SCIENTIFIC CORRESPONDENCE

Telomerase expression in uveal melanoma

Bernhard Heine, Sarah E Coupland, Sören Kneiff, Gudrun Demel, Norbert Bornfeld, Michael Hummel, Harald Stein

Abstract

Background/claims—Accumulating evidence indicates that telomerase activity is repressed in normal human somatic cells but reactivated in cancers and immortal cells, suggesting that activation of telomerase activity has a role in carcinogenesis and immortalisation. To date, telomerase in uveal melanoma and, whether, it may have a role in the development or progression of these tumours has not been described. The expression patterns and the activity of telomerase were investigated in 14 uveal melanoma and these results were correlated with histological and immunohistological features of these tumours.

Methods—A modified PCR based telomeric repeat amplification protocol (TRAP) assay was used to demonstrate telomerase activity in 14 uveal melanomas. In addition, in situ hybridisation was used to demonstrate the expression pattern of the telomerase RNA component (hTR) at the single cell level in eight of these globes.

Results—The TRAP assay revealed moderate telomerase activity in all uveal melanomas examined. In situ hybridisation visualised a moderate to high upregulation of hTR in the melanoma cells but not in the admixed reactive cells. There was no correlation among tumour location, cell type, or growth fraction and the amount of telomerase activity. In addition, the cells of the germinative zone of the lens demonstrated a strong hTR expression.

Conclusion—Telomerase activity is up-regulated in uveal melanomas. The expression of hTR was located to the tumour cells and not the reactive tumour infiltrating cells. Strong telomerase expression was also demonstrated in cells of the germinative zone of the lens.

(Br J Ophthalmol 2000;84:217-223)

telomeres,^{2,3} resulting in the loss of 50–200 nucleotides from the telomere with each cell division.⁴ It has been proposed that this progressive shortening of telomeres in normal somatic cells acts as an internal clock by which the cell counts replication events. Cell senescence acts as an anticancer mechanism and is thought to occur when the telomere length is critically shortened.⁵

Telomerase, a ribonucleoprotein polymerase, is capable of synthesising telomeric DNA onto the ends of chromosomes, compensating the telomeric sequences usually lost with cell divisions.⁶ According to the telomere hypothesis, only those cells which acquire telomerase activity retain the chromosomal stability, altering the cell's biological clock and producing an immortal phenotype.^{1,7} This hypothesis has been supported by data demonstrating telomerase activity in a wide variety of malignant cells and tissues,^{8–15} but not in most normal tissues. Telomerase activity is, however, also described in physiologically proliferating cells such as lymphocytes and basal epithelial cells.^{16–20} Therefore, telomerase activity measured in whole tissue lysates using, for example, a polymerase chain reaction (PCR) based telomeric repeat amplification protocol (TRAP), is not sufficient to confirm the malignant nature of a mixed lesion.

Telomerase is composed of ribonucleic acid (RNA) and protein components. The human telomerase RNA component (hTR) has been recently cloned and shown to be critical for telomerase activity.²¹ Telomerase RNA expression levels determined by northern blot analysis or reverse transcriptase (RT) PCR suffer from similar drawbacks as the TRAP assay since a cellular localisation of the obtained signals is not possible.^{22,23} Applying in situ hybridisation for hTR, it was possible to demonstrate high upregulation of hTR in dysplastic cells whereas reactive proliferating cells exhibit only low to moderate signals.^{10,24–27}

Uveal melanoma is the most frequent primary intraocular tumour in white adults, with an incidence of 0.7 per 100 000.²⁸ The pathogenesis of uveal melanoma is unclear and probably represents a multistep process involving the progressive and clonal accumulation of multiple genetic lesions affecting proto-oncogenes and tumour suppressor genes. Recent studies have demonstrated certain features of these tumours, which may suggest cer-

Department of
Pathology, University
Hospital Benjamin
Franklin, Free
University, Berlin
B Heine
S E Coupland
S Kneiff
G Demel
M Hummel
H Stein

Department of
Ophthalmology,
University Hospital
Essen, Germany
N Bornfeld

Correspondence to:
Dr Bernhard Heine,
Department of Pathology,
UKBF, Hindenburgdamm
30, D-12200 Berlin,
Germany

Accepted for publication
1 October 1999

Telomeres are chromosomal end regions made of several thousand copies of a repeating nucleotide sequence (in human 5'-TTAGGG-3')¹ and are important in stabilising the chromosome during replication. In human cells, DNA polymerase is not able to completely replicate the linear DNA of the

tain genetic changes in their development and progression. These include an overexpression of the cell cycle regulator proteins such as p53,^{29,30} p16,³¹ and cyclin D1,³² as well as chromosomal abnormalities, in particular, monosomy 3.^{33–35} To date, neither the pattern of telomerase expression nor its activity has been investigated in uveal melanoma. In the present study, we investigated telomerase activity in 14 cases of uveal melanomas and confirmed that this activity is derived from the tumour cells by in situ hybridisation and not from admixed reactive cells, such as infiltrating lymphocytes and macrophages. Furthermore, the in situ hybridisation for hTR demonstrated strong signals over equatorial cells of the lens, and occasional ganglion cells of the retina.

Methods

PROCUREMENT AND PREPARATION OF SPECIMENS

Thirteen eyes were enucleated for uveal melanoma; in one patient orbital manifestation of uveal melanoma was examined. Before fixation in 4% formalin, the enucleated eyes were opened and biopsies of the tumour were removed and immediately frozen in liquid nitrogen and stored at -80°C until use. The remaining globes were fixed in formalin and embedded in paraffin for sectioning. Conventional histological stains were assessed for cell type using the modified Callender system.³⁶

IMMUNOHISTOCHEMISTRY

Additional slides were stained for immunohistochemical studies using a monoclonal antibody that are reactive in paraffin sections. An antigen retrieval method using a pressure cooker was performed before immunohistochemical staining.³⁷ Conventional indirect immunohistochemistry was applied following standard protocols.³⁸ The staining consisted of incubation with the primary monoclonal antibody MIB-1 (directed against the Ki-67 antigen, expressed in the late G1, M, G2, and S phase, but not in the early G1 and G0 phases),^{39–41} and it was made visible using alkaline phosphatase anti-alkaline phosphatase (APAAP). In heavily pigmented tumours, the sections were placed in hydrogen peroxide for 18 hours to remove melanin pigmentation from the tumour cells before cover slipping the

slides, as previously described.⁴² Cells were considered positive for MIB-1 only when distinct nuclear staining was identified. The percentage of immunoreactive nuclei in the uveal melanoma was evaluated by counting at least 5×100 cells using the $40\times$ objective (Olympus, BH2). The mean of these values was expressed as a percentage and reflected the "growth fraction" of the tumours.

TRAP ASSAY

To investigate tissue samples for the presence of telomerase activity, $15\ \mu\text{m}$ thick tissue sections were produced from fresh frozen tissue blocks and controlled by histological examination of 4–7 μm sections directly above and beneath the extracted tissue proportion to confirm the presence of the expected lesion. As a positive control for the TRAP assay, the cell line used (Hela cells derived from cervical carcinoma) was cultured in RPMI medium supplemented with 10% FCS. Cell suspensions were centrifuged at 1000 g for 10 minutes and washed twice with phosphate buffered saline. Depending on their size, the sections and cell pellets were homogenised in 200–300 μl of lysis buffer (10 mM TRIS-HCl (pH 7.5), 1 mM MgCl_2 , 1 mM EGTA, 0.1 mM phenylmethylsulphonyl fluoride, 5 mM β -mercaptoethanol, 0.5% CHAPS (Pierce, Oud-Beijerland, Netherlands), 10% glycerol), incubated at 4°C for 30 minutes, and centrifuged at 100 000 g for 30 minutes at 4°C . The supernatants were removed and stored at -80°C until use.

Telomerase activity was determined according to Kim *et al*⁸ using the non-radioactive GeneScan method as described previously^{10,11} with minor modifications using 0.1 mM spermidine (Sigma) replacing T4 protein according to Aldous and Grabill.⁴³ Briefly, an aliquot of 6 μg , 0.6 μg , and 0.06 μg of total protein from each sample was investigated. Two TRAP assays were independently performed from each sample. Each assay was carried out including positive control (Hela cells; dilution from 6 μg , 0.6 μg , 0.06 μg , and 0.006 μg protein) and a negative control (lysis buffer added to the complete TRAP mixture).

Table 1 Clinical details and tumour size, location, cell type, growth fraction, telomerase activity, and hTR expression of the uveal melanomas examined

Case	Age	Location	Size (mm)	Cell type	Degree of pigmentation	Outcome*	MIB 1 (%)	ISH hTR†	TRAP assay (rTA units)
1	61	anterior equator	12×10×3	mixed, spindle epithelioid	strong	awm	10	++	40
2	71	orbit	28×27×22	mixed, spindle epithelioid (original tumour, spindle)	moderate	d	10	+++	35
3	62	anterior	16×14×11	mixed, spindle epithelioid	moderate	awm	20	++	20
4	73	anterior equator	12×12×11	mixed, spindle epithelioid	strong	awm	15	++	30
5	65	equator	12×11×11	mixed, spindle epithelioid	moderate	d	15	++	35
6	71	equator	12×9×12	mixed, spindle epithelioid	mild	a	5	++	35
7	43	equator	14×12×11	mixed, spindle epithelioid	moderate	d	5	++	35
8	50	anterior	9×8×2	mixed, spindle epithelioid	mild	a	5	+	25
9	31	anterior	18×12×7	mixed, spindle epithelioid	moderate	a	10	++	
10	87	anterior posterior	23×17×12	mixed, spindle epithelioid	moderate	a	10	++	
11	55	anterior	18×15×11	mixed, spindle epithelioid	moderate	a	10	++	
12	33	equator	17×10×10	spindle	mild	d	10	+	
13	81	posterior	12×10×5	spindle	mild	awm	5	++	
14	54	posterior	15×10×15	mixed, spindle epithelioid	mild	d	10	++	

*a = alive without metastases; awm = alive with metastases; d = dead.

†Intensity of telomerase signal in hTR: + = mild, ++ = moderate, +++ = strong, ++++ = intense.

Figure 1 Non-radioactive TRAP assay for the detection of telomerase activity in lysates. Fluorescence labelled amplicates were separated on a 8% acrylamide DNA sequencing gel and analysed by the GeneScan software. Positive samples give rise to repetitive 6 base pair (bp) distant peaks of declining height. Specific amplicates start at the length of 39 bp. (A–D) Cell line HELA (positive control) serial diluted. (A) 6 µg protein, (B) 0.6 µg protein, (C) 0.06 µg protein, (D) 0.006 µg protein. (E–G) TRAP positive uveal melanomas (0.6 µg protein each; case nos 3, 4, 5; see Table 1). (H) Negative control (lysis buffer).

ANALYSIS AND QUANTIFICATION OF THE TRAP ASSAY

TRAP positive samples were semiquantified employing the peak areas between 37 bp and 150 bp of the TRAP amplification products as described elsewhere.^{10–11} The sum of corresponding peak areas of a positive control (K562; 0.6 µg protein) obtained from the same GeneScan run was set to 100 units and the peaks of the negative control (lysis buffer) were set to 0 units. The relative telomerase activity (rTA) of a given sample X (0.6 µg protein) was calculated using the following formula:

$$\text{rTAX} = \left[\frac{(X1 - nc)/(pc - nc) + (X2 - nc)/(pc - nc)}{2} \times 100 \right] \text{units}$$

(X1, X2=peak areas of sample X obtained

from two different TRAP assays; pc=peak area, positive control, nc=peak area, negative control).

IN SITU HYBRIDISATION

In situ hybridisation for telomerase expression was carried out as described previously.^{10–11} In brief, the probe for detection of the human telomerase RNA component (hTR) by in situ hybridisation was generated by RT-PCR employing primers deduced from published sequences: TEL-UP 5'-GGTGGCCATTTT-TGTCTAAC-3'; TEL-LOW 5'-TGCATGTGTGAGCCGAGT-3'. Total RNA from phytohaemagglutinin (PHA) activated peripheral blood lymphocytes (1 µg) was used for reverse

transcription and amplification as described above. The resulting 417 bp amplicates were subcloned in pAMP1 (Gibco BRL, Gaithersburg) and sequenced (377A, Perkin-Elmer/Applied Biosystems, Weiterstadt, Germany). Hybridisation was carried out with ^{35}S labelled run off transcript as described elsewhere.⁴⁴ In brief, dewaxed and rehydrated paraffin sections (4 μm thick) were exposed to 0.2 N HCl and 0.125 mg/ml pronase (Boehringer Mannheim, Germany), followed by acetylation with 0.1 M triethanolamine pH 8.0/0.25% (v/v) acetic anhydride and dehydration through

graded ethanol. Slides were hybridised to $2\text{--}4\times 10^5$ cpm of labelled probes overnight at 50°C . After an exposure time between 4–12 weeks, the slides were developed and counterstained with either haemalaun or haematoxylin and eosin.

Results

Clinical details as well as histomorphological data of the tumours examined are summarised in Table 1. The patients with uveal melanoma were 10 females and four males with an age range 31–87 years (mean 60 years). Thirteen

Figure 2 In situ hybridisation for the detection of the telomerase RNA component (hTR). (A) Uveal melanoma (case no 10, antisense probe, original magnification, $\times 200$). Moderate signals over the melanoma cells; the negativity of the endothelium (arrows) serves as an internal negative control. (B) Uveal melanoma (case no 11, antisense probe, original magnification $\times 300$). Accumulation of specific signals over the melanoma cells, no signals over the nuclei of the melanomacrophages (arrows). (C) Lens, equatorial region (case no 13, antisense probe, original magnification $\times 100$). Moderate to strong accumulation of signals over equatorial cells. (D) Lens, equatorial region (case no 13, same case as (C), sense probe, original magnification $\times 100$). No accumulation of signals over the nuclei. (E) Retina (case no 13, same case as (C), antisense probe, original magnification $\times 160$): moderate positivity in scattered ganglion cells of the retina (arrow). (F) Retina (case no 13, same case as (C), sense probe, original magnification $\times 150$). No accumulation of signals over the nuclei.

patients had been treated with primary enucleation; in one patient an orbital exenteration was performed following localised tumour recurrence after globe enucleation. The follow up period varied between 2 and 5 years (mean 3.5 years). Two of the patients had died from their disease; four patients were alive with clinical evidence of liver metastases, and eight patients were alive without evidence of metastases. The majority of the uveal melanomas consisted of mixed epithelioid and spindle melanoma cells with a growth fraction between 5 and 20% (mean 10%).

Moderate telomerase activity could be demonstrated in all choroidal melanomas investigated by the TRAP assay ranging from 20 to 40 units (Fig 1). In situ hybridisation revealed moderate to high expression of hTR over the nuclei of melanoma cells, whereas infiltrating reactive lymphocytes and macrophages did not demonstrate any specific signal accumulation (Fig 2A and B). These results serve as a confirmation that the measured telomerase activity is derived from the malignant tumour cells only. The hTR expression was uniform and did not vary according to the location or the cell type of the tumour. Further, there was neither a correlation between the tumour telomerase activity nor hTR upregulation and the growth fraction of the tumour.

Additional findings included moderate to strong upregulation of hTR in the few equatorial cells of the lens (Fig 2C and D) as well as mild to moderate positivity in scattered ganglion cells of the retina (Fig 2E and F). This positivity in the retina was observed in both tumour adjacent and distant areas. Further, it was not associated with a retinal detachment—that is, retinal ischaemia. Cells which were negative for hTR expression in the eye included the corneal epithelial cells, the corneal endothelium, the endothelial cells lining the trabecular meshwork, the pigmented and non-pigmented layers of the ciliary epithelium, the iris, the melanocytes of the choroid in areas distant from tumour, the remaining cell layers of the retina, including the retinal pigmented epithelium, and the optic nerve.

Discussion

Telomerase is linked to the immortalisation of cells and has been found in most human malignancies. We examined the telomerase expression profile in uveal melanomas and compared it with certain clinical (age, outcome) and morphological features (tumour size, location, cell type, and growth fraction). Furthermore, we performed in situ hybridisation of the whole eye to study the expression of the telomerase RNA component (hTR) at the single cell level in both tumorous and non-tumorous parts of the eye.

All uveal melanomas examined in this study exhibited telomerase activity. The application of in situ hybridisation for hTR confirmed the activity to be derived from the tumour cells and not from the admixed infiltrating lymphocytes or macrophages, frequently seen in uveal melanoma. Further, a positivity for hTR was not observed in normal choroidal melanocytes

distant from tumour, indicating that a telomerase upregulation is present in uveal melanomas. This finding, one in common with all or almost all human malignancies, underlines the importance of a telomerase upregulation in tumorigenesis. The amount of telomerase activity found in uveal melanomas was moderate, with only minimal variation between 20 and 40 units rTA. These findings appear similar to those reported in cutaneous melanoma,^{20–43} although, owing to the different methods used, the results between the two studies are not directly comparable. According to previous studies in the literature,^{45–52} a correlation between rTA and cell type, tumour grade or growth fraction appears to be dependent upon the type of tumour. In the current study, the telomerase expression or rTA of uveal melanomas did not correlate with either morphological (location, cell type) or immunohistological features (growth fraction, determined using the MIB-1 antibody) of the tumour. These findings are in agreement with those of Ogoshi *et al*, who investigated cutaneous melanomas.⁴⁵ They contrast, however, with those observations of authors investigating neoplasms of the brain,^{46–49} thyroid,⁵⁰ lung,⁵¹ and the colon.⁵² Previous studies suggest that telomerase activity may have a role as a prognostic factor in some malignant neoplasms,⁵² as a correlation between the telomerase activity of the tumour cells and the outcome of the patients was observed. Although the number of uveal melanomas examined in the current study is small and the follow up period short, it appears that the amount of rTA does not have any prognostic implication in these tumours.

In vitro data have suggested that a gene located on chromosome 3 acts as a repressor gene of telomerase activity and, thereby, induces senescence in normal cells through the mitotic shortening of telomeres.^{27–53–54} Interestingly, recent studies have demonstrated certain genetic changes associated with uveal melanomas, in particular, monosomy 3, multiplication of chromosome 8q, loss of 9p, as well as the translocation t(3;22)(p13;p11).^{33–55} Further, monosomy 3 in these tumours correlates with a worse prognosis.⁵⁶ Because of this association, we also investigated the genotypic status of chromosome 3 in six of the tumours to determine any relation between monosomy 3 and telomerase activity. Only one of the uveal melanomas demonstrated a genotype -3,+8q, the remaining tumours were disomic for chromosome 3 (results not shown). These preliminary results would suggest that there is no influence of chromosome 3 status on either telomerase expression or activity in uveal melanoma. However, an investigation of a larger number of tumours is required for confirmation of this observation.

In the non-tumorous parts of the enucleated eyes, moderate to high expression of hTR was demonstrated by in situ hybridisation in the pre-equatorial and equatorial lens epithelium—that is, in the germinative zone of the lens. The hTR signal diminished gradually, however, in those elongated cells being trans-

formed into lens fibres, closer to the lens nucleus. This finding is in agreement with previous studies demonstrating maximal mitotic activity in the germinative zone,⁵⁷ indicating that these cells have a high replicative capacity. Although we did not determine telomerase activity in the lens, the strong hTR signals over the lens epithelium of the germinative zone would suggest the necessity of telomerase in these cells to maintain chromosomal stability. They could be compared, therefore, with physiologically proliferating cells such as lymphocytes and basal epithelial cells, where telomerase activity has been reported.^{16–20} Whether there is effect of age on telomerase in the lens epithelium—and whether there may be a relation with telomerase and the development of cataract—remains to be determined.

The telomere hypothesis proposes that differentiated non-dividing cells no longer require telomerase expression. High telomerase activity, for example, was observed in the brain in the embryo and neonate in rats, diminishing rapidly after birth.⁵⁸ In addition, the human brain has been demonstrated to have no telomerase activity and to have long stable telomeres throughout adult life, consistent with their postmitotic status.⁵⁹ Although we cannot completely exclude the possibility of false negative results, the absence of telomerase expression in most parts of the developed adult eye in the current study (see Results) would support the telomere hypothesis. As the retina represents a highly modified extension of the central nervous system, our findings of signals over scattered ganglion cells in the *in situ* hybridisation for hTR, however, contrast with those of the above mentioned investigations examining brain tissue. Maitra *et al*⁶⁰ also observed hTR upregulation in normal ganglion cells after birth as well as in the ganglion cells of four ganglioneuroblastomas and four ganglioneuromas. Despite this, these authors could not demonstrate telomerase activity in the ganglioneuromas examined using the TRAP assay,⁶⁰ confirming that the demonstration of hTR alone in cells does not prove telomerase activity in all cases.²² The biological relevance of persistent hTR expression in postmitotic somatic cells, such as the ganglion cells of the brain and retina, is unknown at present. The possibility that the signals over the ganglion cells in the current investigation represent artefacts cannot be completely excluded. The fact, however, that others⁶⁰ using different probes also demonstrated hTR upregulation in ganglion cells located in the peripheral nervous system and that our sense probe was persistently negative would suggest that the hTR signals over the retinal ganglion cells are specific.

In conclusion, telomerase upregulation is a constant feature in uveal melanomas contributing to the malignant phenotype of these tumours. The amount of telomerase activity in uveal melanomas does not seem to have a prognostic influence on the outcome of this disease. Further, the expression of hTR could be demonstrated in both neoplastic and

non-neoplastic parts of the eyes enucleated for uveal melanoma.

This work was supported by the Deutsche Forschungsgemeinschaft (DFG; Iu 557/2-1).

The authors thank Mrs Elisabeth Oker and Mrs Helga Zimmermann-Höfken for their supportive technical assistance. In addition, the authors are grateful for the examination of some of the uveal melanomas for aberrations of chromosome 3 by Dr. Dieter Lohmann, Institute of Human Genetics, Essen, and for his useful discussions.

- 1 Moyzis RK, Buckingham JM, Cram LS, *et al*. A highly conserved repetitive DNA sequence, (TTAGGG)_n, present at the telomeres of human chromosomes. *Proc Natl Acad Sci USA* 1988;**85**:6622–6.
- 2 Watson JD. Origin of concatemeric T7 DNA. *Nat New Biol* 1972;**239**:197–201.
- 3 Olovnikov AM. A theory of marginotomy. The incomplete copying of template margin in enzymic synthesis of polynucleotides and biological significance of the phenomenon. *J Theor Biol* 1973;**41**:181–90.
- 4 Lindsey GG, Orgeig S, Thompson P, *et al*. Extended C-terminal tail of wheat histone H2A interacts with DNA of the “linker” region. *J Mol Biol* 1991;**218**:805–13.
- 5 Harley CB, Futcher AB, Greider CW. Telomeres shorten during ageing of human fibroblasts. *Nature* 1990;**345**:458–60.
- 6 Greider CW, Blackburn EH. Identification of a specific telomere terminal transferase activity in Tetrahymena extracts. *Cell* 1985;**43**:405–13.
- 7 Bodnar AG, Ouellette M, Frolkis M, *et al*. Extension of lifespan by introduction of telomerase into normal human cells [see comments]. *Science* 1998;**279**:349–52.
- 8 Kim NW, Piatyszek MA, Prowse KR, *et al*. Specific association of human telomerase activity with immortal cells and cancer [see comments]. *Science* 1994;**266**:2011–5.
- 9 Chadeneau C, Hay K, Hirte HW, *et al*. Telomerase activity associated with acquisition of malignancy in human colorectal cancer. *Cancer Res* 1995;**55**:2533–6.
- 10 Heine B, Hummel M, Demel G, *et al*. Demonstration of constant upregulation of the telomerase RNA component in human gastric carcinomas using *in situ* hybridization. *J Pathol* 1998;**185**:139–44.
- 11 Heine B, Hummel M, Müller M, *et al*. Non-radioactive measurement of telomerase activity in human bladder cancer, bladder washings, and in urine. *J Pathol* 1998;**184**:71–6.
- 12 Hiyama E, Yokoyama T, Tatsumoto N, *et al*. Telomerase activity in gastric cancer. *Cancer Res* 1995;**55**:3258–62.
- 13 Hiyama E, Gollahon L, Kataoka T, *et al*. Telomerase activity in human breast tumors. *J Natl Cancer Inst* 1996;**88**:116–22.
- 14 Langford LA, Piatyszek MA, Xu R, *et al*. Telomerase activity in human brain tumours. *Lancet* 1995;**346**:1267–8.
- 15 Tahara H, Kuniyasu H, Yokozaki H, *et al*. Telomerase activity in preneoplastic and neoplastic gastric and colorectal lesions. *Clin Cancer Res* 1995;**1**:1245–51.
- 16 Broccoli D, Young JW, de Lange T. Telomerase activity in normal and malignant hematopoietic cells. *Proc Natl Acad Sci USA* 1995;**92**:9082–6.
- 17 Chiu CP, Dragowska W, Kim NW, *et al*. Differential expression of telomerase activity in hematopoietic progenitors from adult human bone marrow. *Stem Cells* 1996;**14**:239–48.
- 18 Hiyama K, Hirai Y, Kyoizumi S, *et al*. Activation of telomerase in human lymphocytes and hematopoietic progenitor cells. *J Immunol* 1995;**155**:3711–5.
- 19 Hummel M, Heine B, Demel G, Stein H. [Telomerase as a tumor marker?] *Verh Dtsch Ges Pathol* 1997;**81**:312–7.
- 20 Taylor RS, Ramirez RD, Ogoshi M, *et al*. Detection of telomerase activity in malignant and nonmalignant skin conditions. *J Invest Dermatol* 1996;**106**:759–65.
- 21 Feng J, Funk WD, Wang SS, *et al*. The RNA component of human telomerase. *Science* 1995;**269**:1236–41.
- 22 Avilion AA, Piatyszek MA, Gupta J, *et al*. Human telomerase RNA and telomerase activity in immortal cell lines and tumor tissues. *Cancer Res* 1996;**56**:645–50.
- 23 Bodnar AG, Kim NW, Effros RB, *et al*. Mechanism of telomerase induction during T cell activation. *Exp Cell Res* 1996;**228**:58–64.
- 24 Yashima K, Piatyszek MA, Saboorian HM, *et al*. Telomerase activity and *in situ* telomerase RNA expression in malignant and non-malignant lymph nodes. *J Clin Pathol* 1997;**50**:110–7.
- 25 Yashima K, Litzky LA, Kaiser L, *et al*. Telomerase expression in respiratory epithelium during the multistage pathogenesis of lung carcinomas. *Cancer Res* 1997;**57**:2373–7.
- 26 Yashima K, Maitra A, Rogers BB, *et al*. Expression of the RNA component of telomerase during human development and differentiation. *Cell Growth Differ* 1998;**9**:805–13.
- 27 Soder AI, Going JJ, Kaye SB, *et al*. Tumour specific regulation of telomerase RNA gene expression visualized by *in situ* hybridization. *Oncogene* 1998;**16**:979–83.
- 28 Jensen OA. Malignant melanomas of the human uvea: 25-year follow-up of cases in Denmark, 1943–1952. *Acta Ophthalmol Copenh* 1982;**60**:161–82.
- 29 Janssen K, Kuntze J, Busse H, *et al*. p53 oncoprotein overexpression in choroidal melanoma. *Mod Pathol* 1996;**9**:267–72.

- 30 Tobal K, Warren W, Cooper CS, *et al.* Increased expression and mutation of p53 in choroidal melanoma. *Br J Cancer* 1992;66:900-4.
- 31 Ohta M, Berd D, Shimizu M, *et al.* Deletion mapping of chromosome region 9p21-p22 surrounding the CDKN2 locus in melanoma. *Int J Cancer* 1996;65:762-7.
- 32 Coupland SE, Bechrakis N, Schuler A, *et al.* Expression patterns of cyclin D1 and related proteins regulating G1-S phase transition in uveal melanoma and retinoblastoma. *Br J Ophthalmol* 1998;82:961-70.
- 33 Horsman DE, White VA. Cytogenetic analysis of uveal melanoma. Consistent occurrence of monosomy 3 and trisomy 8q. *Cancer* 1993;71:811-9.
- 34 Prescher G, Bornfeld N, Becher R. Nonrandom chromosomal abnormalities in primary uveal melanoma. *J Natl Cancer Inst* 1990;82:1765-9.
- 35 Sisley K, Cottam DW, Rennie IG, *et al.* Non-random abnormalities of chromosomes 3, 6, and 8 associated with posterior uveal melanoma. *Genes Chromosomes Cancer* 1992;5:197-200.
- 36 McLean IW, Foster WD, Zimmerman LE, *et al.* Modifications of Callender's classification of uveal melanoma at the Armed Forces Institute of Pathology. *Am J Ophthalmol* 1983;96:502-9.
- 37 Norton AJ, Jordan S, Yeomans P. Brief, high-temperature heat denaturation (pressure cooking): a simple and effective method of antigen retrieval for routinely processed tissues. *J Pathol* 1994;173:371-9.
- 38 Cordell JL, Falini B, Erber WN, *et al.* Immunoenzymatic labeling of monoclonal antibodies using immune complexes of alkaline phosphatase and monoclonal anti-alkaline phosphatase (APAAP complexes). *J Histochem Cytochem* 1984;32:219-29.
- 39 Gerdes J, Lemke H, Baisch H, *et al.* Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. *J Immunol* 1984;133:1710-5.
- 40 Cattoretti G, Becker MH, Key G, *et al.* Monoclonal antibodies against recombinant parts of the Ki-67 antigen (MIB 1 and MIB 3) detect proliferating cells in microwave-processed formalin-fixed paraffin sections. *J Pathol* 1992;168:357-63.
- 41 Key G, Becker MH, Baron B, *et al.* New Ki-67-equivalent murine monoclonal antibodies (MIB 1-3) generated against bacterially expressed parts of the Ki-67 cDNA containing three 62 base pair repetitive elements encoding for the Ki-67 epitope. *Lab Invest* 1993;68:629-36.
- 42 Kivela T. Immunohistochemical staining followed by bleaching of melanin: a practical method for ophthalmic pathology [letter; comment]. *Br J Biomed Sci* 1995;52:325-6.
- 43 Aldous WK, Grabill NR. A fluorescent method for detection of telomerase activity. *Diagn Mol Pathol* 1997;6:102-10.
- 44 Foss HD, Herbst H, Oelmann E, *et al.* Lymphotoxin, tumour necrosis factor and interleukin-6 gene transcripts are present in Hodgkin and Reed-Sternberg cells of most Hodgkin's disease cases. *Br J Haematol* 1993;84:627-35.
- 45 Ogoshi M, Le T, Shay JW, *et al.* In situ hybridization analysis of the expression of human telomerase RNA in normal and pathologic conditions of the skin. *J Invest Dermatol* 1998;110:818-23.
- 46 Sallinen P, Miettinen H, Sallinen SL, *et al.* Increased expression of telomerase RNA component is associated with increased cell proliferation in human astrocytomas. *Am J Pathol* 1997;150:1159-64.
- 47 Rushing EJ, Yashima K, Brown DF, *et al.* Expression of telomerase RNA component correlates with the MIB-1 proliferation index in ependymomas. *J Neuropathol Exp Neurol* 1997;56:1142-6.
- 48 Hiraga S, Ohnishi T, Izumoto S, *et al.* Telomerase activity and alterations in telomere length in human brain tumors. *Cancer Res* 1998;58:2117-25.
- 49 Kleinschmidt-DeMasters BK, Hashizumi TL, Sze CI, *et al.* Telomerase expression shows differences across multiple regions of oligodendroglioma versus high grade astrocytomas but shows correlation with Mib-1 labelling. *J Clin Pathol* 1998;51:284-93.
- 50 Okayasu I, Osakabe T, Fujiwara M, *et al.* Significant correlation of telomerase activity in thyroid papillary carcinomas with cell differentiation, proliferation and extrathyroidal extension. *Jpn J Cancer Res* 1997;88:965-70.
- 51 Albanell J, Lonardo F, Rusch V, *et al.* High telomerase activity in primary lung cancers: association with increased cell proliferation rates and advanced pathologic stage. *J Natl Cancer Inst* 1997;89:1609-15.
- 52 Okayasu I, Mitomi H, Yamashita K, *et al.* Telomerase activity significantly correlates with cell differentiation, proliferation and lymph node metastasis in colorectal carcinomas. *J Cancer Res Clin Oncol* 1998;124:444-9.
- 53 Ohmura H, Tahara H, Suzuki M, *et al.* Restoration of the cellular senescence program and repression of telomerase by human chromosome 3. *Jpn J Cancer Res* 1995;86:899-904.
- 54 Horikawa I, Oshimura M, Barrett JC. Repression of the telomerase catalytic subunit by a gene on human chromosome 3 that induces cellular senescence. *Mol Carcinog* 1998;22:65-72.
- 55 Blasi MA, Roccella F, Balestrazzi E, *et al.* 3p13 region: a possible location of a tumor suppressor gene involved in uveal melanoma. *Cancer Genet Cytogenet* 1999;108:81-3.
- 56 Prescher G, Bornfeld N, Hirsch H, *et al.* Prognostic implications of monosomy 3 in uveal melanoma. *Lancet* 1996;347:1222-5.
- 57 Cotlier E. The mitotic cycle of the lens epithelium: effect of age and galactose. *Arch Ophthalmol* 1962;68:801.
- 58 Yamaguchi Y, Nozawa K, Savoyesky E, *et al.* Change in telomerase activity of rat organs during growth and aging. *Exp Cell Res* 1998;242:120-7.
- 59 Allsopp RC, Chang E, Kashefi-Azazam M, *et al.* Telomere shortening is associated with cell division in vitro and in vivo. *Exp Cell Res* 1995;220:194-200.
- 60 Maitra A, Yashima K, Rathi A, *et al.* The RNA component of telomerase as a marker of biologic potential and clinical outcome in childhood neuroblastic tumors. *Cancer* 1999;85:741-9.