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Original Paper

N-MYC Amplification, Loss of Heterozygosity on the Short Arm of Chromosome 1 and DNA Ploidy in Retinoblastoma

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Recurrent genetic alterations different from the alteration of the *RB1* gene on chromosome 13q14 have been described in retinoblastoma, including structural alterations on the short arm of chromosome 1 and amplification of the *N-MYC* oncogene. These two genetic alterations are major prognostic factors in neuroblastoma, another embryonic neuro-ectodermal tumour. In order to assess the frequency of these alterations and their possible association with clinical parameters in retinoblastoma, we studied a series of 46 retinoblastoma tumour samples. Ploidy was assessed by flow cytometry, *N-MYC* copy number was evaluated by a spot-blot procedure using the pNb-1 probe and loss of heterozygosity was investigated by PCR analysis at mini- and microsatellites located on the short arm of chromosome 1. Most tumours were in the diploid or near diploid range; only one case exhibited tetraploidy. *N-MYC* amplification was observed in only one of the 45 tumours. Loss of heterozygosity on the short arm of chromosome 1 was observed in 9/43 tumours (21%); in particular, its incidence was higher in metastatic than in localised disease ($P < 0.05$). We suggest that alterations of one or several genes on chromosome 1p might play a role in the oncogenesis or progression of retinoblastoma. Analysis of the long term follow-up of these and additional patients should determine the prognostic value of this parameter.

Key words: retinoblastoma, tumorigenesis, risk factors, LOH-1p
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INTRODUCTION

RETINOBLASTOMA IS a malignant ocular tumour which affects approximately 1/20000 live births in developed countries. Tumours limited to the retina usually carry a good prognosis [1]. When enucleation is necessary, classical adverse prognostic factors are extraretinal extension, such as choroid [2] or optic nerve invasion [3, 4]. These histological criteria are well established poor prognostic factors for the development of extra-ocular dissemination (orbital and/or metastatic) [5]. However, new non-histological criteria might be helpful in therapeutic decisions regarding the need for adjuvant treatment after enucleation, since rare relapses may occur even in the absence of classical adverse prognostic criteria.

This tumour constitutes the prototype for the "two hit" model defined by Knudson. Cloning of the *RB1* gene [6] and further analysis of its structure and expression in retinoblastoma [7] have confirmed its tumour suppressor activity. Since the description of the constitutional and somatic deletion of 13q14 in retinoblastoma patients and tumours, most studies have focused on the analysis of this chromosome region and on the *RB1* gene. However, the *RB1* gene alteration might not be the only event involved in retinoblastoma tumorigenesis. Indeed, it has been shown that reintroduction of the *RB1* gene into WERI-27 retinoblastoma cells reduced but did not completely suppress their tumorigenic potential [8]. Furthermore, recurrent cytogenetic abnormalities, different from the

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13q14 deletion, have been described in retinoblastoma. These include additional copies of 1q, structural alterations on 1p, iso-chromosome 6p, monosomy 16, homogenously staining regions and double-minute chromosomes [9–11]. In some cases, the latter abnormalities have been associated with amplification of the *N-MYC* oncogene [12]. Interestingly, alteration on 1p resulting in deletion of this chromosome fragment and *N-MYC* amplification are frequently observed in neuroblastoma [13], another embryonic neuro-ectodermal tumour [14]. In this tumour, 1p deletion, *N-MYC* amplification and DNA diploidy define a subset of particularly aggressive disease [13, 15, 16]. We studied these genetic parameters in a series of 46 retinoblastoma tumour samples and analysed their prognostic significance.

PATIENTS AND METHODS

Patients

Forty-six tumour samples from 45 patients referred to our institution were analysed between March 1988 and February 1993 (25 boys, 20 girls). Retinoblastoma was unilateral in 24 patients and bilateral in 21 patients. Median age at diagnosis was 20 months (range: 1.5–70 months). Tumour samples were studied at diagnosis in 30 patients: they included 27 specimens derived from ocular tumours, one from a metastatic cervical lymph node, one from an initial orbital involvement and one from a bone metastasis. Tumour samples were studied only at relapse in 15 patients: they included four specimens derived from local ocular relapse (secondary enucleation after conservative treatment with external beam radiotherapy), five from orbital relapse and six from bone and/or bone marrow metastases. Only 1 patient could be successively studied for initial eye tumour and subsequent bone and bone marrow relapse. In the 12 patients with extra-ocular relapse, the median time interval between diagnosis and extra-ocular relapse was 11.5 months (range: 2–122 months). Differential diagnosis between retinoblastoma and secondary sarcoma could be ascertained in all cases, especially for late relapses and when tumours occurred within or near an irradiation field. Extra-ocular relapses have been treated according to previously described protocols [5, 17].

Histopathology

Classical histological criteria of enucleated eyes were analysed looking for optic nerve, choroidal and scleral involvement. For each enucleated eye, 15 paraffin embedded blocks were analysed after inclusion of the whole eye. The degree of differentiation was studied: tumours were considered to be "immature" when no rosette was observed and "differentiated" in all the other cases.

Determination of N-MYC amplification and loss of heterozygosity on the short arm of chromosome 1 (LOH-1p)

Samples used for molecular analysis were obtained by fine needle sampling. In each case, cytological examination ascertained the massive contamination by tumour cells. DNA was isolated from tumour specimens and nucleated blood cells from the same patient using standard procedures [18]. *N-MYC* copy number was evaluated by a spot blot procedure using the pNb-1 probe [19]. LOH-1p was determined after PCR amplification with specific sets of primers at two minisatellite (D1S80 and D1S76) and seven microsatellite loci (D1S214, D1S228, D1S199, D1S233, D1S193, D1S200, D1S203) using matched tumour and constitutional blood DNA as templates. Experimental procedures have been previously described in detail [19, 20].

DNA flow cytometric analysis

DNA histograms were acquired from at least 10000 nuclei analysed on a FACScan (Becton–Dickinson) equipped with a doublet discrimination module as previously described [21]. S-phase fractions was computed using the Cellfit software (Becton–Dickinson) with background subtraction. DNA histograms with a coefficient of variation superior to 8% were rejected and DNA ploidy was classified according to international recommendations in which DNA diploidy corresponds to a DNA index between 0.9 and 1.1 [22]. S-phase fraction was considered as low, intermediate or high when the proportion of S-phase nuclei were less than 5%, between 5 and 10% or more than 10%, respectively. The corresponding cytological smears were also inspected to establish the proportion of viable tumour cells of each sample.

Statistical analysis

The Fisher's exact test was used for statistical comparison of the frequency of LOH-1p according to the extent of the disease.

RESULTS

N-MYC amplification (more than 10 copies) was observed in only one of the 45 tumour specimens derived from the 45

Table 1. Loss of heterozygosity of chromosome 1p (LOH-1p) in 43 retinoblastoma tumour samples

	Tumour samples	
	At diagnosis [29] cases with LOH-1p/cases studied	At relapse [14] cases with LOH-1p/cases studied
Localised disease		
Eye	4*/27†	0/4
Orbit	0	1/4
Metastatic disease		
LN	1/1	0
Bone, BM	0/1	3/6
Total	5/29	4/14

*N-MYC amplification was observed in 1 of these cases. †One of these patients was studied for initial eye tumour and subsequent bone metastasis: no LOH-1p was found in any of these samples.
LN, regional lymph node; BM, bone marrow.

Table 2. Pattern of loss of heterozygosity of chromosome 1p and N-MYC *amplification (NMA) in 9/43 retinoblastoma tumour samples.*

	Cases								
	1*	2	8	9	3	4	5	6	7
Telomere									
Locus 1p									
D1S76	2	1	1,2	1,2	–	2	1	1	2
D1S80	2	1	1,2	1,2	1	1	–	–	2
D1S214	1,2	1,2	–	1	1				
D1S228	1,2	1,2	1,2	–	1,2				
D1S199	1,2	1,2	1	1	1,2	1	1	1	2
D1S233			2	1	1,2	1,2	1,2	1,2	
D1S193						1,2	1,2	1,2	–
D1S200	1,2	1,2	1,2	1	1,2	1,2	1,2	–	
D1S203	–	1,2	–	1	1,2	1,2	1,2	–	1,2
Centromere									

**N-MYC* amplification was observed in this case.

patients: this was an intra-ocular tumour studied at diagnosis and the patient subsequently died from meningeal relapse. All other specimens demonstrated normal *N-MYC* copy number.

DNA ploidy could be evaluated in 30 tumours. A total of 25 tumours were DNA diploid, four were DNA near-diploid (DNA index of 1.12 and 1.14). Only one sample demonstrated a major abnormality of ploidy: it corresponded to a bone relapse which exhibited tetraploidy (DNA index of 2.0). This patient is currently alive with a follow-up of 2.5 years after metastatic relapse. S-phase fraction was high in 14 cases, intermediate in 13 cases and low in 3 cases.

The PCR technique was used to compare constitutional and tumour DNA at nine informative loci dispersed along the short arm of chromosome 1. Tumour samples from 43 patients could be analysed with this procedure. LOH-1p for at least two informative loci was observed in 9 cases (Table 1). The pattern of LOH-1p enabled the definition of two types of deletion (Table 2 and Figure 1). In 7 cases, LOH was observed for distal loci but not for proximal loci suggesting that a terminal deletion had occurred in these tumours. Conversely, in 2 cases (cases 8 and 9), heterozygosity was retained at distal loci whereas LOH was observed at more proximal loci. Together, these two types of LOH do not enable the definition of a single overlapping region.

The incidence of 1p deletion was higher in metastatic tumours (bone, bone marrow or lymph nodes) (4/8) than in localised tumours (eye or orbit) (5/35) ($P < 0.05$) (Table 1). Among the 27 ocular tumours studied at diagnosis, no correlation could be found between LOH-1p and either extraretinal extension or differentiation. LOH-1p analysis and its relation to the histological characteristics of the tumours are summarised in Table 3. Median follow-up for the 27 patients with localised eye disease is 17 months (range: 0–37 months): 3 relapses were observed after enucleation in this patient group. LOH-1p was observed in 1 of these 3 cases; interestingly, in this case, it was associated with *N-MYC* amplification. Finally, both primary and subsequent metastatic sites could be studied in 1 patient: neither of these sites demonstrated LOH-1p.

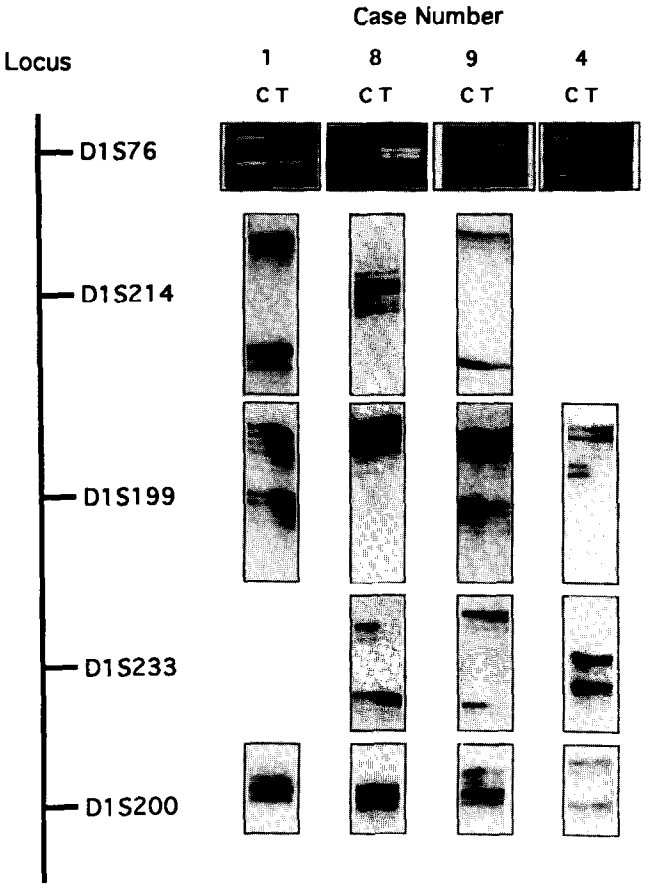

Figure 1. LOH analysis of four retinoblastoma samples. From top to bottom, the different loci are indicated from telomere to centromere. C and T indicate constitutional and tumour DNA, respectively. Case 1: a major allele disequilibrium is observed for locus D1S76, strongly suggesting that one allele of this locus is deleted in the tumour cells and that the sample is slightly contaminated by non-tumour cells. Such a disequilibrium is not observed for the D1S214 and D1S199 loci supporting the hypothesis that this tumour demonstrates a distal deletion on 1p. A more extensive distal deletion is observed for case 4, which demonstrates LOH at the D1S76 and D1S199 loci, but not at the more proximal loci. Case 8: LOH can be observed at the proximal D1S233 locus but not at the distal D1S76 locus. Similarly, for case 9, heterozygosity is maintained at the D1S76 locus but lost at the other displayed loci. In these 2 cases, these results provide evidence for the occurrence of an interstitial deletion.

Table 3. Histological characteristics and LOH-1p analysis in ocular tumours studied at diagnosis

	Tumour extension limited to the retina	Extraretinal extension
LOH-1p:	1/6	3/21
	Immature*	**Differentiated***
LOH-1p:	1/8	3/19

*Tumours were considered as immature when no rosette was observed and differentiated when rosettes were present.
LOH-1p, loss of heterozygosity of chromosome 1p.

DISCUSSION

We systematically studied *N-MYC* amplification, DNA ploidy and LOH-1p analysis in retinoblastoma tumour samples.

Surprisingly, *N-MYC* amplification, which is frequently observed in neuroblastoma and which was found in 3/11 retinoblastoma tumour samples in a previous series [12], was demonstrated in only 1/45 cases. This suggests that *N-MYC* amplification does not have a primary role in retinoblastoma tumorigenesis and that *N-MYC* might not be involved in most of the homogeneously staining regions and double-minute chromosome observed in almost 10% of retinoblastoma tumour samples [11].

In this series, tumours were nearly always DNA diploid or DNA near diploid. Only one DNA tetraploid tumour was observed. These observataions are in agreement with previous cytogenetic analysis which revealed a chromosome number in the 46–48 range in most successfully karyotyped retinoblastomas [9, 11, 23–25].

Deletion of the short arm of chromosome 1 has been observed in numerous cancers, including tumours of neuroectodermal origin, such as neuroblastoma [13], oligodendroglioma [26] and melanoma [27]. Although cytogenetic alterations on 1p have been described in retinoblastoma tumour samples [11, 23, 28], systematic screening of this chromosome region for LOH has not been performed. In this series, LOH-1p was observed in 9/43 retinoblastoma cases (21%).

None of the 4 cases with reported LOH-1p at relapse could be studied in the primary tumour. Most of the deletions involved the distal end of chromosome 1p. They encompassed the 1p36.2 region which encodes a putative neuroblastoma suppressor locus [16], suggesting that this gene might also play a role in tumour progression of a subset of retinoblastomas. Although less probable, we cannot rule out that LOH-1p might be attributed to random loss of genetic material. Further cloning of the neuroblastoma suppressor gene and analysis of its expression in retinoblastoma should enable us to distinguish between these two hypotheses.

Although retinoblastoma usually has a good prognosis, relapse may occur even in tumours limited to the retina [5]. Non-histological criteria might therefore be helpful for therapeutic decision after enucleation for retinoblastoma. *N-MYC* amplification is a well-established prognostic factor in neuroblastoma [15, 29] and DNA ploidy is also used as a prognostic factor in numerous tumours including primitive neuro-ectodermal tumours, such as neuroblastoma [15] or medulloblastoma [30]. Although the only case of *N-MYC* amplification was observed in an ocular tumour from a patient who subsequently died from this disease and the only case of DNA-tetraploidy was observed in a bone metastasis from a currently surviving patient, *N-MYC* amplification and DNA aneuploidy are such rare events in retinoblastoma that it would be difficult to prove any prognostic value from these criteria.

LOH-1p has been associated with poor prognosis in neuroblastoma [16]. It is also suspected to be of prognostic significance in Wilms' tumour [31]. Interestingly, we observed that 1p deletions were more frequent in metastatic than in localised disease, suggesting that this alteration might be linked to the aggressiveness of the tumour. Confirmation of this correlation and evaluation of the impact on patient outcome will need the analysis of additional patients and longer follow-up.

1. Donaldson S, Egbert PR, Lee WH. Retinoblastoma. In Pizzo PA, Poplack DG, eds. *Principles and Practice of Pediatric Oncology* (2nd edition). Philadelphia, Lippincott, 1992, 683–696.
2. Shields CL, Shields JA, Baez KA, Cater J, De Potter P. Choroidal invasion of retinoblastoma: metastatic potential and clinical risk factors. *Br J Ophthalmol* 1993, **77**, 544–548.
3. Kopelman JE, McLean IW, Rosenberg SH. Multivariate analysis of risk factors for metastasis in retinoblastoma treated by enucleation. *Ophthalmology* 1987, **94**, 371–377.
4. Shields CL, Shields JA, Baez KA, Cater J, De Potter P. Optic nerve invasion of retinoblastoma. Metastatic potential and clinical risk factors. *Cancer* 1994, **73**, 692–698.
5. Doz F, Khelfaoui F, Mosseri V, *et al.* The role of chemotherapy in orbital involvement of retinoblastoma: experience of a single institution in 33 patients. *Cancer* 1994, **74**, 722–732.
6. Friend SH, Berbards R, Rogelj S, *et al.* A human DNA segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma. *Nature* 1986, **323**, 643–646.
7. Dunn JM, Philips RA, Becker AJ, Gallie B. Identification of germline and somatic mutations affecting the retinoblastoma gene. *Science (Washington)* 1988, **241**, 1797–1800.
8. Xu HJ, Sumegi J, Hu SX, *et al.* Intraocular tumor formation of RB reconstituted retinoblastoma cells. *Cancer Res* 1991, **51**, 4481–4485.
9. Cano J, Oliveros O, Yunis E. Phenotype variants, malignancy, and additional copies of 6p in retinoblastoma. *Cancer Genet Cytogenet* 1994, **76**, 112–115.
10. Squire J, Philips RA, Boyce S, Godbout R, Rogers B, Gallie B. 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.
11. Venkateswara RP, Helson L, Ellsworth RM, Reid T, Gilbert F. Chromosomal abnormalities in human retinoblastoma. A review. *Cancer* 1986, **58**, 663–671.
12. Lee WE, Murphree AL, Benedict WF. Expression and amplification of the *N-myc* gene in primary retinoblastoma. *Nature* 1984, **307**, 458–460.
13. Brodeur GM, Fong CT. Molecular biology and genetics of human neuroblastoma. *Cancer Genet Cytogenet* 1989, **41**, 153–174.
14. Kyritsis AP, Tsokos M, Triche TJ, Chader GJ. Retinoblastoma—origin from a primitive neuroectodermal cell? *Nature* 1984, **307**, 471–473.
15. Look AT, Hayes FA, Shuster JJ, *et al.* Clinical relevance of tumor cell ploidy and *N-myc* oncogene amplification in childhood neuroblastoma: a Pediatric Oncology Group study. *J Clin Oncol* 1991, **9**, 581–591.
16. Fong CT, Dracopoli NC, White PS, *et al.* Loss of heterozygosity for the short arm of chromosome 1 in human neuroblastomas: correlation with *N-myc* amplification. *Proc Natl Acad Sci USA* 1989, **86**, 3753–3757.
17. Doz F, Neuenschwander S, Plantaz D, *et al.* Etoposide and carboplatin in extra-ocular retinoblastoma: a study by the Société Française d'Oncologie Pédiatrique. *J Clin Oncol* 1995, **13**, 902–909.
18. Sambrook J, Fritsch EF, Maniatis T. In *Molecular Cloning. A Laboratory Manual.* New York, Cold Spring Harbor Laboratory Press, 1989.
19. Schleiermacher G, Peter M, Michon J, *et al.* Two distinct deleted regions on the short arm of chromosome 1 in neuroblastoma. *Genes, Chromosom Cancer* 1994, **10**, 275–281.
20. Peter M, Michon J, Vielh P, *et al.* PCR assay for chromosome 1p deletion in small neuroblastoma samples. *Int J Cancer* 1992, **52**, 544–548.
21. Vielh P. *Flow Cytometry. Guides to Clinical Aspiration Biopsy.* New York, Igaku Shoin, 1991.
22. Shankey TV, Rabinovitch PS, Bagwell B, *et al.* Guidelines for the implementation of clinical DNA cytometry. DNA Cytometry Consensus Conference. *Breast Cancer Res Treat* 1993, **28**, 61–68.
23. Chaum E, Ellsworth RM, Abramson DH, Haik BG, Kitchin FD, Chaganti RSK. Cytogenetic analysis of retinoblastoma: evidence for multifocal origin and in vivo gene amplification. *Cytogenet Cell Genet* 1984, **38**, 82–91.
24. Kusnetsova LE, Prigogina EL, Pogosianz HE, Belkina BM. Similar chromosomal abnormalities in several retinoblastomas. *Hum Genet* 1982, **61**, 201–204.
25. Squire J, Gallie B, Philips RA. A detailed analysis of chromosomal changes in heritable and non heritable retinoblastoma. *Hum Genet* 1985, **70**, 291–301.
26. Bello MJ, Vaquero J, De Campos JM, *et al.* Molecular analysis

of chromosome 1 abnormalities reveals frequent loss of 1p in oligodendroglial tumors. *Int J Cancer* 1994, **57**, 172–175.
27. Dracopoli NC, Harnett P, Bale SJ, *et al.* Loss of alleles from the distal short arm of chromosome 1 occurs late in melanoma tumor progression. *Proc Natl Acad Sci USA* 1989, **86**, 4616–4618.
28. Zwaan CM, De Waal FC, Koole FD, *et al.* A giant congenital orbital tumor: an unusual presentation of retinoblastoma. *Med Pediatr Oncol* 1994, **23**, 507–511.
29. Seeger RC, Brodeur GM, Sather H, *et al.* Association of multiple copies of the *N-myc* oncogene with rapid progression of neuroblastomas. *N Engl J Med* 1985, **313**, 1111–1116.
30. Zerbini C, Gelber RD, Weinberg D, *et al.* Prognostic factors in medulloblastoma, including DNA ploidy. *J Clin Oncol* 1993, **11**, 616–622.
31. Grundy PE, Telzerow PE, Breslow N, Moksness J, Huff V, Paterson MC. Loss of heterozygosity for chromosomes 16q and 1p in Wilms tumors predicts an adverse outcome. *Cancer Res* 1994, **54**, 2331–2333.

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