Electronic letter

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Absence of germline *p16^INK4a^* alterations in *p53* wild type Li-Fraumeni syndrome families

EDITOR—The Li-Fraumeni syndrome (LFS) is a rare familial cancer syndrome that predisposes gene carriers to the development of diverse early onset malignancies, including soft tissue sarcomas, osteosarcomas, adrenocortical carcinomas, brain tumours, breast carcinomas, and leukaemia,[1–3] with other cancer types occurring less frequently.[4–6] Families adhering to the classical definition of the syndrome include those in which one subject, usually the proband, is diagnosed with a sarcoma before 45 years of age, and has a first degree relative with cancer before 45 years of age, and another first or second degree relative in the same parental lineage with any cancer diagnosed under 45 years of age or with sarcoma at any age.[7] Families that do not meet these strict criteria are referred to as LFS-like (LFS-L).[8 9]

The majority of classical LFS families harbour germline mutations of the *p53* tumour suppressor gene.[10–12] However, in the remaining "classical" LFS families, and in most LFS-L families, no alterations in *p53* have been found.[8 9] This lack of an absolute phenotype:genotype concordance could be attributed to incomplete screening of the *p53* gene, inactivation of the p53 protein through interaction with other cellular proteins or viruses, or defects in other genes involved in *p53* mediated cell cycle regulatory pathways.

*p16^INK4a^* is a candidate gene that could account for the cancer predisposition in *p53* wild type LFS families. It is located on chromosome 9p21 and its three exons encode a 156 amino acid, 15.8 kDa protein.[13] *p16^INK4a^* is a cyclin dependent kinase inhibitor that is frequently mutated or deleted in many human cancer cell lines[14–16] and some sporadic malignancies, including sarcomas, breast cancer, leukaemia, and brain tumours, which are all component tumours of LFS.[17] Germline *p16^INK4a^* alterations are associated with familial melanoma.[18–20] p16^INK4a^ shares functional similarities with p53 in that it blocks progression through the cell cycle at G1/S by inhibiting CDK 4/6 mediated phosphorylation of Rb.[13 14] Although *p16^INK4a^* is most frequently inactivated by homozygous deletion, point mutations or somatic methylation of 5' regulatory regions are also important mechanisms of gene inactivation.[17] Methylation has not been reported to date in the germline.

In view of the comparable biological and phenotypic features of *p53* and *p16^INK4a^* inactivation, we proposed that germline inactivation of the *p16^INK4a^* gene could account for the predisposition to cancer development in a proportion of LFS families that harboured wild type *p53*.

Genomic DNA was isolated from 103 subjects ascertained from 82 cancer families. These had all been previously screened for the presence of *p53* mutations in exons 2 and 4-11 by single strand conformational polymorphism (SSCP) analysis and DNA sequencing as previously described.[26–29] Where available, samples from more than one family member were examined to determine whether a germline gene alteration occurred de novo or was inherited. Both *p53* wild type and *p53* mutant samples were included in the *p16^INK4a^* analysis to determine whether germline alterations of one gene precluded the occurrence of alterations of the other. Of the 103 samples, 24 (from 17 kindred) fulfilled the classical definition of LFS, 63 (from 51 kindred) met the criteria of LFS-L, while the remaining 16 (from 14 kindred) exhibited a family history of cancer but did not meet the strict criteria of either LFS or LFS-L. Excluded from the study were 12 subjects

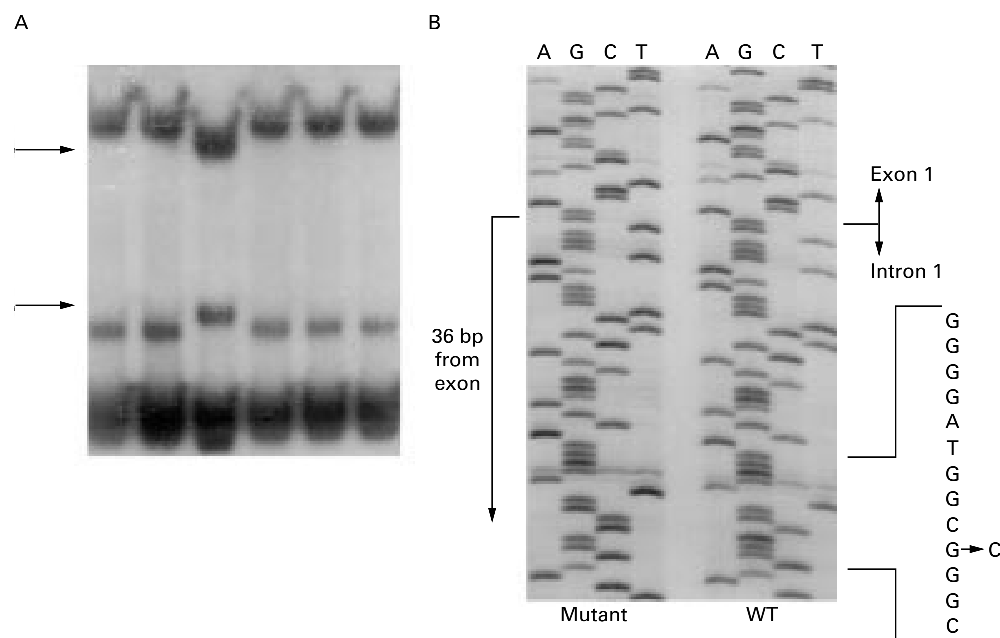

Figure 1 p16^INK4a^ intron 1 alteration. (A) SSCP gel. The arrows indicate the relevant band shifts. Running conditions: 10% glycerol/7.5% acrylamide gel run at 13 W for 17 hours at room temperature. (B) Sequencing gel. WT=wild type, A=adenine, G=guanine, C=cytosine, T=thymine.

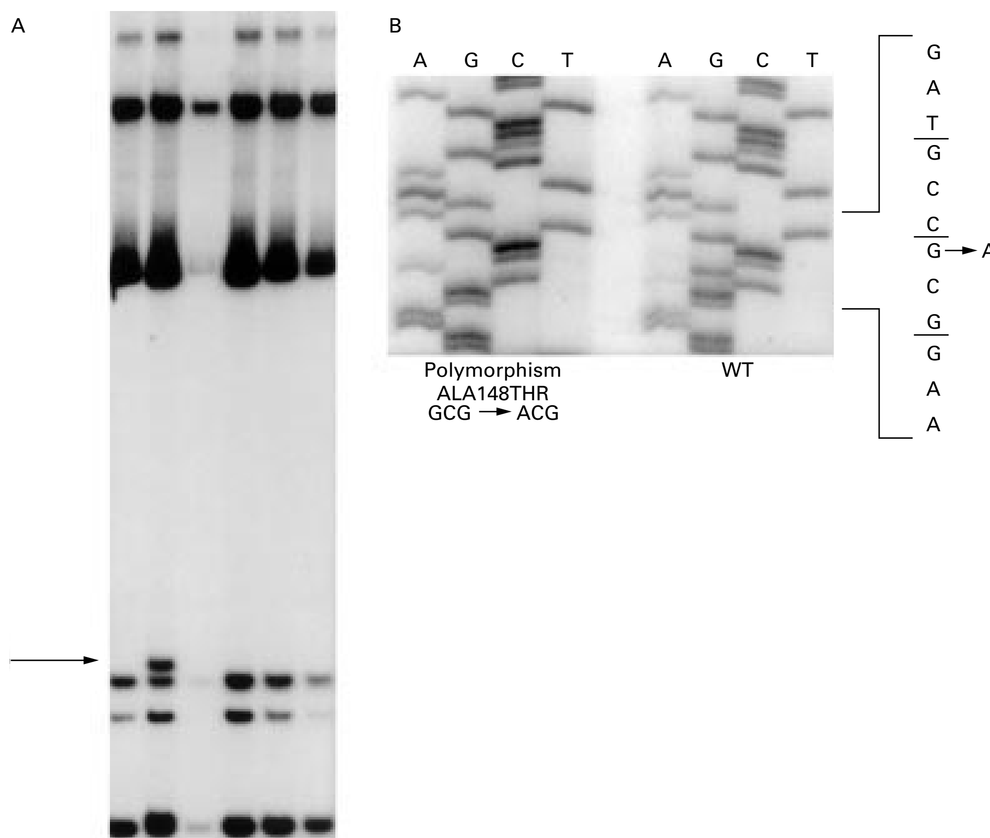

Figure 2 p16^INK4a exon 2 polymorphism. (A) SSCP gel. The arrow indicates the relevant band shift. Running conditions: 10% glycerol/7.5% acrylamide gel run at 6.5 W for 18.5 hours at room temperature. (B) Sequencing gel. WT=wild type, A=adenine, G=guanine, C=cytosine, T=thymine.

for whom complete data regarding tumour type were not available and two who were from a family in whom the proband harboured three *p53* alterations.[29]

All samples (*p53* wild type and *p53* mutant) were screened for *p16^INK4a* mutations using SSCP analysis which has been shown to be both 90% specific and sensitive.[30] Each of the three exons of *p16^INK4a* was amplified using a modification of previously described primers and conditions.[31] In particular, the 461 bp exon 2 fragment was digested with *Sma*I to yield two smaller fragments (179 bp and 282 bp). The samples were denatured for 15 minutes at 85°C and loaded immediately onto a polyacrylamide-TBE non-denaturing gel containing a range of acrylamide (4.5-9.0%) and glycerol (2.0-10.0%) conditions. Electrophoresis was conducted at room temperature for 80-400 Watt hours depending on the concentration of acrylamide and glycerol and both positive and negative controls were applied to each gel. A minimum of two gel conditions were used for each fragment in order to increase the likelihood of detection of subtle band shifts. DNA samples with detectable and reproducible band shifts on SSCP analysis were reamplified with the primers encompassing the abnormal region. Fragments were purified using a Qiaex II extraction kit and directly subcloned into a T-tailed pBSK vector. A minimum of six clones were sequenced by the Sanger dideoxynucleotide method with a Sequenase 2.0 kit (US Biochemical) to determine the precise nature of the sequence alteration. In addition, where a base pair alteration was found, duplicate PCR reactions were performed to rule out the possibility of a PCR generated artefact. For the eight samples that did not amplify on repeated attempts, Southern blot analysis was undertaken to determine whether homozygous or heterozygous deletion of the exon was present.[32]

Table 1 Summary of p53 and p16^INK4a mutation analysis in LFS and LFS-L samples

	Classical LFS Kindreds (17) Subjects (24)	*LFS-like Kindreds (51) Subjects (63)*	*Other Kindreds (14) Subjects (16)*
p53 mutations	13	11	0
p53 polymorphisms	1	3	0
p16 intron 1 alteration	0	2	0
p16 exon 2 polymorphism	1	4	0

Of the 103 subjects examined for *p16^INK4a* alterations, two unrelated subjects harboured an identical single base pair alteration in intron 1, 36 bp downstream of the intron/exon junction (fig 1A, B), and five harboured a common Ala148Thr polymorphism in exon 2 (fig 2A, B). Results from previous studies in our laboratories indicated that 26 patients had a total of 28 *p53* alterations. Of these 26, 13 were within the classical LFS group and 13 were in the LFS-L group. There were a total of 17 missense mutations, two polymorphisms (Arg213Arg), three nonsense mutations, one 6 bp deletion, one 4 bp deletion, one subject with a polymorphism (Arg213Arg) and a 1 bp deletion, and one subject with a polymorphism (Arg72Pro) and a 2 bp deletion. Both the *p16^INK4a* and *p53* mutation analyses are summarised in tables 1 and 2.

All of the *p16^INK4a* alterations were observed in separate kindred. For *p53*, there were two families (denoted † and ‡ in table 2) where two subjects harboured the same mutation and one family (denoted * in table 2) with three mutant gene carriers. However, one member of this third family had a completely different mutation from the other two. Only one subject (509-C) carried both the *p16^INK4a* Ala148Thr polymorphism in exon 2 and a *p53* mutation (Arg196Pro). This subject came from a classical LFS fam-

Table 2 Summary of individual p53 and p16INK4a gene alterations

Sample	p16 alteration	p53 alteration	
		AA substitution	Exon
261-L	Intron 1 G→C (36 bp 5' exon 1)		
274-L	Exon 2 Ala148Thr		
281-L	Exon 2 Ala148Thr		
284-L	Exon 2 Ala148Thr		
306-L	Exon 2 Ala148Thr		
474-L	Intron 1 G→C (36 bp 5' exon 1)		
509-C	Exon 2 Ala148Thr	Arg196Pro	6
262-C		His193Arg	6
265-L*		Tyr220Cys	6
270-L		Arg213Arg	6
304-L		Arg213Arg	6
357-L		Glu258Lys	7
365-L*		Gly245Ser	7
376-C†		Gly245Ser	7
379-C		Arg213UGA	6
405-C		6 bp deletion	5
407-C		Arg72Pro	4
		2 bp deletion	4
425-C		Arg248Gln	7
426-C		Arg175His	5
463-L*		Tyr220Cys	6
464-C†		Gly245Ser	7
466-C		Arg306UGA	8
471-L		1 bp deletion	5
		Arg213Arg	6
472-L		Tyr205Cys	6
475-L		Arg213UGA	6
476-L		Arg290His	8
478-C		Gly245Cys	7
511-C‡		Arg175His	6
512-C‡		Arg175His	6
515-L		Arg110Leu	4
553-L		Pro152Leu	5
583-L		4 bp deletion	8

*,†,‡ Members of three different kindreds.
C = classical LFS family member. L = LFS-like family member.

ily. Southern blot analysis did not show either homozygous or heterozygous deletions (data not shown).

Both *p53* and *p16INK4a* play important roles in the development of multiple types of cancers. Inactivation of *p53* has been found to occur in up to 50% of all cancers, and *p16INK4a* inactivation is observed in up to 26% of all malignancies.[17] Both *p53* and *p16INK4a* exert their growth suppressor effects at the G1/S phase of the cell cycle and inactivation of either is associated with the development of constellations of tumours with some phenotypic similarities. Bearing these features in mind, *p16INK4a* seemed an ideal candidate gene to account for the development of LFS in those kindred that harboured wild type *p53* in the germline.

Of the 103 germline samples analysed for *p16INK4a* point mutations and deletions in this study, two harboured an intron 1 alteration and five a common exon 2 polymorphism. Only one sample showed a *p53* mutation along with the *p16INK4a* exon 2 polymorphism. There was no evidence for *p16INK4a* deletion in any of the samples analysed.

Although we ruled out *p16INK4a* point mutations and deletions, we did not examine methylation as a means of inactivation in our series. However, the likelihood of *p16INK4a* methylation defects occurring in the germline of this sample population is felt to be exceedingly low as these alterations have not been reported to occur in other studies of familial melanoma patients. *p16INK4a* methylation is, however, thought to be an important mechanism of inactivation in sporadic tumours.[33 34] Moreover, a zero numerator does not necessarily mean that no *p16INK4a* mutations will ever be found in a similar population if enough samples are analysed. It has been shown that for a zero numerator, 3 is a good approximation of the upper boundary for a 95% CI.[35] Based on this "rule of 3", we can establish the maximum long run risk associated with the negative observations in LFS, LFS-L, and other cancer families. We can be 95% confident that the chance of a patient similar to our 103 cancer patients having a *p16INK4a* mutation is at most 3/103 or 2.9%. If we consider only LFS patients, then the likelihood that one will have a *p16INK4a* mutation is 3/24 or 12.5%. Similarly, LFS-L patients have a risk of 3/63 or 4.8% and miscellaneous cancer patients have a risk of 3/16 or 18.8%.

Our conclusion that sequence alterations of *p16INK4a* do not appear to account for the development of cancer in LFS or LFS-L families is based on the assumption that neither *p16INK4a* intronic alterations nor polymorphisms have any role in human tumorigenesis. This may, in fact, not be the case. Recent reports suggest that at least one polymorphism in another tumour suppressor gene, namely *p53* (Arg72Pro), may contribute to cancer development,[36] although other reports refute this susceptibility.[37] Moreover, both *p53* and *p21CIP1* intronic alterations that may be associated with cancer predisposition have been reported[38 39] as well as a base substitution in the 3' UTR of *p16INK4a*.[40]

Inactivation of either *p53* or *p16INK4a* ultimately leads to inhibition of cell cycle regulation by preventing phosphorylation of pRB. It may not be surprising that germline alterations of *p16INK4a* were not observed in our study population, as no melanoma families were documented. On the other hand, somatic mutations of the gene had been observed in a wide spectrum of human tumours, including many associated with LFS. Our results indicate that it is not likely that *p16INK4a* plays a significant role in the predisposition to cancer in LFS which support findings of a recent smaller study.[41] Further studies of genes involved in *p53* mediated growth regulation may be fruitful in identifying genetic events involved in cancer predisposition in LFS families who harbour wild type *p53*.

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CAROL PORTWINE*
JODI LEES*
SIGITAS VERSELIS†
FREDERICK P LI†
DAVID MALKIN*

**Division of Oncology, Department of Pediatrics, The Hospital for Sick Children, University of Toronto, 555 University Avenue, Toronto, Ontario, Canada M5G 1X8*
†Divisions of Human Cancer Genetics and Population Sciences, Dana-Farber Cancer Institute, 44 Binney Street, Boston, Massachusetts 02115, USA.

Correspondence to: Dr Malkin, Division of Hematology/Oncology, The Hospital for Sick Children, 555 University Avenue, Toronto, Ontario, Canada M5G 1X8, david.malkin@sickkids.on.ca

1 Li FP, Fraumeni JF Jr. Soft-tissue sarcomas, breast cancer, and other neoplasms. A familial syndrome? *Ann Intern Med* 1969;**71**:747-52.
2 Li FP, Fraumeni JF Jr. Rhabdomyosarcoma in children: epidemiologic study and identification of a familial cancer syndrome. *J Natl Cancer Inst* 1969;**43**:1365-73.
3 Garber JE, Goldstein AM, Kantor AF, Dreyfus MG, Fraumeni JF Jr, Li FP. Follow-up study of twenty-four families with Li-Fraumeni syndrome. *Cancer Res* 1991;**51**:6094-7.
4 Hartley AL, Birch JM, Kelsey AM, Marsden HB, Harris M, Teare MD. Are germ cell tumors part of the Li-Fraumeni cancer family syndrome? *Cancer Genet Cytogenet* 1989;**42**:221-6.
5 Garber JE, Burke EM, Lavally BL, Billett AL, Sallan SE, Scott RM, Kupsky W, Li FP. Choroid plexus tumors in the breast cancer-sarcoma syndrome. *Cancer* 1990;**66**:2658-60.
6 Garber JE, Liepman MK, Gelles EJ, Corson JM, Antman KH. Melanoma and soft tissue sarcoma in seven patients. *Cancer* 1990;**66**:2432-4.
7 Li FP, Fraumeni JF Jr, Mulvihill JJ, Blattner WA, Dreyfus MG, Tucker MA, Miller RW. A cancer family syndrome in twenty-four kindreds. *Cancer Res* 1988;**48**:5358-62.
8 Birch JM, Hartley AL, Tricker KJ, Prosser J, Condie A, Kelsey AM, Harris M, Morris Jones PH, Binchy A, Crowther D, Craft AW, Eden OB, Evans GR, Thompson E, Mann JR, Martin J, Mitchell ELD, Santibanez-Koref MF. Prevalence and diversity of constitutional mutations in the p53 gene among 21 Li-Fraumeni families. *Cancer Res* 1994;**54**: 1298-304.
9 Eeles RA. Germline mutations in the TP53 gene. *Cancer Surv* 1995;**25**:101-24.
10 Malkin D, Li FP, Strong LC, Fraumeni JF Jr, Nelson CE, Kim DH, Kassel J, Gryka MA, Bischoff FZ, Tainsky MA, Friend SH. Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas, and other neoplasms. *Science* 1990;**250**:1233-8.

11 Srivastava S, Zou ZQ, Pirollo K, Blattner W, Chang EH. Germ-line transmission of a mutated p53 gene in a cancer-prone family with Li-Fraumeni syndrome. *Nature* 1990;**348**:747-9.
12 Frebourg T, Kassel J, Lam KT, Gryka MA, Barbier N, Andersen TI, Borresen AL, Friend SH. Germ-line mutations of the p53 tumor suppressor gene in patients with high risk for cancer inactivate the p53 protein. *Proc Natl Acad Sci USA* 1992;**89**:6413-17.
13 Serrano M, Hannon GJ, Beach D. A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. *Nature* 1993;**366**:704-7.
14 Kamb A, Gruis NA, Weaver-Feldhaus J, Liu Q, Harshman K, Tavtigian SV, Stockert E, Day RS III, Johnson BE, Skolnick MH. A cell cycle regulator potentially involved in genesis of many tumor types. *Science* 1994;**264**:436-40.
15 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.
16 Spruck CH III, Gonzalez-Zulueta M, Shibata A, Simoneau, AR, Lin MF, Gonzales F, Tsai YC, Jones PA. p16 gene in uncultured tumours. *Nature* 1994;**370**:183-4.
17 Liggett WH Jr, Sidransky D. Role of the p16 tumor suppressor gene in cancer. *J Clin Oncol* 1998;**16**:1197-206.
18 Hussussian CJ, Struewing JP, Goldstein AM, Higgins PA, Ally DS, Sheahan MD, Clark WH Jr, Tucker MA, Dracopoli NC. Germline p16 mutations in familial melanoma. *Nat Genet* 1994;**8**:15-21.
19 Gruis NA, van der Velden PA, Sandkuijl LA, Prins DE, Weaver-Feldhaus J, Kamb A, Bergman W, Frants RR. Homozygotes for CDKN2 (p16) germline mutation in Dutch familial melanoma kindreds. *Nat Genet* 1995;**10**:351-3.
20 Monzon J, Liu L, Brill H, Goldstein AM, Tucker MA, From L, McLaughlin J, Hogg D, Lassam NJ. CDKN2A mutations in multiple primary melanomas. *N Engl J Med* 1998;**338**:879-87.
21 Jen J, Harper JW, Bigner SH, Bigner DD, Papadopoulos N, Markowitz S, Willson JK, Kinzler KW, Vogelstein B. Deletion of p16 and p15 genes in brain tumors. *Cancer Res* 1994;**54**:6353-8.
22 Mao L, Merlo A, Bedi G, Shapiro GI, Edwards CD, Rollins BJ, Sidransky D. A novel p16INK4A transcript. *Cancer Res* 1995;**55**:2995-7.
23 Liggett WH Jr, Sewell DA, Rocco J, Ahrendt SA, Koch W, Sidransky D. p16 and p16 beta are potent growth suppressors of head and neck squamous carcinoma cells in vitro. *Cancer Res* 1996;**56**:4119-23.
24 Coleman A, Fountain JW, Nobori T, Olopade OI, Robertson G, Housman DE, Lugo TG. Distinct deletions of chromosome 9p associated with melanoma versus glioma, lung cancer, and leukemia. *Cancer Res* 1994;**54**:344-8.
25 Sidransky D. Two tracks but one race? Cancer genetics. *Curr Biol* 1996;**6**:523-5.
26 Mashiyama S, Murakami Y, Yoshimoto T, Sekiya T, Hayashi K. Detection of p53 gene mutations in human brain tumors by single-strand conformation polymorphism analysis of polymerase chain reaction products. *Oncogene* 1991;**6**:1313-18.
27 Peng HQ, Hogg D, Malkin D, Bailey D, Gallie BL, Bulbul M, Jewett M, Buchanan J, Goss PE. Mutations of the p53 gene do not occur in testis cancer. *Cancer Res* 1993;**53**:3574-8.
28 Wagner J, Portwine C, Rabin K, Leclerc JM, Narod SA, Malkin D. High frequency of germline p53 mutations in childhood adrenocortical cancer. *J Natl Cancer Inst* 1994;**86**:1707-10.
29 Quesnel S, Verselis S, Portwine C, Garber J, White M, Feunteun J, Malkin D, Li FP. p53 compound heterozygosity in a severely affected child with Li-Fraumeni syndrome. *Oncogene* 1999;**18**:3970-8.
30 Preudhomme C, Fenaux P. The clinical significance of mutations of the p53 tumor suppressor gene in haematological malignancies. *Br J Haematol* 1997;**98**:502-11.
31 Okamoto A, Demetrick DJ, Spillare EA, Hagiwara K, Hussain SP, Bennet WP, Forrester K, Gerwin B, Seranno M, Beach DH, Harris CC. Mutations and altered expression of p16INK4 in human cancer. *Proc Natl Acad Sci USA* 1994;**91**:11045-9.
32 Sambrook J, Fritsch EF, Maniatis T. *Molecular cloning: a laboratory manual*. 2nd ed. New York: Cold Spring Harbor Laboratory Press, 1989.
33 Herman JG, Merlo A, Mao L, Lapidus RG, Issa JPJ, Davidson NE, Sidransky D, Baylin SB. Inactivation of the CDKN2/p16/MTS1 gene is frequently associated with aberrant DNA methylation in all common human cancers. *Cancer Res* 1995;**55**:4525-30.
34 Fueyo J, Gomez-Manzano C, Bruner JM, Saito Y, Zhang B, Zhang W, Levin VA, Yung WKA, Kyritsis AP. Hypermethylation of the CpG island of p16/CDKN2 correlates with gene inactivation in gliomas. *Oncogene* 1996;**13**:1615-19.
35 Hanley JA, Lippman-Hand A. If nothing goes wrong, is everything all right? Interpreting zero numerators. *JAMA* 1983;**249**:1743-5.
36 Storey A, Thomas M, Kalita A, Harwood C, Gardiol D, Mantovani F, Breuer J, Leigh IM, Matlashewski G, Banks L. Role of a p53 polymorphism in the development of human papillomavirus-associated cancer. *Nature* 1998;**393**:229-34.
37 Rosenthal AN, Ryan A, Al-Jehani RM, Storey A, Harwood CA, Jacobs IJ. p53 codon 72 polymorphism and risk of cervical cancer in UK. *Lancet* 1998;**352**:871-2.
38 Mousses S, Ozcelik H, Lee PD, Malkin D, Bull SB, Andrulis IL. Two variants of the CIP1/WAF1 gene occur together and are associated with human cancer. *Hum Mol Genet* 1995;**4**:1089-92.
39 Avigad S, Barel D, Blau O, Malka A, Zoldan M, Mor C, Fogel M, Cohen IJ, Stark B, Goshen Y, Stein J, Zaizov R. A novel germ line p53 mutation in intron 6 in diverse childhood malignancies. *Oncogene* 1997;**14**:1541-5.
40 Liu L, Dilworth D, Gao L, Monzon J, Summers A, Lassam N, Hogg D. Mutation of the CDKN2A 5' UTR creates an aberrant initiation codon and predisposes to melanoma. *Nat Genet* 1999;**21**:128-32.
41 Burt EC, McGown G, Thorncroft M, James LA, Birch JM, Varley JM. Exclusion of the genes CDKN2 and PTEN as causative gene defects in Li-Fraumeni syndrome. *Br J Cancer* 1999;**80**:9-10.