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Singling-out point mutations

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A highly sensitive molecular technique for detecting and quantifying single-base mutations has been developed by researchers at Johns Hopkins University School of Medicine (http://www.hopkinsmedicine.org). The LigAmp technique is still being refined but it has the potential to become an important tool for diagnosing and managing diseases involving point mutations.

There are already several methods for detecting single-base mutations but, according to study leader James Eshelman, 'the strengths of LigAmp are its sensitivity (~1 mutant molecule in 10,000 wild-type molecules), its quantitative nature, and its ability to detect multiple mutations simultaneously'.

Ligate and amplify

The LigAmp approach (so called because it involves ligation – joining – and amplification steps) essentially scales up single-base differences into something more detectable [1]. It involves two oligonucleotides that match the target gene: one is specific at its 3′ end for the mutant base; the other matches the gene starting just 3′ to the mutant base. Both oligonucleotides bind to both mutant and wild-type versions of the gene, but only the mutant allele binds the upstream oligonucleotide tightly enough for the two oligonucleotides to ligate. The resulting strand can then be amplified and detected using quantitative PCR. Detection depends on a probe sequence built into one of the oligonucleotides, which is amplified to detectable levels only in if ligation has occurred. Ligation and amplification have already been combined in a similar way in the multiplex ligatable probe amplification (MLPA) assay but, says Eshlemen, LigAmp is different as it focuses on point mutations rather than large deletions or duplications.

Eshelman's team tested their method on two important mutations: one in *KRAS2*, a gene implicated in most pancreatic cancers, and the K103N mutation of HIV-1, which confers drug resistance. They were able to detect and quantify both mutations in samples containing mutant and wild-type DNA in a ratio of 1:10,000. They have also shown that the technique is effective for pancreatic juice and plasma taken from patients, highlighting its diagnostic potential. And there is scope for multiplexing: by building different probe sequences into different versions of the upstream oligonucleotides, Eshleman and colleagues detected and quantified two different mutated genes at once, at up to 1:1000 dilution.

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Practicalities

As with any new technique, things are not yet perfect. Mike Makrigiorgos of the Dana Farber–Brigham and Women's Cancer Center (http://www.brighamandwomens.org/bwhcancer/) expressed concerns that LigAmp could prove costly. This was echoed by Vanessa Hayes of the Garvan Institute of Medical Research (http://www.garvan.org.au/). And, although she sees LigAmp as having potential, she thinks it is 'limited by the fact that it cannot detect unknown mutations'.

Furthermore, as Mike Makrigiorgos points out, LigAmp might not work as well for other known single-base mutations as it does for K103N and the *KRAS2* mutation. 'The usefulness

of this particular method will depend on the ability of ligase to distinguish correctly between 'match' and 'mismatch', which is significantly dependent on sequence context'. Eshleman agreed that detection of some mutations could prove more difficult but, considering his team's success so far, he expects that the sensitivity of LigAmp will be 'as high for the vast majority of mutations'. Even with K103N and the *KRAS2* mutation, LigAmp is not foolproof – some nonspecific amplification and false-positive results were reported.

But both Eshleman and Hayes stress that this is normal for such assays, and Eshleman feels that the accuracy of LigAmp results is already 'sufficient for most applications'. He and his team are currently working on refining their technique, for example by testing a panel of different ligases, but he believes that LigAmp is already 'relatively close' to being used as a diagnostic tool.

Reference

1 Shi, C. *et al.* (2004) LigAmp for sensitive detection of single-nucleotide differences. *Nat. Methods* 1 DOI:10.1038/NMETH713 (Epub. ahead of print; http://www.nature.com/nmeth)

New hope for mechanism-based treatment of Parkinson's disease

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US researchers have discovered that rifampicin, an antibiotic used to treat tuberculosis and leprosy, inhibits the formation of α-synuclein fibrils and disaggregates fibrils that have already formed. Because the aggregation of α-synuclein in dopaminergic neurons is a critical step in the pathogenesis of Parkinson's disease (PD), rifampicin or a related compound could provide a new approach to the treatment of PD [1].

α-synuclein and Parkinson's disease

PD is a common neurodegenerative disorder caused by the progressive loss of dopaminergic neurons in the substantia nigra. Current treatments are symptomatic – patients are usually given levodopa to improve motor symptoms. A better approach, notes Anthony Fink, Professor of Chemistry and Biochemistry at the University of California, Santa Cruz, 'would be to get at the root of the disease and prevent any further progression.' And if a

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news

biomarker could be discovered for very early disease, he adds, 'it might even be possible to treat patients in advance of any clinical symptoms.'

Recent discoveries indicate that PD pathogenesis involves the conversion of soluble α-synuclein into abnormal filamentous α-synuclein. In 1997, US researchers reported that α-synuclein is mutated in a familial form of PD, explains Michel Goedert, Joint Head of the Neurobiology Division at the Medical Research Council's Laboratory of Molecular Biology in Cambridge, UK. Inherited PD is very rare, but the genetic defects in these cases can provide clues to the pathogenesis of sporadic PD. The discovery of an α-synuclein mutation thus encouraged Goedert to examine Lewy bodies and neurites, the defining neuropathological lesions of PD, for the presence of α-synuclein. 'Both types of lesion stained strongly for α-synuclein,' says Goedert, 'and it is now clear that this protein is the major component of the filaments in these lesions.'

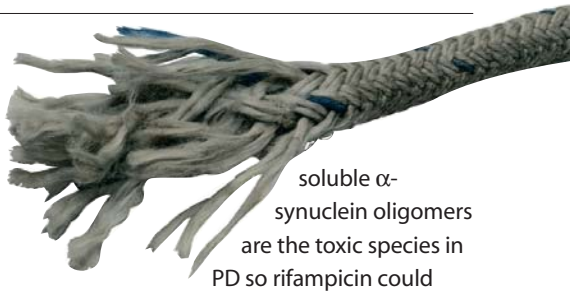
Two further missense mutations and triplications and duplications of the α-synuclein gene, all of which cause the protein to aggregate, have since been discovered in inherited forms of PD. 'Although it is not known why α-synuclein aggregates in sporadic disease,' notes Goedert, 'α-synuclein is clearly a good target for mechanism-based treatment of PD.'

The rifampicin connection

While some researchers are concentrating on enhancing cellular defences against aggregated α-synuclein (for example, by increasing cellular chaperone activity), Fink and co-workers are looking for molecules that can inhibit fibril formation of α-synuclein *in vitro*. 'Compounds that can do this have a common molecular structure, namely a benzene ring with two hydroxyl groups,' explains Fink. This structure, which is found in flavonoids, readily undergoes oxidation to form a quinone. This interacts with the side-chains of α-synuclein, which then forms small stable oligomers rather than large aggregates [2].

In June 2004, Fink's team reported that the flavonoid baicalein inhibits fibrillation of α-synuclein and disaggregates existing fibrils [2]. However, they also pulled rifampicin out of a screen of compounds with related structures and now report that rifampicin has similar effects on α-synuclein [1]. The researchers are currently testing both compounds in cells and in animal models to see if they can prevent neurodegeneration.

'This *in vitro* data is an intriguing but early step in the development of a new therapy for PD', comments Andrew Singleton, an investigator at the US National Institutes of Health, Bethesda who works on the genetic basis of neurodegenerative diseases. 'However, we don't know whether fibrillar α-synuclein or



soluble α-synuclein oligomers are the toxic species in PD so rifampicin could exacerbate rather than treat the disease.'

Goedert also finds Fink's results interesting and not just in the context of PD. 'Rifampicin is certainly worth checking out since it also inhibits the aggregation of other proteins involved in neurodegenerative diseases,' he comments. Indeed, epidemiological data from 1992 indicates that patients treated with rifampicin for leprosy have a lower risk of developing senile dementia than patients not given the drug. Furthermore, a recent clinical study provides some evidence that rifampicin in combination with doxycycline might slow mental deterioration in patients with mild to moderate Alzheimer's disease [3].

References

1 Li, J. *et al.* (2004) Rifampicin inhibits α-synuclein fibrillation and disaggregates fibrils. *Chem. Biol.* 11, 1513–1521

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3 Loeb, M.B. *et al.* (2004) A randomized, controlled trial of doxycycline and rifampin for patients with Alzheimer's disease. *J. Am. Geriatr. Soc.* 52, 381–387

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