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Detection of minimally disseminated disease in the cerebrospinal fluid of children with high-risk retinoblastoma by reverse transcriptase-polymerase chain reaction for GD2 synthase mRNA

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Abstract ***Aim:*** To evaluate minimally disseminated disease (MDD) in cytologically negative cerebrospinal fluid (CSF) specimens of patients with high-risk retinoblastoma by the detection of the synthase of ganglioside GD2 mRNA by reverse transcriptase-polymerase chain reaction (RT-PCR).

Methods: The CSF was evaluated in 26 patients with high risk for CSF relapse: 14 with post-laminar optic nerve invasion, five of them with tumour at the resection margin, five with massive choroidal invasion, three with overt orbital extension and four patients with systemic metastasis. Serial CSF examinations were repeated at different time intervals according to stage and in the event of suspected relapse. GD2 synthase mRNA was evaluated by RT and nested PCR at each procedure.

Results: MDD was present at diagnosis in six cases (23%) and it was significantly associated to massive optic nerve involvement or history of glaucoma ($p < 0.05$). Three of the children with positive MDD had a CSF relapse. Thirteen patients had negative MDD at diagnosis and one had a CSF relapse. In seven children no ARN could be obtained for PCR analysis and two subsequently relapsed. The probability of CSF relapse was 0.50 (95% confidence interval (CI) 0.13–0.88) for children with MDD and 0.08 (95% CI 0.02–0.46) for those with negative RT-PCR examination of the CSF at diagnosis ($p = 0.03$).

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Conclusions: MDD in the CSF detected by RT-PCR for GD2-synthase mRNA occurred in 31.7% of evaluable high-risk children with retinoblastoma with no initial central nervous system (CNS) involvement. It was significantly associated to optic nerve involvement and glaucoma and increased risk of CSF relapse.

1. Introduction

Central nervous system (CNS) dissemination is the most common cause of death of retinoblastoma.[1] It may be present at diagnosis or it may occur as a secondary event in children with no initial CNS involvement. In these children, leptomeningeal dissemination is the most common site of CNS relapse and it is diagnosed by imaging studies and cytological evaluation of the cerebrospinal fluid (CSF).[2,3] Children presenting with orbital dissemination as well as those with postlaminar optic nerve or massive choroidal invasion are at higher risk for this adverse event.[4,5] Chemotherapy is used in these children to prevent this fatal event, however, CSF relapse still occurs in a proportion of these children, usually within the first year of diagnosis.[6,7] Thus, it would be important to identify children at risk early in their evaluation in order to administer more intensive, CNS-directed chemotherapy. However, the timing of disease dissemination to the CNS in retinoblastoma is not known. It has been postulated that CNS dissemination in retinoblastoma may occur through invasion to the optic nerve but there is no predictive pattern of dissemination.[8] As in other paediatric malignancies such as neuroblastoma, the CNS may act as a sanctuary site where active levels of chemotherapy are not sufficient for tumour control leading to relapse.[9] It is possible that minimally disseminated disease (MDD) may be present at diagnosis and, if identified, it may be potentially affected by chemotherapy.[7] However, conventional cytology and imaging studies fail to identify these children, so immunological or molecular determinations of tumour-specific markers may increase the yield of standard examination potentially identifying MDD.[10–12] However, as opposed to other neuroectodermal paediatric tumours such as neuroblastoma,[13,14] only case reports dealing with the use of molecular techniques for detecting MDD for retinoblastoma were published.[15,16] These reports focused on the evaluation of MDD in the bone marrow and peripheral blood, but to our knowledge there are no reports about MDD in the CSF in retinoblastoma. The rarity of this tumour and its low frequency of metastatic dissemination in developed countries constitute an obstacle to study a significant patient cohort of children at risk of metastatic disease. In addition, there is limited information about molecular targets for studying MDD in retinoblastoma.[17] Since it is expressed consistently in retinoblastoma, we and others previously identified the ganglioside GD2 and its synthase as a candidate for MDD evaluation in retinoblastoma and as a cell-surface marker for immunodetection.[17–19] Therefore, this observational study aimed to evaluate the detection of GD2 synthase mRNA by reverse transcriptase-polymerase chain reaction (RT-PCR) in acellular CSF specimens as a marker for MDD in children with high risk retinoblastoma.

2. Patients and methods

All consecutive patients with retinoblastoma belonging to the International Retinoblastoma Staging System (IRSS)[20] stage II–IV consecutively diagnosed from 05/2007 to 02/2012 were included. Children with stage I and high risk features such as massive choroidal invasion and/or retrolaminar invasion in whom serial procurement of CSF was possible during follow-up regular ophthalmological examinations under anaesthesia were included. Children with stage IVb (CNS involvement) were not included. The CSF specimen was procured by lumbar puncture performed with standard technique under general anaesthesia, usually within 10–14 days after enucleation in stages I–II patients and about 1–3 days after admission in children with stage III onwards. Bone marrow examination was done concomitantly.

Cases with stages III and IV retinoblastoma underwent repeated CSF examination after two induction chemotherapy cycles and before consolidation with high dose chemotherapy and autologous stem cell rescue (ASCR) or upon completion of treatment in those not receiving ASCR. Children with stages I–II had a repeated follow-up CSF examination upon completion of adjuvant chemotherapy and at 1 year of diagnosis. Written informed consent was obtained from guardians and the protocol, including the supplementary punctures was approved by our Human Investigations Committee.

2.1. CSF evaluation

CSF specimens were processed immediately upon procurement and examined for cytology. An aliquot of 2–3 ml was placed into a guanidinium thiocyanate (GTC) buffer at a 1:1.5 ratio sample/buffer for PCR studies. The buffer contained 6 M GTC (Promega), 0.0375 M sodium citrate and 0.75% lauroylsarcosine. The sample/GTC mixtures were stored immediately at –70 °C until use.

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CSF was considered positive for malignancy when unequivocal malignant cells were identified at cytology. Immunocytological evaluation for GD2 by a standard indirect fluorescence microscopy technique and/or evaluation of CD45–CD56+ cells by flow cytometry (FC)[19] were done only when malignant cells were identified by cytology or whenever the cell count exceeded three cells/mm^3 regardless of the cytology findings. CSF cell counts were done in duplicate by manual methods using a Levy–Neubauer haemocytometer counting chamber. For cytological evaluation, CSF specimens (500 μl) were centrifuged for 4 min at 1100 rpm and the slides were air-dried, fixed with acetone and stained with May Grünwald Giemsa. Cytospin smears were reviewed by the senior author regardless of the cell counts.

2.2. RT-PCR studies

PCR studies were done blindly without knowing the results of the other tests and their results did not influence childreńs treatment decisions.

2.2.1. RNA extraction

RNA extraction was conducted at 4 °C based on TRIzol extraction methodology (TRIzol LS Reagent; Invitrogen) according to the manufacturer's instructions. Due to CSF scant cellularity, we used ultrapure glycogen (Invitrogen) as a carrier to optimise the extraction as we reported previously.[17]

2.2.2. Primer design

The design of highly specific primers for the human GD2 synthase mRNA sequence (GenBank Accession No. NM_001478) was carried out with PrimerSelect™ 5.05 software (DNAdnastar Inc.) as we previously reported.[17] Primer details are shown in Table 1.

2.2.3. RT-PCR and nested-PCR assays

2.2.3.1. RT-PCR. RNA samples were analysed by a two-step RT-PCR. The first step of cDNA synthesis and PCR was carried out in a final volume of 50 μl using the Illustra™ Ready-To-Go™ RT-PCR Bead kit (GE Healthcare) as we reported elsewhere.[17] The cycling profile for the RT-PCR was carried out as follows: reverse transcription, 1 h at 43 °C; initial denaturation, 5 min at 95 °C; amplification (60 cycles), 1 min at 95 °C, 1 min at 63.7 °C and 30 s at 72 °C; final extension, 10 min at 72 °C.

2.2.3.2. Nested-PCR. We only performed a second round of amplification in GAPDH positive cases. GAPDH-negative cases were considered as not informative for MDD. Nested PCR was done as we previously reported.[17] For the nested-PCR, cycling consisted of: initial denaturation, 1 min at 95 °C; amplification (30 cycles), 30 s at 95 °C, 30 s at 60.2 °C and 30 s at 72 °C; final extension, 5 min at 72 °C.

The sensitivity of the method was 200 pg and 40 pg of total ARN for RT and nested PCR respectively. Patients with positive RT and/or nested PCR were considered as having MDD.

2.3. Treatment

Children with massive choroidal invasion alone did not receive adjuvant therapy. Adjuvant chemotherapy was given to children with post-laminar optic nerve involvement and microscopical scleral involvement and to all children with stage II as reported previously.[21] Children with stages III onwards received neo-adjuvant therapy, secondary enucleation followed by consolidation with orbital radiotherapy and adjuvant chemotherapy in children with stage III and ASCR in stage IV if at least partial response was achieved.[22] Children presenting with buphthalmia were offered neoadjuvant chemotherapy and secondary enucleation followed by adjuvant therapy even when no extraocular dissemination was evident.[23]

2.4. Statistical analysis

Chi square or Fisher exact tests were used for categorical variables and Mann–Whitney test was used for continuous variables. CSF relapse was defined as event and event-free survival curves were calculated according to Kaplan–Meier and survival status was updated to July 2012. Curve comparison was done with the log-rank test.

3. Results

A total of 26 patients with newly diagnosed retinoblastoma (10 bilateral) were evaluated. There were 14

Table 1
Primers for amplification of GD2 synthase and GAPDH mRNA sequences.

mRNA	Forward primer	Reverse primer	Product size (bp)
GD2 reverse transcriptase-polymerase chain reaction (RT-PCR)	5′-TCGGCTACGGCTCTCATCACCAG-3′	5′-CTGAGCGTGGAGCCCGGCG-3′	347
GD2 nested-PCR	5′-GAACCTGGCCGTGTCTCAAGTAAC-3′	5′-CACCACTTATCGGCAGCTGCT-3′	180
GAPDH RT-PCR	5′-GGGGAGCCAAAAGGGTCATCATCT-3′	5′-GACGCCTGCTTCACCACCTTCTTG-3′	457

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children with stage I (nine with postlaminar optic nerve involvement and five with isolated massive choroidal invasion). One of them, with postlaminar involvement, presented with massive buphthalmia without evident extraocular dissemination at imaging studies and she received pre-enucleation chemotherapy followed by planned enucleation and adjuvant therapy. We also included five children with stage II with tumour at the resection margin of the optic nerve after initial enucleation and three patients with stage III (overt orbital extension). There were four patients with stage IVa disease with bone marrow metastasis. During the study period, three patients with stage IVb were diagnosed. Three eligible children were not included because of lack of parental consent for serial CSF examinations.

3.1. Outcome

A total of six patients had a CSF relapse. Malignant cells in the CSF cytology were positive for GD2 by immunocytology in five of them and by FC with a pattern of CD45–CD56+ in the remaining one. The median cell count of relapsing patients was 6.6 cells/mm^3 (range 2.4–151). The initial stage of the relapsing patients was: II = 3, III = 1 and IVa = 2. All children with CSF relapse died of disease.

3.2. MDD evaluation

Six patients (23%) tested positive for GD2 synthase mRNA, revealing MDD. Thirteen patients (50%) were negative both at RT and nested PCR and seven (27%) had no informative results. Clinical features and outcome of patients with and without MDD at diagnosis are shown in Tables 2 and 3. In two patients with MDD at diagnosis, MDD was also detected in subsequent CSF specimens after diagnosis and both had a CSF relapse soon afterwards. Fig. 1 shows the results of one such patient. Overall, three of six children with MDD had a CSF relapse and the remaining three survived disease-free for 18, 26 and 28 months after receiving adjuvant therapy. No MDD was evident in two sequential specimens (6 months and 1 year after diagnosis) in all of them. One patient in the cohort of negative MDD had a CSF relapse and two CSF relapses occurred in the cohort of patients with not informative RT-PCR examinations. In all these cases, only one specimen was evaluated and CSF relapse

Table 2
Description of patients with positive reverse transcriptase-polymerase chain reaction (RT-PCR) for GD2 synthase mRNA.

#	Laterality, Disease stage and extension at diagnosis	Treatment	Positive PCR studies	Outcome	Comments
1	Unilateral Stage IVa Tumour at resection margin Bone marrow invasion	Initial enucleation, conventional chemotherapy, consolidation with ASCR	At diagnosis, after induction chemotherapy and after ASCR before relapse	CSF relapse at 14 months	PCR became negative after induction chemotherapy PCR was positive 1 month before relapse
2	Unilateral Stage II Tumour at resection margin	Initial enucleation, conventional chemotherapy, Orbital radiotherapy	At diagnosis	Alive and disease-free (+22)	Negative PCR examination at 6 months and a year after diagnosis
3	Unilateral Stage III Orbital extension Massive optic nerve invasion	Neo-adjuvant chemotherapy, Enucleation, Adjuvant chemo and radiotherapy	At diagnosis	CSF relapse at 8 months	PCR after neoadjuvant therapy was negative
4	Unilateral Stage I Massive buphthalmia No extraocular extension	Neo-adjuvant chemotherapy, Enucleation, Adjuvant chemotherapy	At diagnosis	Alive and disease-free (+26 months)	Negative PCR examination at 6 months and a year after diagnosis
5	Bilateral Stage III Orbital extension Massive optic nerve invasion	Neo-adjuvant chemotherapy	At diagnosis and after the 1st and 2nd cycle	CSF relapse	Stable disease after chemotherapy PCR was never negative
6	Unilateral Stage III Massive orbital extension	Neo-adjuvant chemotherapy, Enucleation, Adjuvant chemotherapy and radiotherapy	At diagnosis	Alive and disease-free (+18 months)	Negative PCR examination at 6 months and a year

Abbreviations: CSF, cerebrospinal fluid; ASCR, autologous stem cell rescue.

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Table 3
Description of patients with negative minimally disseminated disease (MDD).

Disease extension	Bilateral/ unilateral	Treatment	Outcome	Comments
Stage I (choroidal invasion)	4/1	Observation = 1 Chemotherapy = 4 (#)	All survived event-free	One case had scleral invasion (#) Children with bilateral disease ($n = 3$) received chemoreduction for the treatment of the fellow eye
Stage I (post laminar optic nerve disease)	1/3	Adjuvant chemotherapy = 4	All survived event-free	Three patients had concomitant massive choroidal invasion
Stage II (tumour at the resection margin of the optic nerve)	0/2	Adjuvant chemotherapy and orbital radiotherapy = 2	One patient survived event-free One patient had CSF relapse at 10 months (+)	(+) MDD was negative in two specimens (at diagnosis and 6 months)
IVa (systemic metastasis)	1/1	One patient: Neodajuvant chemotherapy, enucleation, consolidation with ASCR One patient: Orbital exenteration elsewhere, chemotherapy followed by consolidation with ASCR (*)	One patient survived event-free One patient had a CNS relapse (CNS mass) (*)	(*) This patient was treated with orbital exenteration at other center as initial therapy. No lumbar puncture was done at CNS relapse at 12 months because of severe intra-cranial hypertension. The patient had a CSF fistula from the orbital surgery developing after induction chemotherapy, so only one specimen was available for MDD study

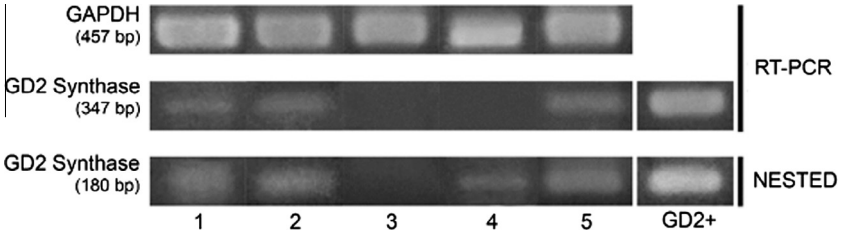

Fig. 1. Results of polymerase chain reaction (PCR) for GD2 synthase in a representative patient. Panel 1 shows cerebrospinal fluid (CSF) examination at diagnosis; panel 2, after two cycles of induction therapy; panel 3, after four cycles of induction therapy; panel 4, 2 months after consolidation with autologous stem cell rescue and panel 5, at the time of overt CSF relapse. Conventional cytology and immunocytology were positive only at panel 5 when the patient had imaging and clinical evidence of leptomeningeal relapse.

occurred before the second specimen was scheduled. Thus, the negative predictive value for CSF relapse of a negative RT-PCR for GD2 synthase at diagnosis was 0.91.

3.3. Clinical factors associated with MDD

MDD was significantly correlated with massive optic nerve involvement (either tumour at the resection margin on initial enucleation or enlarged optic nerve in imaging studies at diagnosis) and a history of glaucoma (Table 4).

3.4. Outcome of patients according to MDD

The 2-year probability of CSF relapse was 0.50 (95% confidence interval (CI) 0.13–0.88) for children with MDD versus 0.08 (95% CI 0.02–0.46) for those with negative RT-PCR examination of the CSF at diagnosis ($p = 0.03$) (Fig. 2).

4. Discussion

With the use of molecular techniques, we detected MDD in the CSF at diagnosis in a subset of children with high-risk retinoblastoma. To our knowledge, this finding, suggesting that there is occult CSF dissemination already at diagnosis in a higher risk subgroup was not reported previously for this malignancy. It was possible from our data to depict a subgroup of patients at higher risk of MDD since those with massive optic nerve invasion and a history of glaucoma were at a significantly higher risk. This finding concurs with clinical experience since recent reports show that up to 2/3 of children with stage III retinoblastoma and optic nerve enlargement by imaging studies presented a CNS relapse.[6] Massive optic nerve involvement has also been previously reported in association with glaucoma,[24,25] and changes in the CSF kinetics in patients with glaucoma reported in other conditions[26] might favour CSF seeding in retinoblastoma.

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Table 4
Comparative analysis between patients with minimally disseminated disease (MDD) detected by a positive reverse transcriptase-polymerase chain reaction (RT-PCR) for GD2 synthase mRNA and those with a negative test.

	Positive RT-PCR for GD2 synthase mRNA ($n = 6$)	Negative RT-PCR for GD2 synthase mRNA ($n = 13$)	p value
Median age at study entry	29.3	20.6	0.9
Bilateral/unilateral	1/5	6/7	0.2
History of glaucoma	5 (83.3%)	5 (38.4%)	0.01
Systemic metastasis	2 (33.3%)	2 (15.4%)	0.3
Massive optic nerve involvement	3 (50%)	2/12* (16.6%)	0.01
Optic nerve enlargement at imaging studies at diagnosis	3/5 (60%)*	0/11*	0.01
Perineural infiltration in enucleated eye	2/4 (50%)*	0/11*	0.05

* Information not available in all patients.

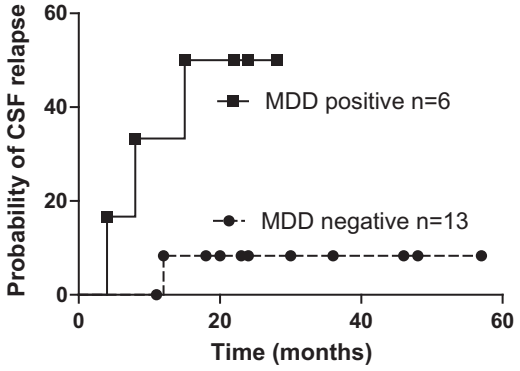

Fig. 2. Probability of cerebrospinal fluid (CSF) relapse according to the presence of minimally disseminated disease (MDD) determined as positivity for GD2 synthase by polymerase chain reaction (PCR) ($p = 0.03$).

Observations from our patient cohort may be useful for inferring patterns of dissemination and clinical relevance of our findings. One of our patients with stage IVa (#1 Table 2 and Fig. 1) had MDD at diagnosis that cleared after induction therapy, but it became positive in the post-transplant evaluation when the child was in clinical complete remission. This heralded the occurrence of an overt CSF relapse a month afterwards. In patient 3, MDD was evident in all the CSF specimens studied and the patient showed leptomeningeal relapse soon afterwards. Another child presenting with massive buphthalmia but no evidence of extraocular disease had MDD at diagnosis. We used pre-enucleation chemotherapy in these children in order to obtain a safer enucleation after reduction of the tumour mass.[23] So, this child had chemotherapy immediately after diagnosis, which might have been beneficial for timely treating of MDD since it became negative with treatment and the child remains disease-free for 26 months. Taking this data together, it is possible that a positive test for MDD may herald clinical CSF relapse if serial specimens are studied.

We acknowledge that the prognostic implications of a positive MDD evaluation at diagnosis, though significantly associated to increased risk of CNS relapse, need to be confirmed in a larger cohort. Even though all our patients presenting with stage II–IVa were sequentially evaluated, the population of stage I and high risk features that could be evaluated serially were limited because of restrictions of procurement of CSF specimens in follow-up examinations. Therefore, even though we included all patients studied with serial CSF evaluations in the period, patients with bilateral disease were over-represented because serial CSF evaluation could be obtained during follow-up examinations under anaesthesia in them. Hence, there might be a selection bias in these lower risk children. In addition, since all children regardless of their MDD status received intensive therapy, this made it impossible to estimate with accuracy the positive predictive value for CSF relapse. In other words, we could not prove that all patients with MDD would eventually relapse because we gave intensive treatment to all of them. On the other hand, we found that, as opposed to a positive CSF by conventional cytology which is almost invariably fatal, a proportion of children with MDD could be salvaged with intensive treatment. Thus, the metastatic potential of some cells or the tumour cell burden may be affected by treatment, so prompt identification of MDD might be used to dictate a more intensive therapy in these selected populations. On the other hand, the negative predictive value of the test was high, even in this high risk population. CSF relapse occurred in only one of 13 children with negative MDD at diagnosis. However, RT-PCR for GD2 synthase mRNA was not informative in about one quarter of our patients and a CSF relapse occurred in two of these children. It is possible that this finding is related to limitations of CSF examination, such as scant cellularity, low volume and evaluation of a limited number of specimens for each patient. Since at the time of study design, the presence of MDD in the CSF was uncertain, we only obtained a limited number of CSF specimens in order to minimise patients' risks. Hence, obtaining a greater CSF volume or repeated samples might increase the number of informative studies and improve the sensitivity of the test.[27] In addition baseline illegitimate GD2 expression in non-malignant cells[28]

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may also give false positive results. Tumours lacking the expression of GD2, as reported in neuroblastoma may also occur[29] which may cause false negative results. Even though the negative predictive value of this test was high, it should be confirmed by studying lower risk patients that were not included in this study because no CSF evaluation was done in them. The results of this study showing that MDD is present in the CSF at diagnosis in high risk patients served as a backbone for our current study that evaluates sequential specimens and the use of more sensitive techniques like real-time PCR for a combination of markers in the higher risk cohorts.

As previously reported in smaller cohorts,[19,30] the use of immunocytological examination for GD2 (coupled with determination of CD45–CD56+ cells by FC) allowed for quick and reliable characterisation of malignant cells in all our cases upon CSF relapse. However, it is not suitable for MDD determination since the presence of a minimum number of cells in the CSF is necessary.[31,32]

To conclude, our results may be considered as a proof of principle that MDD is present in the CSF of children with retinoblastoma without CNS involvement at diagnosis, especially in those who present with massive optic nerve involvement and glaucoma. Even though they had poorer prognosis, some of these children may be salvageable with intensive therapy, justifying a more intensive work up for accurate risk estimation.

Role of the funding sources

The founding sources had no role in the study design, in the collection, analysis and interpretation of data; in the writing of the manuscript; and in the decision to submit the manuscript for publication.

Conflict of interest statement

None declared.

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