Translational evidence for RRM2 as a prognostic biomarker and therapeutic target in Ewing sarcoma

Shunya Ohmura1,2,3, Aruna Marchetto3, Martin F. Orth3, Jing Li1,2, Susanne Jaber4,5, Andreas Ranft4,5, Endrit Vinca1,2, Katharina Ceranski1,2, Martha J. Carreno-Gonzalez1,2, Laura Romero-Perez1,2,3, Fabienne S. Wehweck3, Julian Musa1,2,3, Felix Bestvater3, Maximilian L. Knott3, Tilman L. B. Holting3, Wolfgang Hartmann6, Uta Dirksen4,5, Thomas Kirchner9,10, Florencia Cidre-Aranaz1,2,3 and Thomas G. P. Grunewald1,2,3,11*

Keywords: Ewing sarcoma, RRM2, Targeted therapy, Prognostic biomarker, Paediatric oncology, Triapine, Chemoresistance

Main text

Ewing sarcoma (EwS) is an aggressive bone- or soft tissue-associated malignancy, characterised by the fusion oncoprotein EWSR1-FLI1 [1]. Over the past decades further therapeutic development for this devastating childhood tumour has remained relatively stagnant [2], especially for patients with metastatic or recurrent disease [3, 4]. To develop more effective and specific treatment options we investigated potential therapeutic targets by exploring putative downstream genes of EWSR1-FLI1.

We took advantage of publicly available ‘omics’ data and filtered them in a multi-step approach (Fig. 1a): First, we interrogated a gene expression dataset comprising 50 primary EwS and 929 samples from 71 normal tissue types to identify overexpressed genes (min. log2 fold increase = 2) in EwS, which yielded 292 candidates (Fig. 1b, Supplementary Table 1). Second, we filtered for those genes whose overexpression was significantly negatively correlated with patients’ overall survival in a dataset of matched gene expression and survival data of 166 EwS patients [5] that covered 280 of the 292 overexpressed genes (96%) (Fig. 1c), identifying 22 candidates (Supplementary Table 1). Third, we focused on druggable targets possessing kinase or other enzymatic functions for which specific inhibitors and their pharmacokinetic data were available, but were still not (pre) clinically tested in EwS. This survey identified ribonucleotide reductase regulatory subunit M2 (RRM2) as the single putative target with a prominently negative association with patients’ overall survival (Fig. 1d). The ribonucleotide reductase (RNR) catalyses the conversion of ribonucleoside diphosphates to deoxyribonucleoside diphosphates, the rate-limiting process for de novo deoxyribonucleoside triphosphates synthesis. RNR is composed of two subunits, ribonucleotide reductase catalytic subunit M1 (RRM1) and either RRM2 or ribonucleotide reductase TP53-inducible subunit M2 (RRM2B) [6]. Notably, RRM2B is neither overexpressed in EwS nor negatively correlates with patients’ outcome (Supplementary Figs. 1a,b), and RRM1 is far less overexpressed in EwS compared to RRM2 (Supplementary Figs. 1a,b,c). Similar to primary EwS tumours, assessment of transcriptomes from 18 EwS cell line models (including A-673 and TC-71) also exhibited that, while RRM2 and RRM1 were similarly highly expressed in EwS...
cell line models, RRM2 was on average ~ninefold higher expressed than RRM2B ($P < 0.0001$). These observations, together with the absence of a negative survival association of RRM2B in EwS (Supplementary Fig. 1b), suggested that RRM2B, although being structurally similar to RRM2 [6], may play a subordinate role in EwS.

Prior reports suggested that RRM2 may contribute to the proliferative phenotype of EwS [7, 8]. However, its role in primary EwS tumours remains unclear. To gain first insights into the biological function of RRM2 in EwS, we carried out gene ontology (GO) enrichment analysis of RRM2 co-expressed genes in 166 EwS tumours, which revealed that high RRM2 expression is closely correlated with cell proliferation-associated gene signatures (Fig. 1e), suggesting that high RRM2 expression may contribute to an aggressive clinical course by promoting tumour growth. Next, we analysed the potential association between RRM2 protein levels, clinicopathological prognostic factors, and clinical outcomes in tissue microarrays (TMA) from EwS tumours of 122 patients (Supplementary Table 2, Supplementary Fig. 2a).

In agreement with the findings at the mRNA level (Fig. 1d), high RRM2 protein expression was significantly ($P = 0.0095$) associated with poor overall survival (Fig. 1f). Correspondence analyses of individual cohorts and the joint-cohort (after exclusion of 6 samples (3.6%) from the mRNA-cohort that were in overlap with the TMA cohort) revealed that high RRM2 expression was significantly associated with metastatic disease at diagnosis ($P = 0.0004$) and occurrence of metastatic and/or local relapse ($P = 0.0095$; only available for the TMA cohort) (Supplementary Table 3), supporting that high RRM2 expression promotes an aggressive phenotype. Conversely, RRM2 inhibition by doxycycline (Dox)-inducible shRNA-mediated gene silencing inhibited proliferation and clonogenic growth of three EwS cell lines, and induced cell death in vitro (Supplementary Figs. 2b,c). Consistent with these functional experiments, transcriptome profiling upon RRM2 silencing in two EwS cells demonstrated downregulation of cell cycle and proliferation-associated gene signatures (Supplementary Fig. 2d). Similarly, RRM2 knockout significantly reduced tumour growth of two xenografted EwS cells (Figs. 1g,h). This antineoplastic effect was accompanied by increased apoptosis and DNA damage, as assessed by immunohistochemistry for cleaved caspase 3 (CC3) and γH2A.X, respectively (Fig. 1i, Supplementary Fig. 2e).

Generally, the activity of RNR can be blocked by irreversible RRM1 inhibition using gemcitabine, or by RRM2-specific inhibitors such as hydroxyurea or the more potent compound triapine (alias 3-AP) [6, 9]. Although gemcitabine is used for palliative treatment of EwS patients, EwS tumours rapidly develop a relative resistance [10]. Consistently, we found that long-term treatment of EwS cells with ascending doses of either doxorubicin (A-673, ES7, EW-7, TC-71), gemcitabine (A-673, ES7, TC-71) or triapine (A-673) led to acquisition of relative resistance phenotypes in vitro (Supplementary Fig. 3a), where we noted a relatively fast and strong increase of the relative resistance towards gemcitabine (>2,000-fold increase in IC50 within ~6 weeks), compared doxorubicin (~fourfold increase in ~28 weeks) and triapine (~sevenfold increase in ~20 weeks) (Supplementary Fig. 3b), further suggesting that gemcitabine has limited potential for clinical treatment with curative intent. Thus, we focused on triapine for further functional analyses. First, to assess functional dependency of triapine on RRM2 expression, we performed drug-response assays using triapine in EwS cells with/without RRM2 silencing, which demonstrated that knockdown of RRM2 led to a ~twofold decrease of the IC50 for triapine in A-673 EwS cells, indicating that higher doses of the drug are required to fully block RRM2 activity in case of high RRM2 expression (Supplementary, Figure 3c).
Fig. 1 (See legend on previous page.)
differential effect on sensitivity towards triapine was not observed in A-637 cells expressing a non-targeting control shRNA. Moreover, we observed an ~twofold increase of RRM2 expression in triapine-resistant A-673 (A-673/DR or A-673/TR) compared to parental A-673 EwS cells, suggesting that RRM2 upregulation can be a potential mechanism for acquiring triapine-resistance in A-673 EwS cells (Supplementary Fig. 3d). Dose–response assays revealed that EwS cells were very sensitive towards triapine compared to osteosarcoma cells and non-transformed EwS patient-derived mesenchymal stem cells (mean IC₅₀ values 0.35, 1.63, 101.63 µM, respectively) (Fig. 2a). Likewise, triapine treatment significantly reduced clonogenic growth of EwS cells at clinically relevant doses [11, 12] (Fig. 2b).

Interestingly, doxorubicin or gemcitabine resistant EwS cells (designated EwS/DR or EwS/GR, respectively) still retained triapine sensitivity (Fig. 2c), it should be noted that EwS cells resistant to gemcitabine, triapine, interrupting recovery cycles from methaemoglobin to haemoglobin [15]. To mitigate this toxicity, the small molecule COH29 has been developed that, upon binding to RRM2 subunits, interferes with the molecular interface of RRM1 and RRM2 subunits and thus inhibits its reductase function [14]. Yet, its clinical efficacy and safety remain to be investigated. Another approach for more specific RRM2 inhibition has been undertaken with antisense oligonucleotide-based techniques, exemplified by therapeutic silencing of RRM2 by GTI-2040, which, however, showed little clinical benefit in several clinical trials [16–18]. Hence, despite our data strongly support RRM2 as an actionable and valuable drug target in EwS, and triapine as a potential lead candidate drug for preferential RRM2 inhibition, the development of even more specific RRM2 inhibitors is desirable.

We next explored effective drug combinations with triapine. Based on known functions of RRM2 in DNA synthesis and DNA repair [6] we examined combinatorial applications of triapine with standard chemotherapeutics, doxorubicin, etoposide or vincristine, or poly ADP-ribose polymerase (PARP) inhibitors. Unexpectedly, we observed rather antagonistic effects (Supplementary Figure 4a). To identify rational combinations, we analysed integrated transcriptome profiles of two EwS cells upon RRM2 silencing and triapine treatment, revealing 263 commonly up- and downregulated genes (Supplementary Table 4). GO enrichment analysis demonstrated significant enrichment for cell cycle-associated processes, especially regulation of mitotic cell cycle-associated genes (Fig. 2e), which is consistent with the observation that RRM2 inhibition caused G1/S-phase cell cycle arrest [19, 20]. Thus, we reasoned that RRM2 may synergise with checkpoint inhibitors targeting CHEK1 (checkpoint kinase 1) or WEE1 (WEE1 G2 checkpoint kinase), which were highly significantly (P<0.0001) co-expressed with RRM2 in 166

(See figure on next page.)
EwS tumours (Fig. 2f). In drug combination assays we observed a strong synergism between triapine and a CHEK1 inhibitor (CCT245737) or a WEE1 inhibitor (MK-1775) across four EwS cells (Fig. 2g,h, Supplementary Fig. 4b). Overall, these results provide a rationale for therapeutic combination of triapine with cell cycle checkpoint inhibitors. A recent study pointed out that the drug combination of hydroxyurea and a CHEK1 inhibitor (GDC-0575) can circumvent toxicities caused by the combination of gemcitabine and GDC-0575, which may imply a more manageable combinatory application through RRM2 inhibition and CHEK1 inhibitors [21].
Conclusions
Collectively, our results establish RRM2 as a promising actionable therapeutic target for EwS, even in chemotherapy-refractory cases, and suggest that the combination of triapine with cell cycle checkpoint inhibitors may be highly effective. Moreover, our integrative study of two independent cohorts provides evidence for RRM2 as novel and robust prognostic biomarker that can be readily assessed by immunohistochemistry in routine diagnostics. Thus, our findings may have immediate translational relevance for patients affected by this devastating disease.

Abbreviations
3-AP: 3-Aminopyridine-2-carboxaldehyde thiosemicarbazone; CC3: Cleaved caspase-3; CHEK1: Checkpoint kinase 1; CI: Combination index; Dox: Doxycycline; DR: Doxorubicin-resistant; EwS: Ewing sarcoma; EWSR1: Ewing sarcoma breakpoint region 1; FFPE: Formalin-fixed and paraffin-embedded; FLI1: Friend leukaemia virus integration 1; GO: Gene ontology; GR: Gemcitabine-resistant; GSEA: Gene set enrichment analysis; HPRC: Immunohistochemistry; IRS: Immune reactive score; MSC: Mesenchymal stem cell; NSG: NOD/SCID/gamma; PARP: Poly ADP-ribose polymerase; RNR: Ribonucleotide reductase; RRM1: Ribonucleotide reductase catalytic subunit M1, RRM2: Ribonucleotide reductase regulatory subunit M2, RRM2B: Ribonucleotide reductase regulatory subunit M2; TPS3: Inducible subunit M2; TMA: Tissue microarray; TR: Triapine-resistant; WE1: WE1 G2 checkpoint kinase; WGCNA: Weighted gene co-expression network analysis; ZIP: Zero Interaction Potency.

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s12943-021-01393-9.

Additional file 1. Methods.
Additional file 2: Supplementary Figure 1. RRM1, RRM2, and RRM2B expression in EwS tumours, normal tissues and EwS cell line models, and their association with overall survival in 166 EwS patients. Supplementary Figure 2. RRM2 silencing inhibits cell proliferation and clonogenic growth in EwS in vitro, and representative immunohistochemical staining. Supplementary Figure 3. Development of chemo-resistance in EwS cell lines, functional dependency of triapine on RRM2, and adverse effects of triapine treatment in vivo. Supplementary Figure 4. Drug interaction and combination efficiency of triapine with chemotherapeutics, PARP inhibitors, CHEK1 inhibitor or WE1E1 inhibitor.

Additional file 3: Supplementary Table 1. Overexpressed genes in EwS compared to normal tissues and their prognostic relevance. Supplementary Table 2. Clinicopathological characteristics of EwS patients for the mRNA (discovery) and TMA-cohort (validation). Supplementary Table 3. Multivariate analysis for RRM2 expression and clinical parameters in the mRNA (discovery), TMA-cohort (validation) and joint-cohort. Supplementary Table 4. Commonly regulated genes upon RRM2 silencing and pharmacological inhibition by triapine. Supplementary Table 5. Oligonucleotide sequences.

Acknowledgements
We are grateful to M. Melz for constructing TMA.s, to S. Stein and F. Zahnow for experimental assistance, and to A. Heier and A. Sendelhofert for technical assistance in establishing immunohistochemical staining procedures.

Authors’ contributions
S.O. and T.G.P. conceived the study, wrote the manuscript and designed the figures and tables. S.O., M.F.O., and T.G.P. performed bioinformatic and statistical analyses. S.O. and F.W. scored tissue-microarrays. S.J., A.R., U.D., and W.H. provided gene expression data, tissue-microarrays and helped in statistical analysis of clinical data. M.F.O., J.L., F.C.A., L.R.P., M.M.L.K., T.L.B.H., I.M. contributed to experimental procedures. F.B. and T.K. provided laboratory infra-structure and histological guidance. T.G.P. supervised the study and data analysis. All authors read and approved the final manuscript.

Authors’ information
Not applicable.

Funding
This work was mainly supported by a grant from the Deutsche Forschungsgemeinschaft (DFG-391665916 to S.O.). In addition, the laboratory of T.G.P. is supported the Matthias-Lackas Foundation, the Dr. Leopold and Carmen Ellinger Foundation, the Dr. Rolf M. Schwiete Foundation, the Boehringer-Ingelheim Foundation, the German Cancer Aid (DKH-70112257, DKH-70114111), the Gert and Susanna Mayer Foundation, the SMARCB1-association, and the Barbara and Wilfried Mohr foundation. The working group of U.O. (S.J. and A.R) is supported by the German Cancer Aid (DKH-70113419, DKH-70112418), the Gert and Susanna Mayer Foundation, and the Barbara and Hubertus Trettiner Foundation. T.L.B.H. was supported by a Mildred-Scheel scholarship from the German Cancer Aid.

Availability of data and materials
Original microarray data used in this study were deposited at the National Centre for Biotechnology Information (NCBI) GEO under accession numbers GSE166415 and GSE166419. Custom code is available from the corresponding author upon reasonable request.

Declarations
Ethics approval and consent to participate
Human tissue samples were retrieved from the tissue archives of the Institute of Pathology of the LMU Munich (Germany) or the Gerhard-Domagk Institute of Pathology of the University of Münster (Germany) upon approval of the institutional review board. All patients provided informed consent. Tissue-microarrays (TMAs) were stained and analysed with approval of the ethics committee of the LMU Munich (approval no. 550–16 UE). Animal experiments were approved by the local authorities and performed in accordance with ARRIVE guidelines, recommendations of the European Community (86/609/EEC), and United Kingdom Coordinating Committee on Cancer Research (UKCCCR) guidelines for the welfare and use of animals in cancer research.

Consent for publication
Not applicable.

Competing interests
The authors declare no conflict of interest.

Author details
1Hopp Children’s Cancer Center Heidelberg (KiTZ), Heidelberg, Germany. 2Division of Translational Pediatric Sarcoma Research (B410), German Cancer Research Center (DKFZ) & Hopp-Children’s Cancer Center (KiTZ), Im Neuenheimer Feld 280, 69210 Heidelberg, Germany. 3Max-Eder Research Group for Pediatric Sarcoma Biology, Institute of Pathology, Faculty of Medicine, LMU Munich, Munich, Germany. 4Pediatrics III, West German Cancer Centre, University Hospital Essen, Essen, Germany. 5German Cancer Consortium (DKTK), partner site Essen, Essen, Germany. 6Department of General, Visceral and Transplantation Surgery, Heidelberg University Hospital, Heidelberg, Germany. 7Light Microscopy Facility, German Cancer Research Center (DKFZ), German Cancer Consortium (DKTK), Heidelberg, Germany. 8Division of Translational Pathology, Gerhard-Domagk-Institute for Pathology, University Hospital Münster, Münster, Germany. 9Institute of Pathology, Faculty of Medicine, LMU Munich, Munich, Germany. 10German Cancer Consortium (DKTK), partner site Munich, Munich, Germany. 11Institute of Pathology, Heidelberg University Hospital, Heidelberg, Germany.

Received: 19 March 2021   Accepted: 12 July 2021
Published online: 27 July 2021
References

1. Grünewald TGP, Cidre-Aranaz F, Suzuki D, Tomazou EM, de Álava E, Kovar H, et al. Ewing sarcoma Nat Rev Dis Primers. 2018;4(1):3.

2. Gaspar H, Hawkins DS, Dirksen U, Lewis U, Ferrari S, Le Deley MC, et al. Ewing Sarcoma: Current Management and Future Approaches. Through Collaboration. J Clin Oncol. 2015;33(27):3036–46.

3. Leavey PJ, Mascarenhas L, Marina N, Chen Z, Kraus B, Misier J, et al. Prognostic factors for patients with Ewing sarcoma (EWS) at first recurrence following multi-modality therapy: A report from the Children’s Oncology Group. Pediatr Blood Cancer. 2008;51(3):334–8.

4. Stahl M, Ranft A, Paulussen M, Boiling T, Vieth V, Bielack S, et al. Risk of recurrence and survival after relapse in patients with Ewing sarcoma. Pediatr Blood Cancer. 2011;57(4):549–53.

5. Musa J, Cidre-Aranaz F, Aynaud MM, Orth MF, Knott MML, Mirabeau O, et al. Cooperation of cancer drivers with regulatory germline variants shapes clinical outcomes. Nat Commun. 2019;10(1):4128.

6. Aye Y, Li M, Long MJ, Weiss RS. Ribonucleotide reductase and cancer: biological mechanisms and targeted therapies. Oncogene. 2015;34(16):2011–21.

7. Goss KL, Gordon DJ. Gene expression signature based screening identifies ribonucleotide reductase as a candidate therapeutic target in Ewing sarcoma. Oncotarget. 2016;7(39):63003–19.

8. Goss KL, Koppenhaver SL, Harmoney KM, Terry WW, Gordon DJ. Inhibition of CHK1 sensitizes Ewing sarcoma cells to the ribonucleotide reductase inhibitor gemcitabine. Oncotarget. 2017;8(50):87016–32.

9. Finch RA, Liu M, Grill SP, Rose WC, Loomis R, Vasquez KM, et al. Triapine (3-aminopyridine-2-carboxaldehyde-thiosemicarbazone): A potent inhibitor of ribonucleotide reductase activity with broad spectrum antitumor activity. Biochem Pharmacol. 2000;59(8):983–91.

10. Stadler WM, Desai AA, Quinn DJ, Bukowsk R, Poiesz B, Kardinal CG, et al. A phase II/III study of GTI-2040 and capecitabine in patients with renal cell carcinoma. Cancer Chemother Pharmacol. 2008;61(4):689–94.

Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Ready to submit your research? Choose BMC and benefit from:

- fast, convenient online submission
- thorough peer review by experienced researchers in your field
- rapid publication on acceptance
- support for research data, including large and complex data types
- gold Open Access which fosters wider collaboration and increased citations
- maximum visibility for your research: over 100M website views per year

At BMC, research is always in progress.

Learn more: biomedcentral.com/submissions