Determination of the prognostic value of loss of heterozygosity at the retinoblastoma gene in osteosarcoma

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Abstract. The retinoblastoma (RB) tumour suppressor gene is implicated in the development of several malignancies including osteosarcoma. Recent studies postulated its loss of heterozygosity (LOH) to be a poor prognostic factor at diagnosis of osteosarcoma (OS). It remains unclear whether LOH of the RB gene is suitable as a prognostic factor at diagnosis in patients with osteosarcoma. In this study we aimed to determine the early prognostic value of RB-LOH as well as the ability of denaturating high performance liquid chromatography (DHPLC) to detect LOH at this gene locus in comparison to classical PAGE. We therefore analysed 41 samples of OS on restriction fragment length polymorphisms in introns 1, 17 and 25, and variable numbers of tandem repeats (VNTRs) in intron 20. PCR fragments were separated on 1.5% agarose gel electrophoresis. VNTRs with length differentiation of only a few base pairs were analysed by 8% PAA/Spreadex gels and additionally by DHPLC. One-hundred percent concordance was observed between the results obtained by classical PAGE and DHPLC. The latter improved intron 20 analysis as a sensitive and high throughput method for detecting LOH. Overall we found 16 RB-LOH in 41 OS (39%). Three tumours exhibited additional microsatellite instability. There was no significant correlation of the event-free- and overall-survival rate or response to chemotherapy with RB-LOH found in our study. LOH positivity was associated with a significantly younger age at diagnosis. In conclusion RB-LOH could not be verified as a poor prognostic factor for osteosarcoma in the present study.

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Introduction

Osteosarcoma is a highly malignant bone tumour, mainly occurring in adolescents and young adults, with incidence peaking around the age of 14 years. At the time of diagnosis there are micrometastases in at least 80% of cases. Therefore the usual therapy today is neoadjuvant polychemotherapy followed by subsequent surgery and further postoperative chemotherapy. Tumour site and size, primary metastases, response to chemotherapy, and surgical remission are of independent prognostic value in osteosarcoma (1). It would be desirable to estimate the individual risk of each patient in advance in order to adapt the initial therapy to the malignancy of the tumour. For this reason there has been ongoing research for a molecular prognostic marker. A promising candidate for this is the retinoblastoma (RB) gene.

The RB gene is a recessive gene, located on chromosome 13q14. It is the prototype of a tumour suppressor gene and is well described (2-8). Although the gene was named for its prominent role in the genesis of retinoblastoma, RB-inactivation seems to be important for the development of a variety of other malignancies (9-15), such as osteosarcoma (8,16-18). Inactivation of the RB gene, caused by mutations of the coding region and promoter region, as well as loss of heterozygosity (16,19-21) have been reported. During the process of tumour development, loss of heterozygosity (LOH), wherein one allele of a tumour suppressor gene becomes deleted, is a common event, often constituting the second hit predicted by Knudson's 'two-hit' hypothesis (22).

It has been known for many years that retinoblastomas and osteosarcomas share in part a common aetiology (4,23). Hereditary retinoblastoma patients who are cured of their tumours are at high risk of developing secondary neoplasms, particularly osteosarcoma. Patients with inherited retinoblastoma have a cumulative incidence rate of 6% for osteosarcoma as second malignancy (24). As osteosarcoma is otherwise rare, this finding suggests a link between the RB gene and osteosarcoma development. A previous study in

Table I. Patient profile and clinical data.

No.	Subtype	Age (years)	Sex	Size	Regression	Primary metastasis	Events	Overall survival (years)	Event-free survival (years)	Status
1	Osteo-chond	15	f	≥1/3	1	No	Relapse/metastasis	9.2	2.9	Died of disease
2	Osteo	7	f	<1/3	1	No	Relapse/metastasis	19.0	1.8	Alive
3	Chond	14	m	<1/3	3	No	Relapse/metastasis	12.2	3.2	Alive
4	Osteo	15	m	<1/3	2	No	Relapse/metastasis	14.5	1.5	Died of disease
5	Chond	20	f	≥1/3	3	No	Relapse/metastasis	6.2	2.9	Died of disease
6	Chond	40	m	<1/3	6	Yes	Relapse/metastasis	14.4	2.0	Died of disease
7	Osteo	20	m	<1/3	1	No	Event-free	10.8	10.8	Alive
8	Osteo	18	m	<1/3	4	No	Relapse/metastasis	1.6	1.1	Died of disease
9	Osteo	20	m	≥1/3	3	No	Event-free	3.4	3.4	Alive
10	Osteo	17	m	<1/3	4	No	Event-free	10.1	10.1	Alive
11	Chond	15	f	≥1/3	3-4	No	Event-free	9.8	9.8	Alive
12	Chond	20	m	<1/3	6	No	Relapse/metastasis	12.2	2.7	Alive
13	Osteo	11	f	<1/3	3	No	Relapse/metastasis	10.5	3.5	Alive
14	Polym	16	m	≥1/3	3	No	Event-free	8.0	8.0	Alive
15	Osteo	12	m	<1/3	3	No	Event-free	6.9	6.9	Alive
16	Osteo	13	f	≥1/3	5	No	Relapse/metastasis	3.9	2.4	Died of disease
17	Osteo	8	m	≥1/3	5	No	Relapse/metastasis	10.8	2.4	Alive
18	Osteo	16	m	<1/3	n.k.	No	Relapse/metastasis	3.2	2.0	Died of disease
19	Central	21	f	<1/3	n.k.	Yes	Relapse/metastasis	10.8	1.8	Alive
20	Chond	8	m	<1/3	2	No	Relapse/metastasis	11.2	9.1	Alive
21	Chond	25	f	<1/3	4	No	Relapse/metastasis	4.0	1.8	Died of disease
22	Dediff	15	f	≥1/3	1	Yes	Event-free	8.5	8.5	Alive
23	Osteo	10	m	≥1/3	3	No	Event-free	7.7	7.7	Alive
24	Sklerotic	14	f	n.k.	2	No	Relapse/metastasis	6.1	1.8	Alive
25	Osteo	11	f	<1/3	4	No	Event-free	7.0	7.0	Alive
26	Polym	18	m	<1/3	3	No	Event-free	7.9	7.9	Alive
27	Osteo	19	m	≥1/3	1	Yes	Event-free	5.5	5.5	Alive
28	Chond	15	m	<1/3	2	No	Event-free	7.3	7.3	Alive
29	Osteo	13	f	≥1/3	3	No	Event-free	6.8	6.8	Alive
30	Small cell	9	f	≥1/3	4	No	Event-free	4.7	4.7	Alive
31	Osteo	14	f	n.k.	2	No	Event-free	5.6	4.4	Alive
32	Osteo-chond	11	m	≥1/3	3	No	Relapse/metastasis	5.3	2.7	Alive
33	Osteo	12	f	≥1/3	1	Yes	Relapse/metastasis	3.2	1.8	Died of disease
34	Osteo	14	f	≥1/3	3	No	Event-free	4.3	4.3	Alive
35	Osteo-chond	16	m	≥1/3	4	Yes	Relapse/metastasis	0.8	0.0	Died of disease
36	Osteo	16	m	n.k.	1	No	Event-free	1.3	1.3	Died of disease
37	Osteo	26	m	n.k.	3	No	Event-free	5.5	5.5	Alive
38	Osteo	5	m	n.k.	3	No	Event-free	5.3	5.3	Alive
39	Small cell	14	m	n.k.	2	No	Event-free	6.3	6.3	Alive
40	Polym	9	m	n.k.	n.k.	No	Relapse/metastasis	1.9	0.9	Died of disease
41	Periostal	9	f	n.k.	n.k.	Yes	Relapse/metastasis	3.4	1.6	Died of disease

Event-free and overall survival are given in years after end of therapy. Regression was classified according Salzer-Kuntschik. The age is that at time of diagnosis. Patients 40 and 41 are not COSS patients. Osteo, osteoblastic; chond, chondroblastic; polym, polymorphic; central, low malignant central OS; dediff, dedifferentiated; f, female; m, male; n.k., not known.

Table II. Polymorphic sites of the RB gene.

Location	Primer sense/antisense	Annealing	Size	Endonucleases	Refs.
Intron 20	B103: 5'-AATTAACAAGGTGTGGTGGT-3'				(50)
	#310: 5'-AAGTAAGAAAATCAAGCACTT-3'	52˚C	250-300 bp VNTR	*Bst*NI	
Intron 1	Int 1A: 5'-CAGGACAGCGGCCCGGAG-3'				(16,51)
	Int 1B: 5'-CTGCAGACGCTCCGCCGT-3'	65˚C	180 bp RFLP	*Bam*HI (130+50 bp)	
Intron 17	17A: 5'-TTCCAATGAAGAACAAATGG-3'				(16)
	17B: 5'-GCAATTGCACAATCCAAGTT-3'	55˚C	945 bp RFLP	*Xba*I (630+315 bp)	
Intron 25	25A: 5'-TCCATTTATAAATACACATG-3'				
	25B: 5'-TAACGAAAAGACTTCTTGCA-3'	50˚C	167 bp RFLP	*Dra*I (129+38 bp)	(16)

Polymorphic sites at the RB-locus, analysed in the study. VNTR, variable number of tandem repeats; RFLP, restriction fragment length polymorphism.

primary osteosarcomas reported LOH at the RB locus in 40-70% of biopsies at diagnosis correlating with a poor outcome, and therefore suggested RB-LOH to be a poor prognostic factor (20).

Based on these previous studies we wanted to determine the early prognostic value and the clinical applicability of RB-LOH using conventional PCR followed by PAGE. Four markers: RFLPs in intron 1/*Bam*HI, intron 17/*Xba*I, intron 25/*Dra*I and VNTR in intron 20, were used, all known to be highly polymorphic. Likewise we introduced the denaturated high performance liquid chromatography (DHPLC) as an improved method for the detection of LOH.

Materials and methods

Clinical data. Clinical characteristics (gender, age at diagnosis, tumour size, histological subtype and response to chemotherapy) of the investigated osteosarcomas were derived from the Co-operative Osteosarcoma Study Group (COSS) (Table I). The handling of the data was in agreement with the permission of the ethics commission from 1996 during COSS 96 study protocol and the corresponding research analysis. Response to preoperative chemotherapy was assessed histopathologically according to the six-grade scale of Salzer-Kuntschik *et al* (25,26). Except for 2 cases, all patients received chemotherapy prior to surgery according to the COSS protocols.

Samples. We investigated pairs of specimens (tissue and blood) derived from 41 osteosarcoma patients (39 constitutional heterozygous, 2 homozygous). Seventeen primary untreated tumour tissues, 15 recurrences and 9 resections after chemotherapy were analysed. Tumour tissues were frozen immediately after surgery and stored at -80˚C until use. All tumour specimens were evaluated for sufficient content of vital tumour cells microscopically by the pathologists. Cell lines SaOS, CEM, and La-N-1 as well as commercial placental DNA served as controls.

DNA isolation. Genomic DNA was extracted from fresh peripheral blood or frozen cells ($1x10^7$ cells for each sample) using silica gel based spin columns (QiaAmp DNA blood mini/midi kit; Qiagen, Hilden, Germany) according to the 'blood and body fluid protocol' or the 'tissue protocol' of the manufacturer.

LOH analysis by PCR. PCR was carried out for the RFLP-markers intron 1/*Bam*HI, intron 17/*Xba*I, intron 25/*Dra*I and VNTR-marker in intron 20, in a reaction volume of 50 μl containing 250-500 ng DNA in each reaction, 100 pmol each primer (intron 1: 20 pmol), and 1.5 mM $MgCl_2$ (intron 1: 2 mM) for 35 cycles, using the TagBead Hot Start Polymerase kit (Promega, Mannheim, Germany). In the cases of intron 1 and 20, 5-10% DMSO was added. Annealing temperatures, primers, endonucleases and sizes of PCR fragments are listed in Table II. PCR products were digested by appropriate endonucleases, using conditions specified by the manufacturer. Digested DNA fragments were separated on 1.5% agarose gels in the cases of introns 1, 17 and 25. VNTRs of intron 20 were separated either on 8% polyacrylamide/Spreadex-polymer NAB (Elchrom Scientific-Herolab, Wiesloch, Germany) gels (PAGE) or on DHPLC. Agarose gels were stained with ethidium bromide and acrylamide/Spreadex gels were silver-stained. Results were classified as LOH positive (LOH^+) when one of two alleles was completely lost or in cases where the intensity of one of the two alleles was reduced by >50%. Patients were classified as LOH negative (LOH^-) (het), when

Table III. Summary of the results.

No.	LOH overall	Intron 1	Intron 17	Intron 25	Intron 20	Regression	Material
1	-	n.i.	n.i.	n.i.	MI	4	Resection after chemo.
2	-	n.i.	n.i.	n.i.	het	1	Biopsy before chemo.
3	+	n.d.	n.d.	n.d.	LOH	2	Resection after chemo.
4	-	het	het	n.i.	het	3	Resection after chemo.
5	-	het	n.i.	het	het	1	Biopsy before chemo.
6	-	n.i.	het	het	n.i.	3	Biopsy before chemo.
7	-	n.i.	het	n.i.	het	1	Resection after chemo.
8	-	n.i.	n.i.	n.i.	het	3	Resection after chemo.
9	+	n.d.	n.d.	n.d.	LOH	1	Metastasis/relapse
10	-	n.i.	n.i.	n.i.	het	4	Resection after chemo.
11	-	het	het	het	het	1	Biopsy before chemo.
12	-	het	n.i.	n.i.	het	3	Resection after chemo.
13	-	n.i.	het	n.i.	n.i.	3	Biopsy before chemo.
14	+	n.i.	het	n.d.	LOH+MI	3	Biopsy before chemo.
15	-	n.i.	het	n.d.	het		Metastasis/relapse
16	-	n.i.	n.i.	n.i.	het	6	Metastasis/relapse
17	-	het	het	het	n.i.	5	Resection after chemo.
18	+	n.d.	n.d.	n.d.	LOH	2	Metastasis/relapse
19	-	n.i.	n.i.	n.i.	het	2	Resection after chemo.
20	-	n.i.	het	n.i.	het	3	Resection after chemo.
21	+	n.d.	het	n.d.	LOH	1	Biopsy before chemo.
22	-	het	n.i.	het	het	6	Resection after chemo.
23	+	n.d.	n.d.	n.d.	LOH	2	Biopsy before chemo.
24	+	LOH	LOH	n.d.	n.i.	1	Biopsy before chemo.
25	+	LOH	het	het	het	3	Biopsy before chemo.
26	+	n.i.	n.d.	n.d.	LOH	No data	Biopsy before chemo.
27	-	n.i.	n.i.	n.i.	het	3	Resection after chemo.
28	+	LOH	LOH	n.d.	MI	4	Resection after chemo.
29	-	n.i.	n.i.	n.i.	het	3	Metastasis/relapse
30	n.i.	n.i.	n.i.	n.i.	n.i.	2	Metastasis/relapse
31	n.i.	n.i.	n.i.	n.i.	n.i.	2	Biopsy before chemo.
32	+	n.i.	het	n.d.	LOH	3	Metastasis/relapse
33	-	het	het	het	het	3	Resection after chemo.
34	+	n.d.	n.d.	n.d.	LOH	4	Biopsy before chemo.
35	+	n.d.	n.d.	n.d.	LOH	No data	Biopsy before chemo.
36	+	het	het	n.d.	LOH	3	Biopsy before chemo.
37	-	n.i.	het	het	het	4	Biopsy before chemo.
38	-	het	het	het	het	No data	Metastasis/relapse
39	+	n.d.	n.d.	n.d.	LOH	4	Metastasis/relapse
40	-	n.i.	n.i.	n.i.	het	3.5	Resection after chemo.
41	+	n.d.	n.d.	n.d.	LOH	5	Resection after chemo.
All	16	3	2	0	13		

Loss of heterozygosity (LOH) and microsatellite instability (MI) in osteosarcoma: summary of the results. het, heterozygous; n.i., not informative; n.d., not done.

tumour DNA showed two alleles with identical intensity almost like the constitutional DNA. In the case of microsatellite instability (MI) one or both alleles in the tumour are a few base pairs longer or shorter than in the constitutional blood.

VNTR analysis by DHPLC. Five microliters of each PCR reaction was used for DHPLC analysis on the WAVE DNA Fragment Analysis System (Transgenomic, San Jose, USA). All conditions were adapted from zur Stadt *et al* (27). A linear 2% triethylammoniumacetate (TEAA)-acetonitril (ACN) gradient at the column oven temperature of 50˚C (native conditions) was applied for optimal identification of VNTR's. Under these DHPLC conditions PCR fragments were detected within 10-12 min after injection of the sample. The resulting chromatogram contains a pattern of peaks, representing PCR products of various size.

Statistical analysis. Survival analysis was performed using the Kaplan-Meier method (28). Differences between categories were estimated by the log-rank test with date of histological diagnosis as starting point. Statistical analysis and graphics were made using SPSS statistical package Base 12.0 program for Windows (SPSS GmbH Software, Munich, Germany).

Results

LOH at the RB locus. Pairs of specimens (blood and tissue) from 41 osteosarcoma patients of different histological subtypes (Table I) were screened for RB-LOH on four informative polymorphisms (Table II).

Overall we found RB-LOH in 16 out of 41 (39%) osteosarcomas summarised in Table III. RB-LOH was detected in 9 out of 17 biopsies as well as in 3 out of 15 resections and in 4 out of 9 metastasis. We found 13 out of 16 LOH cases in the VNTR polymorphic site of intron 20, while in intron 1, 3 RB-LOH and in intron 17, 2 RB-LOH cases were found. In the present study no RB-LOH cases were found in intron 25 (Fig. 1).

In three cases, microsatellite instability (MI) was found in intron 20. One of these shows MI in addition to RB-LOH in intron 20 (Fig. 1D, lanes 12 and 13). Another one shows MI in intron 20 in addition to LOH in two introns (1 and 17) at the same time. Overall we found more than one LOH in two patients (no. 24 and 28; Table III).

We found RB-LOH in different sources of specimens: in biopsies, metastases and resections. We therefore demonstrate that RB-LOH can be detected not only in untreated biopsies of osteosarcoma tumour specimens but also in tissue samples derived during or after chemotherapy. To avoid systemic error we also made the Kaplan-Meier analysis of survival of RB-LOH for biopsies only (diagrams not shown), and found no significant correlation.

Regarding the Kaplan-Meier analysis (Fig. 3) of the prognostic significance of RB-LOH there seemed to be a slightly worse outcome for patients with LOH^+, but it is not at all significant for the overall survival (Fig. 3A) (log-rank 0.508) and hardly visible for the event-free survival (Fig. 3B) (log-rank 0.776).

To test whether or not any prognostic marker can be seen in these 41 patients we examined the response to chemotherapy as a well-known prognostic factor. The visible difference in survival between patients with a good and those with a poor response to chemotherapy is almost significant with a log-rank of 0.0597 (Fig. 3C). The relative size of the tumour and the presence of primary metastases are also visible as prognostic factors, but a prognostic value of RB-LOH could not be proven after an average time of 5.98 years.

Patients with LOH show a distinctly younger age than patients without LOH (Table IV). At the time of diagnosis, patients without LOH had an average age of 17.1 years while patients with LOH had an average age of 12.4 years. The correlation (according to Pearson) is significant (2-sided, level 0.05).

DHPLC for RB-LOH analysis at intron 20 VNTRs. All specimens analysed with two methods at the VNTR site of intron 20 (PAGE and DHPLC) showed identical results. Three representative examples are indicated in Fig. 2. We demonstrated that DHPLC is a sensitive and high throuput methodology for detecting this type of genetic alteration with a better and clear identification compared to classical PAGE.

Discussion

Recent studies have analysed RB-LOH in osteosarcoma, but its prognostic value remains unclear. Earlier investigators found LOH of the RB gene in approximately 40-70% of cases (16,18,20,29,30) and some of them associate RB-LOH with a worse prognosis (16,20,29,30). One of them (16) examined introns 1, 17, 20 and 25, but did not mention the treatment that the investigated 63 patients received. Another one (20) had the advantage that all 34 patients were treated in the same way. However only one intron (intron 20) was examined. In addition, the authors announced a prospective study about the prognostic value of RB-LOH, but this has not yet been published. Patino-García *et al* (30) tested introns 2 and 20 in 41 patients that were treated alike. The research of Goto *et al* (29) comprises 32 patients treated alike that were tested in only one microsatellite site. Out of these, 7 were not informative. Toguchida *et al* (18) and Belchis *et al* (19) also studied RB-LOH, but made no conclusion about the prognosis. So far the early prognostic value of RB-LOH for osteosarcoma remains unclear.

Our study is the first to examine 4 introns in parallel in patients that were treated according to the same protocol (COSS, Germany). One advantage of this approach is the low number of not-informative patients (2 out of 41), when several loci are examined. The total number of RB-LOH found in the four introns differs between 13 in intron 20 and 0 in intron 25. This finding underlines the necessity for testing more than one polymorphic site for RB-LOH analysis.

To our knowledge we are the first to improve RB-LOH diagnostic by introducing the DHPLC technique for the analysis of VNTRs in intron 20 of the RB gene. RB-LOH of VNTRs can only be identified by small differences of a very low number of base pairs of VNTR fragments. Using conventional PAGE these small base pair differences are sometimes hard to see, even if using the Spreadex supplement to PAGE. We demonstrate that DHPLC improved the intron 20

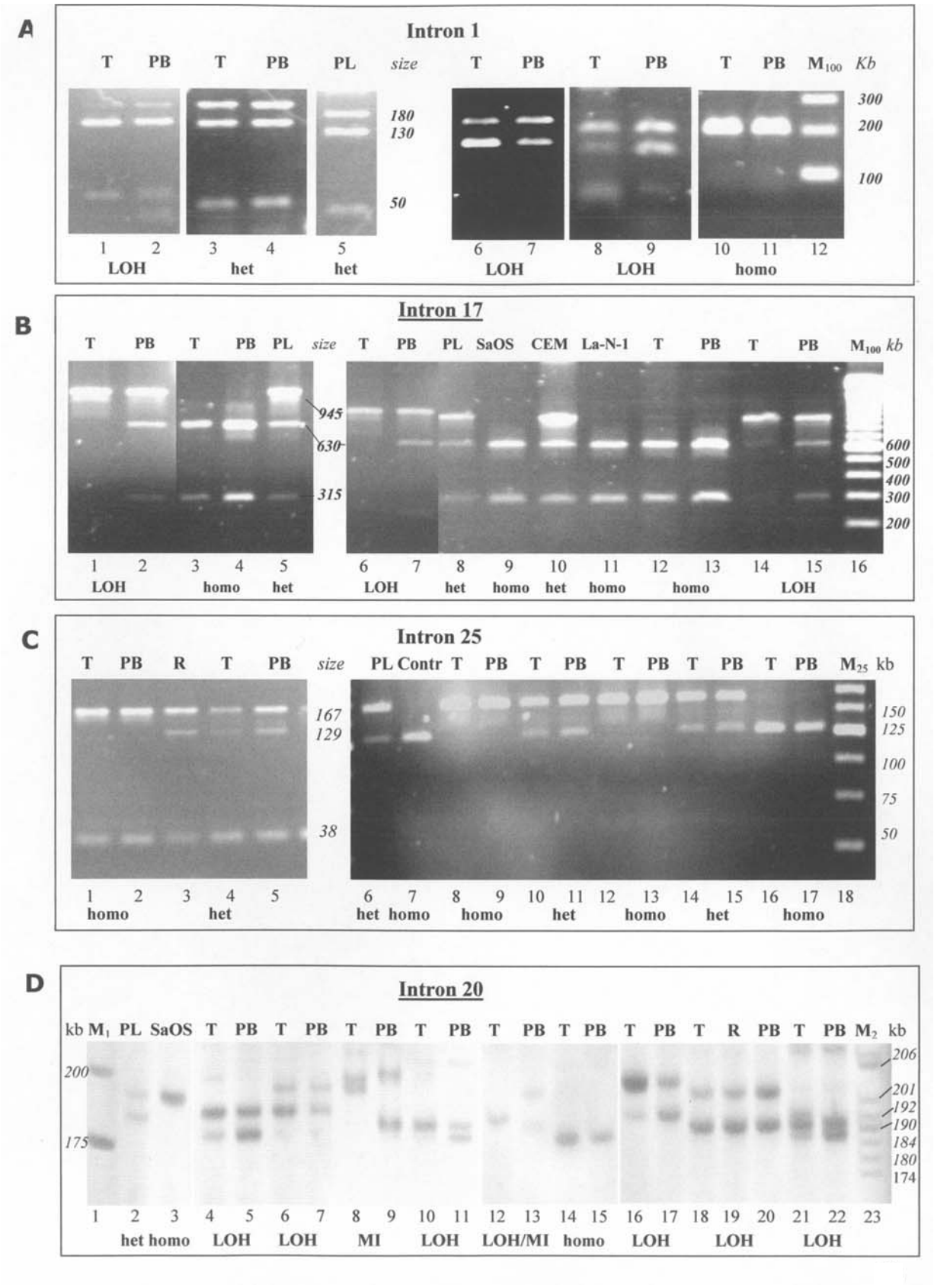

Figure 1. LOH at the RB locus in osteosarcoma. T, tumour; PB, peripheral blood (constitutional); PL, placental DNA; LOH, loss of heterozygosity; het, heterozygous; homo, homozygous; M, DNA size marker: numbers to left or right site giving the fragments in base pairs (bp). A, *Bam*HI polymorphism in intron 1. Digestion of the 180-bp PCR products in the heterozygous conditions (lane 5) with *Bam*HI results in three bands: 180, 130, 50 bp, in the case of LOH one band is lacking or reduced to 50% (lanes 1, 6 and 8). B, *Xba*I polymorphism in intron 17. Digestion of the 945-bp PCR product in the heterozygous condition (lane 5) with *Xba*I results in three bands of 945, 630 and 315 bp. Lacking or reduction of 50% of one of the bands indicates LOH (lanes 1, 6 and 14). C, *Dra*I polymorphism in intron 25. Digestion of the 167-bp PCR product in the heterozygous condition (lane 5) results in three bands of 167, 129 and 38 bp. In our investigation no LOH was detected. D, variable number of tandem repeats (VNTR) in intron 20. Fragment size of this polymorphism range between 150 and 300 bp with sometimes very small difference between the two bands. *Bst*NI digestion make it easier to distinguish between double bands. LOH is indicated by loss or reduction of one band to 50% (lanes 4, 6, 10, 12, 16, 18, 19 and 21). Additionally to LOH, MI is indicated in lanes 8 and 12.

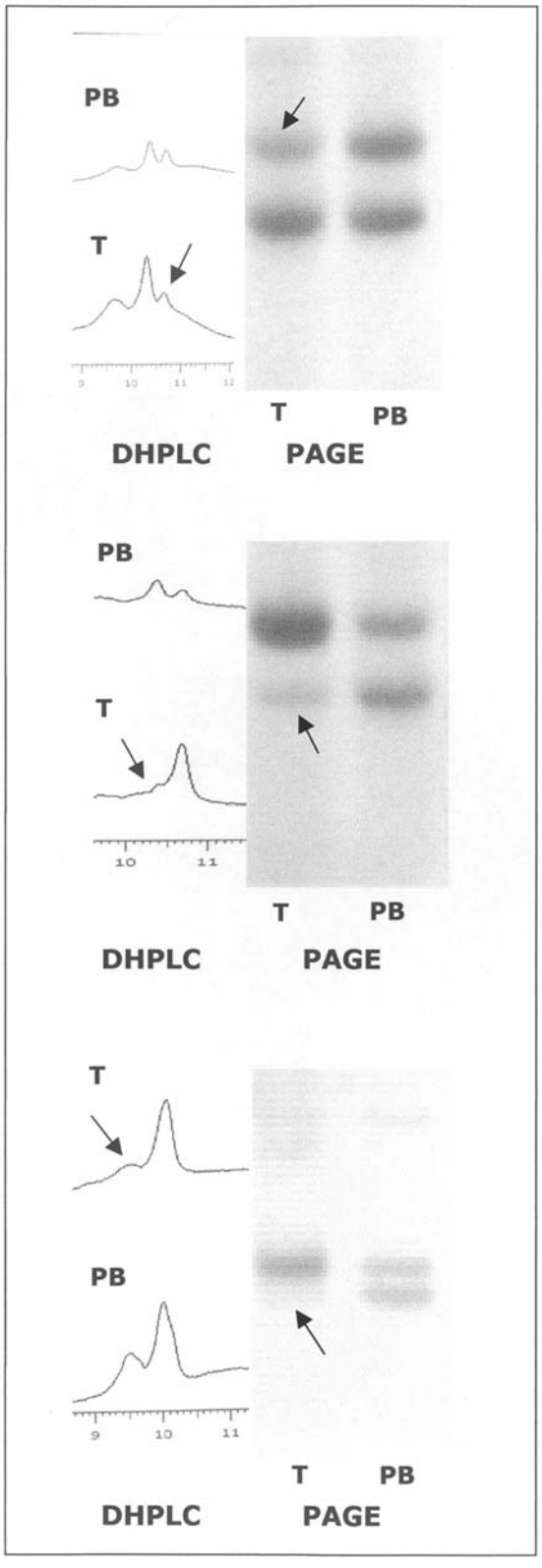

Figure 2. Comparison of VNTR detection results by PAGE and DHPLC. Examples in three cases. LOH is indicated by arrows in each case. T, tumour; PB, peripheral blood (constitutional).

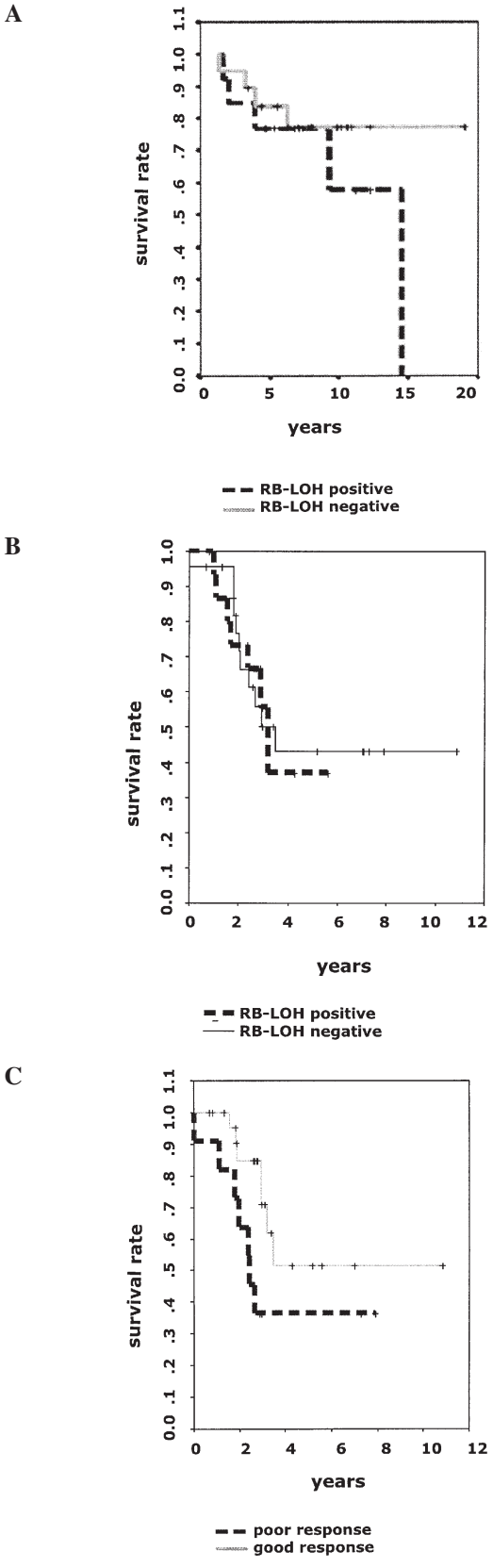

Figure 3. Prognostic significance for osteosarcoma patients. Kaplan-Meier survival curves for overall survival (A) and event-free survival (B and C). A, overall survival function in comparison to RB-LOH. Patients with LOH seem to have a worse prognosis, but the negative trend is statistically irrelevant (log-rank test 0.508). B, event-free survival function in comparison to LOH. The data for LOH^+ and LOH^- patients look almost identical (log-rank test 0.776). C, event-free survival compared to histological response to chemotherapy. Patients with good response (regression grade 1-3) have less metastases (log-rank test 0.0597) than those with poor response (regression grade 4-6).

analysis by making a definitive clear identification of these small differences possible as shown in Fig. 2. It can replace gel electrophoresis for separation of PCR products of a wide range. DHPLC also allows a higher throughput of samples with less handling by the operator than PAGE. It is therefore

Table IV. Summary of the age analysis and RB-LOH.

RB-LOH	Mean value age at diagnosis (years)	N	Standard error	Median
LOH^-	17.13	23	6.97	16.00
LOH^+	12.44	16	3.81	13.00
Not informative	14.00	2	0.00	14.00
Overall	15.15	41	6.12	15.00

RB-LOH compared to patient age at diagnosis.

less time consuming since there is no gel handling, loading, and staining.

Moreover we found microsatellite instability which has initially been described for colorectal carcinoma (31) and which have been correlated with a high mutational rate and DNA repair processes (32). Meanwhile MI has been reported to be implicated in various cancers (33-40) as well as in other diseases, such as chronic obstructive pulmonary disease (COPD) (41), pulmonary sarcoidosis (42) and bronchial asthma (43). For osteosarcoma MI had previously only been described twice (19,44) but its function in this disease is still unknown. Belchis *et al* comment on MI as a possible poor prognostic factor while Martin *et al* did not comment on the role of MI for sarcomas. Paulson *et al* (45) has suggested a correlation between MI and a poor prognosis in breast cancer. The significance of MI as well as the combination of RB-LOH and MI for osteosarcoma has yet to be determined. These data collectively suggest that the DNA repair deficiency associated with MI could be an early event in tumour development as well as in other diseases.

With an overall 39% RB-LOH rate we detected a lower LOH rate than previous studies for osteosarcoma. We could not verify the findings of Feugeas *et al* and three other previous investigators that link RB-LOH to a worse prognosis (16,20,29,30). Our data show no statistically significant correlation of RB-LOH with event-free or overall survival. One reason may be the selection of patients in our study or the aggressive treatment according to the COSS protocols they received, but the study of Ozaki *et al* (46), who analysed a comparable group of patients, contradicts this. They screened patients with comparative genomic hybridisation (CGH) and found several chromosomal gains and losses. They concluded that loss of 13q14 was a negative prognostic marker, when excluding primary metastasis. This might be caused by the RB gene or e.g. by LEU1 (leukemia associated gene 1) or LEU2 (46). Another possibility is, that the influence of RB-LOH would be seen after a longer surveillance of the patients. This might be a possibility, but seems improbable after an average follow-up of 5.98 years. Possibly this prognostic marker could not be clearly identified within our patient collective simply by chance or the prognostic value of RB-LOH is indeed not as high as assumed before. Contradicting results were found by other investigators who examined erbB2 as a possible prognostic marker for osteosarcoma. Onda *et al* proposed erbB2 to be a negative prognostic marker (47) when finding an increased expression of it, but in contrast Akatsuda *et al* (48) pointed out that an increased expression of erbB2 is to be considered as a positive prognostic marker. Due to the small number of cases in most series, such conflicting results are common.

The survival of younger patients in our study is better (log-rank test 0.0922) than the survival of the older patients (data not shown). Although the age at diagnosis is not commonly seen as a prognostic marker, our patients that were 15 years old or younger seemed to have a better prognosis than older patients. At the same time the younger patients showed LOH more frequently, therefore we analysed the survival for both groups separately for the influence of RB-LOH (Table IV). In the younger patients, a negative influence of the presence of RB-LOH was observed, but this was not statistically significant. The same was valid for patients over 15 years of age.

It is remarkable that on average, patients with RB-LOH were younger at time of diagnosis. Our finding is in agreement with the studies of Wadayama *et al* (16) and Toguchida *et al* (3), although it is not highlighted in these studies. Feugeas *et al* (20) do not comment on the age, but their data show, that their patients with RB-LOH were also younger with an average age of 15 years, compared to 19.4 years for patients without RB-LOH (calculated from their Tables II and III). Regarding patients with hereditary retinoblastoma, it is known that LOH-initiated tumours are associated with a significantly younger age at diagnosis (49) and it is postulated to be a poor prognostic factor for this disease. Since cancer is a genetic disease developing step by step over time, one possible explanation is that a loss of heterozygosity at the RB locus shortens the time from the first mutations to clinical occurrence of cancer and, therefore, generally leads to younger patients.

In conclusion we could not verify the poor prognostic value of RB-LOH for osteosarcoma patients treated according to the COSS protocol in the present study.

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