Autoantibody profiling of alveolar rhabdomyosarcoma patients unveils tumor-associated antigens with diagnostic and prognostic significance

Elena Poli a, Manuela Cattelan b, Ilaria Zanetti a, Angela Scagnellato a, Giuseppe Giordano b,c, Angelica Zin a, Gianni Bisogno a, and Paolo Bonvini d

ABSTRACT
Alveolar rhabdomyosarcoma (ARMS) is a highly aggressive subtype of childhood cancer for which efficacious treatments are needed. Immunotherapy represents a new therapeutic opportunity to pursue, but it requires the identification of worthwhile tumor antigens. Herein, we exploited the capacity of ARMS autoantibodies to recognize tumor self-antigens, probing human protein microarrays with plasma from ARMS patients and healthy subjects. We assessed the autoantibody response in ARMS, validated data with independent techniques, and estimated autoantibodies diagnostic and prognostic significance by receiver-operator characteristic curves (ROC), univariate and multivariate analysis. Of the 48 tumor antigens identified, General Transcription Factor II–I (GTF2I) and Protocadherin Gamma Subfamily C5 (PCDHGC5) were selected as candidate targets to validate tumor-restricted antigen expression and autoantibody reactivity through an independent technique and wider cohort of cases. GTF2I and PCDHGC5 overexpression was observed in tumor tissues compared to normal counterparts, and anti-GTF2I and anti-PCDHGC5 autoantibodies were found able to distinguish ARMS patients from healthy subjects as well as cases with different histology. Moreover, low levels of PCDHGC5 autoantibodies characterized patients with worse event-free survival and proved to be an independent negative prognostic factor. This approach provided the first comprehensive autoantibody profile of ARMS, giving novel insights into the immune response of this malignancy and paved the way toward novel potential antibody-based therapeutic applications suitable to improve the survival of ARMS patients.

Introduction
Rhabdomyosarcoma (RMS) is a malignant tumor of mesenchymal origin that accounts for over half of all soft tissue sarcomas in children and adolescents. There are two main histotypes: the embryonal subtype (ERMS) which includes 80% of all cases and the alveolar (ARMS) which represent the 15–20%.
Pathognomonic fusions between PAX3 or PAX7 and FOXO1 genes are found in the majority of ARMS, whereas a minority of them and all ERMS are fusion-negative and display a higher level of genomic instability and mutational load. ARMS are more aggressive, prone to metastatize and characterized by a worse 5-year survival rate compared to ERMS (48% vs. 73%, respectively).

The chance of cure for children with RMS has increased over the years, thanks to the adoption of multimodal tailored treatments consisting of surgery, chemotherapy and/or radiation. However, survival for children with metastatic or refractory/relapsed disease remains dismal, highlighting the need for more effective treatments. Only a minority of non-responding patients can be cured with second-line therapies, which is still based on cytotoxic drugs, while novel molecular targeted agents have not shown a convincing activity against RMS, so far. In this context, immunotherapy holds promise as effective and possibly less toxic treatment. The success of immunotherapy in the treatment of cancer in adults has sparked the interest in harnessing this approach in pediatric oncology as well. However, this strategy is difficult to apply to pediatric solid tumors where immune checkpoint molecules are frequently downregulated and checkpoint inhibitors hardly exert benefits when used as monotherapy. An alternative strategy is to use antibodies targeting the so-called tumor-associated antigens (TAAs): proteins enriched or mislocalized on cancer cells while expressed at low levels on normal tissues. Malignant transformation creates novel antigens, either tumor-specific or -associated (TSAs and TAAAs, respectively), which are recognized by the immune system as ‘foreign’ and used to target neoplastic cells for destruction. Gene mutations potentially generate neoantigens, but the low mutational burden in RMS implies that the vast majority of tumor antigens are aberrantly expressed, non-mutated self-antigens.

Autoantibodies have the potential to illuminate the full epithelial space of the tumor cells, being able to perceive mutations, post-translational modifications and novel splice variants of cancer proteins, likewise quantitative differences in expression. Among several technologies available to unveil tumor-associated autoantibodies (TAABs), protein microarrays represent a suitable and easy-to-use platform for high-throughput detection of circulating autoantibodies in blood. Protein
microarrays may also serve for the determination of autoantibodies in early disease detection, the simultaneous characterization of specificity and diversity of the autoantibody response among patients and the identification of tumor antigens at proteome level that can facilitate the development of antibody-based targeted therapies.

Herein, we have profiled the autoantibody repertoire present in the plasma of 19 PAX3-FOXO1-positive ARMS patients and 15 controls using human protein microarrays followed by qualitative and quantitative validation of tumor antigens able to distinguish patients from healthy subjects, as well as patients belonging to different risk groups. Potential antigens predictors of survival were also investigated, to provide evidence that autoantibodies may be an important tool for risk stratification and prognosis of rhabdomyosarcoma patients. Our study suggests a new method to identify promising immunotherapeutic targets that may represent an initial step for a successful immunotherapy.

Materials and methods

Patients

Tumor biopsies and plasma samples obtained from forty-eight RMS patients were analyzed in this study. Samples were collected between July 2004 and March 2015 at the time of diagnosis, or at the moment of disease relapse, from RMS patients enrolled in the pediatric sarcoma protocols RMS 4.99, EpSSG RMS 2005 and EpSSG MTS-2008. Studies on human samples were approved by Padua Hospital Ethics Committee and patients were included in this study after obtaining institutional review board approval (No. 191P, 20 June 2000; No. 988P, 31 March 2005). Diagnosis of RMS was reviewed by the Italian Association of Pediatric Hematology and Oncology (AIEOP) reference pathologists and supported by molecular investigation of MyoD1 and PAX3/7-FOXO1A transcripts. The basic clinical and molecular features of all the participants are summarized in Table S1. Briefly, all patients were Caucasian with a median age of 9.3 ± 5.74 years, 28 were females and 20 were males. We considered only patients diagnosed with the two major histological variants: alveolar fusion-positive RMS (n = 30) and embryonal ones (n = 18). In ten cases the primary tumor arose in a favorable site (n = 5, urogenital non-prostate/prostate; n = 5, head and neck non-parameningeal), in 36 cases tumor was localized in an unfavorable site (n = 11, head and neck parameningeal; n = 3, urogenital bladder/prostate; n = 13, extremities; n = 9, all “other sites”) and in 2 cases the site of onset was unknown. According to the Intergroup Rhabdomyosarcoma Study (IRS) Group system, 2 patients were classified in IRS group I (completely-excised tumors), 2 in IRS group II (grossly-resected tumors with microscopic residual disease and/or regional lymph node involvement), 18 in IRS group III (gross residual disease after incomplete resection or biopsy), 25 in IRS group IV (metastatic disease), while for 1 this information wasn’t available. In 26 cases the size of the primary tumor at the time of diagnosis was more than 5 cm, in 11 was less or equal to 5 cm, while in 11 this data wasn’t available. In this study, fifteen pediatric healthy subjects (HS), 12 males and 4 females with a median age of 9.48 ± 3.75 years, were also included and used as controls.

Blood samples

Forty-eight blood specimens were collected in a sodium citrate tube at the time of diagnosis, prior to any type of treatment, and eleven at the time of relapse before starting second line treatment. Plasma samples were obtained by centrifugation of peripheral blood at 820 x g for 10 min, followed by a further centrifugation step at 16.000 x g for 10 min to avoid any type of contamination by blood cells. Plasmas were aliquoted and stored at −80°C until use.

Protein arrays

Plasma samples obtained from 19 ARMS patients (Table S2) and 15 healthy subjects were probed on ProtoArray™ Human Protein Microarray v5.1 (Thermo Fischer Scientific). ProtoArray hybridization procedure required slides equilibration at 4°C for 15 min followed by incubation in blocking buffer [50 mM HEPES pH 7.5, 200 mM NaCl, 0.08% Triton®X-100, 25% Glycerol, 20 mM reduced glutathione, 1 mM DTT, 1X Synthetic BlockTM (Thermo Fischer Scientific)] for 1 h at 4°C with gentle agitation. Microarrays were then washed 5 times for 5 min at 4°C with circular shaking with washing buffer [1X PBS, 0,1% Tween20, 1X Synthetic BlockTM (Thermo Fischer Scientific)] prior to be probed with plasma samples for 90 min at 4°C with circular shaking. Human bound antibodies were detected incubating Alexa Fluor-647 goat anti-human IgG antibody (1 µg/mL in washing buffer) for 90 min at 4°C with circular shaking. Fluorescence signals intensity was measured by ScanArrayLite laser scanner (Perkin Elmer) setting excitation wavelength at 635 nm, photomultiplier tube (PMT) gain at 600, laser power 65% and pixel size at 10 µm. For background determination a protein microarray slide, incubated with secondary Alexa Fluor 647-labeled antibody only, was used.

Indirect ELISA assay for autoantibodies detection

Plasmatic autoantibodies (Abs) against GTF2i and PCDHC5 were assessed by a home-made indirect ELISA assay. Human recombinant GTF2i (Abnova) and PCDHC5 (Abnova) diluted in 50 mM carbonate-bicarbonate buffer (Sigma-Aldrich, St Louis, MO, USA) to a final concentration of 0.5 µg/mL were coated overnight at 4°C on immunlon 4HBX microtiter plates with extra-high binding surface (Dynex Technologies Inc., Chantilly, VA). Serially diluted purified human IgG (5 –640 ng/mL) (Sigma-Aldrich, St Louis, MO, USA) was used to provide the standard curve. Blocking, washing, probing, development and quantification procedures were performed as previously described.19

Total IgG measurement

To normalize GTF2i and PCDHC5 autoantibodies levels, total plasma IgG level of each plasma sample was assessed in
triplicate by Human IgG ELISA kit (RayBiotech, Inc., Norcross, GA) according to the manufacturer’s instructions.

**Cell lines**

The human rhabdomyosarcoma (RMS) cell line RH30 was purchased from ATCC (Manassas, VA), while RH4 and RH28 cells were a gift of Dr P.J. Houghton (St Jude Children’s Hospital, Memphis, TN). Cell lines were grown in Dulbecco’s Modified Eagle’s Medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, Life Technologies Co., Carlsbad, CA, USA), glutamine (2 mM), penicillin (100 U/mL) and streptomycin (100 μg/mL) at 37°C in a humidified atmosphere containing 5% CO₂.

**Quantitative RT-PCR**

Total RNA was extract from 3 cell lines and 11 frozen tumor biopsies of ARMS patients analyzed in this study. Extraction procedure involved the use of the TRIzol reagent (Invitrogen), while retrotranscription to cDNA the Super-Script II enzyme (Invitrogen), according to the manufacturer’s instructions. As normal controls mesenchymal stem cells and fetal and adult skeletal muscle samples were used (Thermo Fisher scientific). Quantitative RT-PCR (qRT-PCR) was performed on ViiA7 thermal cycler (Applied Biosystems), using SYBR Green chemistry (Applied Biosystems) and standard protocol of amplification. Gene-specific primer sequences were 5’-GCAGGCCTTCGAAACTGAT-3’ (forward) and 5’-CTGAAAGATGGTGCCCTTCTTG-3’ (reverse) for GTF2i, 5’-GCCGTCCTCATAAGGGATT-3’ (forward) and 5’-AACGTTCAATGATCCCAGAG-3’ (reverse) for PCDHG5, as 5’-TCCTGACTTCAACACGCGA-3’ (forward) and 5’-GGTCTCTACTCTTCGGAGGC-3’ (reverse) for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The relative expression of GTF2i and PCDHG5 was calculated by the 2^(-ΔΔCt) method, using GAPDH as reference gene and the fetal skeletal muscle as external calibrator.

**Cell lysis and immunoblotting**

Cells were washed twice in ice-cold 1X PBS and incubated on ice for 30 min with a lysis buffer containing Tris–HCl 50 mM at pH 7.5, NaCl 150 mM, EDTA 2 mM, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, aprotinin 20 μg/mL, leupeptin 20 μg/mL and PMSF 1 mM. The lysates were clarified by centrifugation at 14,000 rpm for 30 min at 4°C and protein concentrations were determined by the bicinchoninic acid assay (Thermo Fisher scientific) using bovine serum albumin (BSA) as a standard. Cell lysates (60 μg) were diluted with Laemmli loading buffer 5X (2% SDS, 100 mM DTT, 60 mM Tris at pH 6.8, 0.01% blue bromophenol and 10% glycerol), denatured at 95°C for 5 min and fractionated by 10% polyacrylamide gel for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins were electrotransferred onto nitrocellulose membranes overnight at 4°C. Membranes were probed with GTF2i (Thermo Fisher scientific) and PCDHG5 (Abnova) primary antibody diluted in a milk solution (GE Healthcare) for 3 h at room temperature with gentle shaking and then for 1.5 h with HRP-conjugated secondary antibody (GE Healthcare). Protein bands were visualized by ECL chemiluminescence detection system (Perkin Elmer) and acquired with iBright FL1500 Imaging System (Thermo Fisher scientific).

**Immunofluorescence**

RH30 and RH4 cells were seeded on 4-well chamber slide (Falcon) and incubated at 37°C for 48 h. Once reached subconfluence, cells were fixed with 4% paraformaldehyde for 20 min, and permeabilized with 0.2% Triton X-100 in PBS1X for 10 min. Slides were then incubated for 10 min in 100 mM glycine and for further 10 min in 10% FBS in PBS1X. Primary antibody against GTF2i (Thermo Fisher scientific) and in 2% PBS/1X PBS were probed at 37°C for 60 min, followed by secondary antibody Alexa Fluor® 546 conjugate (Thermo Fisher scientific) in 1X PBS at 37°C for 60 min. Slides were washed and mounted in 1:1 glycerol/1X PBS supplemented with DAPI (6,6-diamino-2-phenylindole, dihydrochloride) (Thermo Fisher scientific). The images were acquired with a Leica DFC420C digital camera, mounted on a Leica DM4000B microscope, at 20X magnification. Image analysis was performed with Leica IM1000 software (Leica Microsystems).

**Flow cytometry**

One million of ARMS cells were incubated with anti-human PCDHG5 antibody (Abnova) for 30 min at room temperature and then with anti-mouse Alexa Fluor® 488 conjugate (Thermo Fisher scientific) at the same conditions. Unlabeled cells were first acquired for each cell lines to ensure labeling specificity. Samples were analyzed by FC500 flow cytometer (Beckman Coulter, USA) and data were examined using CXP Analysis software (Beckman Coulter, USA).

**Statistical analysis**

Microarrays were analyzed by GenePixTMPro 6.0 software. Data were filtered to remove signals related to reactive antigens in a limited number of samples. For this purpose, Z-scores were calculated and only antigens with Z-factor ≥0.4 in at least 4 patients and 4 healthy subjects were considered. Intra- and inter-array normalization of data were computed exploiting the presence of Human and Anti-Human IgG control proteins spotted in each of the 48 subarray according to a pre-specified 8-point concentration gradient (from 0.125 μg/mL to 16 μg/mL). A robust linear model (RLM) was fitted to the log2-transformed signals of the control proteins including as independent variables an indicator for the subject, the type of antigen and the subarray in which the antigen was spotted.20 The estimates of the robust regression coefficients for each subject and each subarray were employed for normalization of the mean signal of each antigen spotted in twice. To remove batch effects from Protoarray data we employed an empirical Bayes procedure that estimates the variance for each antigen in microarrays belonging to the same batch and rescales the antigen consequently. Obtained normalized signals were used
to evaluate which antigens were truly different between patients and healthy subjects, using Mann-Whitney test. For each antigen the distance between groups was calculated by euclidean metric and used to perform an unsupervised hierarchical cluster analysis, represented by a dendrogram. A heatmap was also generated to display the intensity values related to the antibody response against each antigen through an increasing intensity color scale. Also a principal component analysis (PCA) was performed to explore data and visualize the distances between patients and controls. With respect to ELISA and expression data all the comparison were performed using Mann-Whitney test. Receiver-operator characteristic curves (ROCs) were generated to determine the discriminatory capacity of GTF2i and PCDHGC5 autoantibodies levels to predict diagnosis and histological subtype classification. The area under the ROC curves was analyzed by the Hanley and McNeil method. Survival analysis curves were estimated by the Kaplan–Meier method and overall differences were compared by the log-rank test. The outcome considered were event-free survival (EFS), calculated from the date of diagnosis to the date of the first event (tumor progression or relapse) or the last follow-up, and overall survival (OS), calculated from the date of diagnosis to the date of death for any cause or the last follow-up. Cox uni- and multivariate proportional hazard analysis were carried out to estimate the prognostic impact of GTF2i and PCDHGC5 autoantibodies. All Statistical analyses were carried out using GraphPad Prism 8, IBM SPSS Statistics 23 and R statistical software. All p-values were two-tailed and, considered statistically significant at the alpha level of 0.05.

Results

Profiling of circulating ARMS-associated autoantibodies

To identify RMS tumor antigens recognized by patients’ circulating autoantibodies, 19 ARMS plasma samples, collected at the onset of the disease (Table S2), and 15 healthy donors specimens were used to probe protein microarray slides. The immune response profile was obtained for each patient and control and reactivity values of the 9374 spotted proteins were computed. A protein gradient of purified human IgG printed on each array served to verify antibody probing and proper antigen detection, as well as to perform intra- and inter-array robust linear model (RLM) normalization. The data were filtered to remove the noise of antigens reactive in a very limited number of subjects, setting up positive signals to cutoff z-score ≥0.4 and considering only antigens above cutoff value in at least 4 patients and 4 controls. This procedure led to discard 1300 out of 18750 spots and to identify 48 antigens exhibiting a statistical different pattern of immunoreactivity (p < 0.05) in children with ARMS compared to pediatric healthy subjects. Among these 48 proteins, 4 displayed higher signal intensity in ARMS patients, while all the others showed higher reactivity to plasma autoantibodies in healthy subjects (Figure S1, Table S3). The identification of a limited number of reactive antigens in ARMS patients might be consistent with the infiltration-excluded nature of rhabdomyosarcoma.21 A heat map based on a hierarchical clustering algorithm using an euclidean distance for the 48 antigens was obtained, allowing a clear grouping of ARMS patients except one, while controls splitted apart, probably due to the high heterogeneity in the natural autoantibody response as previously reported (Figure 1(a)).22 To substantiate these findings a principal component analysis (PCA) was performed maximizing as much as possible the variance of the graphically represented data. The PCA analysis highlighted the partition between groups and the first two main components (PC1 and PC2) were able of explaining 56.2% of the data variability (Figure 1(b)).

Therefore, we proceeded with Immuno Response Binding Profile (IRBP) analysis in patients with localized (IRS-III, n = 10) and metastatic (IRS-IV, n = 9) tumors at diagnosis, as we supposed them being diversely exposed to the immune system. Overall, 98 proteins were found to be differently reactive in localized ARMS patients compared to controls, but only 3 of them displayed stronger autoantibody reactivity in ARMS patients. Moreover, cases and controls did not cluster clearly, suggesting a scarce immunogenicity and a high heterogeneity in the autoantibody response of localized ARMS (Figure S2A). Conversely, autoantibody response profiling of metastatic patients identified 55 proteins with statistically significant p-value and either higher (n = 29) or lower (n = 26) signal intensity compared to controls, which clustered metastatic patients while leaving healthy subjects divided in two main branches (Figure S2B). The PCA analysis performed on the localized- and metastatic-associated autoantibody signatures supported these findings, as a minimum degree of overlap between localized patients and healthy subjects was obtained, while a net separation of patients with metastasis at diagnosis and controls was observed.

Tumor-associated antigen expression in ARMS cancer cells

Putative tumor antigens GTF2i and PCDHGC5, ranked by p-value and fold change (Table S3), were selected for validation. Since stronger autoantibody reactivity is expected to be associated to protein overexpression we assessed GTF2i and PCDHGC5 expression by qRT-PCR in ARMS cell lines (n = 3) and primary tumor tissues (n = 11) and observed an increase in both types of specimens when compared to normal controls (mesenchymal stem cells, fetal and adult skeletal muscle tissues) (Figure 2(a)). In the case of PCDHGC5 expression, although not statistically significant, we observed higher expression levels in ARMS cell lines and tumor biopsies also respect to ERMS cell lines. Different GTF2i and PCDHGC5 levels in ARMS patients and healthy subjects were confirmed by querying R2-AMC-Oncoinformatics (http://r2.amc.nl) web-based database, which consists of gene expression datasets from several pediatric cancers, including rhabdomyosarcoma (Figure S3). Higher GTF2i and PCDHGC5 protein expression was also determined by blotting cell extracts of fusion-positive ARMS cell lines and adult muscle control tissues (Figure 2(b)), as well as their proper nuclear and plasma membrane localization was demonstrated when cells were stained with specific antibodies and assessed by immunofluorescence and flow cytometry, respectively (Figure 2(c)).
We developed specific ELISA assays to confirm the ability of anti-GTF2i and PCDHGC5 autoantibodies to discriminate between ARMS patients and healthy subjects, since high-throughput methods, including protein microarrays, are likely to return a high number of false positives. We validated the presence of GTF2i and PCDHGC5 autoantibodies using plasma samples from the same cohort of patients profiled with protein microarrays and recombinant GTF2i and PCDHGC5 proteins coated on microtiter plates with extra-high binding surface. We confirmed that higher titers of autoantibodies were present in patients' plasma samples and differences were statistically significant (Figure 3(a); p = 0.003 and p < 0.0001, respectively). When the cohort of patients was expanded, including additional 11 alveolar and 18 embryonal (ERMS) rhabdomyosarcoma cases (Table S1), GTF2i and PCDHGC5 autoantibodies still distinguished ARMS patients from controls (p = 0.005 and p < 0.0001, respectively), but also ARMS from ERMS cases (Figure 3(b); p = 0.029 and p = 0.022, respectively), whereas no significant associations with disease stage, gender, age, tumor site and size were found (Figure S4).

To assess the role of GTF2i and PCDHGC5 autoantibodies as diagnostic biomarkers of ARMS, receiver-operating characteristic (ROC) curves were generated. As expected, a good discrimination capacity between ARMS patients and controls (Figure 4(a), GTF2i, p = 0.006; PCDHGC5, p = 0.0001), likewise between ARMS and ERMS cases (Figure 4(b), GTF2i, p = 0.029;
PCDHGC5, \( p = 0.022 \) was demonstrated. When tested in combination, the diagnostic performance of GTF2i and PCDHGC5 autoantibodies, calculated by binary logistic regression, improved further the capacity of distinguishing ARMS cases from controls \( (p < 0.0001, \text{AUC} = 0.89) \) and RMS cases with different histology \( (p = 0.001, \text{AUC} = 0.71) \) (Figure 4(c)).

**Prognostic value of GTF2i and PCDHGC5 autoantibodies levels**

Finally, to explore the possibility that GTF2i and PCDHGC5 autoantibodies might be relevant to predict patient outcome, 40 RMS patients (23 ARMS and 17 ERMS), with clinical follow up data available, were analyzed. Patients median follow-up
time was 3.4 years (range: 0.24–10.01 yrs), while median time for event occurrence 3.6 years (range: 0.08–4.36 years). Twenty-six patients (65%) experienced an event such as relapse (local, distal or at regional lymph nodes), progression of disease or death and 21 died (20 of disease and one for treatment-related toxicity). When GTF2i and PCDHG5 autoantibodies median values (6217 ng/ml and 5655 ng/ml, respectively) were used as cutoff, Kaplan-Meier analysis proved that low levels of PCDHG5 autoantibodies were associated with an inferior event-free survival (log-rank test: $p = 0.003$) (Figure 5). Overall survival was not statistically significant, but patients with low levels of PCDHG5 autoantibodies had a 3-year overall survival rate of 38.8% compared to 70% of patients with high levels (Figure S5). The 3-year survival rate of patients with low and high GTF2i autoantibody levels was 50% and 60%, respectively. To substantiate these findings, univariate and multivariate Cox regression analyses were performed in 36 patients with complete clinical data. Low PCDHG5 autoantibody levels and the presence of metastatic disease at diagnosis were found significantly associated with EFS both in uni- and in multivariate analysis resulting the only two independent negative prognostic factors (PCDHGC5: $p = 0.001$, HR = 6.755, 95%CI = 2.270–20.102; IRS group: $p = 0.001$, HR = 0.165, 95%CI = 0.057–0.483) (Table 1). This provided evidence that low levels of autoantibodies were useful to identify patients with higher risk of recurrence and progression.

**Evaluation of PCDHG5 autoantibody levels at disease relapse**

As previously stated, plasma autoantibodies may indicate tumor burden and have the potential to monitor treatment response and recurrence. We investigated in 11 RMS cases (7 ARMS and 4 ERMS) if autoantibodies could be used as relapse biomarkers. The mean time between the end of first-line
treatment and relapse was 11.2 months: four patients had a local recurrence, 4 a regional lymph nodes relapse, 1 metastasis and 2 both local and metastatic relapses. When PCDHGC5 autoantibodies were quantified, a variable longitudinal change between diagnosis and relapse was observed (Wilcoxon matched-pairs signed rank test, \( p = 0.32 \)). The majority of cases (64%) showed an increase in the autoantibodies level at relapse, while the remaining (36%) a stable response or even a sharp decrease (Figure 6).

Discussion
Immunotherapy is paving the way of a new era of cancer treatment, boosting and strengthening patients’ own immunity to control tumors. This approach enables the elimination of malignant cells, previously ‘invisible’ to the immune system and has been shown to provide therapeutic results both in primary and recurrent/refractory tumors.23,24 Specific recognition and elimination of malignant cells by antibodies has been...
Figure 5. Correlation between humoral immune response against GTF2i and PCDHGCS antigens and RMS patients’ outcome. Kaplan-Meier survival analysis representing event-free survival (EFS) of 40 RMS patients (23 ARMS and 17 ERMS) divided by the median values of GTF2i and PCDHGCS autoantibodies level.

Table 1. Uni- and multivariate Cox regression for event-free survival in 36 RMS patients.

| Clinical features | Patients | Uni. p-value | Multiv. p-value | HR | 95%CI(HR) |
|------------------|----------|--------------|----------------|----|-----------|
| Gender           |          |              |                |    |           |
| Male             | 14       | 0.395        |                |    |           |
| Female           | 22       |              |                |    |           |
| Age              |          |              |                |    |           |
| ≥10 yrs or <     | 15       | 0.168        |                |    |           |
| 1 yrs            | 21       |              |                |    |           |
| <10 yrs          |          |              |                |    |           |
| Tumor            |          |              |                |    |           |
| Favorable        | 7        | 0.178        |                |    |           |
| Unfavorable      | 29       |              |                |    |           |
| Site             |          |              |                |    |           |
| Tumor            |          |              |                |    |           |
| > 5 cm           | 26       | 0.380        |                |    |           |
| ≤5 cm            | 10       |              |                |    |           |
| Size             |          |              |                |    |           |
| PCDHGCS          |          |              |                |    |           |
| >5655 ng/ml      | 19       | 0.015        | 0.001          | 6.755 | 2.270–20.102 |
| ≤5655 ng/ml      | 17       |              |                |    |           |
| IRS group        |          |              |                |    |           |
| IV               | 17       | 0.030        | 0.001          | 0.165 | 0.057–0.483 |
| I, II, III       | 19       |              |                |    |           |
| Histology        |          |              |                |    |           |
| ARMS P3/F+       | 19       | 0.353        |                |    |           |
| ERMS             | 17       |              |                |    |           |

ARMS P3/F+, alveolar rhabdomyosarcoma PAX3-FOXO1-positive; ERMS, embryonal rhabdomyosarcoma; Univ., univariate analysis; Multiv., multivariate analysis; HR, hazard ratio; CI, confidence interval.

proposed for a long time and nowadays antibodies are used clinically in an effort to realize a potential antitumor target therapy. Antibodies can target cancer cells by engaging differentially expressed surface antigens, such as rituximab targets CD20 in non-Hodgkin B cell lymphoma, trastuzumab interferes with HER2 signaling in breast cancer and cetuximab inhibits EGFR in colorectal cancer. Cancer cells generate antigenic changes that are recognized by the immune system as foreign (neoadtigens), triggering an adaptive immune response that includes the production of tumor-associated autoantibodies (TAAbs). Serum autoantibodies against cancer antigens are considered a novel type of biomarkers of disease onset and progression, but also valuable instruments in oncological therapy. The advantage of using autoantibodies as cancer biomarkers is explained by their easy accessibility in the blood, their hampering response and longer half-life compared to antigens. Autoantibodies provide the most direct and comprehensive route to the identification of cancer antigens with clinical drug development potential, being able of characterizing the full epitope space of the cancer proteome, which also include splice variants, post-translational modifications and amino acid substitutions. In this context, autoantibodies give indications of continuous tumor cells remodeling and can be used to define a unique cancer signature that monitors both disease progression and evolution. Magnitude and spectrum of autoantibodies production help measuring the adaptive immune response to cancer and its overall immunogenicity, two critical aspects of a successful immunotherapy.25,26

Ideal autoantibody target candidates are tumor-specific antigens (TSAs): mutated proteins exclusively expressed by cancer cells.27 However, common TSAs are a significant challenge in pediatric solid tumors, that are characterized by low mutational burden, high level of intratumor heterogeneity and non-inflammatory tumor microenvironment.28 Targeting tumor-associated antigens (TAAs) may be a feasible alternative to TSAs, as TAAs consist of non-mutated proteins aberrantly expressed or modified by truncation, misfolding or abnormal posttranslational modifications in cancer cells, but not in normal tissues. This has been shown for HER2, IGF-1 R and VEGFA, in children with newly diagnosed or relapsed RMS treated with antagonist monoclonal antibodies29-31 or in RMS cancer cells successfully treated with natural killer cells when exposed to antibodies targeting cell surface receptors EGFR and CXCR4.32,33 Based on this, we profiled the autoantibody response in alveolar RMS patients, probing protein microarrays with patients’ plasma samples collected at diagnosis prior to surgery or any systemic treatment, in order to identify tumor antigens capable of distinguishing cases from controls, likewise patients belonging to different clinical risk groups. Protein microarrays have enabled to discover many tumor-associated antigens in cancer patients, as those with glioma, malignant mesothelioma, lung and prostate adult cancers, but they’ve never been tested in pediatric solid tumors. Herein, a variable, though substantially low antibody response was observed in ARMS patients. Indeed, among the differentially-reactive antigens obtained from the comparison between cases and controls, only 4 were more highly reactive in all patients, 3 when considering patients with localized disease and 29 in high-risk metastatic patients. Only the metastatic profile allowed a clear distinction between cases and controls, which might be attributed to a high heterogeneous natural antibody response in healthy subjects, a scarce immunogenicity of localized RMS tumors and a higher chance of exposure of metastatic RMS cells to the immune system. The non-inflamed and immunosuppressive microenvironment may help supporting these hypothesis, as the RMS tumor immune
microenvironment is known to be characterized mainly by tumor-associated CD163+ macrophages (TAMs) and perivascular tertiary lymphoid structures (TLS), where CD4+ T cells and CD20+ B cells are trapped. In accordance with our findings, a lack of clear distinction between patients and controls has been observed in low-grade gliomas when exploiting cancer autoantibodies, whereas a difference between high-grade gliomas and controls was detected, due perhaps to the increased invasion capacity of the latter, the higher number of antigens exposed to the humoral system and, hence, the high number of TAAs detected by autoantibodies.

Changes in immunoreactivity and autoantibody response can be used for diagnostics, prognostics and therapeutic purposes. However, high-throughput microarrays data need to be independently validated, particularly when used to provide guidance for therapeutic strategies. Therefore, among the common antigens we selected GTF2I (General Transcription Factor II-I, TFII-I) and PCDHG5 (Protocadherin Gamma Subfamily C5) for further validation. GTF2I is a signal-induced transcription factor, activated upon phosphorylation by a variety of extracellular signaling pathways, including B and T cell receptors. Its overexpression may result from mutations in coding regions regulating its degradation, alternative splicing patterns, gain-of-function mutations or gene fusion events. GTF2I appears to be important not only as signal-dependent regulatory factor, but also for DNA translesion synthesis, a DNA damage tolerance mechanism that maintains genomic stability and allow survival of cancer cells upon drug treatment. PCDHG5 is a transmembrane protein belonging to the cadherin superfamily of intercellular adhesion and cell-cell communication proteins, whose dysregulation has been associated with either cancer progression or suppression. Ectopic expression of PCDHG5 has been reported in astrocytomas, while its hypermethylation in a variety of other tumor types, including sarcomas. As potential novel tumor antigens, GTF2I and PCDHG5 expression and immunoreactivity was confirmed in ARMS cell lines and tumor specimens, while the autoantibody response was validated in a larger cohort of patients, including cases of ERMS. Both GTF2I and PCDHG5 autoantibodies resulted significant markers of ARMS histology, even better when used in combination, although neither GTF2I nor PCDHG5 are described as target genes of PAX3-FOXO1 fusion protein. With respect to prognosis, PCDHG5 autoantibodies and the presence of distal metastasis at diagnosis were associated with event occurrence in univariate and multivariate analysis, resulting independent predictors in event-free survival. In particular, PCDHG5 autoantibody levels, below median cutoff value, characterized patients with higher risk of recurrence as it was for tumor stage (IRS-IV clinical risk group). Although not statistically significant, low levels of PCDHG5 autoantibodies were observed in patients with poorer outcome as well, high-lightening the positive correlation between antibody levels and patients’ survival. High titers of tumor-specific antibodies could inform about a functional immunoresponse in RMS patients. Whether they exert a protective role and control disease spreading remains to be ascertained.

Finally, it is known that humoral immune response may vary according to changes in tumor burden: decreasing after tumor excision and treatment, while increasing at recurrence. In few ARMS cases for whom plasma was available at relapse, PCDHG5 autoantibodies were assessed and found to increase in the majority of cases (64%), with levels even twice of those detected at diagnosis. Although changes were not suggestive of progression due to the lack of preceding blood samples, they revealed the presence of the disease.
Relapsed RMS patients were characterized by low levels of PCDHGC5 autoantibodies at the onset of disease. The increase at the time of relapse may be explained with the augmented exposure of tumor cells to the immune system, which results when tumor cells enter into the circulation and spread afar.

In conclusion, this study was designed to ameliorate the understanding of ARMS tumorigenesis, leading to an improvement in survival especially for non-responder patients. Throughout the identification of surface antigens like PCDHGC5, we searched for novel immunotherapeutic targets able to fight RMS in a different way. In the future, we’ll use this approach to identify antibody signature changes that are specific for various types of therapies and reflect patients’ response to them. We’ll define the correlation between antibody response and clinical outcome, as we recognize that production of autoantibodies against tumor-associated antigens leads to prolonged progression-free and overall survival.

To the best of our knowledge, this is the first study that screens tumor antigens in alveolar RMS patients, using circulating autoantibodies as natural detection system. Autoantibody data analysis must be completed and additional larger cohort studies must be performed to give a clearer picture of the functionality of the humoral immune system in ARMS patients. Nonetheless, TAAs identified in this study opens new avenues for exploitation of promising novel targets of successful immunotherapy approaches.

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The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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**Abbreviations**

- AUC: Area Under the Curve
- CI: Confidence Interval
- ERMS: Embryonal rhabdomyosarcoma
- ELISA: Enzyme-Linked Immunosorbent Assay
- EpSSG: European Soft Tissue Sarcoma Study Group
- EFS: Event-free survival
- FBS: Fetal bovine serum
- GTF2i: General Transcription Factor II-I
- HR: Hazard ratio
- HS: Healthy subjects
- IRBP: Immuno Response Binding Profile
- IRS: International Rhabdomyosarcoma Study Group
- AIEOP: Italian Association of Pediatric Hematology and Oncology
- OS: Overall survival
- PBS: Phosphate buffered saline
- PCA: Principal component analysis
- PCDHGC5: Protocadherin Gamma Subfamily C5
- ROC: Receiver-Operator Characteristic curve
- RMS: Rhabdomyosarcoma
- RLM: Robust linear model
- SKM: Skeletal muscle
- TAAs: Tumor-associated antigens

**Abbreviations**

- DAPI: 6,6-diamino-2-phenylindole, dihydrochloride
- ARMS: Alveolar rhabdomyosarcoma
- Abs: Antibodies
TAAbs
Tumor-associated autoantibodies

TSAs
Tumor-specific antigens

Yrs
Years

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