Etoposide upregulates survival favoring sphingosine-1-phosphate in etoposide-resistant retinoblastoma cells

V Kakkassery[1 u 2], S Skosyrski[1], RA Lüth[3], B Kleuser[3], M van der Giet[4], R Tate[5], J Reinhard[6], A Faissner[6], SC Joachim[2], N Kociok[1]

[1] Department of Ophthalmology, Charité Universitätsmedizin, Berlin, Germany

[2] Department of Ophthalmology, Ruhr-University, Bochum, Germany

[3] Department of Nutrition Science, University Potsdam, Germany

[4] Department of Nephrology, Campus Benjamin Franklin, Charité Universitätsmedizin, Berlin, Germany

[5] Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, Glasgow, United Kingdom

[6] Department of Cell Morphology and Molecular Neurobiology, Faculty of Biology and Biotechnology, Ruhr-University, Bochum, Germany

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Correspondence to:

Vinodh Kakkassery, MD

Universitätsklinikum Knappschaftskrankenhaus Bochum - Ruhr University Bochum

In der Schornau 23-25

44892 Bochum, Germany

Phone: +49-234-299-3101

Fax: +49-234-299-3109

E-mail: vk@rub.de

Abstract

Introduction: Improved knowledge of retinoblastoma chemotherapy resistance is needed to raise treatment efficiency. The objective of this study was to test whether etoposide alters glucosyl-ceramide, ceramide, sphingosine and sphingosine-1-phosphate (sphingosine-1-P) levels in parental retinoblastoma cells (WERI Rb1) or their etoposide-resistant subclones (WERI EtoR).

Material and methods: WERI Rb1 and WERI EtoR were incubated with 400 ng/ml etoposide for 24 hours. Levels of glucosyl-ceramides, ceramides, sphingosine, sphingosine-1-P were detected by Q-TOF mass spectrometry. Statistical analysis was done by ANOVA followed by Tukey post-hoc test ($p<0.05$)

The mRNA expressions of sphingolipid pathway enzymes in WERI Rb1, WERI EtoR and four human retinoblastoma tissue samples were analyzed by quantitative real time PCR.

Results: Pathways enzymes mRNA expression confirmed similarities of human sphingolipid metabolism in both cell lines and tissue samples, but different relative expression. Significant up-regulation of sphingosine was seen in both cell lines ($p<0.001$). Only sphingosine-1-P up-regulation was significantly increased in WERI EtoR ($p<0.01$), but not in WERI Rb1 ($p>0.2$).

Conclusion: Both cell lines upregulate pro-apoptotic sphingosine after etoposide incubation, but only WERI EtoR produces additional survival favorable sphingosine-1-P. These data may suggest a role of sphingosine-1-P in retinoblastoma chemotherapy resistance, although this seems not to be the only resistance mechanism.

Introduction

Retinoblastoma is the most common malignant eye tumor of the childhood with a lethality rates that reaches up to 95% if left untreated (1, 2). Currently, the most effective treatment in retinoblastoma is the surgical removal/enucleation of the affected eye, with obvious disadvantages for the child (2). In bilateral involvement, chemotherapy is administered in order to facilitate partial tumor regression and size reduction, but chemotherapy resistance frequently leads all too often to limited efficiency (3-10). Therefore, new adjuvant therapy is needed to raise therapy efficiency in chemotherapy resistant retinoblastoma.

Recent advances in research have elucidated a central role of sphingolipids in cellular apoptosis and survival signaling mechanisms (11). Of particular interest is the apoptotic effect of up-regulation of glucosyl-ceramide, ceramide, and sphingosine expression, whereas sphingosine-1-phosphate (sphingosine-1-P) upregulation induces cell survival (11) (see **fig 1**). The apoptotic effect of sphingolipids has been demonstrated in various tumors including stomach, lung and kidney cancer, non-Hodgkin-lymphoma, colon adenomacarcinoma, astrocytoma, glioblastoma, and breast cancer (12-21). Furthermore, glucosyl-ceramide, ceramide and sphingosine up-regulation has been associated with increased chemotherapy efficiency in *in vitro* and *in vivo* tumor models (22-24). In contrast, sphingosine-1-P down-regulation has been accompanied with increased chemotherapy efficiency in tumor cells (25-28).

Therefore, we hypothesized, that chemotherapy treatment in resistant retinoblastoma cells induces sphingosine-1-P expression, thus reducing chemotherapy efficiency. The objective of this study was to test, whether etoposide alters the level of glucosyl-

ceramide, ceramide, sphingosine, and sphingosine-1-P in parental retinoblastoma cells (WERI Rb1) or their etoposide-resistant subclone (WERI EtoR).

Methods

Authentication verifications of WERI Rb cell lines

DNA fingerprinting of both human WERI Rb cell lines was confirmed by carried out DNA profiling using 8 different and highly polymorphic short tandem repeat (STR) loci. Authentication verifications were done by Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Cultures (www.dsmz.de). Generated STR profiles of used cell lines showed a full match of the respective parental reference STR profiles as indicated by a search of the database of cell banks ATCC (USA), JCRB (Japan), RIKEN (Japan), KCLB (Korea) and DSMZ. Samples of WERI Rb1 and WERI EtoR were taken from authenticated cell cultures.

Also, both WERI Rb cell lines were tested for presence of mitochondrial DNA sequences from rodent cells as mouse, rat, Chinese and Syrian hamster. Results revealed no detection of any mitochondrial sequences from mouse, rat or Chinese and Syrian hamster cells in the human samples at a detection limit of $1:10^5$. The samples are derived of pure human cell cultures.

WERI Rb1 and WERI Rb1 etoposide-resistant subclones (WERI EtoR)

The retinoblastoma cell line WERI Rb1 and the etoposide-resistant WERI Rb1 (WERI EtoR) subclone were a courtesy of Dr. H. Stephan (University Hospital, Essen, Germany) (29). Both cell lines were cultivated in cell culture medium containing DMEM supplemented with 15% FBS, 4 mM L-glutamine, 50 mM ß-mercaptoethanol, 10 mg insulin/mL, and penicillin/streptomycin at 37 °C at 10% CO_2 and 80% humidity (30). DAPI/propidium iodide assays to determine apoptosis/necrosis rate confirmed chemotherapy sensitivity of WERI Rb1, but resistance of WERI EtoR after 400 ng/ml

etoposide incubation for 24 hours (**fig 2**).

Glucosyl-ceramide, ceramide, sphingosine and sphingosin-1-P quantification

WERI Rb1 and WERI EtoR were incubated with DMEM supplemented with 400 ng/ml etoposide or vehicle control for 24 hours. Ceramides, sphingosine and S1P were extracted and quantified as recently described (31-33). Briefly, lipid extraction of cells was performed using C17-sphingosine, C17-S1P and C17-ceramide as internal standards. Sample analysis was carried out by rapid resolution LC-MS/MS using a QTOF 6530 mass spectrometer (Agilent Technologies, Waldbronn, Germany) operating in the positive ESI mode. The precursor ions of sphingosine (m/z 300.289,) C17-sphingosine (m/z 286.274), S1P (m/z 380.256), C17-S1P (m/z 366.240), C16-ceramide (m/z 520.508) and C17-ceramide (m/z 534.524) were cleaved into the fragment ions of m/z 282.280 for sphingosine, m/z 268.264 for C17-sphingosine, m/z 264.270, m/z 250.252 for S1P, m/z 264.270 for C17-S1P and m/z 264.270 for ceramides, respectively. Quantitation was performed with Mass Hunter Software.

Quantitative PCR (RT-PCR) for sphingolipid enzyme miRNA in retinoblastoma samples and cell lines

To confirm the expression of sphingolipid enzyme mRNA, we used tumor tissue from four anonymous diagnosed retinoblastoma patients after enucleation. Biodata have been summarized in tab 1. Ethic committee approval of Charité Universitätsmedizin (Berlin, Germany) was obtained and the study was conducted following the guidelines of the Helsinki declaration. To obtain mRNA, 5×10^6-1×10^7 sensitive WERI Rb1 (n=5) and resistant WERI EtoR cells (n=5) were snap frozen in liquid nitrogen and stored at -80°C

until RNA isolation. Total RNA was extracted from four different retinoblastoma tumor tissues. The Gene Elute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, Munich, Germany) was used for total RNA extraction following manufacturer's instructions. Purity and concentration of RNA was quantified in a BioSpectrometer (Eppendorf, Hamburg, Germany). For cDNA synthesis, 1 µg total RNA was reverse-transcribed to cDNA using the First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Darmstadt, Germany).

Quantitative Real-time PCR

The gene expression levels in the samples were determined by reverse-transcription quantitative real-time PCR (RT-qPCR) using PowerUp^{TM} SYBR Green chemistry (Thermo Fisher Scientific, UK). PCR primers for the genes of interest were designed using Primer-BLAST (34). Where possible, the primers spanned exons and all of PCR products were within the 136-150 bp size range. Primer sequences are listed in tab 2. Beta-2-microglobulin (B2M; NM_004048.2) was chosen as the reference gene for normalization purposes. 2.5 ng of cDNA was used per 10 µl RT-qPCR with 500 nM of the sense and antisense primers and run on a StepOnePlus real-time PCR system (Applied Biosystems, UK). PCRs were carried out in triplicate per primer set per sample. The resultant cycle threshold values (C_T) were used to obtain the delta C_T ($\Delta C_T = C_T$ target gene – C_T B2M reference gene) for each sample. The normalized expression levels of the genes of interest were calculated as $2^{\wedge(-\Delta CT)}$.

Statistical analysis

Data are presented as mean ± SD and mean ± SEM unless otherwise stated. Statistical Analysis was done by ANOVA followed by a Tukey post-hoc test using Statistical

Software (V10.0, Statsoft, Tulsa, Ok, USA). P-values below 0.05 were considered statistically significant.

Results

Retinoblastoma tissue biodata

Four human retinoblastoma patients' samples were included in this study. One patient had no treatment before enucleation, one had chemotherapy before, one had beam radiation before and one had combined chemotherapy/beam radiation before. Three patients experienced bilateral retinoblastoma and one patient only unilateral retinoblastoma. None of the four patients have been diagnosed with metastasis or had risk factors for metastasis. All data have been displayed in **tab 1**.

Etoposide-induced upregulation of apoptotic sphingosine in WERI Rb1 and WERI EtoR

WERI Rb1 vehicle control, WERI Rb1 400 ng/ml etoposide, WERI EtoR vehicle, and WERI EtoR 400 ng/ml etoposide were incubated with DMEM supplemented with etoposide or vehicle control and harvested after 24 hours. Levels for apoptotic glucosyl-ceramide, ceramide and sphingosine were evaluated by mass spectroscopy for all treatment groups. Statistical significant upregulation of apoptotic sphingosine was seen after etoposide treatment in WERI Rb1 and WERI EtoR ($p<0.01$, **fig 3 A**, absolute values and SD values **tab 3**). Slight downregulation of ceramide and glu-ceramide were seen after etoposide treatment in WERI Rb1 and WERI EtoR, but this was only statistical significant for ceramide in WERI Rb1 after etoposide treatment ($p<0.5$; **fig 3 B and D**, absolute values and SD values **tab 3**).

Etoposide-induced upregulation of survival favoring sphingosine-1-P only in etoposide resistant WERI EtoR

WERI Rb1 vehicle control, WERI Rb1 400 ng/ml etoposide, WERI EtoR vehicle, and WERI EtoR 400 ng/ml etoposide were incubated with DMEM supplemented with etoposide or vehicle control for 24 hours and were harvested. Levels for survival favoring sphingosine-1-P were evaluated by mass spectroscopy. Statistical significant upregulation of sphingosine-1-P was seen after etoposide treatment in therapy resistant WERI EtoR, but not in therapy sensitive WERI Rb1 ($p<0.01$, **fig 3 C**, absolute values and SD values **tab 3**)

Quantitative sphingolipid enzyme expression in human retinoblastoma samples and in WERI Rb1 and WERI EtoR

mRNA expression of sphingolipid metabolism enzymes was measured by quantitative PCR in four human retinoblastoma samples and in WERI Rb1 and WERI EtoR. **Fig 4** displays the relative expression of sphingosine kinase 1 (SPHK1), sphingosine kinase 2 (SPHK2), sphingosphingosine-1-phosphate receptor 1 (S1P1), sphingosphingosine-1-phosphat receptor 2 (S1P2), sphingosphingosine-1-phosphate receptor 3 (S1P3), sphingosphingosine-1-phosphate receptor 4 (S1P4), sphingosphingosine-1-phosphate receptor 5 (S1P5), sphingosphingosine-1-phosphate phosphatase 1 (SGPP1), sphingosphingosine-1-phosphate phosphatase 2 (SGPP2), ceramide synthase 1 (LASS1), ceramide synthase 2 (LASS2), ceramide synthase 3 (LASS3), ceramide synthase 4 (LASS4), ceramide synthase 5 (LASS5), ceramide synthase 6 (LASS6), acid ceramidase 1 (ASAH1), acid ceramidase 2 (ASAH2), alkaline ceramidase 1 (ACER1), alkaline ceramidase 2 (ACER2), alkaline ceramidase 3 (ACER3), ceramide kinase (CERK), ceramide kinase like (CERKL), sphingomyelin synthase 1 (SGMS1), sphingomyelin synthase 2 (SGMS2), acid sphingomyelinase 1 (SMPD1), neutral

sphingosphingomyelinase 2 (SMPD2), neutral sphingomyelinase 3 (SMPD3), neutral sphingomyelinase 4 (SMPD4), alkaline sphingomyelinase (ENPP7)). No expression or almost no expression has been detected for ACER1, ASAH2, ENPP7, S1P1 S1P4 and S1P5 in retinoblastoma tissue, WERI Rb1 and WERI EtoR. For ASAH1, CERK, LASS1, LASS2, LASS6, S1P3, SGPP1, SGPP2, SMPD2 and SPHK1 lowest expression was seen in retinoblastoma tissue and highest in WERI EtoR.

Taken together, the similar sphingolipid enzyme mRNAs, and especially SPHK1 and SPHK2, are at all expressed in four human retinoblastoma samples and in WERI Rb1 and WERI EtoR. Differences have been found between native tissue and both cell lines in relative expression of these enzymes.

Discussion

The objective of this study was to test, whether etoposide alters the level of glucosyl-ceramide, ceramide, sphingosine, and sphingosine-1-P in parental retinoblastoma cells (WERI Rb1) or their etoposide-resistant subclone (WERI EtoR). Data from this study demonstrate significant upregulation of sphingosine expression following etoposide treatment in WERI Rb1 and WERI EtoR and therefore presume an apoptotic signal in both cell lines. Up-regulation of survival favoring sphingosine-1-P was only seen in therapy-resistant WERI EtoR following etoposide treatment, but not in chemotherapy-sensitive WERI Rb1. Therefore, we assume a survival signal in WERI EtoR, which might explain etoposide-resistance. In accordance with the sphingosine data, small decrease in glucosyl-ceramide and ceramide levels was detected following etoposide therapy, which might be a result of a consumptive shift to sphingosine in WERI Rb1 and WERI EtoR. As etoposide-induced downregulation of apoptotic ceramide and at the same time cell death in WERI Rb1, this suggests that there might be other apoptotic mechanisms leading to etoposide-vulnerable WERI Rb1, which is not in WERI EtoR. Taken together, these data may indicate a functional role of sphingosine-1-P in retinoblastoma chemotherapy resistance, but seems not to be the only resistance mechanism.

Experiments to demonstrate sphingolipid differences following treatment were conducted with previously described etoposide-sensitive WERI Rb1 and etoposide-resistant WERI EtoR (29). Key analysis of our study, measurements of sphingosine, sphingosine-1-P, glucosyl-ceramide and ceramide, were performed by Q-TOF mass spectrometry, as described before (31-33). Realizing the role of sphingolipid enzymes, and especially SPHK1 and SPHK2, in sphingosine-1-P chemotherapy resistance of different tumor cells, we investigated their mRNA expression in four human

retinoblastoma samples and in WERI Rb1 and WERI EtoR in this study. Therefore, we have not only seen upregulation of sphingosine-1-P in retinoblastoma chemotherapy-resistant cells, but also demonstrated similarity of sphingolipid metabolism in the used WERI Rb1 and WERI EtoR cell models as well in human retinoblastoma tissue.

Our results are consistent with findings from other study groups for sphingosine-1-P and SPHK1 and 2 in other diverse tumors. For example, SPHK1-siRNA-knockout experiments showed a reduction of sphingosine-1-P-levels and a reduced proliferation in prostatic tumor cells, pancreatic tumor cells and leukemia cells (25-28, 35). Furthermore, sphingosine-1-P expression upregulation was seen after treatment in daunorubicin-resistant leukemia (28, 35). Interestingly, increased SPHK1 expression have been also demonstrated in human samples of stomach tumors, lung tumors, kidney tumors, non-Hodgkin-lymphoma, colon carcinoma, astrocytoma, glioblastoma and breast cancer (12-21). In particular, in breast cancer, high SPHK1 expression is associated with unfavorable prognosis (20, 21). Furthermore, in-vitro-SPHK1-overexpression leads to transformation of benign NIH3T3 fibroblasts into fibrosarcomas (36). This phenomenon is reversible after competitive SPHK1 inhibition with N,N'-dimethyl-sphingosine (36). Moreover, SPHK1 expression levels are positive correlated with chemotherapy resistance in prostatic tumor cells, pancreatic tumor cells and leukemia cells (25-28). Also, increased doxorubicin-induced apoptosis rates are demonstrated in breast cancer, gliobastoma and colon tumor SPHK2-siRNA-knockout-celllines (16, 37).

Further experiments manipulating sphingolipid metabolism enzymes in chemotherapy-resistance retinoblastoma cells are planned in order to better understand the significance of sphingosine-1-P function in retinoblastoma chemotherapy resistance and chemotherapy vulnerability in these cells.

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Conflict of Interest for all authors: NONE

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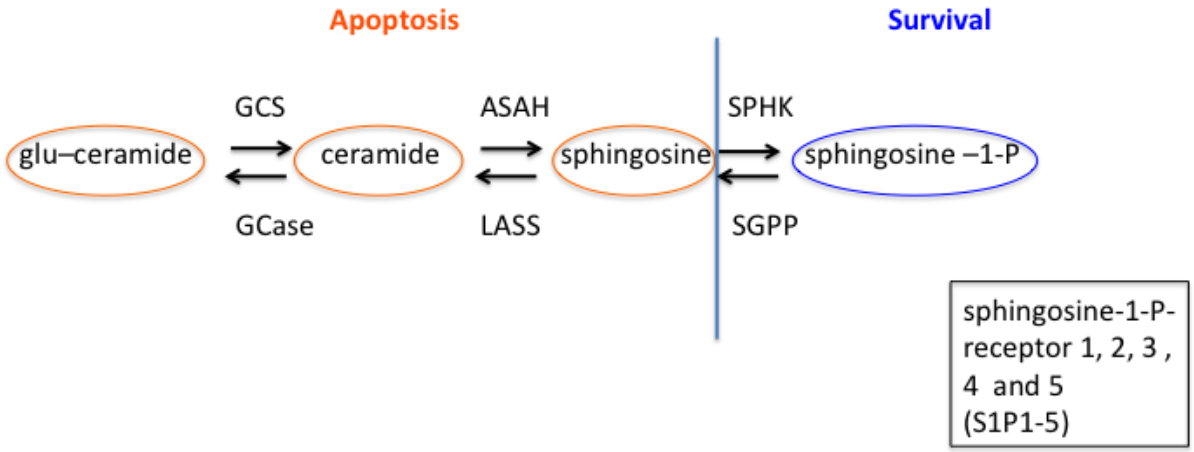

Fig 1 Schematic cascade of sphingolipid metabolism

Three sphingolipids (glu-ceramide, ceramide, sphingosine) induce apoptosis, whereas sphingosine-1-P sends a survival signal. The figure involves the enzymes Glu-Ceramide-Synthase (GCS) Glu-Ceramidase (GCS), Ceramide-Synthase (LASS), Ceramidase (ASAH), Sphingosinekinase (SPHK) and Sphingosinephosphatase (SGPP). The box below lists sphingosine-1-P-receptors.

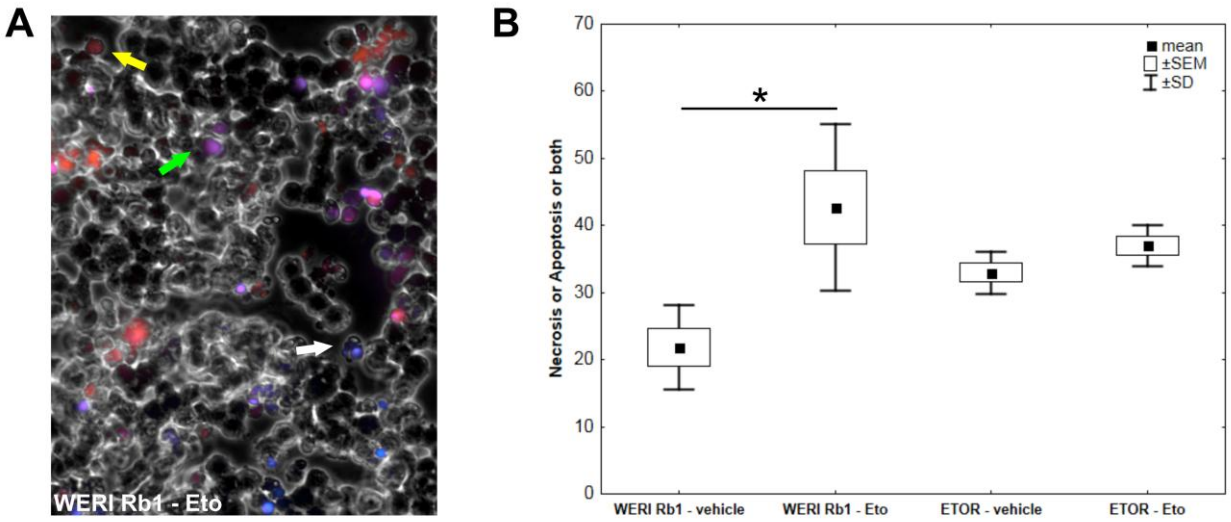

Fig 2 DAPI/propidium iodide assays

A) Assay example shows apoptosis/necrosis rate in retinoblastoma cells after etoposide treatment. The yellow arrow marks a propidium iodide positive necrotic cell. The white arrow marks a DAPI positive apoptotic cell. The green arrow marked a DAPI/propidium iodide negative, alive cell.

B) Assay example shows a higher vulnerability of WERI Rb1 to etoposide (x axis: treatment group and vehicle control of each cell line; y axis: percentage of necrotic or apoptotic cells to all cells; n=5). Statistical significant differences and p-values are integrated in the figure.

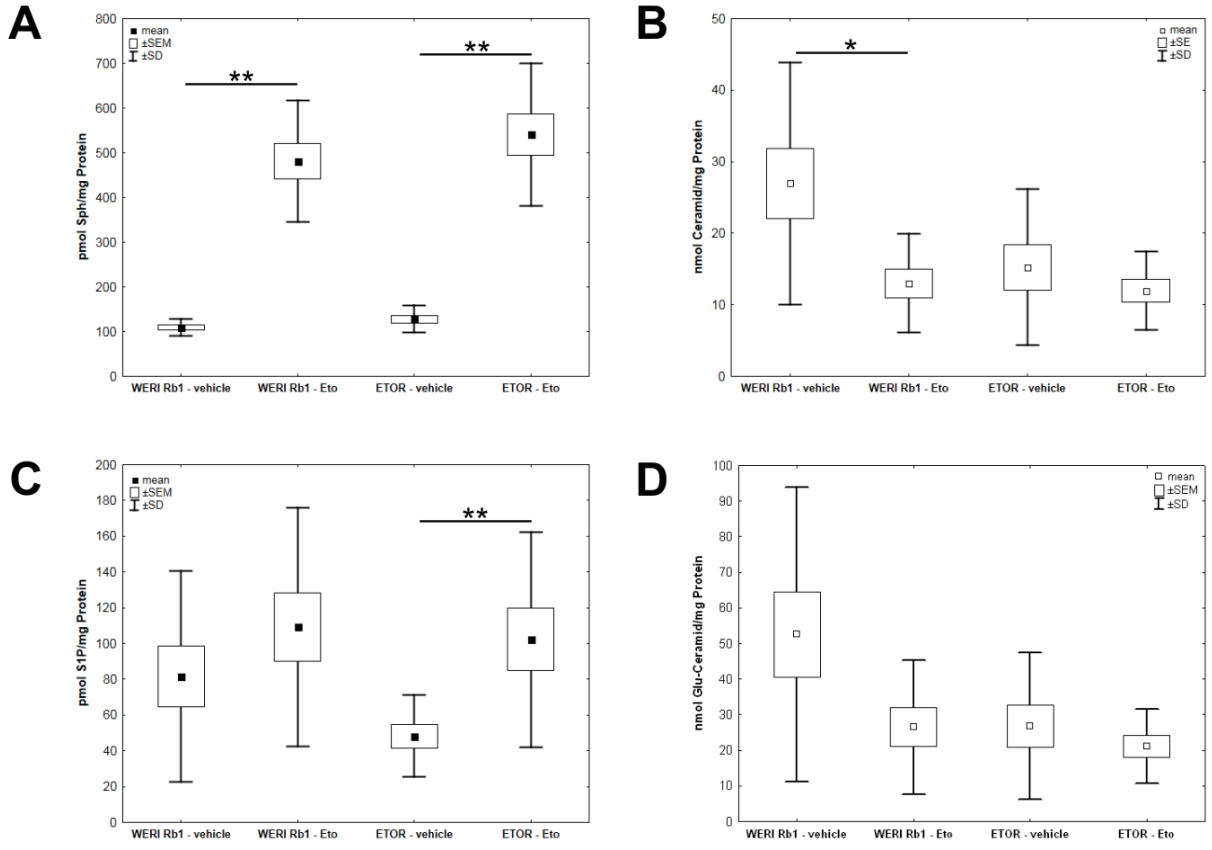

Fig 3 Sphingolipid measurements

Graphic shows statistical significant up-regulation of apoptotic sphingosine WERI-Rb1 and etoposide-resistant WERI EtoR (A, $p < 0,01$), but only statistical significant up-regulation of survival-favoring sphingosine-1-P in etoposide-resistant WERI EtoR ($p < 0,01$). Further (A) small down-regulation of ceramide (B) and glu-ceramide (D) was seen in WERI Rb1 and WERI EtoR after etoposide treatment was seen both celll lines ($p < 0,05$ for ceramide downregulation in WERI Rb1 after etoposide treatment, all other $p > 0,1$). Treatment or vehicle group (WERI Rb1 or WERI EtoR) are on the x-axis and amount of sphingosine (A, pmol per mg protein), ceramide (B, nmol per mg protein), sphingosine-1-P (C, pmol per mg protein) and glu-ceramide (D, nmol per mg protein) are

on the y-axis. Mean values (mean), standard diviation values (SD) and standard error of the mean (SEM) has been shown as box plots. Statistical analysis was done by Anova with Tukeys post-test. P values < 0,05 has been marked with * and p values < 0,01 has been marked with **.

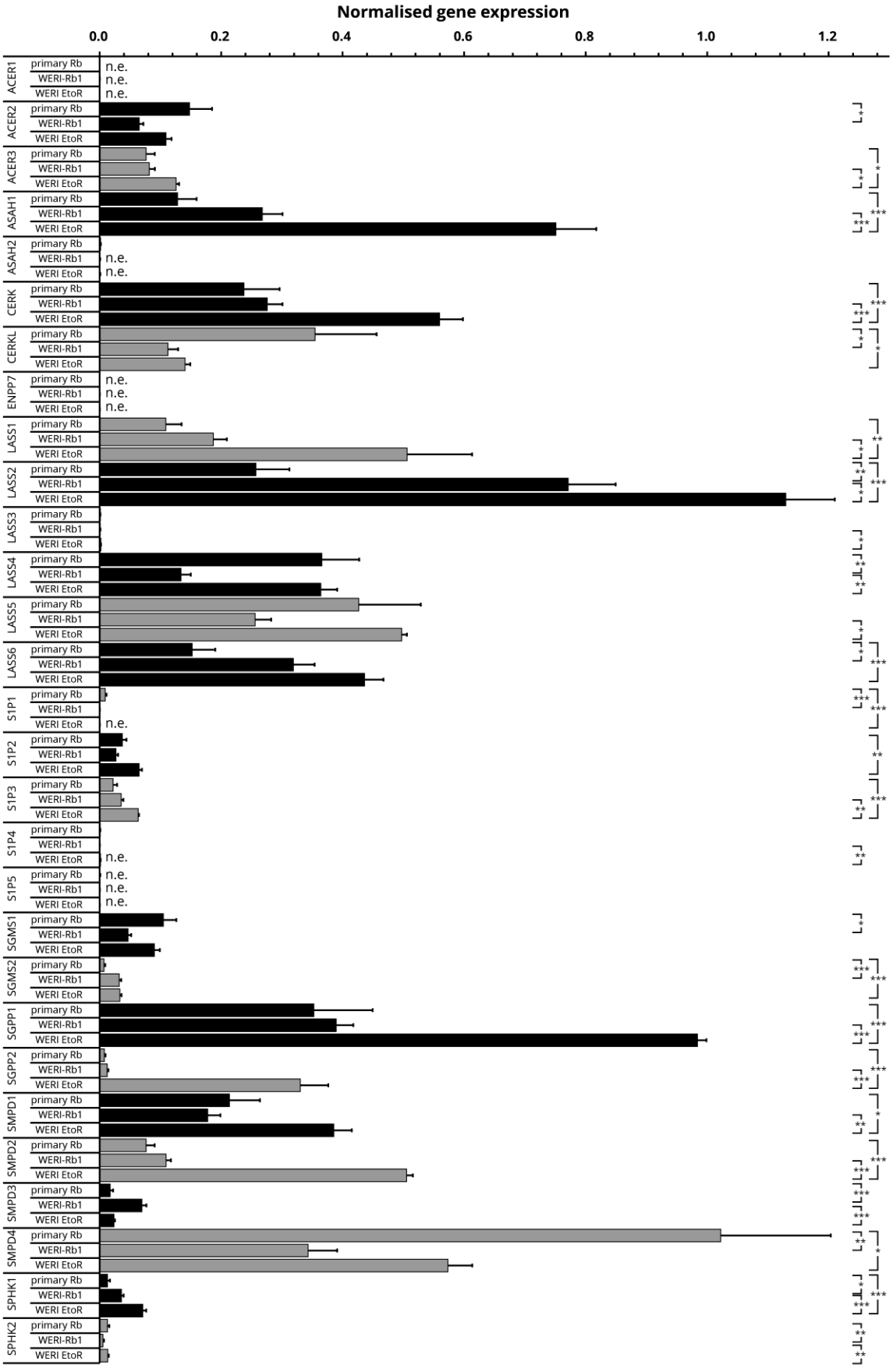
Normalised gene expression
0.0
0.2
0.4
0.6
0.8
1.0
1.2
ACER1
ACER2
ACER3
ASAH1
ASAH2
CERK
CERKL
ENPP7
LASS1
LASS2
LASS3
LASS4
LASS5
LASS6
S1P1
S1P2
S1P3
S1P4
S1P5
SGMS1
SGMS2
SGPP1
SGPP2
SMPD1
SMPD2
SMPD3
SMPD4
SPHK1
SPHK2
primary Rb
WERI-Rb1
WERI EtoR
n.e.

Fig. 4 Normalized gene expressions of proteins of the ceramide pathway in retinoblastoma tissue, WERI-Rb1 and WERI EtoR

mRNAs with CT values above 40.0 are considered as not expressed (n.e.). Normalization was done using the expression of ß2-microglobulin as reference. Expression levels are calculated from RT-qPCR ΔC_T means as $2^{(-\Delta CT)}$. Statistical analysis was done by Anova with Tukeys post-test. p values < 0.05 are set as significant (*); p < 0.01 as **, p < 0.001 as ***.

patient	clinical TNM classification	pathological TNM classification	age	histology	previous treatment	unilateral/ bilateral	isolated/ heritable	risk factors
1	ycTxNxMxRxLxVx	ypT1NxMXRxLxVx	2m	endophytic	non	bilateral	unknown	none
2	ycT1NxMxRxLxVx	ypT1NxMXRxLxVx	6y 6m	exophytic	5 cycles chemotherapy	bilateral	isolated	none
3	ycT1NxMxRxLxVx	ypT1NxMXRxLxVx	1y 8m	exophytic	7 cycles chemotherapy external beam radiotherapy	bilateral	unknown	none
4	ycT2NxMxRxLxVx	ypT1NxMXRxLxVx	6y 5m	endophytic	external beam radiotherapy	unknown	unknown	none

Tab. 1 Clinical and histopathological features of the four patients with retinoblastoma

Clinical and pathological TNM classification, age, histology, previous treatment, previous treatment, unilateral or bilateral cases, isolated or heritable, and risk factors for metastasis have been listed.

Sequence Name	Sequence
LASS1_FOR	GTACATCGTGGCGTTTGCAG
LASS1_REV	AAGCGCTTGTCCTTCACCAG
LASS2_FOR	AGCCCAAGCAGGTGGAAGTA
LASS2_REV	ATCTCCAGCTGGCTTCTCGG
LASS3_FOR	GGAAGCTTGCTGGAGATTTGC
LASS3_REV	CAGTACTGGGATGGCAGCAG
LASS4_FOR	CGTGGAGGAAGAGGCATTGAG
LASS4_REV	TGTCCACGTGACATTGGGTG
LASS5_FOR	GGTTTCGCCATCGGAGGAAT
LASS5_REV	TGTCGGATGTCCCAGAACCA
LASS6_FOR	GCGCCATAGCCCTCAACATT
LASS6_REV	CCCAGTCCAGTTGCTTGGAG
SGPP1_FOR	TCTTATTCCCTGCTGGTGTTCTC
SGPP1_REV	GTTGAAGTTGTCAATCAGGTCCAC
SGPP2_FOR	CATACGGTCCTGGATGTGCTG
SGPP2_REV	TGGCACAACTATGACACACACG
SGMS1_FOR	TTCAGCGACTGAAGGAGTGTG
SGMS1_REV	TGGTGTGCCTGAATCATGCTG
SGMS2_FOR	CTCTACCTGTGCCTGGAATGC
SGMS2_REV	CGCTGAAGAGGAAGTCTCCAC
SMPD1_FOR	TGGCTGCTCAGTTCTTTGGC
SMPD1_REV	TGGTACACACGGTAACCAGGA
SMPD2_FOR	TTTGCTGGAGGAGGTGTGGAG
SMPD2_REV	AAGCTCCTGGATTGGATGTTTGG
SMPD3_FOR	GTGTAACGACGATGCCCTGG
SMPD3_REV	GGATGGCGCTGTCCTCTTG
SMPD4_FOR	GGGGCGGTACCAGATCATCA
SMPD4_REV	TGTGGTTGATGGCAGACGAC
ENPP7_FOR	TGCTGCACACAGAATCTGCTC
ENPP7_REV	GGCGTTATGCGACCTCAGAC
ASAH1_FOR	CTGTCCTCAACAAGCTGACCG
ASAH1_REV	TGTCTCAGAGGCCGCATTCT
ASAH2_FOR	GGGCCATTTGTAGCAGCCTT
ASAH2_REV	AATGCACATGCTAGGCCCAC
ACER1_FOR	GTGGACTGGTGTGAGAGCAAC
ACER1_REV	ACGTAAATGTAGCGGGAGCG
ACER2_FOR	GTGTCCTGTCTGCGGTTACG
ACER2_REV	AGGCCCAGCTTAAACACACG

ACER3_FOR	CCGAGTTCTGGAATACAGTGAGTAA
ACER3_REV	GAAGCACCAGGATCCCATTCC
CERK_FOR	CTGCACGGTCTGATTGGGAG
CERK_REV	CCACGGTGGAGTAACACACG
CERKL_FOR	TCGATGGATGTCCCCTAACCA
CERKL_REV	TCCCTGTGCCCTCCTTTCTT
SPHK1_FOR	TGGCTGAGGCTGAAATCTCCT
SPHK1_REV	CGTTCACCACCTCGTGCAT
SPHK2_FOR	TTCCGGAAGAAAGGGATCTGGG
SPHK2_REV	GACCTTCAGCTCTCCAACACTG
S1P1_FOR	TGAGCGAGGCTGCGGTT
S1P1_REV	CGCTACTCCAGACGAACGCTA
S1P2_FOR	GCCTAGCCAGTTCTGAAAGCC
S1P2_REV	GAGGTCGTCTCCTGCGTTTC
S1P3_FOR	GACGGAGGAGCCCTTTTTCAAC
S1P3_REV	GGCATTCACAGACGATTAGCTCC
S1P4_FOR	CGCGACGCTGGGTCTACTAT
S1P4_REV	CCCTCCCGTAGGAACCACTG
S1P5_FOR	CTCACTCGGTTCAAGGCAGC
S1P5_REV	GGAGCTTGCCGGTGTAGTTG
B2M_FOR	CTACACTGAATTCACCCCCACTG
B2M_REV	AATGCGGCATCTTCAAACCTCC

Tab. 2 Quantitative Real-time PCR primer for sphingolipid enzymes

PCR primer sequences used for quantitative PCR of sphingolipid gene expression: sphingosine kinase 1 (SPHK1), sphingosine kinase 2 (SPHK2), sphingosphingosine-1-phosphate receptor 1 (S1P1), sphingosphingosine-1-phosphat receptor 2 (S1P2), sphingosphingosine-1-phosphate receptor 3 (S1P3), sphingosphingosine-1-phosphate receptor 4 (S1P4), sphingosphingosine-1-phosphate receptor 5 (S1P5), sphingosphingosine-1-phosphate phosphatase 1 (SGPP1), sphingosphingosine-1-phosphate phosphatase 2 (SGPP2), Ceramide synthase 1 (LASS1), ceramide synthase 2 (LASS2), ceramide synthase 3 (LASS3), ceramide synthase 4 (LASS4), ceramide

synthase 5 (LASS5), ceramide synthase 6 (LASS6), acid ceramidase 1 (ASAH1), acid ceramidase 2 (ASAH2), alkaline ceramidase 1 (ACER1), alkaline ceramidase 2 (ACER2), alkaline ceramidase 3 (ACER3), ceramide kinase (CERK), ceramide kinase like (CERKL), sphingomyelin synthase 1 (SGMS1), sphingomyelin synthase 2 (SGMS2), acid sphingomyelinase 1 (SMPD1), neutral sphingomyelinase 2 (SMPD2), neutral sphingomyelinase 3 (SMPD3), neutral sphingomyelinase 4 (SMPD4), alkaline sphingomyelinase (ENPP7).

sphingolipids	WERI RB ETOR Vehicle	WERI RB ETOR Etoposid	WERI Normal Vehicle	WERI Normal Etoposid
nmol Glu-ceramide /mg Protein	26,77 (+/-20,54)	21,1 (+/-10,43)	52,56 (+/-41,40)	26,45 (+/-18,89)
nmol ceramide /mg Protein	15,22 (+/-10,90206)	11,95 (+/-5,45785)	26,95 (+/-16,8696)	13 (+/-6,87694)
nmol sphingosine /mg Protein	0,12 (+/-0,03)	0,54 (+/-0,16)	0,11 (+/-0,02)	0,48142 (+/-0,13)
nmol sphingosine-1-P /mg Protein	0,05 (+/-0,02)	0,1 (+/-0,06)	0,08 (+/-0,06)	0,11 (+/-0,07)

Tab. 3 Absolute Sphingolipid values

Absolute values of sphingosine, ceramide, sphingosin-1-P and, Glucosyl-ceramide for each treatment group (WERI Rb1 vehicle, WERI Rb1 etoposide, WERI EtoR vehicle, WERI EtoR etoposide) were given in nmol per mg total protein.