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Engraftment and growth of patient-derived retinoblastoma tumour in severe combined immunodeficiency mice

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Abstract

The development of an *in vivo* model of retinoblastoma could be important for studying its biological behaviour and developing novel therapeutic strategies. We examined the ability of patient-derived retinoblastoma cells to grow and disseminate in severe combined immunodeficiency CB-17-SCID mice after subcutaneous (s.c.) inoculation without conditioning treatment. 24/30 (80%) of patient-derived tumours engrafted and grew as s.c. nodules in SCID mice. Whilst most xenografted tumours appeared to be localised, by PCR assay a positive DNA band of human minisatellite region (YNZ.22) was determined in the bone marrow of 19/25 (76%), in the spleen of 14/25 (56%) and in the liver of 16/25 (64%) mice, respectively, indicating dissemination to distant organs. Cytogenetic analysis demonstrated i(6p) in 5/12 (42%) and trisomy 1 or 1q abnormalities in 8/12 (67%) of the xenografted tumour samples studied, respectively, suggesting that retinoblastoma tumour cells maintain their cytogenetic abnormalities following adoptive growth in SCID mice. In this report we demonstrate the ability to propagate human primary retinoblastoma cells in SCID mice after s.c. inoculation and suggest the possibility of using the SCID mouse model to study the intrinsic biological behaviour of human retinoblastoma and to develop novel therapeutic strategies in the treatment of this disease.

Keywords: Human retinoblastoma; SCID mice; *In vivo* growth pattern

1. Introduction

Retinoblastoma, the most common primary ocular tumour in children, occurs in two forms. Patients with multifocal disease carry a germline mutation of the *RB1* gene, whereas only approximately 10% of patients with unilateral disease carry the germline mutation. Historically, retinoblastoma was until recently routinely treated by surgical enucleation of the afflicted eye(s) and/or with external beam radiation therapy. Chemotherapy, widely used for most other childhood tumours, was used infrequently. However, it has been recognised that patients who carry the germline mutation have a high risk of developing secondary malignancies, and that the risk is elevated further in those exposed to external beam radiation therapy and this has led many investigators to try to use chemotherapy as an alternative. However, the rational development of clinical protocols has been hampered by a lack of phase II clinical studies indicating which agents are effective against retinoblastoma. Pre-clinical data are also lacking, and such studies, and others regarding retinoblastoma biology, have been hindered by the lack of experimental models. Retinoblastoma cell lines have been difficult to develop, and the few that are available (WERI, Y-79) have the potential problems of harbouring genetic or phenotypic changes that have evolved during long-term *in vitro* culture, and they, therefore, may not reflect the biological behaviour of primary retinoblastoma tumour cells.

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Therefore, the development of an *in vivo* model in which patient-derived retinoblastoma tumour cells engraft and grow might be useful.

A limited number of reports have demonstrated that the human retinoblastoma cell lines Y-79 or WERI and primary retinoblastoma tumour cells can be transplanted and adoptively grown in athymic nude mice or SCID mice [1–5]. Recently, we and others have shown that some human primary leukaemia cells as well as solid tumour cells can be transplanted and adoptively grown in severe combined immunodeficiency (SCID) mice [6–9]. This has allowed the propagation of human tumour cells that in prior studies failed to grow under *in vitro* culture conditions or in nude mice. This ability to propagate human malignant cells in SCID mice may provide a new tool for studying the biological characteristics of specified human cancers.

To determine whether, and to what degree, human retinoblastoma cells would grow and disseminate within the SCID mouse, we have subcutaneously (s.c.) inoculated SCID mice with patient-derived retinoblastoma cells. In this report, we demonstrate the ability to propagate human primary retinoblastoma cells in SCID mice after s.c. inoculation and present the characteristics and biological behaviour of human retinoblastoma in a SCID mouse model.

2. Patients and methods

2.1. Retinoblastoma tumour samples

Retinoblastoma tumour tissue was obtained from eyes enucleated due to retinoblastoma after clinical pathological material was obtained. 30 patients were studied. The tumour tissue was immediately transferred to α-MEM (modified Eagle's medium) containing 15% fetal calf serum (FCS), penicillin (100 U/ml), streptomycin (100 mg/ml) and L-glutamine 2 mmol/L (Sigma) and sent to the laboratory. The tissue was gently grounded in a 70 mm Nylon cell strainer (Becton Dickinson Labware, Franklin Lakes, NJ, USA) within a tissue culture dish containing α-MEM medium to make single cell suspension. Ficoll Hypaque density gradient separation was used to remove non-viable cells and the number of viable retinoblastoma tumour cells was determined by trypan blue exclusion.

2.2. SCID mice

SCID (CB17-SCID/SCID) mice were purchased from Taconic Farms and maintained in the MSKCC animal laboratory in micro-isolator cages under sterile conditions in a specific pathogen-free environment without the use of any antibiotics. Female SCID mice between 6 and 8 weeks of age were used.

2.3. Inoculation of human retinoblastoma cells into SCID mice

A median of 2.0×10^6 ($0.2–10\times10^6$) viable retinoblastoma cells were resuspended in 200 μl of ice cold matrigel matrix (Collaborative Biomedical, Bedford, MA, USA) liquid and injected s.c. with a tuberculin syringe into the right flank of the SCID mouse. The dose of tumour cells injected per mouse from an individual patient was dependent on the number of retinoblastoma cells recovered from the tumour tissue (Table 1). The animals did not receive any conditioning treatment before inoculation. Retinoblastoma tumour growth was assessed by weekly measurements of the diameters of s.c. nodules, and the volume was calculated by the following formula: $d^2\times D\times\pi/6$ with d as the smaller and D as the larger diameter [10]. Animals were sacrificed by cervical dislocation when the volumes of s.c. nodules were greater than 4.0 cm^3 or more than 6 months after inoculation. The gross anatomy was evaluated and samples from peripheral blood, sternum, femur, spleen, liver, lung–heart complex, kidney, brain and tumour nodules were subsequently removed for analysis by histological and/or fluorescence *in situ* hybridisation (FISH) analysis. Organ weights of spleen, liver and tumour nodules were also measured. For secondary passage of retinoblastoma cells, the tumour nodule removed from SCID mice was cut into small pieces with a scalpel, and then gently grounded in the 70 mm Nylon cell strainer in the α-MEM medium. Single cell suspensions were collected and counted with trypan blue to determine the number of viable cells. A similar cell dose and method was used as for the first passage.

2.4. Histopathology

Tissue sections from sacrificed SCID mice were fixed in 10% neutral buffered formalin, dehydrated and embedded in paraffin, sectioned and stained according to standard histological techniques.

2.5. *Fluorescence* in situ *hybridisation (FISH)*

FISH was performed as previously described [6]. In brief, single cell suspensions from tumour nodules or organs were washed and fixed in a mixture of methanol and acetic acid (3:1 v/v). The cells were put onto slides and air dried. Subsequently, the slides were washed and denatured at 72°C in 70% formamide/2X SSC. Denatured probes (10 mg/ml biotin-labelled; Oncor, Gaithersburg) in Hybrisol VI hybridisation solution (Oncor) were deposited on slides. After hybridisation, biotinylated probes for the human X chromosome were detected by applying 20 ml of detection reagent containing avidin/FITC (Oncor), and incubating in a humidified chamber at 37°C for 30 min. The slides were

Table 1
Characteristics of patients and their retinoblastoma cell growth in SCID mice

Patients	Age/sex (months)	Laterality	Localised disease	Eye histology	Prior chemotherapy	No cells inoculated $\times 10^6$	Growth in SCID mice
Rb1	13/F	Unilateral	Yes		None	3.0	1/1
Rb2	58/F	Unilateral	Yes	on	None	3.0	1/1
Rb3	6/F	Unilateral	Yes		None	2.0	0/1
Rb4	13/M	Bilateral	Yes		None	2.0	1/1
Rb5	22/F	Unilateral	Yes		None	0.8	2/2
Rb6R	12/F	Bilateral	Yes		VCE	3.0	1/1
Rb6L			Yes	as	VCE Ctx T	0.2	1/1
Rb7	116/M	Unilateral	Yes		None	1.0	2/2
Rb8	34/F	Unilateral	Yes		None	2.0	2/2
Rb9	23/F	Unilateral	Yes		None	3.0	1/1
Rb10	98/F	Unilateral	Yes		None	0.3	0/1
Rb11	1/M	Unilateral	Yes	ch	None	5.0	1/1
Rb12	101/M	Unilateral	Yes		None	1.6	0/1
Rb13	25/F	Unilateral	Rec	on, ch	None	2.0	2/2
Rb14	16/M	Unilateral	Yes		None	1.0	1/1
Rb15	26/F	Unilateral	Yes		None	2.8	1/1
Rb16	11/F	Bilateral	Yes		None	10	0/1
Rb17	120/F	Unilateral	Yes		None	1.1	0/1
Rb18	22/F	Unilateral	Yes		None	1.0	1/1
Rb19	21/M	Unilateral	Yes		None	0.1	1/1
Rb20	2/F	Unilateral	Yes		None	2.0	1/1
Rb21	26/F	Unilateral	Yes		C	1.0	1/1
Rb22	18/M	Unilateral	Yes		None	6.7	1/1
Rb23	8/M	Bilateral	Yes	as, ch	Ctx, C	1.7	1/1
Rb24	58/M	Unilateral	Yes		None	2.0	1/1
Rb25	7/F	Unilateral	Yes		None	2.0	1/1
Rb26	14/M	Unilateral	Yes		None	2.0	1/1
Rb27	5/F	Bilateral	Yes		C	0.1	1/1
Rb28	13/M	Unilateral	Yes		None	1.0	1/1
Rb29	20/F	Unilateral	Yes		C	1.6	1/1
Rb30	10/F	Bilateral	Yes		None	1.5	0/1

C, carboplatin; VCE, vincristine, carboplatin, etoposide; T, thiotepa; Ctx, cyclophosphamide, Rec, recurrence, intraocular tumour involvement; on, optic nerve; as, anterior segment; ch, choroid.

then washed and propidium iodide (2 mg/ml; Sigma) was applied for counterstaining. A Zeiss fluorescence microscope (Axioskop, Carl Zeiss, Pelham, NY, USA) was used for visualisation and at least 200 cells were scored.

2.6. PCR analysis

The dissemination of human retinoblastoma cells in SCID mice after inoculation was evaluated by PCR analysis of DNA aliquots extracted from peripheral blood, bone marrow, spleen and liver of the sacrificed animals. Two primers specific for the human minisatellite region YNZ.22 [11] were used. An oligonucleotide probe recognising 24 nucleotides in the middle of the amplified sequence was used to demonstrate the specificity of the PCR products by Southern blot hybridisation.

2.7. Long-term in vitro culture of retinoblastoma cells

Mononuclear cells isolated by Ficoll Hypaque from patient-derived retinoblastoma samples or recovered from retinoblastoma tumour nodules were washed and plated into α-MEM medium containing 15% FCS, penicillin (100 U/ml), streptomycin (100 μg/ml) and 2 mmol/l L-glutamine (Sigma) at a density of $1–5\times10^5$ cells/ml. The medium was replaced twice a week.

2.8. Cytogenetic analysis

Samples of tumour cells recovered from tumour nodules were processed for culture and chromosome preparations according to methods routinely employed in the laboratory [12]. Air dried metaphases were G-banded, a minimum of 10 metaphases were examined for each sample.

3. Results

3.1. Characteristics of patients

Table 1 summarises the clinical characteristics of the 30 patients with retinoblastoma in this study. The ages of patients ranged from 1 to 120 months. 24 patients

were diagnosed as unilateral retinoblastomas and 6 patients were bilateral retinoblastomas. Specimens Rb6R and Rb6L were obtained from same patient (Rb6) during enucleations on two different dates. All patients initially had localised disease, but patient Rb13 later developed an orbital recurrence. 5 patients (Rb6, 21, 23, 27 and 29) had received carboplatin and/or vincristine, carboplatin, etoposide chemotherapy and patient RB6 was also treated with external beam irradiation prior to enucleation.

3.2. Engraftment and growth pattern of human retinoblastoma in SCID mice

Retinoblastoma cells from 24/30 (80%) patients grew as s.c. nodules in SCID mice (Table 1). The retinoblastoma cells derived from 8 patients (Rb5, 6L, 11, 18, 21, 22, 24 and 28) displayed a rapid growth pattern in SCID mice and the mean times for the tumour volumes to reach 2.0 cm^3 and 4.0 cm^3 in the animals bearing human retinoblastoma were 14.1 ± 3.4 ($n=8$) and 18.1 ± 3.9 ($n=8$) weeks after inoculation, respectively. The retinoblastoma cells derived from another group of 11 patients (Rb1, 4, 7, 8, 9, 13, 20, 23, 26, 27 and 29) as well as tumour cells from patient Rb6R displayed a slower growth pattern in SCID mice. The mean time for the tumours volume to reach 2.0 cm^3 and 4.0 cm^3 were 27.1 ± 7.5 ($n=12$) and 35.2 ± 5.6 ($n=6$) weeks after inoculation, respectively. Five patient-derived retinoblastoma cells (Rb2, 14, 15, 19 and 25) displayed a very slow growth pattern in SCID mice. Two of these retinoblastoma tumours (Rb2 and 25) reached 2.0 cm^3 of tumour volume only after 70 and 80 weeks, respectively. The times for the growth of other three tumours (Rb14, 15 and 19) to reach volumes of 0.5, 0.65 and 0.9 cm^3 were 70, 43 and 38 weeks, respectively. No correlation between extent of the patient's intraocular tumour (pathological evidence of optic nerve, choroidal, scleral or anterior segment involvement) and growth characteristics of the xenografted tumour was apparent. Samples derived from 6 patients (20%) (Rb3, 10, 12, 16, 17, and 30) did not induce tumour growth in the SCID mice.

3.3. In vitro growth of cells recovered from tumour nodules

Retinoblastoma cells were harvested from tumours in the SCID mice with a recovery rate of $4.6 \pm 2.2 \times 10^7$ cells/g of tumour tissue ($n=15$, 11 from passage 1 and 4 from passage 2) after sacrifice of the animals. To study the *in vitro* growth potential, cells from 7 samples harvested from passage 1 (Rb6Rp1, 6Lp1, 15p1, 22p1, 23p1, 24p1, and 28p1) and 5 samples from passage 2 (Rb5p2, 6Lp2, 9p2, 13p2, and 21p2) were cultured *in vitro*. Cells from most of the samples died within 3 to 4 weeks, while some survived 2 or 3 months. No permanent cell lines could be established.

3.4. Engraftment of the human retinoblastomas in the secondary SCID mice

After adoptive growth of retinoblastoma cells derived from patients Rb5, 6R, 7, 8, 9, 11, and 13 in SCID mice, specimens were obtained from s.c. tumour tissue and passaged in secondary SCID mice by s.c. inoculation using the same amount of cells and the same methodology utilised during the first passages. All 7 specimens generated s.c. tumour growth in the secondary SCID mice. The retinoblastoma cells derived from 2 patients (Rb5 and 11) displayed a similar growth pattern when compared with their tumour growth in the first SCID mice passages. However, retinoblastoma cells from the other 5 patients grew more rapidly in the secondary SCID mice compared with their first passage. The mean time of achieving 2.0 cm^3 and 4.0 cm^3 of tumour volumes in the secondary SCID mice were 18.8 ± 5.1 and 24.2 ± 6.5 weeks, respectively, in contrast to the time needed in their first passages (26.3 ± 5.3 weeks and 32.5 ± 5.9 weeks, respectively).

3.5. Dissemination of human retinoblastoma cells

Table 2 demonstrates the dissemination pattern of the human retinoblastoma cells in SCID mice by histopathology, FISH, PCR and organ weight determination. The mice bearing tumours derived from patients RB19, 20, 27 and 29 were not examined for dissemination and, therefore, are not included in Table 2. In general, the human retinoblastoma cells displayed a localised growth pattern after s.c. inoculation in SCID mice. In 20 animals examined bearing tumours derived from the individual retinoblastoma samples, 12 (Rb2, 6R, 7p2, 8; 11p2, 13p2, 15, 23, 24, 25, 26 and 28) developed splenomegaly, and 7 (Rb1, 4, 5, 6Rp2, 7, 24 and 25) developed hepatomegaly. However, there were no tumour cells detectable in the various tissues examined (bone marrow, spleen, liver, kidney, lung, brain and eyes), except in the tumour nodules of these sacrificed animals by histopathological assay (Table 2). However, in the animals bearing tumours, tumour cells were detected by FISH analysis in other tissues at relatively low frequencies: 1–5% in the peripheral blood cells in 3/25 (12.0%) mice, 1–8% in the bone marrow cells in 13/27 (48.1%) mice, 1–6% in the spleen cells in 5/27 (18.5%) mice and 1–4% in the liver cells of 9/27 (33.3%) mice, respectively. Tissue analysis with FISH showed that human retinoblastoma cells ($98.5 \pm 1.6\%$; $n=24$) represented the majority of cells present in the tumour nodules.

PCR analysis was even more sensitive than the FISH assay in detecting the dissemination of the human

Table 2
Engraftment and dissemination of patient-derived retinoblastoma cells in SCID mice

No.	Time of sacrifice (weeks)	Organ weight (g)			Histopathology								FISH (%) Human cells					PCR				
		Tu	Sp	Li	Tu	Bm	Sp	Li	Ki	Lu	Br	Eye	Tu	Pb	Bm	Sp	Li	Tu	Pb	Bm	Sp	Li
Rb1	47(D)	6.8	0.035	1.32[a]	+	–	–	–	–	–	–	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Rb2	70(D)	1.1	0.048[a]	1.24	+	–	–	–	–	–	–	ND	96	0	2	0	1	+	–	–	–	+
Rb3	45(S)	NG	0.029	1.13	NG	–	–	–	–	–	–	ND	NG	ND	ND	ND	ND	NG	–	–	–	–
Rb4	22(S)	1.8	0.031	1.32[a]	+	ND	–	–	ND	ND	ND	ND	96	0	0	0	1	ND	ND	ND	ND	ND
Rb5	21(S)	4.3	0.045	1.38[a]	+	–	–	–	–	–	–	–	96	0	1	0	2	+	ND	–	+	+
Rb5p2	28(S)	6.4	0.029	1.05	+	–	–	–	–	–	–	ND	97	0	1	2	1	+	–	+	+	+
Rb6R	44(S)	3.6	0.054[a]	1.04	+	–	–	–	–	–	–	–	98	0	1	0	0	+	–	+	+	+
Rb6Rp2	44(S)	3.8	0.106[a]	1.35[a]	+	–	–	–	–	–	–	–	100	0	1	0	0	+	–	+	+	+
Rb6L	24(S)	5.1	0.030	1.14	+	–	–	–	ND	ND	ND	ND	99	0	0	0	0	+	–	+	–	+
Rb7	34(S)	5.0	0.037	1.26[a]	+	–	–	–	–	–	–	ND	95	0	0	0	0	+	–	+	–	+
Rb7p2	13(S)	1.3	0.057[a]	1.39[a]	+	–	–	–	–	–	–	ND	99	0	0	1	0	+	–	+	+	+
Rb8	38(S)	5.3	0.084[a]	1.19	+	–	–	–	–	–	–	–	99	1	1	0	2	+	–	+	+	+
Rb8p2	31(S)	4.9	0.054[a]	0.96	+	–	–	–	–	–	–	ND	99	0	4	6	0	+	–	+	+	+
Rb9	35(S)	4.6	0.045	1.11	+	–	–	–	–	–	–	ND	100	0	4	0	0	+	–	+	+	+
Rb9p2	21(S)	3.8	0.042	0.98	+	–	–	–	–	–	–	–	99	0	0	6	0	+	–	+	+	+
Rb10	41(S)	NG	0.042	1.23	NG	ND	ND	ND	ND	ND	ND	ND	NG	0	0	0	0	NG	–	–	–	–
Rb11	11(S)	9.6	0.032	1.22	+	–	–	–	–	–	–	–	99	5	1	3	2	+	–	+	+	+
Rb11p2	16(S)	8.0	0.056[a]	1.08	+	–	–	–	–	–	–	ND	100	0	8	0	2	+	–	+	+	+
Rb12	34(S)	NG	0.043	1.18	NG	–	–	–	–	–	–	–	NG	0	0	0	0	NG	–	+	–	–
Rb13	24(S)	3.6	0.033	1.15	+	–	–	–	–	–	–	–	100	ND	6	0	1	+	ND	+	+	–
Rb13p2	20(S)	4.1	0.060[a]	1.19	+	–	–	–	ND	ND	ND	ND	99	2	0	0	0	+	–	–	–	–
Rb14	48(S)	1.0	0.047	1.02	+	–	–	–	–	ND	ND	ND	100	0	0	0	0	+	–	+	+	+
Rb15	43(S)	0.6	0.060[a]	1.19	+	–	–	–	–	–	–	–	100	0	0	0	0	+	–	–	–	–
Rb16	28(S)	NG	0.042	1.09	NG	ND	ND	ND	ND	ND	ND	ND	NG	0	0	0	0	NG	–	+	–	–
Rb17	30(S)	NG	0.039	1.13	NG	ND	ND	ND	ND	ND	ND	ND	NG	ND	ND	ND	ND	NG	ND	ND	ND	ND
Rb18	25(S)	6.0	0.043	1.05	+	–	–	–	ND	ND	ND	ND	100	0	2	0	0	+	–	+	+	+
Rb21	20(S)	4.3	0.040	1.24	ND	ND	ND	ND	ND	ND	ND	ND	100	ND	4	0	4	+	ND	+	–	–
Rb22	21(S)	4.6	0.042	1.20	+	ND	ND	ND	ND	ND	ND	ND	99	0	0	0	0	ND	ND	ND	ND	ND
Rb23	21(S)	2.1	0.060[a]	1.26	+	–	–	–	–	–	–	–	97	0	0	0	0	ND	ND	ND	ND	ND
Rb24	17(S)	2.6	0.104[a]	1.38[a]	+	–	–	–	–	ND	ND	ND	96	0	0	0	0	+	–	+	–	–
Rb25	81(S)	1.3	0.068[a]	1.36[a]	+	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Rb26	38(S)	2.1	0.089[a]	1.08	+	ND	ND	ND	ND	ND	ND	ND	98	ND	ND	ND	ND	ND	ND	ND	ND	ND
Rb28	16(S)	2.3	0.198[a]	0.71	ND	ND	ND	ND	ND	ND	ND	ND	97	ND	ND	ND	ND	ND	ND	ND	ND	ND
Rb30	41(S)	NG	0.032	0.98	NG	ND	ND	ND	ND	ND	ND	ND	NG	ND	ND	ND	ND	NG	ND	ND	ND	ND
NC			0.046	1.10																		
($n=8$ X ± 2 S.D.)			± 0.01	± 0.14																		

ND, not done; NG, no growth; NC, normal control; S, sacrificed; D, died; Tu, tumour; Bm, bone marrow; Pb, peripheral blood; Sp, spleen; Li, liver; Ki, kidney; Lu, lung; Br, brain; p, passage; R, right; L, left; S.D., standard deviation.

[a] Organ enlarged.

retinoblastoma cells in SCID mice. In the animals bearing tumours, the positive DNA band of human minisatellite region (YNZ.22) was detectable in the bone marrow of 19/25 (76%) mice, in the spleen of 14/25 (56%) mice and in the liver of 16/25 (64%) mice, respectively, by PCR analysis. However, there were no positive signals detectable in the blood samples in any of 22 mice examined by the PCR assay.

In the 6 animals in whom retinoblastoma cells did not induce s.c. tumours, splenomegaly and hepatomegaly were not noted. FISH analysis did not reveal any human cells in the organs of 3 animals examined (Table 2). However, in the samples of bone marrow derived from 2 animals without tumour growth (Rb12 and Rb16), positive signals were determined by the PCR assay (Table 2). These results suggest that rare retinoblastoma cells may migrate to and reside in the distant organs after s.c. inoculation despite no manifest evidence of a tumour.

3.6. Cytogenetic analysis of recovered retinoblastoma cells from tumour tissues

Due to the insufficient number of cells available, patient-derived samples from the retinoblastoma tumours were not initially karyotyped, but were first passaged in SCID mice and only the cells recovered from the tumours were karyotyped. Although del(13q) was detected in only one of the tumour cells studied, alterations in several of the chromosomes often reported

Table 3
Cytogenetic characteristics of retinoblastoma cells after adoptive growth in SCID mouse

Patient	Karyotype
Rb5p2	43-47,XX,**der(2)t(1;2)(q11;q37),der(16)t(12;16)(q13;q12)**
Rb6Lp1	39-45,XX,**dup(1)(q12q31)**,hsr(1)(q31),del(3)(p21), del(7)(q32),del(9)(p13),del(9)(q13q22),**+dmins**,inc
Rb7p1	47-49,XY,+7,add(9)(q34),der(16)t(1;16)(q22;q23),+19,+mar
p3	43-48,XY,**+add(1)(p13)**,+7,-9,-16,+19,+mar
Rb8p1	47-49,XX,**+del(1)(p23),**add(2)(q37),+del(3)(q21),+5, **i(6)(p10),+der(6)i(6)(p10)add(6)(p25),del(13)(q12q14),** der(15)t(1;15)(q21;p11),add(16)(q24),-21
Rb9p1	46,XX,+1x2,**add(1)(p22)x2,i(6)(p10)**,add(16)(p13),inc
Rb11p1	47,XY,der(11)t(5;11)(q21;q25),+18,~50dmin
Rb13p1	43-46,XX,**+i(1)(q10),i(6)(p10)**,inc
Rb18p1	46,XX,**+der(6)t(1;6)(q21;p15)**
Rb21p1	48-55,XX,**i(6)(p10)**,+13,add(19)(q13),inc
Rb22p1	42-46,XY,der(1)t(1;11)(q32;q13),der(5)t(5;11)(q31;p15), **+del(6)(q25q31)**,add(7)(p13),-11, inc
Rb23p1	48-50,XY,**der(1)t(1;1)(p36;q11)**,der(3)t(3;4)(q23;q21), inc
Rb28p1	45-47,XX,**+i(1)(q10),add(1)(p11)**,+2,+18

der, derivation; t, translocation; dup, duplication; hsr, homogeneously staining region; del,deletion; dmis, double-minute; inc, incomplete karyotype; add, addition; mar, marker chromosome; i, isochromosome.

to be involved in non-random rearrangement in retinoblastoma were seen in our tumour cell lines. The summary of clonal rearrangements seen is presented in Table 3 and a representative G-banded karyotype is shown in Fig. 1. Cytogenetic analysis of retinoblastoma cells from the tumours recovered from SCID mice demonstrated complex, but recurrent clonal abnormalities of chromosomes 1, 6 and 16. The clonal rearrangements in addition to several abnormalities included partial deletion of the long arm of chromosome 6 or complete loss of the long arm resulting in i(6p) in 5 samples (Rb 8p1, 9p1, 13p1, 18p1 and 21p1), partial trisomy of chromosome 1q in 8 samples (Rb 5p2, 6Lp1, 7p3, 8p1, 9p1, 18p1, 23p1 and 28p1) and relative deficiency of 1p in 5 samples (Rb7p3, 8pl, 9pl, 3pl and 28pl) and either complete loss of chromosome 16 or deletion of 16q in 4 samples (Rb 5p2, 7p3, 8p1 and 9p1).

4. Discussion

This study has demonstrated that most patient-derived retinoblastoma tumours can engraft and grow into tumours in SCID mice after s.c. inoculation, and that small numbers of cells can disseminate to distant organs. In contrast, long-term *in vitro* cell culture could not be established from these cells. The successful engraftment and dissemination of human retinoblastoma in this SCID mouse model may provide a potentially important tool for both the study of retinoblastoma biology and the pre-clinical evaluation of novel therapeutic strategies.

Retinoblastoma appears to be more tumorigenic in this model than leukaemia. Whilst 24/30 (80%) of the retinoblastoma specimens led to tumour formation, unpublished data from our group indicate that only 7/31 (23%) samples from patients with newly diagnosed acute myelogenous leukaemia and 7/26 (27%) samples from patients with newly diagnosed acute lymphocytic leukaemia formed tumours in this same model (data not shown). However, in contrast to the relatively localised growth pattern of the retinoblastoma cells in the SCID

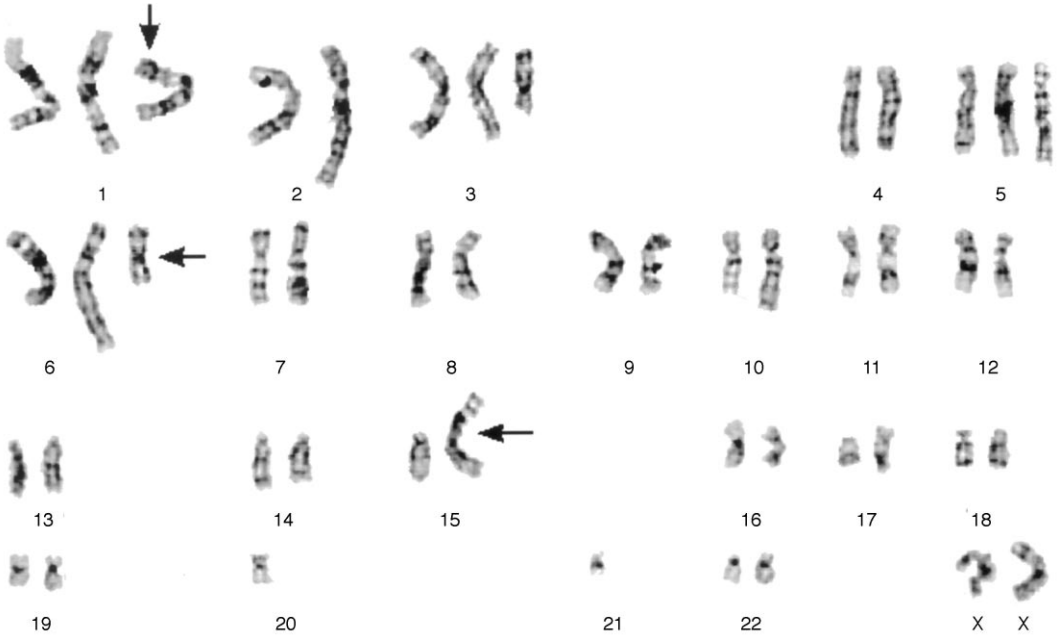

Fig. 1. A representative G-banded karyotype from a retinoblastoma sample (RB8p1) to show clonal chromosomal abnormalities in Table 3. This karyotype in addition to clonal abnormalities also contain random abnormalities 10 and loss of chromosome 20. The recurrent abnormalities often seen in retinoblastoma are shown by arrows.

mice, many human leukaemias have an aggressive growth pattern, with the majority of the animals having extensive dissemination and infiltration of distant organs such as the liver, spleen, lung, kidney, brain, blood and bone marrow [6,7,13,14]. These growth patterns are similar to the clinical characteristics of the respective diseases, and suggest that the xenografted cells maintain some intrinsic biological characteristics of the original tumour cells.

Retinoblastoma cells recovered from the SCID mice demonstrated recurrent cytogenetic abnormalities commonly seen in retinoblastoma. Isochromosome (6p) or del(6q) was detected in 5/12 tumours (42%) studied; trisomy of chromosome 1 or 1q in 8/12 samples (67%), whereas trisomy of 1q and relative deficiency of 1p was noted in 4/12 samples (33%). It was interesting to note that some tumour samples 4/12 (33%) also showed either loss of 16q or monosomy of chromosome 16 [15–17]. In addition four tumours also showed abnormalities of both chromosomes 1 and 16. These findings suggest that retinoblastoma tumour cells maintain their cytogenetic abnormalities following adoptive growth and passage in SCID mice. Whilst retinoblastoma tumour formation is initiated by mutations in the *RB1* gene located at 13q14, the functional role of genes located at 6p, 6q, or 1p and 1q and 16q is unknown. It is, however, likely that the mutation of potential tumour suppressor genes at these chromosomal sites may confer a growth advantage to the cells, and that such mutations are associated with tumour progression [15,17–21]. Because of the limited number of primary tumour cells, cytogenetic analyses were not performed. Therefore, it is unclear whether there was any correlation between cytogenetic abnormalities and ability of the cells to engraft in the SCID mouse model. However, a correlation with growth rate and cytogenetic abnormalities was suggested. Six of the seven samples that demonstrated aggressive growth that were karyotyped had either trisomy 1q (Rb5p2, 6Lp1, 18p1, and 28p1) or i(6p) (Rb21p1) or del (6q) (Rb22p1) cytogenetic abnormalities (Table 3). In another five samples that grew relatively slowly in the mice, two (Rb7p1 and 8p1) had trisomy 1q, one (Rb23p1) had trisomy 1, and one (Rb13p1) had +i(1)(q10) and i(6)(p10). These findings may support the hypothesis that increased gene dosage on 6p and 1q or loss of genes from 6q and 1p may lead to a tumour growth advantage and progression [17–21]. Although the exact nature of genes located at these chromosome sites is not known as yet, some of these regions are non-randomly involved in rearrangements in a variety of tumour types both lympho-haematopoietic and solid tumours indicating that the genes located at these sites may be involved in tumour progression and, therefore, may not be specific to retinoblastoma. Unfortunately, no karyotype analysis was performed on the tumours with an indolent growth pattern.

Chromosomal abnormalities consistently associated with retinoblastoma tumours previously described, and also seen in our xenografted tumours, suggest that mutations in addition to those of *RB1* gene are required for malignant transformation and progression. Reports that genetically supplementing an intact wild-type *RB* gene into retinoblastoma tumour cells may not reduce or only partially reduce their *in vitro* growth potential and tumorigenicity in immunodeficient mice also suggests that genetic abnormalities in addition to the *RB1* gene are important in the multistep tumorigenicity of retinoblastoma tumours [1,20–24]. Little is known of these other genes, partially due to the fact that primary retinoblastoma tumour tissue is usually available in small quantities. Xenografted tumours and growth of human retinoblastoma in SCID mice may be a useful source of tumour tissue which, together with animal models may help in the elucidation of other genes involved in tumour growth promotion and progression.

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References

1. Szekely L, Wang Y, Klein G, Wiman KG. RB-reconstituted human retinoblastoma cells from RB and positive intraocular and intracerebral but not subcutaneous tumors in SCID mice. *Int J Cancer* 1995, **61**, 683–691.
2. Zhou Y, Li J, Xu K, Hu SX, Benedict WF, Xu H-J. Further characterization of retinoblastoma gene-mediated cell growth and tumor suppression in human cancer cells. *Proc Natl Acad Sci USA* 1994, **91**, 4165–4169.
3. Xu HJ, Sumegi J, Hu SX, *et al*. Intraocular tumor formation of RB reconstituted retinoblastoma cells. *Cancer Res* 1991, **51**, 4481–4485.
4. Cowell JK, Ramani P, Song Y, Evans M, Morgan G. The use of SCID mice for the growth of retinoblastoma cell lines and for the establishment of xenografts from primary tumours. *Eur J Cancer* 1997, **33**, 1070–1074.
5. Yi X, Ni Z, Wang W. Establishment and characteristics of a model of nude mouse xenograft of human retinoblastoma. *Chin J Ophthalmology* 1995, **31**, 145–148.
6. Yan Y, Salomon O, McGuirk J, *et al*. Growth pattern and clinical correlation of subcutaneously inoculated human primary acute leukemia in SCID mice. *Blood* 1996, **88**, 3137–3146.
7. McGuirk J, Yan Y, Childs B, *et al*. Differential growth patterns in SCID mice of patient derived chronic myelogenous leukemias. *Bone Marrow Transplant* 1998, **22**, 367–374.
8. Lapidot T, Sirard C, Vormoor J, *et al*. A cell initiating human acute myeloid leukemia after transplantation into SCID mice. *Nature* 1994, **367**, 645.

9. Salomon O, Cheung N, Nguyen H, Myers D, O'Reilly RJ, Reisner YA. A new model for human solid tumors and acute myeloid leukemia in SCID/BALB chimeric mice. *Blood* 1992, **82**, 289a.
10. Renner C, Pfreundschuh M. Treatment of heterotransplanted Hodgkin's Tumor in SCID mice by a combination of human NK or T cells and bispecific antibodies. *J Hematotherapy* 1995, **4**, 447–451.
11. Mackinnon S, Barnett L, Bourhis JH, Black P, Heller G, O'Reilly RJ. Myeloid and lymphoid chimerism after, cell depleted bone marrow transplantation: evaluation of conditioning regimens using polymerase chain reaction to amplify human minisatellite regions of genomic DNA. *Blood* 1992, **21**, 3235–3241.
12. Verma RS, Babu A. *Human chromosomes: principles and techniques*. McGraw-Hill Inc., 1995, 6.
13. Kamel-Reid S, Letarte M, Sirard C, *et al*. A model of human acute lymphoblastic leukemia in immunedeficient SCID mice. *Science* 1989, **246**, 1597.
14. Sawyers CL, Gishizky ML, Quan S, Golde DW, Witte ON. Propagation of human blastic myeloid leukemias in the SCID mouse. *Blood* 1992, **79**, 2089–2098.
15. Horsthemke B. Genetics and cytogenetics of retinoblastoma. *Cancer Genet Cytogenet* 1992, **63**, 1–7.
16. Benedict WF, Banerjee A, Mark C, Murphree AL. Non-random chromosomal changes in untreated retinoblastomas. *Cancer Genet Cytogenet* 1983, **10**, 311–333.
17. Squire J, Phillips RA, Boyce S, Godbout R, Rogers B, Gallie BL. Isochromosome 6p, a unique chromosomal abnormality in retinoblastoma: verification by standard staining techniques, new densitometric methods, and somatic cell hybridization. *Hum Genet* 1984, **66**, 46–53.
18. Horsthemke B, Greger V, Becher R, Passarge E. Mechanism of i(6p) formation in retinoblastoma tumor cells. *Cancer Genet Cytogenet* 1989, **37**, 95–102.
19. Squire J, Gallie B, Phillips RAA. detailed analysis of chromosomal change in heritable and nonheritable retinoblatoma. *Human Genet* 1985, **70**, 291–301.
20. Kusnetsova LE, Prigognia EC, Pogosianz HE, Belkina BM. Similar chromosomal abnormalities in several retinoblastomas. *Hum Genet* 1984, **61**, 201–204.
21. Klein G. The role of gene dosage and genetic transposition in carcinogenesis. *Nature* 1981, **294**, 313–318.
22. Medraperia SA, Whittum-Hudson JA, Prendergast RA, Chen P-L, Lee W-H. Intraocular tumor suppression of retinoblastoma gene-reconstituted retinoblastoma cells. *Cancer Res* 1991, **51**, 6381–6384.
23. Muncaster MM, Cohen BL, Phillips RA, Gallie BL. Failure of to reverse the malignant phenotype of human tumor cell lines. *Cancer Res* 1992, **52**, 654–661.
24. Riley DJ, Lee EY, Lee WH. The retinoblastoma protein: more than a tumor suppressor. *Ann Rev Cell Biol* 1994, **10**, 1–29.