats the stated purpose of maximizing efficiency. The sensitivity problem may be more relevant for HSCR, where mutations are distributed throughout the coding sequence [8], than for MEN2, where mutations are found in only a few exons.

Second, as convenient as it is, the Phast System could be much more so. As currently configured, the System can accommodate no more than 24 samples. Thus, short of purchasing two machines, one can analyze in a single loading only 1/2 to 1/3 the number of samples that can be fit on a full-size sequencing gel (with a 48- or 72-well comb, respectively). For a diagnostic laboratory that does not require high throughput, this may not be an issue. But for laboratories performing large numbers of analyses on controls, patients, and their families, turnaround time could be greatly enhanced by increasing the sample capacity. Also, my colleagues and I have found that after a while the separation control unit (where the gels sit during electrophoresis) no longer cools to 4°C. After consulting with the manufacturer, we were still not able to overcome this problem and settled for analyses at 8°C. As the report in this issue demonstrates [11], temperature variation can have a significant impact on sensitivity. By conceding the use of temperatures below 8°C, we may have missed some mutations.

Finally, in my opinion, Pharmacia would do well to adapt the Phast System for use with novel mutation detection methods, of which there is no shortage. In particular, I am thinking of the enzyme mismatch cleavage (EMC) method developed by Cotton’s group [18] and the Cleavase\textsuperscript{®} system (Third Wave Technologies, Madison, WI). Both methods use endonucleases that cleave perturbations in DNA conformation so as to distinguish between wild-type and mutant species. EMC is based on the recognition of mismatches, whereas Cleavase recognizes (and cuts) specific types of single-stranded DNA conformers as generated by SSCP. The two methods share several advantages: They are highly sensitive, are no more labor-intensive than ordinary SSCP, and, best of all, can be used to analyze fragments of \( >1 \text{ kb} \)—some two- to fourfold longer than SSCP or DGGE. SSCP in particular is most sensitive when applied to fragments of \( \sim150 \text{ bp} \) [19]. Both the EMC and Cleavase methods would be readily amenable to Phast System analysis except for the lack of commercially available acrylamide Phastgels containing urea to act as a denaturant. Using the currently available non-denaturing gels, the Phast System cannot run at a sufficiently high temperature to maintain the samples in their denatured state. Denaturing conditions are undesirable for the nondenaturing technique of SSCP but are essential for the newer methods. The optimization of these approaches for automated electrophoresis and silver-staining—which would also make end-labeling superfluous (although at the cost of knowing the exact location of the mutation)—would provide mutation hunters with another easy, nonradioactive entree into the state-of-the-art.

A final caveat: This so-called state-of-the-art is changing in a hurry, so the mutation detection debate may soon be relegated to the history books. Enterprising biotechnologists at Affymetrix (Santa Clara, CA) have figured out how to array 10\textsuperscript{4} or more oligonucleotides on silicon chips, relegating to the history books. Enterprising biotechnologists at Affymetrix (Santa Clara, CA) have figured out how to array 10\textsuperscript{4} or more oligonucleotides on silicon supports. By cohybridizing reference and test samples against these arrays and quantifying differences in hybridization patterns, one can scan huge genomic regions for all possible heterozygous mutations. This approach has recently been applied to the human mitochondrial genome and the familial early-onset breast cancer gene, \textit{BRCA1}, with impressive results [20, 21]. We can now dare to imagine extremely rapid, inexpensive, and automated mutation detection on a vast scale and, best of all, in a gel-free world. Amen!

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