EUROPEAN JOURNAL OF CANCER 44 (2008) 1269–1274

available at www.sciencedirect.com

ScienceDirect

journal homepage: www.ejconline.com

A comparison of *CDKN2A* mutation detection within the Melanoma Genetics Consortium (GenoMEL)

Mark Harland[a], *Alisa M. Goldstein*[b], *Kairen Kukalizch*[a], *Claire Taylor*[c], *David Hogg*[d], *Susana Puig*[e], *Celia Badenas*[e], *Nelleke Gruis*[f], *Jeanet ter Huurne*[f], *Wilma Bergman*[f], *Nicholas K. Hayward*[g], *Mitchell Stark*[g], *Hensin Tsao*[h], *Margaret A. Tucker*[b], *Maria Teresa Landi*[b], *Giovanna Bianchi Scarra*[i], *Paola Ghiorzo*[i], *Peter A. Kanetsky*[j], *David Elder*[k], *Graham J. Mann*[l], *Elizabeth A. Holland*[l], *D. Timothy Bishop*[a], *Julia Newton Bishop*[a,*], *members of GenoMEL, the Melanoma Genetics Consortium*

[a]*Division of Epidemiology and Biostatistics, Leeds Institute of Molecular Medicine, Cancer Research UK Cancer Centre at Leeds, St James's University Hospital, Leeds, UK*
[b]*Genetic Epidemiology Branch, Division of Cancer Epidemiology and Genetics, National Cancer Institute, NIH, DHHS, Bethesda, MD, USA*
[c]*Mutation Detection Facility, Cancer Research UK, St James's University Hospital, Leeds, UK*
[d]*Department of Medical Biophysics, Institute of Medical Sciences, University of Toronto, Toronto, Ontario, Canada*
[e]*Dermatology Department and Genetic Service, Hospital Clinic, IDIBAPS, Barcelona, Spain*
[f]*Department of Dermatology and Center for Human and Clinical Genetics, LUMC, Leiden, The Netherlands*
[g]*Human Genetics Laboratory, Queensland Institute of Medical Research, Brisbane, Australia*
[h]*Wellman Center for Photomedicine, Department of Dermatology, Massachusetts General Hospital, Boston, MA, USA*
[i]*Department of Oncology, Biology and Genetics, University of Genoa, Italy*
[j]*Department of Biostatistics and Epidemiology and Center for Clinical Epidemiology and Biostatistics, University of Pennsylvania, Philadelphia, PA, USA*
[k]*Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, PA, USA*
[l]*Westmead Institute for Cancer Research, University of Sydney at Westmead Millennium Institute, Westmead, Australia*

ARTICLE INFO

Article history:
Received 18 January 2008
Received in revised form
7 March 2008
Accepted 10 March 2008
Available online 3 April 2008

Keywords:
CDKN2A
Melanoma
Mutation detection
Sequencing
Polymorphism
Audit
DHPLC
False negative

ABSTRACT

CDKN2A is the major melanoma susceptibility gene so far identified, but only 40% of three or more case families have identified mutations. A comparison of mutation detection rates was carried out by "blind" exchange of samples across GenoMEL, the Melanoma Genetics Consortium, to establish the false negative detection rates. Denaturing high performance liquid chromatography (DHPLC) screening results from 451 samples were compared to screening data from nine research groups in which the initial mutation screen had been done predominantly by sequencing. Three samples with mutations identified at the local centres were not detected by the DHPLC screen. No additional mutations were detected by DHPLC. Mutation detection across groups within GenoMEL is carried out to a consistently high standard. The relatively low rate of *CDKN2A* mutation detection is not due to failure to detect mutations and implies the existence of other high penetrance melanoma susceptibility genes.

© 2008 Elsevier Ltd. All rights reserved.

* *Corresponding author:* Tel.: +44 113 206 4573; fax: +44 113 234 0183.
E-mail address: j.a.newton-bishop@leeds.ac.uk (J.N. Bishop).
0959-8049/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.ejca.2008.03.005

1. Introduction

GenoMEL (the Melanoma Genetics Consortium; http://www.genomel.org) is comprised of groups on four continents working on high penetrance genes in multiple case melanoma families. The major susceptibility locus for melanoma is *CDKN2A* on chromosome 9p. The majority of causal mutations at this locus, many of which are single base pair substitutions in exons 1α and 2, affect the function of the protein p16INK4a. Some of the mutations in exon 2 also impact on the alternative splice product of the locus, p14ARF. Since p16INK4a was first described as a melanoma susceptibility gene,[1–3] increasing numbers of mutations at the locus have been described.

Less common types of germline mutation have been reported, including a promoter variant that creates an alternative initiation codon[4,5] and a deep intronic mutation common in England.[6] A comprehensive screen of the intronic regions of *CDKN2A* identified two additional putative intronic mutations. However, in English pedigrees at least, these do not appear to explain predisposition to melanoma in a significant proportion of families.[7]

Recently, rare causal mutations have been identified in exon 1β; these mutations impact p14ARF alone. Specifically, a germline deletion not affecting p16INK4a was reported in 2001,[8] a 16 base pair insertion in exon 1β was detected in a Spanish melanoma family,[9] and a number of pedigrees with exon 1β splice site variants have been described.[10,11] Finally, a recent screen of 146 English melanoma families identified a small number of pedigrees with germline deletions at the 9p21 locus.[12]

Within GenoMEL, the overall proportion of families with identifiable mutations is relatively low and there is considerable variation between centres.[13,14] In a study from Italy, 33% of pedigrees with two or more cases of melanoma had mutations,[15] whereas a Spanish study showed that 17% of melanoma families had *CDKN2A* mutations.[16] In Australia, lower percentages have been reported, e.g. 8.4% of two or more case families.[17] The variation between centres may result from the founder effects and the variable presence of other as yet unidentified susceptibility genes such as the putative gene at 1p22.[18] There may also be an effect of the environment. Clustering in families in areas of high sun exposure such as Australia may result from enhanced contribution of lower penetrance susceptibility genes such as MC1R. Indeed, compared to Europe, there is almost a doubling of the penetrance of *CDKN2A* mutations in Australia which is thought to be due to a higher ultraviolet radiation flux.[19]

Another possibility, however, is that groups had failed to identify significant numbers of mutations at the *CDKN2A* locus, particularly since early mutation detection studies often used the single-stranded conformational polymorphism (SSCP) analysis rather than sequencing. GenoMEL, therefore, designed an audit to evaluate the overall quality of mutation detection across the entire *CDKN2A* locus. We also investigated the utility of denaturing high performance liquid chromatography (DHPLC) as a screening approach to be used by GenoMEL in large numbers of samples. Samples that had initially been genotyped at the centre of origin by sequencing (eight centres) or by SSCP (one centre) were sent to Leeds, UK, for screening with DHPLC. The study also therefore provides a comparison of sequencing with DHPLC.

2. Materials and methods

2.1. Samples and general organisation

The core groups within GenoMEL agreed to send samples to the Division of Epidemiology and Biostatistics of the Cancer Research UK Clinical Centre at Leeds, UK. The participating groups were from Barcelona, Spain (BCN), Leiden University Medical Center, the Netherlands (LUMC), Queensland Institute of Medical Research, Australia (QIMR). Massachusetts General Hospital, Boston, USA (MGH), the National Cancer Institute, Washington, USA (NCI/USA), an NCI group collaborating with Emilia-Romagna, Italy (NCI/Italy), the University of Genoa, Italy (U Genoa), the University of Pennsylvania, Philadelphia, USA (U Penn) and Westmead Institute for Cancer Research, New South Wales, Australia (WICR). The samples were labelled by the study number alone, and therefore the Leeds group was blind to the mutation status of the sample.

All groups provided DNA from two melanoma cases from families with three or more melanoma patients that had been screened by this group, whether a mutation had been detected or not. In each case, the initial mutation detection screen carried out at the centre of origin was by sequencing, with the exception of WICR, where the primary screen was by SSCP for *CDKN2A* exon 1 and by sequencing for exon 2.

The samples were processed by the Leeds group and the results sent to the NCI in Washington Bethesda, MD, where DHPLC audit results were pooled with the original groups' results. Only coding mutations were assessed; polymorphisms were not considered in this analysis.

2.2. Statistical analysis

The DHPLC results from Leeds were compared to the results from the original centres using two units of evaluation: "sample" and "exon". Sample summarized the results over the five different exons evaluated. Exon separately examined *CDKN2A* exons 1α, 1β, 2 and 3 and *CDK4* exon 2. Two measures of evaluation were used: failure and discrepancy. Failure was defined as the percentage of samples or exons that failed the DHPLC assay. Discrepancy was the proportion of inconsistencies between DHPLC and the original centre's results. To confirm discrepancies and eliminate any sample handling errors at any point in the process, all samples with initial evidence for discrepant results were sequenced at the University of Toronto (D. Hogg).

2.3. PCR amplification

The four exons of *CDKN2A* (exons 1α, 1β, 2 and 3) and *CDK4* exon 2 were amplified from genomic DNA by PCR, using previously described primers (Table 1).[2,3,20] PCR was carried out in a total volume of 25 μl, using 25 ng genomic DNA, 0.2 mM dNTPs, 50 μM each primer, 5% (v/v) DMSO, 1.5 mM $MgCl_2$ and 1 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Warrington, UK), in the reaction buffer supplied by the manufacturer. PCR amplification conditions were as fol-

Table 1 – PCR primers and DHPLC conditions

Exon	Primers	Fragment size (bp)	DHPLC temperature
CDKN2A Exon 1α	F-CAGCACCGGAGGAAGAAAG R-GCGCTACCTGATTCCAATTC	351	65, 68, 69
CDKN2A Exon 2	F-GGAAATTGGAAACTGGAAGC R-GGAAGCTCTCAGGGTACAAATTC	499	60, 65, 70
CDKN2A Exon 3	F-CCATTGCGAGAACTTTATCC R-TGGACATTTACGGTAGTGGG	329	56, 62
CDKN2A Exon 1β	F-CACCTCTGGTGCCAAAGGGC R-CCTAGCCTGGGCTAGAGACG	351	61, 65, 69
CDK4 Exon 2	F-GCTGCAGGTCATACCATCCT R-ATCATCACACCCCACCTATAGG	371	62

lows: an initial denaturation at 94 °C for 10 min; followed by 30 cycles of denaturing at 94 °C (30 s), annealing at 55 °C (30 s) and extension at 72 °C (30 s), with a final 7 min extension at 72 °C. PCR fragments were isolated by agarose gel electrophoresis and purified prior to sequencing using the QIAquick Gel Extraction Kit (Qiagen, Paisley, UK).

2.4. *DHPLC analysis*

The DNA samples were screened for sequence changes in *CDKN2A* exons 1α, 1β, 2 and 3, and *CDK4* exon 2, by DHPLC analysis. The DHPLC system had previously been optimised using the Leeds family samples. Each of the 22 separate variants observed in the Leeds melanoma samples[21,22] could be clearly detected by DHPLC using the conditions described below.

Temperatures for mutation detection were calculated using the DHPLC Melt program available at http://insertion.stanford.edu/melt.html[23] Melting temperatures were also determined empirically by running a wild type sample at progressively increasing temperatures until a reduction in the retention time of 1 min was observed. For *CDKN2A* exons 1α, 1β and 2, analysis was carried out at the temperatures determined by DHPLC Melt. For exon 3, one of the two temperatures used was 1 °C higher than that predicted by DHPLC Melt (Table 1).

DHPLC was carried out using a Transgenomic WAVE Nucleic Acid Fragment Analysis system and DNASep column (Transgenomic, Crewe, UK). The composition of buffer A was 0.1 M triethylammonium acetate (TEAA); buffer B contained 0.1 M TEAA and 25% (v/v) acetonitrile. Analysis was carried out at a flow rate of 0.9 ml/min and a buffer B gradient increase of 2%/min for 4 min. Start and end concentrations of buffer B were determined empirically for each fragment.

PCR products were prepared for DHPLC by denaturing at 95 °C for 5 min and then cooling to 65 °C to allow the formation of heteroduplexes. Data analysis were by the visual inspection of chromatograms by two independent observers.

2.5. *Sequencing analysis of DHPLC 'positive' samples*

All PCR fragments, which displayed an aberrant DHPLC chromatogram, were sequenced to identify the underlying nucleotide change. Sequencing reactions were carried out using the ABI PRISM BigDye v2 Terminator Cycle Sequencing Kit. Data collection were performed using a 3100 Genetic Analyser (Applied Biosystems, Warrington, UK) running Applied Biosystems Data Collection Software (version 1). Data analysis were carried out by the visual inspection of electropherograms, and using Applied Biosystems Sequence Navigator analysis software (version 1.0.1). DNA sequencing was performed in both directions, initiated from the forward and reverse primers used in the initial PCR amplification of each fragment.

3. Results

A total of 537 samples were screened by DHPLC for sequence variation in five exons (*CDKN2A* exons 1α, 1β, 2 and 3, and *CDK4* exon 2). Of the 2685 products processed by DHPLC, 106 (4%) failed the assay. DNA that had been extracted from buccal samples showed a much higher failure rate than DNA extracted from blood; 18/40 (45%) of DHPLC assays on buccal-derived DNA failed, compared to 88/2645 (3%) of assays on blood-derived samples.

The DHPLC audit identified 40 different mutations (138 in total) and 10 different polymorphisms (149 in total) in the five exons investigated. A total of 37/1343 (3%) of DHPLC assays were found to give a false positive screening result in that the DHPLC traces were judged to be atypical, but subsequent sequencing showed that the samples were wild type (Table 2). Where comprehensive local sequencing data were available, a comparison was made between the DHPLC results and the sequencing data. The DHPLC audit results could be compared to the local screening data for (at least) *CDKN2A* exons 1α and 2, in a total of 451 samples.

Three mutations, identified by the local centre's primary screen, were not detected by the audit DHPLC (3/1343 = 0.2% by exon, or 3/451 = 0.7% by sample). A Gly101Trp mutation, in *CDKN2A* exon 2, was not detected by DHPLC in a sample originating from the National Cancer Institute, USA (NCI/USA), despite this mutation being clearly detected in several other samples in this investigation. Sequencing confirmed the presence of the Gly101Trp mutation in this sample. The *CDKN2A* exon 1 variant Trp15OPA was not identified in two samples originating from the Westmead Institute for Cancer Research, Australia (WICR), where this variant had been detected by SSCP in the primary screen. Sequencing confirmed

Table 2 – Comparison of audit DHPLC results with primary screening results

Exon	Number of exons screened[a]	DHPLC positive[b]	Causal mutations detected	Polymorphisms detected	False positives[c]	Mutations missed[d]
CDKN2A exon 1α	348	47	37	3	7	2 (Trp15OPA)
CDKN2A exon 2	310	132	83	37	12	1 (Gly101Trp)
CDKN2A exon 3	244	112	7	104	1	0
CDKN2A exon 1β	179	13	6	0	7	0
CDK4 exon 2	262	20	5	5	10	0
Total	1343	324	138	149	37 (3%)	3 (0.2%)

a The number of samples where the local primary screening data were available for comparison with the audit DHPLC results for each exon analysed.
b The number of samples displaying an aberrant DHPLC trace.
c The number of aberrant DHPLC traces which on sequencing appeared to be of wild type.
d The number of mutations identified in the local primary screen that were not detected by the audit DHPLC.

the presence of the Trp15OPA mutation in these samples; however, repeated DHPLC analysis demonstrated that this variant was not detectable using the DHPLC conditions employed. The application of an additional melting temperature (69 °C) for the exon 1 DHPLC subsequently enabled the detection of the Trp15OPA variant.

The centralised DHPLC audit did not identify any additional mutations that had not been reported by individual groups after the local primary mutation screen.

4. Discussion

We carried out an assessment of mutation detection by blind exchange of samples from nine melanoma research groups within GenoMEL. The assessment was carried out with the intention of (1) standardising mutation detection across a range of research groups worldwide, (2) identifying mutations that may have been missed in the primary mutation screen and (3) validating DHPLC as a screening technique.

The DHPLC technique is sensitive, rapid and relatively inexpensive,[24–27] and was therefore considered to be well suited to an audit of this nature. Different screening techniques are known to have different limitations in identifying mutations.[28–30] The use of DHPLC for the audit screen has the further advantage of complementing a primary screen using complete sequencing, the screening technique employed at most GenoMEL centres.

The DHPLC failure rate was low, only 3% of assays on blood-derived DNA samples could not be analysed. However, the failure rate for buccal-derived samples was considerably higher, at 45%. The overwhelming majority of DHPLC failures were in fact failures of the initial PCR reaction, resulting in the absence of PCR product for DHPLC analysis. DHPLC is to some extent sensitive to the quality of DNA used in the initial PCR reaction. Because the number of buccal samples was so few ($n = 8$), we did not attempt to re-optimise exon-specific PCR conditions.

A small number (3%) of DHPLC assays were found to give a false positive screening result. The majority of these were found to be the result of over-cautious scoring of DHPLC traces, as samples that gave a weak or atypical DHPLC trace were typically recorded as positive, as is appropriate for a screening technique. Although unlikely, there is the possibility that these DHPLC positive traces represent a true mutation that is undetectable by sequencing.

Our observed high concordance between primary and audit screens (99.8%) was based on a large sub-set of genotyping data from across GenoMEL. In some cases, a direct comparison could not be made between primary and audit screens, as a number of research groups had not routinely screened the whole of *CDK4* exon 2 or *CDKN2A* exon 1β, due to the low reported frequency of mutations in these genes.[20,31–33] Also, some samples had been screened by a specific test for a mutation already identified in an additional family member, and hence the primary screen did not cover the entire exon.

Although the DHPLC assay can be optimised to detect known variants in a particular exon, there is always the possibility that the technique could be insensitive to a previously unidentified variant. Employing as broad a set of variants as possible to optimise the DHPLC assay can reduce this risk. One of the benefits of undertaking a mutation detection audit across a number of research groups is that it enables the pooling of many different *CDKN2A* variants, and therefore increases confidence in the ability of the technique to identify variants in future studies.

The DHPLC analysis of *CDKN2A* exon 3 was somewhat problematic. Approximately 50% of all exon 3 DHPLC traces were positive, but only 7/244 (3%) carried a causal mutation. The presence of two common 3'UTR polymorphisms (500 c > g and 540 c > t) substantially increased the cost of subsequent sequencing to identify the underlying mutation. In addition, the frequency of the polymorphisms in the population (15% and 20%, respectively) is such that they are occasionally found as homozygous variants, which are not detected by DHPLC.[34] Therefore, DHPLC is not ideally suited to the analysis of this exon. Perhaps, an improved approach to a high throughput screening of *CDKN2A* exon 3 and its flanking regions would be the use of mutation specific tests for the three known variants in this part of the gene (the two 3'UTR polymorphisms in the exon and the causal melanoma associated intronic variant IVS2-105 a > g). No other germline causal variant has been identified in exon 3, and indeed the size of the translated portion (4 amino acids) and the demonstrated lack of function of exon 3[35] make the existence of additional mutations unlikely.

DHPLC was evaluated as an alternative technique for mutation detection. This study has shown that the technique can be used to carry out primary *CDKN2A* mutation screening for future large-scale studies where issues of speed and expense are critical. DHPLC has been shown to have only a slightly lower rate of mutation detection than direct sequencing, which is generally regarded to be the most sensitive screening technique.[36,37] Out of a total of 50 different variants identified in this study, a single variant (Trp15OPA) was initially opaque to the audit DHPLC. This represents a sensitivity of 98% compared to sequencing. Following further optimisation, the sensitivity was subsequently increased to 100%. The technique's relatively low cost (approximately 8 times cheaper than sequencing) and high throughput make it ideal for screening large numbers of samples in which the expected mutation frequency is low, for example a population based screen for *CDKN2A* mutations.

This audit has also shown that the variation in mutation detection frequencies between the different groups is not a result of variation in sequencing approaches; in fact, the standard of screening across groups is uniformly high. Rather the variation in mutation frequency between groups reflects differences between populations, either through differing genetic backgrounds or through environmental contributions. GenoMEL continues to address this issue to better understand the mutation frequency variation across populations.

Conflict of interest statement

None declared.

Acknowledgements

This work was supported by Grants R01 CA83115 and R01 CA88363 from the National Cancer Institute; Grants 01/1546 and 03/0019 from Fondo de Investigaciones Sanitarias; V2003-REDC03/03 and /07 from the Italian Association for the Cancer Research (AIRC). This work was also partially supported by research grants and scholarships of the Australian National Health and Medical Research Council, The Cancer Councils of Australia, the Australian Cancer Research Foundation, the Melanoma Foundation and Cancer Research Fund of the University of Sydney, and also by the Intramural Research Program of the National Institutes of Health, NCI, DCEG.

REFERENCES

1. Nobori T, Miura K, Wu DJ, Lois A, Takabayashi K, Carson DA. Deletions of the cyclin-dependent kinase-4 inhibitor gene in multiple human cancers. *Nature* 1994;**368**:753–6.
2. Hussussian CJ, Struewing JP, Goldstein AM, et al. Germline p16 mutations in familial melanoma. *Nat Genet* 1994;**8**:15–21.
3. Kamb A, Shattuck-Eidens D, Eeles R, et al. Analysis of the p16 gene (CDKN2) as a candidate for the chromosome 9p melanoma susceptibility locus. *Nat Genet* 1994;**8**:23–6.
4. Liu L, Dilworth D, Gao L, et al. Mutation of the CDKN2A 5' UTR creates an aberrant initiation codon and predisposes to melanoma. *Nat Genet* 1999;**21**:128–32.
5. Harland M, Holland EA, Ghiorzo P, et al. Mutation screening of the CDKN2A promoter in melanoma families. *Genes Chromosomes Cancer* 2000;**28**:45–57.
6. Harland M, Mistry S, Bishop DT, Newton Bishop JA. A deep intronic mutation in CDKN2A is associated with disease in a subset of melanoma pedigrees. *Hum Mol Genet* 2001;**10**:2679–86.
7. Harland M, Taylor CF, Bass S, et al. Intronic sequence variants of the CDKN2A gene in melanoma pedigrees. *Genes Chromosome Cancer* 2005;**43**:128–36.
8. Randerson-Moor JA, Harland M, Williams S, et al. A germline deletion of p14(ARF) but not CDKN2A in a melanoma-neural system tumour syndrome family. *Hum Mol Genet* 2001;**10**:55–62.
9. Rizos H, Puig S, Badenas C, et al. A melanoma-associated germline mutation in exon 1beta inactivates p14ARF. *Oncogene* 2001;**20**:5543–7.
10. Hewitt C, Lee Wu C, Evans G, et al. Germline mutation of ARF in a melanoma kindred. *Hum Mol Genet* 2002;**11**:1273–9.
11. Harland M, Taylor CF, Chambers PA, et al. A mutation hotspot at the p14ARF splice site. *Oncogene* 2005;**24**:4604–8.
12. Mistry S, Tayor CF, Randerson-Moor J, et al. The prevalence of 9p21 deletions in UK melanoma families. *Genes Chromosome Cancer* 2005;**44**:292–300.
13. Goldstein AM, Chan M, Harland M, et al. High-risk melanoma susceptibility genes and pancreatic cancer, neural system tumors, and uveal melanoma across GenoMEL. *Cancer Res.* 2006;**66**:9818–28.
14. Goldstein AM, Chan M, Harland M, et al. Features associated with germline CDKN2A mutations: a GenoMEL study of melanoma-prone families from three continents. *J Med Genet* 2007;**44**:99–106.
15. Mantelli M, Barile M, Ciotti P, et al. High prevalence of the G101W germline mutation in the CDKN2A (P16(ink4a)) gene in 62 Italian malignant melanoma families. *Am J Med Genet* 2002;**107**:214–21.
16. Ruiz A, Puig S, Malvehy J, et al. CDKN2A mutations in Spanish cutaneous malignant melanoma families and patients with multiple melanomas and other neoplasia. *J Med Genet* 1999;**36**:490–3.
17. Holland EA, Schmid H, Kefford RF, Mann GJ. CDKN2A (P16(INK4a)) and CDK4 mutation analysis in 131 Australian melanoma probands: effect of family history and multiple primary melanomas. *Genes Chromosome Cancer* 1999;**25**:339–48.
18. Gillanders E, Hank Juo SH, Holland EA, et al. Localization of a novel melanoma susceptibility locus to 1p22. *Am J Hum Genet* 2003;**73**:301–13.
19. Bishop DT, Demenais F, Goldstein AM, et al. Geographical variation in the penetrance of CDKN2A mutations for melanoma. *J Natl Cancer Inst* 2002;**94**:894–903.
20. Zuo L, Weger J, Yang Q, et al. Germline mutations in the p16INK4a binding domain of CDK4 in familial melanoma. *Nat Genet* 1996;**12**:97–9.
21. Harland M, Meloni R, Gruis N, et al. Germline mutations of the CDKN2 gene in UK melanoma families. *Hum Mol Genet* 1997;**6**:2061–7.
22. Newton Bishop JA, Harland M, Bishop DT. The genetics of melanoma: the UK experience. *Clin Exp Dermatol* 1998;**23**:158–61.
23. Jones AC, Austin J, Hansen N, et al. Optimal temperature selection for mutation detection by denaturing HPLC and comparison to single-stranded conformation polymorphism and heteroduplex analysis. *Clin Chem* 1999;**45**:1133–40.

24. Liu W, Smith DI, Rechtzigel KJ, Thibodeau SN, James CD. Denaturing high performance liquid chromatography (DHPLC) used in the detection of germline and somatic mutations. *Nucleic Acids Res* 1998;**26**:1396–400.
25. Orlow I, Roy P, Barz A, Canchola R, Song Y, Berwick M. Validation of denaturing high performance liquid chromatography as a rapid detection method for the identification of human INK4A gene mutations. *J Mol Diag* 2001;**3**:158–63.
26. Frueh FW, Noyer-Weidner M. The use of denaturing high-performance liquid chromatography (DHPLC) for the analysis of genetic variations: impact for diagnostics and pharmacogenetics. *Clin Chem Lab Med* 2003;**41**:452–61.
27. Herbert O, Trossaert M, Boisseau P, Fressinaud E, Gerson F. Evaluation of denaturing high-performance liquid chromatography (DHPLC) in the screening of mutations in hemophilia B patients. *J Thromb Haemost* 2004;**2**:2267–9.
28. Gross E, Arnold N, Goette J, Schwarz-Boeger U, Kiechle M. A comparison of BRCA1 mutation analysis by direct sequencing, SSCP and DHPLC. *Hum Genet* 1999;**105**:72–8.
29. Ellis LA, Taylor CF, Taylor GR. A comparison of fluorescent SSCP and denaturing HPLC for high throughput mutation scanning. *Hum Mutat* 2000;**15**:556–64.
30. van der Heiden IP, van der Werf M, Lindemans J, van Schaik RH. Sequencing: not always the "gold standard". *Clin Chem* 2004;**50**:248–9.
31. Platz A, Hansson J, Ringborg U. Screening of germline mutations in the CDK4, CDKN2C and TP53 genes in familial melanoma: a clinic-based population study. *Int J Cancer* 1998;**78**:13–5.
32. Newton Bishop JA, Harland M, Bennett DC, et al. Mutation testing in melanoma families: INK4A, CDK4 and INK4D. *Br J Cancer* 1999;**80**:295–300.
33. Soufir N, Lacapere JJ, Bertrand G, et al. Germline mutations of the INK4a-ARF gene in patients with suspected genetic predisposition to melanoma. *Br J Cancer* 2004;**90**:503–9.
34. Ruas M, Peters G. The p16INK4a/CDKN2A tumor suppressor and its relatives. *Biochim Biophys Acta* 1998;**1378**:F115–77.
35. Parry D, Peters G. Temperature-sensitive mutants of p16CDKN2 associated with familial melanoma. *Mol Cell Biol* 1996;**16**:3844–52.
36. Cotton RG. Mutation detection and mutation databases. *Clin Chem Lab Med* 1998;**36**:519–22.
37. Sevilla C, Moatti JP, Julian-Reynier C, et al. Testing for BRCA1 mutations: a cost-effectiveness analysis. *Eur J Hum Genet* 2002;**10**:599–606.