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A SURVIVAL ANALYSIS ON THE IMMUNE LANDSCAPE OF PAEDIATRIC SOLID TUMOURS.

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Abstract.

Introduction:

The functional orientation of the tumor microenvironment has been shown in large immunogenomic investigations to play a predictive role in adult solid tumors; however, the paediatric equivalent of this variable has received little attention.

Method:

For four paediatric tumor types (408 patients), Wilms tumor (WLM), neuroblastoma (NBL), osteosarcoma (OS), clear cell sarcoma of the kidney (CCSK), and rhabdoid tumor of the kidney (RT), we carried out a thorough study of public RNAseq data (TARGET). We evaluated the Immunologic Constant of Rejection's (ICR) capability to detect an active Th1/cytotoxic response. Additionally, we carried out gene set enrichment analysis (ssGSEA), grouped more than 100 immunological features with good characterization into distinct immune subtypes, and compared the results.

Result:

Higher ICR scores were linked to better OS and high-risk NBL without MYCN amplification survival, but worse WLM survival. The same four major modules previously discovered in adult tumors (TCGA) were revealed by clustering immunological characteristics. These modules classified paediatric patients into six immunological subtypes (S1–S6), each of which had a different prognosis for survival. The S2 cluster, which has low enrichment of the wound healing signature, high Th1, and low Th2 infiltrates, and the S4 cluster, which has the opposite characteristics, demonstrated the best overall survival. Increased T-cell infiltration and worse outcomes were linked to the WNT/Beta-catenin pathway in OS.

Conclusion:

We showed that extracranial paediatric tumors might be categorized by their immunological makeup, revealing parallels with tumors seen in adults. To find diagnostic and prognostic biomarkers and to find potential immune-responsive tumors, immunological factors may be investigated.

Recommendations:

Close disease surveillance and genetic evaluation are recommended for patients with certain solid tumors or particular predisposing conditions.

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1. INTRODUCTION.

One of the top causes of death in children globally is cancer, and the incidence that has been documented tends to increase over time [1]. The most common cause of childhood deaths outside of infancy in the US [2] and other high-income nations [3] is cancer. The global incidence rates of pediatric cancer range from 50 to 200 per million children [1]. Leukaemias, brain tumors, lymphomas, neuroblastomas, and nephroblastomas (Wilms tumor, WLM) are the most prevalent types of childhood cancers [4]. Nearly 50% of cancer cases are solid tumors [5]. The two tumor types that are virtually exclusively found in children are WLM and neuroblastoma, the most common paediatric extra-cranial tumor [6].

The greatest cause of death worldwide is cancer [7]. In 112 of the 183 countries, it is the leading or second cause of death for those under the age of 70, with 23 more nations trailing at the third or fourth spot for cancer-related deaths (World Health Organisation [8, 9]). According to estimates, female breast cancer (11.7%), lung cancer (11.4%), colorectal cancer (10.0%), prostate cancer (7.3%), and stomach cancer (5.6%) account for more than 44% of all cancer incidence worldwide, including both sexes.

Additionally, solid tumors contribute to the greatest mortality rates for both male and female cancers globally, with lung cancer alone accounting for 18.0% of cancer-related fatalities, followed by colon cancer (9.4%), liver cancer (8.3%), stomach cancer (7.7%), and female breast cancer (6.9%) [8]. This illustrates the severity of the influence of solid cancers on the worldwide cancer burden. Despite therapeutic improvements, most tumors are identified at an advanced stage, which results in a dismal prognosis.

The molecular foundation of juvenile malignancies has become increasingly clear in recent decades as a result of the intensive growth of molecular research at that time. The published research demonstrates a significant variability of molecular changes that explain the onset of the malignant process, therapeutic response, and disease progression. These results unequivocally show that the molecular profile of children's tumors is markedly different from that of adult cancers. Therefore, it is not possible to immediately apply adult molecular marker knowledge and experience to the paediatric population. The identified discrepancies call for the creation of a new diagnostic and treatment strategy for this group of patients since they are not only related to the genomic basis of the disease but also to its actual anatomical site and histological features.

Different immune system components recognize various targets. In general, antibodies are capable of binding a wide range of antigens, including proteins, peptides, and carbohydrates [10], whereas native T lymphocytes are capable of recognizing peptides when they are given in the context of the class I major histocompatibility complex (MHC). NK cells can engage with stress chemicals directly on cell surfaces or recognize antibody-coated cells via their Fc receptor [11]. Understanding those targets and their paediatric significance is crucial because both T and NK cells have been modified by gene transfer or CRISPR/Cas technologies to be equipped with molecularly altered cell surface receptors that lead them to specific targets. With a brief mention of non-targeted immunotherapies, we have specifically chosen to emphasize novel targeted immunotherapies in paediatric solid tumors.

The treatment landscape for cancer patients has transformed due to the introduction of immune checkpoint inhibitors that unleash natural anti-tumor immunity, especially in grownups [12]. One of the key contributing aspects to the ability to respond to immunological checkpoint inhibition is neoantigen availability, which is a result of all non-synonymous mutations in the tumor [13,14] Mutational load. Children's cancers exhibit a modest mutational load overall [15]; thus, Immune checkpoint inhibitors have exhibited very little effectiveness in this situation [16]. In light of this, example, the FDA has authorized the use of checkpoint PD1 blockades as a therapeutic for treating kids with insufficient mismatch repair,

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unstable microsatellites, or tumors with hypermutations [17], without regard to the Histology of tumors. Immune-mediated cancer, however, It's possible that cell recognition and death happen separately. modified antigen, as well as various forms, are available. the potential of immunotherapy in tumors with low mutational burden [18], which includes malignancies in children.

T-cell or NK-cell-based adoptive therapy [19], vaccines targeting non-mutated antigens, and oncolytic therapy, either alone or in conjunction with other treatments, are a few examples of these. Inhibitors of checkpoints [20] [21]. These methods have revealed intriguing outcomes in pre-clinical models [19-21], although immunotherapy clinical successes have only been attained in tumor-type neuroblastoma with the likeli- hood of the reported spontaneous remissions cell-mediated immunity [22,23]. In high-danger patients receiving dinutuximab, which inhibits the NBL-associated antigen GD2, in addition to conventional therapy Granulocyte-monocyte colony-stimulating factor (GM-CSF) and interleukin-2 (IL-2), increased event-free and overall survival [24], but is not generally curative who will eventually relapse and pass away [19].

Furthermore, genetic information on the challenges of treating refractory or relapsed solid tumors is sparse, which limits our understanding of the molecular makeup of such entities. It is crucial to carry on with research and targeted medication trials employing tumor molecular profiling in children and adolescents to further increase pediatric cancer cure rates.

2. MATERIALS AND METHODS.

Except where otherwise noted, program names are R packages and all analysis was done in R version 3.6.1.

2.1. Data acquisition and normalization.

The TARGET paediatric dataset, which is available on the GDC portal website, contains RNA-seq data for five paediatric tumors: Wilms tumor (WLM), neuroblastoma (NBL), osteosarcoma (OS), rhabdoid tumor (RT), and clear cell sarcoma of the kidney (CCSK). These data were downloaded and processed using TCGAbiolinks (v. 2.14.1) [25]. Using TCGAbiolinks to con- vert gene symbols to official HGNC symbols and excluding genes without symbols or gene information, a pan-cancer expression matrix containing 20,155 genes was produced. One primary tumor (TP) sample was examined for each patient, with blood-derived samples, metastatic tumors, and recurrent primary tumors being excluded.

Using the TCGA analyze_Normalization function from TCGAbiolinks, RNA-seq gene counts were normalized. This included within-lane normalization procedures to correct for the effect of GC content, between-lane normalization procedures to correct for distributional differences between lanes as well as quantile normalization using TCGAbiolinks. The pan-cancer matrix was divided according to cancer type after normalization and log2-transformed. The GDC portal was used to collect the clinical data for the TARGET research.

2.2. ICR classification.

The ConsensusClusterPlus (v.1.42.0) [26] was used to cluster the patients from each cancer type and pan-cancer using the gene expression data of the ICR signature with the following parameters: 5000 repetitions, a maximum of six clusters, and Ward.D2 agglomerative hierarchical clustering. The Calinski-Harabasz index was used to calculate the ideal number of clusters.

The three clusters that were discovered were labeled "ICR High," "ICR Medium," and "ICR Low," with "ICR High" exhibiting the highest ICR gene expression and "ICR Low" the lowest. Each sample's ICR score was determined by averaging the normalized, log2-transformed gene expression values for the ICR genes.

Complex Heatmap (v. 2.6.2) was used to draw heatmaps [27].

The full RNA expression matrix was dimension reduced using the t-distributed stochastic neighbor embedding (t-SNE) visualization using Rtsne (v. 0.15) [28] (sets perplexity=15, theta=0.5). ICR clusters and cancer types were noted on t-SNE plots.

2.3. Survival analysis.

Clinical records include survival information as well as clinical parameters, including, among others, the age at diagnosis, the tissue type, the vital condition, the illness stage, and the disease metastasis. We used the time to death and the time to the last follow-up, as well as vital status, for the overall survival analysis. Relapse, advancement, second malignant neoplasm death, and death without remission were all considered events for event-free survival. Using the ggsurv function from survminer (v. 0.4.8), we conducted a survival analysis and showed the Kaplan-Meier curves [29]. Patients with follow-up durations of less than 1 day were excluded, and survival data were censored after 10 years of follow-up. The X2 test was used to calculate the Hazards Ratio (HR) between the ICR Low and ICR High groups or between the six immunological subtypes and the accompanying p-values. Utilizing survivability (v. 2.41-3), confidence intervals (97.5% CIs) were determined.[30].

Utilizing Survival, Cox proportional hazards regression analysis was carried out and represented as a forest plot. A new variable was introduced to the multivariate analysis: cancer kind. To test the proportional hazards assumption (PHA), we used the cox. zph tool. The high-risk NBL without MYCN amplification cohort's clinical characteristics that affect immunological subtype survival were adjusted using the same manner.

The (Mitosis-Karyorrhexis Index) MKI (High, Intermediate, Low), Ploidy (diploid and hyperploid), and Age group (0-18m,18m-5y, and above 5y) are the clinical factors. A forest plot (v.1.7.2) was used to create the forest plots [31].

2.4. Immune cell subpopulation and oncogenic pathway enrichment analysis.

On the log2-transformed, normalized gene expression data using GSVA (v.1.38.2), we did single sample gene set enrichment analysis (ssGSEA) to assess the enrichment of specific gene sets that affect immune cell types or specific oncogenic pathways.

Immune cell-specific signatures were applied in a manner slightly different from that described by Bindea et al. [32]. Immature dendritic cells (iDC), plasmacytoid dendritic cells (pDC), myeloid dendritic cells (mDC), and dendritic cells (DC) took the place of the dendritic cell (DC) hallmark. The regulatory T-cell (Treg) signature was also applied, as explained by Angelova et al. [33]. Specific tumor-related gene sets that affect particular pathways were chosen from a variety of sources, and gene sets that affect cancer-related immune signatures were used, as previously explained by Torsson et al. [34]. The method used to calculate the correlation between continuous gene set enrichment scores and survival is detailed above. The p-values were determined using the Cox algorithm, and differences between the HRs of the signatures were displayed in a heatmap using ComplexHeatmap (v. 2.2.0) software. All tumor-specific signatures with a p-value greater than 0.1 were disregarded.

2.5. Comprehensive paediatric immune subtypes.

105 of the 108 immunological signatures that had previously been reported were used in the ssGSEA [34]. The analysis did not include tree signatures since there was insufficient data on gene expression. Using corrplot (v.0.90), the Spearman correlation between the obtained enrichment scores was determined. Visual identification of signature modules was done before patients were grouped by the ssGSEA enrichment of the five signatures that represented the previously identified modules by Torsson et al. K-means clustering (km=6, repeats=10,000) was used to cluster the samples using ComplexHeatmap. The ideal number of clusters was calculated using the gap statistics.

The percentage of each immunological subtype inside each cancer type and the percentage of each cancer type in the immune subtypes were displayed using stacked bar charts from ggplot2 (v. 3.3.3). Density plots from ggplot2 were used to display the log2 values of HLA-1 and HLA- 2 from the filtered normalized RNAseq matrix in addition to the median enrichment scores of a few immunological signatures from among the 105 signatures [34].

2.6. Gene expression correlation.

The Pearson correlation of the ICR genes' expression across all cancer types and in the general population was calculated using the corrplot (v.0.90). Due to the limited sample size (n=13), CCSK was left out of this correlation analysis. On the enrichment matrix of 105 tumor immune expression profiles [34], Spearman correlation was carried out and shown using corrplot (v. 0.84). Using Pearson correlation, correlation matrices of NK-cells/CD8T enrichment scores and the enrichment score (ES) of particular oncogenic pathways were computed and visualized by ComplexHeatmap.

2.7. Immune checkpoints expression.

Immune checkpoints were listed and categorized as activating or inhibitory. ComplexHeatmap was used to plot the median values of the log2 transformation of the normalized gene expression counts for these genes.

2.8. CIBERSORTx immune cells fractions.

We used the CIBERSORTx website to analyze the normalized gene expression data of the 408 paediatric samples to evaluate the immune cell fractions between various immunological subtypes. 22 different immune cell types' relative distributions inside the leukocyte compartment (LM22) were estimated. Using ggplot2, cell fractions were shown in bar charts and boxplots. To make comparisons easier, we combined the proportions of comparable immune cells into "Aggregates" [34]. The total number of lymphocytes is made up of naive B-cells, B-cells that are memory, T-cells that are follicular helper, regulatory, Treg, T-cells that are gamma delta, T-cells that are CD8, NK-cells that are resting, NK-cells that are active, and plasma cells. Monocytes, Macrophages M0, Macrophages M1, and Macrophages M2 fractions are added together to form Macrophages. Dendritic cells are made up of both the resting and active components of these cells. Mast cells are made up of the combined portions of resting and active mast cells.

3. RESULTS.

3.1. The prognostic value of ICR differs across paediatric cancer types.

From the TARGET dataset (https://ocg.cancer.gov/programs/target), we examined the expression profiles of patient samples from four different solid paediatric cancer types: WLM, NBL, OS, RT, and CCSK. after the patients listed below have been excluded: We analyzed 408 patient samples (WLM (n=118), NBL (n=150), OS (n=68), RT (n=59), and CCSK (n=13) from patients with WLM, NBL, OS, RT, and CCSK. Of the 20 OS patients who were older than 18 years old, one NBL patient did not have MYCN status information, and one RT patient's sam- ple clustered with NBL samples based on the full transcriptome. The NBL cohort was separated into three groups based on the annotated COG (Children's Oncology Group) risk group and the MYCN gene amplification status: high-risk NBL with MYCN amplification (n=33), high-risk NBL without MYCN amplification (n=91), and Intermediate and low-risk NBL (NBL-ILR) (n =26), because these subgroups were shown to have distinct immune infiltration [35,36].

We treated each subgroup as a different cancer type in our study since dimension-reduction using t-distributed Stochastic Neighbour Embedding (tSNE) based on the complete transcriptome also revealed the separation of NBL subgroups (Wei et al. 2018). To assess the strength of the T1/cytotoxic intratumoral response, we used the expression of ICR genes.

In the majority of the TARGET paediatric solid tumor cohorts, the ICR genes showed a substantial overall connection with one another; however, the correlation was decreased in high-risk NBL with MYCN amplification and in WLM. Three ICR clusters—"ICR High," "ICR Medium," and "ICR Low"—were created from samples from each kind of cancer. A clear difference in the distribution of ICR scores across cancer types was detected, even though dimension reduction of the expression data does not reveal samples being separated by ICR clusters within each tumor type. WLM and CCSK had lower ICR scores, but

RT had the highest ICR ratings. Large immune orientation differences between samples within NBL were reflected by significant differences in ICR scores across NBL subgroups ($p<0.00001$) for high-risk NBL without MYCN amplification vs. Intermediate and low-risk NBL and high-risk NBL with MYCN amplification, respectively. When compared to high-risk NBL without MYCN amplification, ICR scores in high-risk NBL with MYCN amplification, as well as intermediate and low-risk NBL, were significantly lower. This finding is in line with earlier studies that found lower T-cell infiltrates in high-risk NBLs with MYCN amplification [36.37] and larger T-cell infiltrates in high-risk NBLs without MYCN amplification [36].

Comparing the survival between the ICR clusters revealed that in Osteosarcoma (OS), the ICR Low group had significantly lower overall survival ($p0.001$) and event-free survival estimates ($p<0.05$) compared to the other groups combined. Overall survival analysis of continuous ICR scores demonstrated a significant association of ICR scores with a high survival rate in Osteosarcoma ($p<0.016$). In high-risk NBL lacking MYCN amplification, the same pattern was observed, with ICR High being linked to improved overall survival as opposed to ICR Low. As has been shown in adult kidney tumors [28], this pattern was inverted in WLM. Rhabdoid tumors and clear cell sarcomas of the kidney did not significantly correlate with survival (Kaplan Meier curve for CCSK not shown due to the small number of samples, $n=13$).

We then looked at the tumor intrinsic characteristics that correlate with immune infiltrate, as determined by ICR score, in OS and high-risk NBL without MYCN amplification because ICR was highly prognostic in these two types of tumors.

Barrier genes, mismatch repair, proliferation, and G2M checkpoints are among the signatures inversely correlated with survival in both tumors, while PI3K Akt, mTOR signaling, immunogenic cell death, and apoptosis are among the intrinsic tumor pathways that correlated with ICR score in these tumors. In contrast to high-risk NBL without MYCN amplification, osteosarcoma revealed a very substantial inverse connection between Wnt/beta-catenin signaling and ICR.

The Wnt/beta-catenin pathway was significantly related to a worse OS prognosis ($p0.05$) when we next looked at the relationship between intrinsic tumor characteristics and survival in these tumors. In high-risk NBL without MYCN amplification, we did not notice this same connection. Numerous pathways, including Myc targets, glycolysis, mTORC1, DNA repair, mismatch repair, E2F targets, G2M checkpoints, and proliferation, were linked to a worse prognosis in this subgroup of neuroblastoma displaying significant differences in immunological orientation between samples within NBL. When compared to high-risk NBL without MYCN amplification, ICR scores in high-risk NBL with MYCN amplification, as well as intermediate and low-risk NBL, were significantly lower. This result is in line with earlier studies that found lower T-cell infiltrates in high-risk NBLs with MYCN amplification [36, 37] and larger T-cell infiltrates in high-risk NBLs without MYCN amplification [36].

3.2. The functional orientation of infiltrating immune cells influences the clinical outcome of paediatric cancers.

Using the gene expression signatures of previously published datasets [32,33], as described in the methods section, we compared the enrichment of leukocyte subpopulations within and among cancer types (pan-cancer) to further explore the various immune characteristics of paediatric tumor types. In the pan-cancer analysis, markers like NK-cells, Tcm, TFH, Tem, CD8+ T-cells, and neutrophils were significantly linked to higher overall survival, but T helper and T2 cells were linked to a worse prognosis.

Osteosarcoma had an immunologically active phenotype in comparison to other cancer types, as evidenced by greater mean enrichment of transcripts for dendritic cells (DC), macrophages, neutrophils, and mDC.

TFH, DC, neutrophils, macrophages, monocytes, T1, and regulatory T cells (Treg) enrichment scores were associated with significantly im-

proved prognosis, whereas B cell and gamma delta T-cell enrichments were associated with significantly worse survival in this cancer type.

In neuroblastoma, T-cells, CD8+ T cells, T17, NKT cells, T1 cells, Treg cells, and DCs were significantly more enriched in the high-risk NBL without MYCN amplification group compared to the high-risk NBL with MYCN amplification group ($p < 0.05$). TFH was also significantly higher in all three subgroups and significantly positively correlated with survival. Additionally, in the high-risk NBL without MYCN amplification group, gamma delta T cell (Tgd) enrichment demonstrated a strong correlation with survival ($p < 0.05$).

T2 cells and NK CD56 bright cells, on the other hand, were significantly more enriched in the high-risk NBL with MYCN amplification group compared to the high-risk NBL without MYCN amplification group (p 0.05), and in intermediate and low-risk NBL, there was a significant association between NK CD56 bright cells and a worse prognosis (p 0.05).

While RT displayed the greatest ICR score, WLM and CCSK were characterized by modest immune infiltrates as seen by low ICR scores.

In WLM, lower infiltration was linked to better survival the opposite correlation has previously been seen in adult kidney cancer [38]. Immune subpopulations were found to have a generallylow enrichment in WLM, although there was no discernible correlation with survival.

While the pattern was reversed in T2 and T helper cells, consistent with similar observations in adult cancer, pan-cancer expression patterns consistent with enrichment of several immune cells were associated with favorable prognosis, including NK-cells, Tcm, TFH, Tem, CD8 T cells, and Neutrophils. We were unable to find consistent significant predictive indicators in the leukocyte populations across all malignancies, though, because of the limited sample sizes of some cohorts.

3.3. Identification of distinct immune subtypes of paediatric tumors.

We broadened our analysis to include a set of previously described immune signatures to better understand the influence of the cancer immune phenotypes in pediatric solid cancer. On 105 immune signatures, we used ssGSEA to cluster the immunological signatures into defined modules of strongly correlated immune signatures. Five major signature clusters, or modules, were found.

Unexpectedly, we were able to locate one of the typical signatures listed in Torsson et al. [34] (IFN-, TGF-ß, Macrophages, Lymphocytes, Wound healing) in each of these modules. This finding shows that immunological signature modules in paediatric cancer displayed a pattern of correlation that was similar to those found in adult solid tumors, reflecting the durability of these modules.

The 400 patients were then divided into 6 immunological subtypes (S1 to S6) with different immunologic orientations based on the enrichment scores of these 5 sample immune gene signatures. Patients from various tumor types are represented in each subtype, and each tumor type is made up of various immunological subtypes. For each of the four sample immune signatures and each of the seven additional immune indicators known to affect the immunological orientation, density plots were created. This made it possible for us to categorize the immunological subtypes according to their enrichment capabilities. The T2 dominant subtype S1 contains the lowest TGF-ß, Macrophage, Lymphocyte, and IFN-ß signal and the greatest T2. Since S2 has the highest T1-T2 ratio, highest HLA1 expression, and low- est wound healing enrichment, it has been dubbed the inflammatory subtype. We refer to this sub- type as immunologically silent since strong TGF-ß sticks out in S3, in addition to the low enrichment of T1, and T17. The highest wound healing enrichment is found in the S4 subtype, which also has a significant number of T2 and Treg cells. The S5 subtype is referred to as Macrophage dominating because it appears to be immunologically compromised by high Macrophage presence despite having enhanced TGF-ß and IFN-,

ICR signals, and high T1 and T17 enrichment. The last immune subtype, S6, also known as the lymphocyte-suppressed subtype, is enriched in practically all characteristics of a high immune infiltration (highest ICR), including the presence of macrophages and counter-regulatory signals from T2 and Treg. We refer to it as immunological (or lymphocyte) suppression since it also exhibits significant levels of immune checkpoints and exhaustion markers.

Using CIBERSORTx, we examined the immune cell fractions amongst immunological subtypes. High proportions of macrophages were discovered in S5, with elevated proportions of M2 macrophages in S4, high proportions of mast cells in S3, and high proportions of lymphocytes in S2—the cell type with the best survival rate. Additionally, it was quite obvious that S6 had high lymphocyte proportions, which are indicated by elevated immunological checkpoints and exhaustion markers that inhibit the effect of T cells and place the tissue in an exhausted state.

We created the heatmaps in the Supplementary to better understand how each immune subtype contributes to the overall immune response for each tumor type. For instance, the S6, S5, and S4 immune subtypes, which are distinguished by the highest ICR enrichment scores, predominate in the rhabdoid tumor.

The high ICR scores shown in the RT are a result of this, in turn. In addition to the signals from T2, Treg, downregulated HLA1, Macrophage presence, immunological checkpoints, and exhaustion markers, as previously revealed, the immune-suppressed S6 subtype is characterized by the enrichment of nearly all attributes of a high immune infiltration. While a significant M2 macrophage presence suppresses S5 (Macrophage Dominant). Finally, the increased expression of genes involved in wound healing suppresses S4. This explains why a high ICR score in these RT patients is not linked to a better prognosis.

3.4. Immune subtype classification segregates tumors into distinct risk categories.

We stratified the model for the cancer type and performed Cox proportional hazard regression analysis, which revealed significant differences in overall survival between subtypes. Cox proportional hazard models showed significant violations of the model when adding the cancer type as a covariate. The Inflammatory subtype (S2) had the best prognosis, but the Wound Healing Dominant subtype (S4) had the worst survival. The immune subtype with the highest survival among the immune subtypes, S2, had the lowest enrichment of the wound healing signature, demonstrating a link between the expression of the wound healing signature and prognosis in paediatric tumors.

High T1 and low T2 infiltrations were seen in S2 in addition to the poor enrichment of wound healing, but in S4 the opposite was shown (high T2 and low T1 infiltration). This observation supports the T1 orientation of the tumor microenvironment's beneficial prognostic impact in this situation. We conducted a multivariate analy- sis using a Cox proportional hazards model with the cluster (immune subtype) and cancer type as covariants to determine whether the difference in survival across immune subtypes is caused by the tumor type distribution between clusters. We again found a significant difference in survival between S2 and S4 ($p=0.02$), between S5 and S4 ($p=0.013$), and between S6 and S4 ($p=0.0325$), demonstrating the prognosis.

We analyzed overall survival between immune subgroups within each tumor to assess the prognostic usefulness of our immunological stratification within each tumor type. We discovered significant differences between all immune subtypes against S4 for high-risk NBL without MYCN amplification tumors, which is intriguing and suggests the existence of subgroups with unique immunological characteristics within the high-risk NBL without MYCN amplification cohort. Both Wilms and Rhabdoid tumors showed the same survival pattern for S4, and an obvious difference in survival between the S3 and S5 subtypes

was identified in osteosarcoma (p=0.09). Because there were insufficient samples for CCSK, the K-M curve could not be plotted. These findings demonstrate the immune variability inside tumors and emphasize the significance of comprehending the immunological characteristics of paediatric tumors and their subgroups to improve the therapeutic effect.

We performed multivariate analysis to correct for the contribution of other clinical parameters in the survival of NBL; a significant difference in survival was found between S2 (p=0.0319) and S6 (p=0.0452) compared to S4. NBL is a heterogeneous tumor and different clinical parameters contribute to the survival of NBL [36,39]. As previously mentioned, a significant difference across immune subtypes within the high-risk NBL without MYCN amplification was observed. Similarly, we looked at sub-setting different cancer kinds based on various clinical factors, but no significant findings were made.

3.5. Immune checkpoint expression pattern varies across different immune subtypes.

We conducted a survival analysis for checkpoint expression across our immune subgroups and throughout the entire spectrum of cancer to better understand the predictive function of immunological checkpoints in paediatric tumors. In a comprehensive investigation of cancer, the CD276 gene was significantly linked to survival. Weobserved that immunological checkpoints with significant prognostic associations, such as CD276, KIRD3DL1, VTCN1, and C10orf54 (VISTA), are weakly enriched in S4, whereas those with great prognostic associations, such as LAG3, CD70, TNFSF4, IDO1, KIRD3DX1, CD28, and TNFSF9, were strongly expressed in S4. We created an HR heatmap annotated by immune subtypes to better understand the predictive impact of the expression of immunological checkpoints within each immune subtype. C10orf54 (VISTA) and CD86 were found in S4 to have a distinct pattern of connection with a worse prognosis (p 0.05–0.1). While TNFRSF9 was linked to lower survival in S4 and S3, it was not. Expression of CD70 and LAG3 was significantly associated with poor survival in pan-cancer (p 0.05). Some immunological checkpoints exhibit a reverse pattern of survival with various subtypes, including TNFRSF4 across S2 and S6, TNFRSF14 across S1 and S3, and C10orf54 linked with reverse favorable prognosis in S2. These findings reflect the differences in immune checkpoint expression prognosis among various tumor types.

In comparison to other immune subtypes, the Leukocyte Dominant Subtype (S6) showed high expression of immunological checkpoints. This could be explained by the immunological subtype's state of fatigue, which has the highest lymphocyte enrichment.

3.6. Activation of oncogenic pathways is associated with the differential immune disposition.

By examining the relationship between overall survival and the expression of tumor intrinsic pathways in pan-cancer and across the immune subtypes, as well as by comparing the enrichment of tumor intrinsic pathways between the 6 immune subtypes, we also looked at tumor intrinsic differences between immune subtypes. Between immunological subtypes, a large range of pathways were differentially enriched. When compared to other groups, S4 demonstrated a very high enrichment for Myc targets, DNA repair, and oxidative phosphorylation. Wnt/beta-catenin and TGF-ß, however, demonstrated a comparable pattern of immunological subtype enrichment with greater enrichment in S3 and S5. It's interesting to note that most of the pathways exhibit mirrored expression levels between S2 and S4. For instance, TGF-ß and barrier gene enrichment in S4 was significantly higher than in S2 (p 0.05), whereas p38 MAPK Sig- nalling, ErbB2 ErbB3 Signalling, NOS1 Signa- ture, and SHC1/pSTAT3 Signatures were sig- nificantly higher in S4 than in S2 (p 0.05). Only the immunological subtype S4 shows a very high correlation between some oncogenic pathways—including mTORC1, Myc targets, NOS1, ERK5, and PI3K AKT—with a worse prognosis.

4. DISCUSSION.

In this study, we dissented immune-cancer interactions about clinical outcomes to offer a thorough assessment of the immunological landscape of paediatric tumors. In the context of adult cancer, we've previously demonstrated that the Immunologic Constant of Rejection (ICR), a signature that detects the presence of active immune-mediated tumor rejection, has prognostic and predictive value [38,40]. To test if this signature can also predict survival in paediatric tumors, we applied it to children with solid tumors. In a per-cancer analysis of the five paediatric solid tumor types observed in the TARGET cohort, we discovered that high ICR and the disposition of an immune active phenotype were linked with a good prognosis in high-risk NBL without MYCN amplification and osteosarcoma. When the adult and paediatric osteosarcoma are studied jointly, this connection with survival is also seen (C. Zhang et al. 2020).

WNT/-catenin was discovered to be related to poor immune infiltrate and cancer immunosurveillance in several adult tumors when we tried to untangle the mechanism involved in tumor immune evasion [41].

In this study, we discovered an inverse relationship between immune infiltrate (ICR score) in OS and prognostic value in the same cohort. The higher the WNT/-catenin pathway enrichment, the worse the prognosis. In addition to the link with overall survival and low immune infiltrate, Xie et al.'s meta-analysis revealed that overexpression of -catenin is a sign of metastasis in patients with osteosarcoma [42]. For the treatment of various tumors, several clinical trials are currently being conducted that combine immunotherapy with inhibitors of the WNT/-catenin signaling pathway (Y. Zhang and Wang 2020).

We showed that high-risk NBL without MYCN amplification was linked with noticeably greater immune infiltrates, as was the case in OS, and we illustrated the predictive significance of the ICR signature in this subgroup of NBL. In the high-risk NBL without MYCN amplification subgroup of NBL patients, but not in the other groups, a recent signature focused on effector genes (5 granzymes paired with perforin) that affect the level of cytotoxic immune cell activity demonstrated a connection with survival [36].

We observed the same results in paediatric tumors, where the wound healing dominant subtype (S4) displayed the worst survival among the paediatric immune subtypes. High enrichment of the wound healing signature was linked to worse outcomes in adult tumors [34, 43].

High enrichment of the proliferation signature and increased expression of angiogenic genes go hand in hand with wound healing enrichment. A large percentage of macrophages, particularly M2 macrophages, as detected by CIBERSORTx were also present in S4.

Additionally, S2 showed high T1 and low T2 infiltrates, whereas S4 showed the opposite pattern, suggesting that T1 infiltrates are associated with a better prognosis in these paediatric tumors, similar to the findings in other adult cancer types [38,40,34,44,45]. In contrast, and in line with earlier findings in adult cancer [34], the T2 was heavily infused in the wound healing subtype (S4), which is characterized by a worse prognosis and high proliferation rate.

Additionally, as we have previously shown, S6, S5, and S4 predominate most in the rhabdoid tumor.

These immune subtypes are repressed in a variety of ways, including elevated levels of T2, Treg, immunological checkpoints, and fatigue markers in S6, elevated levels of M2 macrophage presence in S5, or elevated wound healing gene expression in S4. The greatest ICR scores were reported in S6, S5, and S4 when looking at the ICR scores across the 6 immunological subtypes. These findings underline the significance of this more comprehensive immune classification, which captures the various immunological features of the tumor's immune microenvironment that the ICR score missed.

We observed a significantly larger abundance of DNA repair pathways in the S4 subtype compared to the other subtypes when we looked beyond the subtype at how underlying cancer intrinsic path-

ways interact with the immune system. The concept of synthetic lethality, which might be investigated experimentally in these tumors, offers therapeutic options to kill cancer cells without harming normal cells in a variety of cancers [46-48].

5. CONCLUSION.

Finally, we showed that extracranial solid paediatric tumors can be divided into categories based on their immunological profile, revealing unexpected parallels between paediatric and adult tumors. Further investigation of immunological characteristics can reveal diagnostic and prognostic indicators as well as potential immune-responsive tumors. The importance of categorizing paediatric solid tumors into various immunological phenotypes is highlighted by the notable variations in immune checkpoint expression across immune subtypes and the various associations of immune checkpoints with survival. This is the first paediatric pan-cancer immunogenomic study.

6. LIMITATIONS.

The limitations of this study include a small sample population who were included in this study. The findings of this study cannot be generalized for a larger sample population. Furthermore, the lack of a comparison group also poses a limitation for this study's findings.

7. RECOMMENDATIONS.

Close disease surveillance and genetic evaluation are recommended for patients with certain solid tumors or particular predisposing conditions.

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9. LIST OF ABBREVIATIONS.

WLM- Wilmstumour
NBL- Neuroblastoma
OS- Osteosarcoma
CCSK- Clear cell sarcoma of the kidney
RT- Rhabdoid tumour
ICR- Immunologic Constant of Rejection
ssGSEA- Single-sample Gene Set Enrichment Analysis
MYCN- v-myc myelocytomatosis viral related oncogene, neuroblastoma derived (avian)
TCGA- The Cancer Genome Atlas
WNT- Wingless-related integration site
MHC- Major histocompatibility complex
NK- Natural killer
CRISPR- Clustered Regularly Interspaced Short Palindromic Repeats
FDA- Food and Drug Administration
GM-CSF- Granulocyte macrophage colony-stimulating factor
IL- Interleukin
RNA- Ribonucleic acid
t-SNE- t-distributed Stochastic Neighbor Embedding
HR- Hazards Ratio
PHA- Proportional hazards assumption
MKI- Mitosis-Karyorrhexis Index
GSVA- Gene set variation analysis
iDC- Immature dendritic cells
mDC- Myeloid dendritic cells
ILR- Intermediate and low risk
TFH- Follicular helper T
TGF- Transforming growth factor

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11. Conflict of interest.

The authors report no conflicts of interest in this work.

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