

Technical advance

Open Access

## CYP3A4 and CYP3A5 genotyping by Pyrosequencing

Adam A Garsa<sup>1</sup>, Howard L McLeod<sup>1,2</sup> and Sharon Marsh\*<sup>1,2</sup>

Address: <sup>1</sup>Washington University School of Medicine, Department of Medicine, Division of Oncology, St. Louis, MO 63110, USA and <sup>2</sup>The Siteman Cancer Center, St. Louis, MO 63110, USA

Email: Adam A Garsa - adam\_garsa@yahoo.com; Howard L McLeod - hmcleod@im.wustl.edu; Sharon Marsh\* - smarsh@im.wustl.edu

\* Corresponding author

Published: 09 May 2005

Received: 28 September 2004

BMC Medical Genetics 2005, 6:19 doi:10.1186/1471-2350-6-19

Accepted: 09 May 2005

This article is available from: <http://www.biomedcentral.com/1471-2350/6/19>

© 2005 Garsa et al; licensee BioMed Central Ltd.

This is an Open Access article distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/2.0>), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

### Abstract

**Background:** Human cytochrome P450 3A enzymes, particularly CYP3A4 and CYP3A5, play an important role in drug metabolism. CYP3A expression exhibits substantial interindividual variation, much of which may result from genetic variation. This study describes Pyrosequencing assays for key SNPs in CYP3A4 (CYP3A4\*1B, CYP3A4\*2, and CYP3A4\*3) and CYP3A5 (CYP3A5\*3C and CYP3A5\*6).

**Methods:** Genotyping of 95 healthy European and 95 healthy African volunteers was performed using Pyrosequencing. Linkage disequilibrium, haplotype inference, Hardy-Weinberg equilibrium, and tag SNPs were also determined for these samples.

**Results:** CYP3A4\*1B allele frequencies were 4% in Europeans and 82% in Africans. The CYP3A4\*2 allele was found in neither population sample. CYP3A4\*3 had an allele frequency of 2% in Europeans and 0% in Africans. The frequency of CYP3A5\*3C was 94% in Europeans and 12% in Africans. No CYP3A5\*6 variants were found in the European samples, but this allele had a frequency of 16% in the African samples. Allele frequencies and haplotypes show interethnic variation, highlighting the need to analyze clinically relevant SNPs and haplotypes in a variety of ethnic groups.

**Conclusion:** Pyrosequencing is a versatile technique that could improve the efficiency of SNP analysis for pharmacogenomic research with the ultimate goal of pre-screening patients for individual therapy selection.

### Background

The human cytochrome P450 3A (CYP3A) subfamily of enzymes plays an important role in drug metabolism. The four CYP3A genes lie within a 218 kb region of chromosome 7q22.1 in the following order: CYP3A5, CYP3A7, CYP3A4, and CYP3A43. CYP3A enzymes, primarily CYP3A4 and CYP3A5, catalyze the metabolism of a multitude of exogenous and endogenous compounds. As the most abundant group of CYPs in the liver and small intestine, CYP3A enzymes strongly affect the oral bioavailability and clearance of many drugs, and it is estimated that

CYP3A enzymes are involved in the metabolism of over half of the drugs currently approved by the Food and Drug Administration [1-4].

Interindividual variation in CYP3A expression is substantial. Protein expression in liver and small intestine varies up to 40-fold, leading to variation in drug metabolism [2,5]. Genetic variation within the CYP3A genes may contribute to interindividual variability in drug metabolism. It has been suggested that approximately 90% of interindividual differences in hepatic CYP3A activity are due to

genetic variation [6]. Single nucleotide polymorphisms (SNPs) are the most common form of genetic variation in the CYP3A genes.

*CYP3A4\*1B* is a 5' untranslated region -392A>G transition in *CYP3A4* [7]. A number of associations between *CYP3A4\*1B* and clinical phenotypes have been found. Rebbeck et al. have shown that prostate cancer patients are more likely to have the *CYP3A4\*1B* allele than healthy controls, and this has been confirmed in other studies [7-9]. Additionally, homozygous wild-type (*CYP3A4\*1A/\*1A*) individuals have an increased risk for developing leukemia after epipodophyllotoxin therapy [10].

Relatively little is known about the effects of the other commonly studied *CYP3A4* SNPs, *CYP3A4\*2* and *CYP3A4\*3*. *CYP3A4\*2* is a SNP in exon 7 (15713T>C) that results in a Ser222Pro change. *In vitro* kinetic studies have shown that *CYP3A4\*2* has a 6- to 9-fold reduced intrinsic clearance for nifedipine compared to wild-type [11]. *CYP3A4\*3* is a 1334T>C transition causing a Met445Thr change. Although this SNP occurs within a conserved region, no difference in testosterone, progesterone, or 7-benzyloxy-4(trifluoromethyl)coumarin metabolism was found [12].

*CYP3A5\*3C* is an IVS3-237A>G (6986A>G) transition within intron 3 of *CYP3A5* [13]. This transition creates an alternative splice site in the pre-mRNA, leading to the production of aberrant mRNA with a premature stop codon [13]. This SNP leads to polymorphic expression of *CYP3A5*. *CYP3A5\*3C* homozygotes lack *CYP3A5* expression, while individuals with at least one *CYP3A5\*1* wild-type allele express *CYP3A5* [13]. Polymorphic expression of *CYP3A5* may account for some of the interindividual variation in clearance of *CYP3A* substrates. Indeed, *CYP3A5* genotype is predictive of tacrolimus doses for lung and kidney transplant recipients [14,15].

*CYP3A5\*6* is a 14690G>A synonymous mutation that causes the formation of a splice variant mRNA. Exon 7 is deleted, resulting in a frameshift and a truncated protein [13]. Very little is known about the effects of this SNP, although *CYP3A5\*6* was found to have no effect on midazolam clearance in a small sample size [16].

For further analysis of these SNPs and their relations to clinical outcomes, an accurate, rapid, and cost efficient method of SNP evaluation is needed. This study describes the use of Pyrosequencing to assay key *CYP3A4* and *CYP3A5* SNPs.

## Methods

### Genotyping

PCR was performed on DNA from 95 healthy European volunteers and 95 healthy African volunteers, after IRB approval and written informed consent [17,18]. PCR primers were designed using Primer Express Version 2.0 (ABI, Foster City, CA, USA) and Pyrosequencing Primer SNP Design Version 1.01 software [19]. Primer sequences and PCR conditions are described in Table 1. PCR was carried out using 1–5 ng genomic DNA, 0.6 nmol each of forward and reverse oligonucleotide PCR primers (one of which is biotinylated) (Integrated DNA Technologies, Coralville, Iowa, USA) and 1X AmpliTaq Gold PCR Master Mix (Applied Biosystems, CA, USA), containing 255U (0.05 U/ml) AmpliTaq Gold DNA polymerase, Gene Amp PCR Gold Buffer (30 mmol/L Tris-HCL, 100 mmol/L KCL, pH 8.05), 400 mM dNTP and 5 mmol/L MgCl<sub>2</sub>. Pyrosequencing was carried out as described [20] using internal primer diluted in 1X Annealing Buffer (20 mmol/L Tris-Acetate, 2 mmol/L MgAc<sub>2</sub>), 2X Binding Wash Buffer II pH 7.6 (10 mmol/L Tris-HCL, 2M NaCl, 1 mmol/L EDTA, 0.1% Tween20), Streptavidin Sepharose Beads (Amersham Biosciences, Uppsala, Sweden), 0.2 M NaOH, 70% Ethanol, and a PSQ HS96 SNP reagent kit (Pyrosequencing AB, Uppsala, Sweden). Samples were analyzed on a PSQ HS96A instrument with pyrosequencing software (Biotage, Uppsala, Sweden). A Tecan pipetting robot (Tecan, Research Triangle Park, NC, USA) was used for all of the steps apart from the addition and transfer of the sepharose beads.

### Statistics

Pairwise linkage disequilibrium ( $|D'|$ ), haplotype inference, and Hardy-Weinberg equilibrium were determined using the Polymorphism and Haplotype Analysis Suite [21,22]. Tag SNPs were determined using SNPtagger [23,24].

## Results and discussion

Genotyping data from the European and African samples are shown in Table 2. All results are in Hardy-Weinberg equilibrium. *CYP3A4\*1B* allele frequencies were 4% for Europeans and 82% for Africans. No *CYP3A4\*2* alleles were found in either the European or African population samples. *CYP3A4\*3* had an allele frequency of 2% in Europeans and 0% in Africans. The frequency of *CYP3A5\*3C* was 94% in Europeans and 12% in Africans. No *CYP3A5\*6* variants were found in the European samples, but this allele had a frequency of 16% in the African samples. There were no individuals homozygous for both *CYP3A5\*3C* and *CYP3A5\*6* in either population.

The *CYP3A4* and *CYP3A5* genes lie in close proximity (136 kb) to one another on chromosome 7q22.1, so haplotypes were determined across both genes for each

**Table 1: Primer sequence, PCR and Pyrosequencing conditions for CYP3A4 and CYP3A5 SNP analysis.**

| SNP       | Forward Primer <sup>a</sup> (5'-3') | Reverse Primer <sup>a</sup> (5'-3') | Number of Cycles | Annealing Temperature (° C) | Internal Primer <sup>bc</sup> (5'-3') | Sequence to Analyze <sup>d</sup> |
|-----------|-------------------------------------|-------------------------------------|------------------|-----------------------------|---------------------------------------|----------------------------------|
| CYP3A4*1B | aggacagccatagagacaagg               | *atcaatgttactgggagtc                | 55               | 55                          | F-ccatagagacaaggca                    | A/GGAGA                          |
| CYP3A4*2  | aacaatccacaagaccctt                 | *atcttcaatgtactacaatcactga          | 55               | 55                          | F-ttggatccattccttc                    | TCTC/TAAT                        |
| CYP3A4*3  | cgtggaaccagattcagca                 | *gaaggagaagttctgaaggactctg          | 55               | 65                          | F-ccagaaactgcattgg                    | CAT/CGAGG                        |
| CYP3A5*3C | *cccacgtatgtaccaccagc               | attagggtgtgacacagcaaga              | 55               | 65                          | R-ccaaacagggagaga                     | TAC/TTG <sup>e</sup>             |
| CYP3A5*6  | *tctttgggcctacagcatg                | aaagaataatagccacatacttattgagag      | 55               | 62                          | R-agaaccaaattttagaa                   | CTTC/TTTAG <sup>e</sup>          |

<sup>a</sup>\* = biotin molecule attached

<sup>b</sup>F = forward primer

<sup>c</sup>R = reverse primer

<sup>d</sup>Simplex entry nucleotide information for Pyrosequencing

<sup>e</sup>Assays on reverse complement strand

**Table 2: Genotype and allele frequencies for CYP3A4 and CYP3A5 SNPs in European and African populations. Figures in brackets are 95% confidence intervals.**

| SNP       | European       |           |              |         |                  |                  | African        |           |              |         |                  |                  |
|-----------|----------------|-----------|--------------|---------|------------------|------------------|----------------|-----------|--------------|---------|------------------|------------------|
|           | n <sup>a</sup> | Wild Type | Heterozygous | Variant | p                | q                | n <sup>a</sup> | Wild Type | Heterozygous | Variant | p                | q                |
| CYP3A4*1B | 93             | 86        | 7            | 0       | 0.96 (0.92–0.98) | 0.04 (0.02–0.08) | 88             | 3         | 25           | 60      | 0.18 (0.11–0.27) | 0.82 (0.73–0.89) |
| CYP3A4*2  | 56             | 56        | 0            | 0       | 1                | 0                | 87             | 87        | 0            | 0       | 1                | 0                |
| CYP3A4*3  | 94             | 90        | 4            | 0       | 0.98 (0.95–0.99) | 0.02 (0.01–0.05) | 93             | 93        | 0            | 0       | 1                | 0                |
| CYP3A5*3C | 95             | 0         | 12           | 83      | 0.06 (0.03–0.10) | 0.94 (0.90–0.97) | 95             | 72        | 23           | 0       | 0.88 (0.83–0.92) | 0.12 (0.08–0.17) |
| CYP3A5*6  | 92             | 92        | 0            | 0       | 1                | 0                | 91             | 64        | 25           | 2       | 0.84 (0.78–0.89) | 0.16 (0.11–0.22) |

<sup>a</sup> n = number of passed samples out of 95 samples tested

**Table 3: Haplotype analyses for CYP3A4 and CYP3A5.**

|      | CYP3A4*1B | CYP3A4*2 | CYP3A4*3 | CYP3A5*3C | CYP3A5*6 | % European       | % African        |
|------|-----------|----------|----------|-----------|----------|------------------|------------------|
| Hap1 | A         | C        | T        | G         | G        | 90               | 0.6 <sup>a</sup> |
| Hap2 | A         | C        | T        | A         | G        | 5.5              | 15               |
| Hap3 | A         | C        | C        | G         | G        | 1.8              | 0                |
| Hap4 | G         | C        | T        | A         | G        | 1.8 <sup>a</sup> | 57               |
| Hap5 | G         | C        | T        | G         | G        | 1                | 12               |
| Hap6 | G         | C        | T        | A         | A        | 0                | 11               |
| Hap7 | A         | C        | T        | A         | A        | 0                | 3                |

<sup>a</sup> inferred frequency

population (Table 3). In Europeans, haplotype 1 was the predominant haplotype, with a 90% frequency. Haplotypes 2, 3, and 5 were also observed, with frequencies of 5.5%, 1.8%, and 1%, respectively. Haplotype 4 was not observed, but it has an inferred frequency of 1.8%. The African population had five observed haplotypes: Haplotype 4 (57%), Haplotype 2 (15%), Haplotype 5 (12%), Haplotype 6 (11%), and Haplotype 7 (3%). Haplotype 1 was not observed, but it has an inferred frequency of

0.6%. No loci are significantly linked in either population (data not shown). In addition, no haplotype tag SNPs could be identified in either population. However, genotyping Europeans for CYP3A5\*3C could be used to identify the haplotype of 95.5% of the population. Similarly, genotyping Africans for CYP3A4\*1B and CYP3A5\*3C could be used to identify the haplotype of 84% of the population.

The frequency of *CYP3A4\*1B* in Europeans (4%) is consistent with other studies [25]. The *CYP3A4\*1B* frequency in Africans (82%) is much higher than in Europeans, and it is also higher than the 35–67% frequency seen in African Americans [25]. The rare *CYP3A4\*2* allele was not found in either of our population samples. To date *CYP3A4\*2* has only been described in a Finnish Caucasian population, with an allele frequency of 2.7% [11]. The *CYP3A4\*3* allele frequency in Europeans (2%) is consistent with frequencies reported in other studies [25].

*CYP3A5\*3C* frequency shows dramatic interethnic variation. In Europeans, the *CYP3A5\*3C* variant is the predominant allele (94% frequency), but this allele has a much lower frequency in the African population (12%). *CYP3A5\*6* frequency also shows interethnic variation. The *CYP3A5\*6* allele was not found in Europeans, but it was found in the African population at a frequency of 16%. Other studies have also failed to find *CYP3A5\*6* in Europeans, but this has been found in African Americans at a frequency of 13–16% [16,26].

Haplotype also shows interethnic variation. Haplotype 1 is the predominant haplotype in Europeans, with haplotypes 2–5 occurring at low frequencies. In contrast, haplotype 4 is the most common haplotype in Africans, and haplotypes 2, 5, and 6 all occur at frequencies greater than 10%. However, the presence of homozygous variants was rare for *CYP3A4* in Europeans and for *CYP3A5* in Africans. Consequently, the limits of *in silico* determining haplotype frequencies in these populations should be taken into account. Larger population studies are necessary for a more accurate understanding of *CYP3A4*-*CYP3A5* haplotypes. Interethnic variation highlights the need to analyze clinically relevant SNPs and haplotypes in a variety of ethnic groups. An understanding of the genetic variation that exists in various populations will aid in tailoring health care to different populations.

## Conclusion

Restriction fragment length polymorphism (RFLP) is the predominant method of SNP analysis used to assay *CYP3A4* and *CYP3A5* SNPs in previous studies [14,27]. Pyrosequencing offers several advantages over RFLP. For RFLP analysis, a SNP must alter a restriction enzyme cutting site. This limitation precludes many SNPs from RFLP analysis. Pyrosequencing assays can be designed for the vast majority of SNPs, making it a versatile alternative. Pyrosequencing also requires less time than RFLP. Post-PCR, Pyrosequencing steps take approximately 30 minutes for 96 samples, whereas enzyme digestion (1–2 h) and gel electrophoresis for RFLP take significantly longer. Additionally, Pyrosequencing assays are readily transferable to any lab with the appropriate equipment and require no on-site optimization. This procedure could improve

the efficiency of SNP analysis for pharmacogenomic research with the ultimate goal of pre-screening patients for individual therapy selection.

## Competing interests

The author(s) declare that they have no competing interests.

## Authors' contributions

AG performed the experimental analysis and drafted the manuscript. AAG, HLM and SM interpreted the results. HLM and SM conceived of the study design and implementation and helped draft the manuscript. All authors read and approved the final manuscript.

## Acknowledgements

This work was supported by the NIH Pharmacogenetics Research Network (U01 GM63340); <http://pharmacogenetics.wustl.edu>, the General Clinical Research Center (P30 CA091842-01), R21 CA102461-01, and P01 CA101937-01. The authors thank Hilary Kannall, Christi Ralph and Chris Rose for technical assistance, and Derek Van Booven for computational expertise. This data has been deposited in the Pharmacogenetics Knowledge Base <http://www.PharmGKB.org>.

## References

- Shimada T, Yamazaki H, Mimura M, Inui Y, Guengerich FP: **Interindividual variations in human liver cytochrome P-450 enzymes involved in the oxidation of drugs, carcinogens and toxic chemicals: studies with liver microsomes of 30 Japanese and 30 Caucasians.** *J Pharmacol Exp Ther* 1994, **270**:414-423.
- Paine MF, Khalighi M, Fisher JM, Shen DD, Kunze KL, Marsh CL, Perkins JD, Thummel KE: **Characterization of interintestinal and intrainestinal variations in human CYP3A-dependent metabolism.** *J Pharmacol Exp Ther* 1997, **283**:1552-1562.
- de Waziers I, Cugnenc PH, Yang CS, Leroux JP, Beaune PH: **Cytochrome P 450 isoenzymes, epoxide hydrolase and glutathione transferases in rat and human hepatic and extrahepatic tissues.** *J Pharmacol Exp Ther* 1990, **253**:387-394.
- Wrighton SA, VandenBranden M, Ring BJ: **The human drug metabolizing cytochromes P450.** *J Pharmacokinet Biopharm* 1996, **24**:461-473.
- Watkins PB: **Cyclosporine and liver transplantation: will the midazolam test make blood level monitoring obsolete?** *Hepatology* 1995, **22**:994-996.
- Ozdemir V, Kalowa W, Tang BK, Paterson AD, Walker SE, Endrenyi L, Kashuba AD: **Evaluation of the genetic component of variability in CYP3A4 activity: a repeated drug administration method.** *Pharmacogenetics* 2000, **10**:373-388.
- Rebeck TR, Jaffe JM, Walker AH, Wein AJ, Malkowicz SB: **Modification of clinical presentation of prostate tumors by a novel genetic variant in CYP3A4.** *J Natl Cancer Inst* 1998, **90**:1225-1229.
- Paris PL, Kupelian PA, Hall JM, Williams TL, Levin H, Klein EA, Casey G, Witte JS: **Association between a CYP3A4 genetic variant and clinical presentation in African-American prostate cancer patients.** *Cancer Epidemiol Biomarkers Prev* 1999, **8**:901-905.
- Tayeb MT, Clark C, Sharp L, Haites NE, Rooney PH, Murray GI, Payne SN, McLeod HL: **CYP3A4 promoter variant is associated with prostate cancer risk in men with benign prostate hyperplasia.** *Oncol Rep* 2002, **9**:653-655.
- Felix CA, Walker AH, Lange BJ, Williams TM, Winick NJ, Cheung NK, Lovett BD, Nowell PC, Blair IA, Rebeck TR: **Association of CYP3A4 genotype with treatment-related leukemia.** *Proc Natl Acad Sci U S A* 1998, **95**:13176-13181.
- Sata F, Sapone A, Elizondo G, Stocker P, Miller VP, Zheng W, Raunio H, Crespi CL, Gonzalez FJ: **CYP3A4 allelic variants with amino acid substitutions in exons 7 and 12: evidence for an allelic variant with altered catalytic activity.** *Clin Pharmacol Ther* 2000, **67**:48-56.

12. Eiselt R, Domanski TL, Zibat A, Mueller R, Presecan-Siedel E, Hustert E, Zanger UM, Brockmoller J, Klenk HP, Meyer UA, Khan KK, He YA, Halpert JR, Wojnowski L: **Identification and functional characterization of eight CYP3A4 protein variants.** *Pharmacogenetics* 2001, **11**:447-458.
13. Kuehl P, Zhang J, Lin Y, Lamba J, Assem M, Schuetz J, Watkins PB, Daly A, Wrighton SA, Hall SD, Maurel P, Relling M, Brimer C, Yasuda K, Venkataramanan R, Strom S, Thummel K, Boguski MS, Schuetz E: **Sequence diversity in CYP3A promoters and characterization of the genetic basis of polymorphic CYP3A5 expression.** *Nat Genet* 2001, **27**:383-391.
14. Thervet E, Anglicheau D, King B, Schlageter MH, Cassinat B, Beaune P, Legendre C, Daly AK: **Impact of cytochrome p450 3A5 genetic polymorphism on tacrolimus doses and concentration-to-dose ratio in renal transplant recipients.** *Transplantation* 2003, **76**:1233-1235.
15. Zheng H, Webber S, Zeevi A, Schuetz E, Zhang J, Bowman P, Boyle G, Law Y, Miller S, Lamba J, Burckart GJ: **Tacrolimus dosing in pediatric heart transplant patients is related to CYP3A5 and MDRI gene polymorphisms.** *Am J Transplant* 2003, **3**:477-483.
16. Floyd MD, Gervasini G, Masica AL, Mayo G, George ALJ, Bhat K, Kim RB, Wilkinson GR: **Genotype-phenotype associations for common CYP3A4 and CYP3A5 variants in the basal and induced metabolism of midazolam in European- and African-American men and women.** *Pharmacogenetics* 2003, **13**:595-606.
17. McLeod HL, Syvanen AC, Githang'a J, Indalo A, Ismail D, Dewar K, Ulmanen I, Sludden J: **Ethnic differences in catechol O-methyltransferase pharmacogenetics: frequency of the codon 108/158 low activity allele is lower in Kenyan than Caucasian or South-west Asian individuals.** *Pharmacogenetics* 1998, **8**:195-199.
18. Ameyaw MM, Collie-Duguid ES, Powrie RH, Ofori-Adjei D, McLeod HL: **Thiopurine methyltransferase alleles in British and Ghanaian populations.** *Hum Mol Genet* 1999, **8**:367-370.
19. **Pyrosequencing primer design** [<http://techsupport.pyrosequencing.com/>]
20. Rose CM, Marsh S, Ameyaw MM, McLeod HL: **Pharmacogenetic analysis of clinically relevant genetic polymorphisms.** *Methods Mol Med* 2003, **85**:225-237.
21. Schaid DJ, McDonnell SK, Wang L, Cunningham JM, Thibodeau SN: **Caution on pedigree haplotype inference with software that assumes linkage equilibrium.** *Am J Hum Genet* 2002, **71**:992-995.
22. **Polymorphism and Haplotype Analysis Suite** [<http://ilya.wustl.edu/~pgrn/>]
23. Ke X, Cardon LR: **Efficient selective screening of haplotype tag SNPs.** *Bioinformatics* 2003, **19**:287-288.
24. **SNPtagger** [<http://well.ox.ac.uk/~xiayi/haplotype/index.html>]
25. Lamba JK, Lin YS, Schuetz EG, Thummel KE: **Genetic contribution to variable human CYP3A-mediated metabolism.** *Adv Drug Deliv Rev* 2002, **54**:1271-1294.
26. Hustert E, Haberl M, Burk O, Wolbold R, He YQ, Klein K, Nuessler AC, Neuhaus P, Klattig J, Eiselt R, Koch I, Zibat A, Brockmoller J, Halpert JR, Zanger UM, Wojnowski L: **The genetic determinants of the CYP3A5 polymorphism.** *Pharmacogenetics* 2001, **11**:773-779.
27. Kadlubar FF, Berkowitz GS, Delongchamp RR, Wang C, Green BL, Tang G, Lamba J, Schuetz E, Wolff MS: **The CYP3A4\*1B variant is related to the onset of puberty, a known risk factor for the development of breast cancer.** *Cancer Epidemiol Biomarkers Prev* 2003, **12**:327-331.

### Pre-publication history

The pre-publication history for this paper can be accessed here:

<http://www.biomedcentral.com/1471-2350/6/19/prepub>

Publish with **BioMed Central** and every scientist can read your work free of charge

"BioMed Central will be the most significant development for disseminating the results of biomedical research in our lifetime."

Sir Paul Nurse, Cancer Research UK

Your research papers will be:

- available free of charge to the entire biomedical community
- peer reviewed and published immediately upon acceptance
- cited in PubMed and archived on PubMed Central
- yours — you keep the copyright

Submit your manuscript here:  
[http://www.biomedcentral.com/info/publishing\\_adv.asp](http://www.biomedcentral.com/info/publishing_adv.asp)

