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Blastemal NCAM$^+$ALDH1$^+$ Wilms' tumor cancer stem cells correlate with disease progression and poor clinical outcome: A pilot study

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ABSTRACT

Background: Cancer Stem Cells (CSCs) have been suggested as the culprit responsible for tumor resistance to treatment and disease recurrence. Wilms' tumor (WT) is a paradigm for studying the relation between development and tumorigenesis, showing three main histological elements: undifferentiated blastema, epithelia and stroma, mimicking human kidney development. NCAM + ALDH1 + cells were previously found to contain the cancer stem like-cell population in WT. Thus far, the correlation between histologic characterization of this cell population, clinicopathologic parameters and prognostic outcome has yet been investigated in WT.

Procedures: Paraffin-imbedded primary WT specimens from twenty-four patients were immunostained for NCAM and ALDH1. Positivity and histologic compartment localization were determined by two independent observers, blinded to the clinical outcome. Clinicopathologic parameters and prognostic outcomes were determined based on the patients' medical records. The association of NCAM and ALDH1 co-localization with clinicopathologic characteristics was analyzed by χ^2-test. Survival analysis was carried out by the log-rank test using Kaplan-Meier method.

Results: Blastemal co-localization of NCAM and ALDH1 was observed in 33% of WTs. Metastases, ICE chemotherapy protocol, blastemal predominance following preoperative chemotherapy, recurrence and patient demise were found to significantly correlate with blastemal NCAM + ALDH1 + cell staining ($p < 0.05$). A significant inverse correlation between blastemal double positive cells, disease-free survival and overall survival was also observed.

Conclusions: WT blastemal NCAM + ALDH1 + CSCs significantly correlate with adverse clinicopathologic parameters and poorer prognosis. These results underscore the role of CSCs in disease progression. Additionally, this pilot study supports the addition of these markers for risk stratification of WTs.

1. Introduction

Pediatric malignant solid tumors are a group of childhood cancers that are comprised of embryonic cellular lineages, recapitulating embryonic development. Interruptions to normal development in these lineages are believed to underlie the genesis of many, if not all of these tumors [1,2]. Throughout human kidney development, the nephrogenic stem cells are located within the metanephric mesenchyme, and

Abbreviations: NCAM, neural cell adhesion molecule; ALDH1, aldehyde dehydrogenase1; CSCs, cancer stem cells; WT, Wilms' tumor; ICE, ifosfamide, carboplatin and etoposide; CM, cap-mesenchyme; COG, children's oncology group; SIOP, international society of pediatric oncology; FH, favorable histology; PDXs, patient derived xenografts; DP, double positive

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specifically the cap-mesenchyme (CM) of the nephrogenic zone [3,4]. Wilms' tumor (WT), the most common pediatric malignancy of the urinary tract, is believed to arise from malignant transformation of abnormally persistent renal stem cells that maintain self-renewal capacity and in parallel undergo partial differentiation to form the different cellular compartments observed in the tumor [5]. WT serves as a prototype for studying the relation between development and tumorigenesis [6,7]. This hypothesis is strengthened by the appearance of three main elements in the tumor: undifferentiated blastema (resembling the undifferentiated embryonic metanephric mesenchyme), tubular epithelia (thought to arise by an attempt of the blastemal cell to differentiate toward renal tubules) and stroma, which are histologically similar to the different cellular components of the developing human kidney [8]. WT blastemal component has been implicated as the most aggressive cellular compartment in the tumor. For instance, proliferation capacity of the blastemal but not of the epithelial or stromal components had a significant adverse prognostic value [9,10]. Moreover, the blastemal component was shown to be the most likely to metastasize and was found to constitute most if not all of WT metastatic lesions before and after chemotherapy treatment [11].

Malignant renal tumors accounts for 7% of all pediatric malignancies. Of these Wilms' tumor (WT), is the second most common intra-abdominal cancer and the most common malignancy of the genitourinary tract in children [12]. The mean age at diagnosis is 41.5 months for boys and 46.9 months for girls, bilateral disease accounts for 4–7% of cases and is diagnosed earlier (31 months) [13].

Distant metastasis can be found in about 10% of WT through hematogenous spread, most commonly to the lungs (85%), liver (10%) and only very rarely to the bones or brain. WT can be divided into two main prognostic groups based on tumor histopathology: favorable histology (FH) and unfavorable histology (focal or diffused anaplasia). The latter is associated with poor prognosis, mainly in combination with a higher stage [14].

With advances in multimodal treatment approaches, survival rate for WT patients has reached 90% for localized and 75% for metastatic disease [8]. However, there are subgroups for whom survival rates are around ˜50%. These include patients with poor prognostic factors and disease recurrence [15].

Treatment of WT currently depends on risk stratification based on prognostic factors applied by the Children's Oncology Group (COG) and International Society of Pediatric Oncology (SIOP). The most important prognostic marker is tumor histology (e.g. favorable histology versus anaplasia), with tumor anaplasia considered the highest-risk. Additionally, predominantly blastemal component of the tumor (as opposed to tri-lineage or epithelial predominant histology), especially following preoperative chemotherapy, is also considered higher risk [16]. Tumor stage is the second most important prognostic factor. Additional prognostic factors that contribute to risk stratification include patient age, tumor weight, molecular abnormalities such as 1q gain and loss of heterozygosity (LOH) of 1p and 16q [17].

Current treatment involves a multimodal approach that includes surgery, chemotherapy and radiation. Chemotherapy protocols include different combinations of vincristine, dactinomycin, doxorubicin, cyclophosphamide, etoposide and carboplatin. However, these treatment protocols are not cell- nor target-specific [18].

According to the cancer stem cell model, there exists a small cell subpopulation within the tumor bulk, termed cancer stem cells (CSCs), which possess the unique capacity to self-renew and thus exclusively maintain tumor growth and progression. These CSCs are capable of both expanding the CSCs pool and differentiating into the heterogeneous nontumorigenic cancer cells that comprise most of the tumor bulk [19].

Due to their relatively quiescent nature, CSCs have been shown to be resistant to therapies directed at eradication of the rapidly dividing cells within the tumor that comprise the majority of tumor cells. Thus, their persistence after such treatments (i.e. conventional chemotherapy, radiation) put forward the CSCs as the culprit responsible for tumor recurrence [19–23].

We have previously found that neural cell adhesion molecule 1 (NCAM1)-positive and aldehyde dehydrogenase 1 (ALDH1)-positive enzymatic activity ($NCAM^+ALDH1^+$) cells, located within Wilms' tumor blastemal component, contain the cancer stem like-cell population in WT [2].

WT CSCs were isolated from tri-lineage human WT propagated in immunodeficient mice as WT Patient Derived Xenografts (WT-PDX), based on the expression of NCAM1 - a marker of both the human fetal kidney (hFK) cap-mesenchyme and WT blastema, and ALDH1 enzymatic activity ($NCAM^+ALDH1^+$). These propagated WT-PDX showed blastemal-predominance (NCAM+) histology upon passages in mice. Targeting of the NCAM + cells with an ADC, lovertuzumab, resulted in complete eradication of these predominant blastemal WT-PDXs [2].

Correlation between the CSC population and disease prognosis has been found in several malignancies. For instance, in breast cancer, $CD44^+/CD24^-$ CSC population was shown to be associated with distant metastasis and decreased disease-free survival [24] and $ALDH1^+$ CSCs were associated with poor clinical outcome [25]. In glioma patients, $CD133^+$ cells were associated with an adverse prognosis [26]. However, their association with poorer clinicopathologic and prognostic parameters has yet been determined in pediatric tumors in general and specifically in WT.

Herein we studied the connection between the presence and co-localization of the WT CSC markers, NCAM and ALDH1 within primary WTs and several clinical and prognostic parameters.

2. Materials and methods

2.1. Patient characteristics

Forty five patients who underwent nephrectomy between 2001 and 2018 at the Sheba Medical Center and Hadassah Ein Karem were enrolled in this study. Of these, only for twenty four a complete follow-up was available and therefore were included in this study. All cases were reviewed according to WHO classification criteria, using standard tissue sections and appropriate immunohistochemical slides. Twenty-four medical records were reviewed for clinical information; histologic parameters were determined from the H&E-stained slides. Clinicopathologic parameters evaluated for each tumor included patient gender, age at initial diagnosis, tumor size, histologic subtype, tumor grade, tumor stage, tumor recurrence, distant metastasis, LOH of 1p or 16q, use of neoadjuvant chemotherapy, blastemal predominance following neoadjuvant treatment, treatment protocols, disease free survival (DFS) and overall survival (OS). Moreover, all specimens were characterized for all routine diagnostic immunophenotypic parameters.

As for the other 21 cases excluded from the final analysis, these were omitted either due to loss to follow up, otherwise limited data insufficient for analysis, and/or lack of available blocks for immunostaining. Nevertheless, this group did not significantly differ from the final cohort in clinical parameters nor in demographic characteristics (Supplementary Table S1).

2.2. Immunohistochemical staining

Immunostaining was performed as previously described [27]. Briefly, 4-μm thick sections, were cut from primary WT, hFK and hAK for immunohistochemistry. Sections were processed within 1 week to avoid oxidation of antigens. Cuts were mounted on super frost/plus glass (Menzel, Glazer, Braunschweig, Germany) and processed by the labeled – (strept) avidin-biotin (LAB-SA) method using a histostain plus kit (Zymed San Francisco, CA, USA). Heat-induced antigen retrieval was performed by controlled microwave treatment using an H2800 model processor (Energy Bean Sciences, INC) in 10 mM citrate buffer, PH 6.0 for 10 min at 97 °C followed by 3% H2O2 for 10 min. The slides

were subsequently stained using the labeled strepavidin-biotin (LAB-SA) method using a Histostain plus kit (Zymed, San Francisco, CA, USA). Anti-human NCAM (LifeSpan Biosciences, Inc. Seattle, WA, USA) and anti human ALDH1 antibodies (BD Biosciences #611195) were used. Negative control incubations were performed by substituting non-immune serum for the primary antibody. Biotinylated second antibody was applied for 10 min followed by incubation with horseradish peroxidase –conjugated streptavidin (HRP-SA) for 10 min. The immunoreaction was visualized by an HRP-based chromogen/substrate system, including DAB (brown) chromogen (liquid DAB substrate kit – Zymed). The sections were then counterstained with Mayer's hematoxylin, dehydrated and mounted for microscopic examination.

2.3. NCAM and ALDH1 quantification and localization – immunohistochemistry evaluation and scoring

Representative paraffin tumor blocks were selected according to initial evaluation of haematoxylin/eosin-stained slides. For each sample, two cores (inside the tumor) were analyzed. Antigen expression was evaluated by two independent investigators (I.B. and N.P.S.) in a blind manner, using light microscopy. Observers were unaware of the clinical and pathological variables. Following individual assessments a consensus was reached. The extent, cell type and subcellular distribution of NCAM and ALDH1 co-localization were evaluated. According to our previous work the localization of single markers, either NCAM or ALDH1 alone was not scored as these does not represent the CSCs [2]. The extent of NCAM + ALDH1 + double-positive cells was scored as follows: 0 = none; 1 = 1–5% positive cells; 2 = 6–50% positive cells; 3 = > 50% positive cells. For each WT the mean value of two biopsies was used in further analysis. The staining intensity was not taken into account, i.e. cells were scored as either positive or negative. Staining was assessed at × 10, × 20 and x40. In cases where scoring was more difficult magnification of x60/x100 was used. For NCAM1 expression, immunopositivity in the cell membrane was considered positive [28,29], while, for ALDH1 only cytoplasmic staining was deemed positive [30].

2.4. Immunofluorescence (IF) staining and confocal microscopy

Please see supplementary materials and methods section

2.5. Statistical analysis

All measured variables and derived parameters were listed individually and, if appropriate, tabulated by descriptive statistics.

For categorical variables summary tables were provided giving sample size, absolute and relative frequency.

For continuous variables summary tables were provided giving sample size, arithmetic mean, and standard deviation.

Chi-square test or Fisher's Exact test (as is appropriate) was applied for testing the statistical significance of the differences between the study groups.

The two-sample *t*-test or Non-parametric Wilcoxon-Mann-Whitney Rank sum test for independent samples (as is appropriate) was applied for testing the statistical significance of the differences between the study groups.

Overall Survival (OS) and Disease-Free Survival (DFS) curves were calculated using Kaplan-Meier method (survival function curve). OS was defined as the time from diagnosis to death by any cause or until the most recent follow-up. DFS was measured as the time from diagnosis to the occurrence of progression or relapse after complete remission

All tests were two-tailed, and a p value of 5% or less was considered statistically significant.

All the statistical analyses were carried out using SPSS Statistics (IBM SPSS Statistics, Version 25, 2017, IBM Corp, Armonk, NY, USA.

Table 1
Patient characteristics.

Clinicopathologic features			*n = 24*
Age (at diagnosis, years, mean ± SD)			3 ± 1.6
Gender (male/female)			14/10(58%/42%)
Tumor	Stage (at diagnosis)	I	3(13%)
		II	5(21%)
		III	9(38%)
		IV	3(13%)
		V	4(17%)
	Metastases (yes/no)		7(30%)
	Histology	Triphasic	13(54%)
		Blastemal	6(25%)
		Epithelial	3(13%)
		Stromal	1(4%)
	non-favorable/favorable histology (anaplasia/no anaplasia)		2/22 (8%/92%)
	Renal failure		3(13%)
	Presentation	mass	24(100%)
		hematuria	3(13%)
		pain	2(8%)
		Pulmonary embolism	1(4%)
	LOH 16q/1p		2(8%)
	Treatment protocol	DD4A	12(50%)
		EE4A	9(38%)
		Radiotherapy	12(50%)
		HU1 (ICE)	5(21%)
		Glivec	1(4%)
		M protocol (CE)	6(25%)
Neo-adjuvant			8(33%)
Decent (Jewish/arab muslim)			14/10(58%/42%)
Relapse			7(29%)
Demise			3(13%)

Due to the size of our cohort, statistical analysis was performed by two independent biostatisticians.

3. Results

3.1. Patient characteristics

Our cohort was initially comprised of foury-five Wilms' tumor (WT) cases of which twenty-one were lost to follow-up and only for twenty-four a full follow-up was available. We have assessed the clinicopathologic features of the twenty-four Wilms' tumor patients as detailed in Table 1 and supplemental table S1. The mean age at time of diagnosis was 2.9 years. A full follow-up period was available for all patients with a mean of 60 months (ranging from 12 to 180 months). There were 10 (42%) female and 14 (58%) male patients. Histological analysis showed favorable histology for 22 (92%) and unfavorable histology for 2 (8%) of the 24 WT cases. Triphasic histology was observed in 13 (54%), blastemal predominance in 6 (25%), epithelial predominance in 3 (13%) and stromal predominance in 1 (4%) of patients.

Of the 24 patients, 3 (13%) were stage I, 5 (21%) were stage II, 9 (38%) were stage III, 3 (13%) were stage IV, and 4 (17%) were stage V.

Among the 24 patients, recurrence occurred in 7 (29%) of cases during the follow-up period and 13% had succumbed to their disease.

3.2. Expression of NCAM + ALDH1 + in Wilms' tumor and human fetal kidney (hFK) shows different staining patterns

Blastemal co-expression of NCAM and ALDH1 in WT advanced generation PDXs was previously shown to mark the WT CSCs [2]. In order to determine expression patterns of NCAM and ALDH1 in WT as well as in its non-malignant counterpart, the human fetal kidney (hFK), we have stained 24 primary WTs, 9 hF K and 4 human adult kidneys (hAK, adjacent to the tumor) for NCAM and ALDH1. NCAM expression was mainly membranous, while ALDH1 staining was mainly

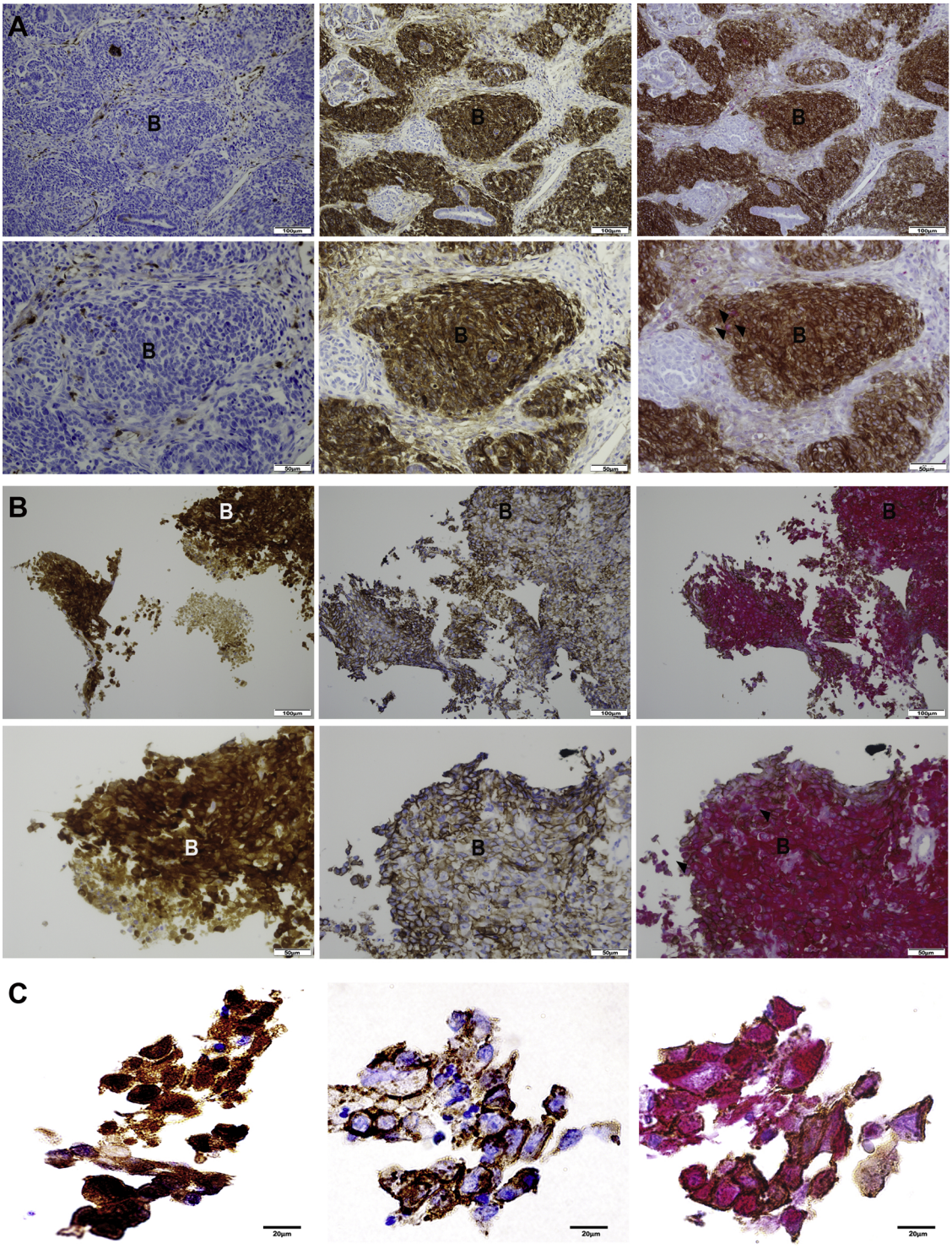

Fig. 1. Representative Immunostaining of NCAM and ALDH1 in WT blastema. (A–C) Immunohistochemical staining for NCAM alone (left panel-brown), ALDH1 alone (middle panel-brown) and both NCAM and ALDH1 (right panel-brown and red respectively) in serial sections of representative WTs showing, double positive blastemal staining. Arrow heads indicate representative cells with co-localization of NCAM and ALDH1; B-Blastema; Scale bars are indicated in the images. In sections A and B magnifications are x20 (upper panels) and x40 (lower panels) of the same area in the tumor. Section C shows magnification of x100 of serial tumor sections.

cytoplasmic as previously described (Fig. 1 and Supplemental Figures S1, S2 and S3) [31]. Blastemal double positive (DP) cells were observed in 8 of tumors (33%), epithelial DP cells in 2 tumors (8%) and stromal DP cells in 1 tumor (4%) (Fig. 2A-C and Table 2). In the hFK no overlapping expression of NCAM, which stained the cap mesenchyme and s/comma shaped bodies of the developing kidney and ALDH1, which stained mature epithelial tubules, was observed (Fig. 2D). No DP cells were observed in adjacent normal human kidney (supplemental Figure S4). These results are in line with previous findings supporting the WT CSCs as distinct from the hFK stem cells residing in the Cap-Mesenchyme of the developing kidney [32].

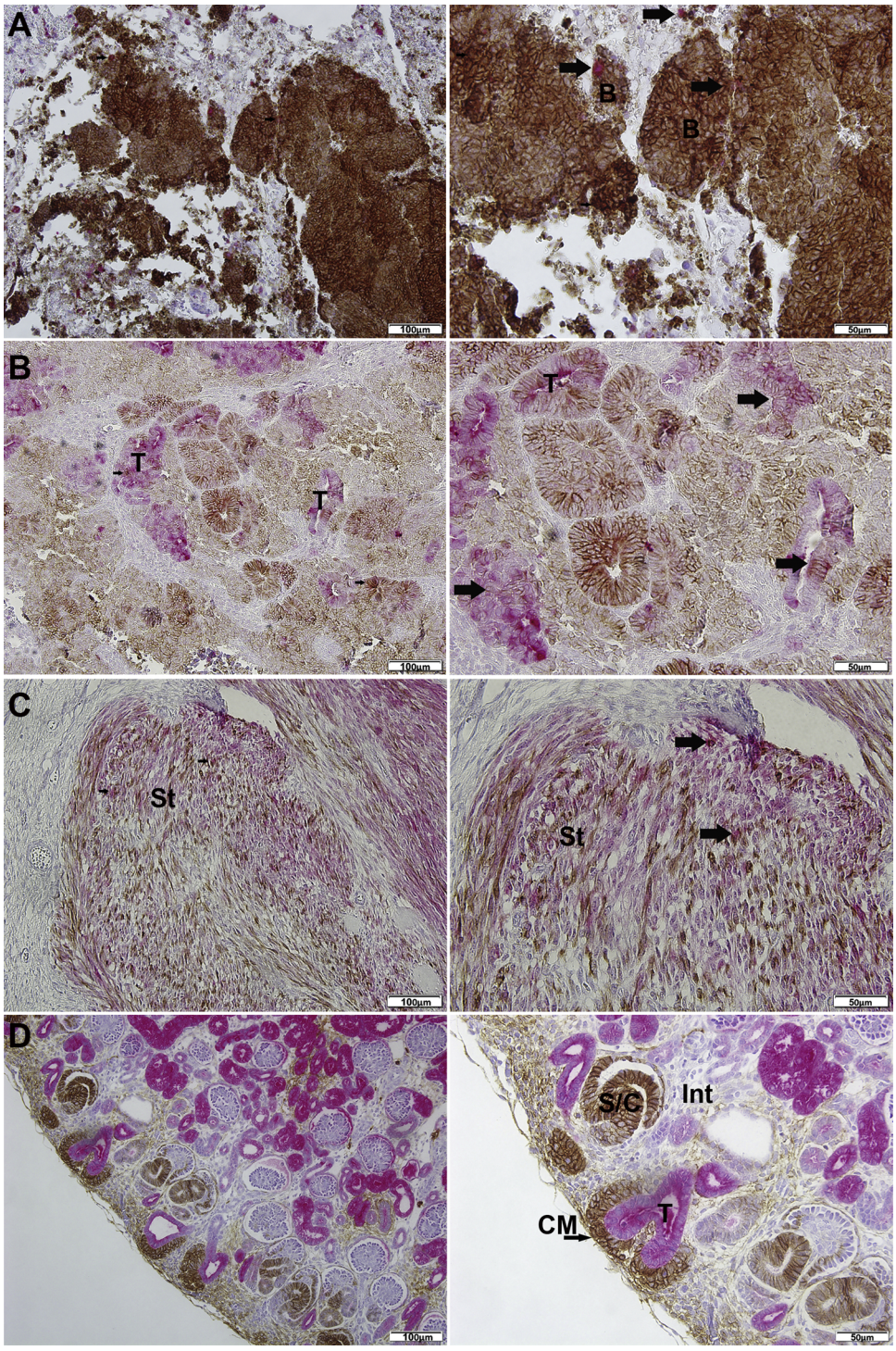

Fig. 2. NCAM$^+$ALDH1$^+$ localizes to the undifferentiated blastema only in Wilms' tumor and not in the human Fetal Kidney (hFK). Immunohistochemical staining for NCAM (brown) and ALDH1 (red) in representative primary WT and hFK demonstrating (A) WT blastemal NCAM$^+$ALDH1$^+$ staining; (B) WT epithelial NCAM$^+$ALDH1$^+$staining; (C) WT stromal NCAM$^+$ALDH1$^+$ staining; (D) NCAM and ALDH1 staining in the hFK showing NCAM (brown) to be expressed by the cap mesenchyme and s/comma-shaped bodies as well as the Interstitium and ALDH1 to localize to mature tubular structures, there is no co-localization of these two proteins in the hFK; B-Blastema; T-Tubules; St-Stroma; S/C-S/Comma shaped bodies; Int-Interstitium. Scale bars are indicated in the images. In each panel magnifications are x20 (left) and x40 (right) of the same area in the tumor.

Table 2
Expression patterns of NCAM and ALDH1 in Wilms' Tumor.

Blastemal NCAM + ALDH1 +	Epithelial NCAM + ALDH1 +	Stromal NCAM + ALDH1 +	No NCAM + ALDH1 +
8/24 (33%)	2/24 (8%)	1/24 (4%)	13/24 (54%)

3.3. Localization of NCAM + ALDH1+ cells to WT blastema correlates with disease prognosis while epithelial or stromal confinement does not

The aggressive nature of the WT CSCs has been previously shown in WT-PDXs model [2]. However, the correlation between WT CSCs and clinicopathologic elements has yet been established.

In order to determine the association between NCAM + ALDH1 + WT CSCs and disease prognostic factors, immunohistochemical staining for NCAM and ALDH1 cellular co-localization was performed, analyzed and correlated with clinicopathologic features of the twenty-four WT

Table 3
Association Between Clinicopathological Variables and NCAM + ALDH1 + Positivity in Wilms' Tumors.

	Blastemal NCAM + ALDH1 +			Epithelial NCAM + ALDH1 +			Stromal NCAM + ALDH1 +		
	Positive $n=8$	Negative $n=16$	p value	Positive $n=2$	Negative $n=22$	p value	Positive $n=1$	Negative $n=23$	p value
Gender									
Females	2	8	0.24	1	9	0.80	1	9	0.22
Males	6	8		1	13		0	14	
Age (months)									
> 24	6	12	1.00	2	15	0.34	0	18	0.08
< 24	2	4		0	7		1	5	
Origin									
Israeli	4	10	0.55	2	12	0.21	1	13	0.37
Palestinian	4	6		0	10		0	10	
Presentation									
Mass	8	13		2	19		1	20	
Pain	0	1	0.19	0	1	0.87	0	1	0.98
Hematuria	0	1		0	1		0	1	
Pulmonary Embolism	0	1		0	1		0	1	
Stage									
I	0	3		1	2		0	3	
II	2	3		0	5		0	5	
III	2	7	0.69	1	8	0.13	0	9	0.29
IV	2	1		0	3		0	3	
V	2	2		0	4		1	3	
Histopathology									
Favorable	6	16	0.03	2	20	0.65	0	3	0.75
Unfavorable (Anaplastic)	2	0		0	2		1	20	
Renal Failure									
Yes	1	2	0.95	0	3	0.57	1	2	0.008
No	7	14		2	19		0	21	
LOH-16q_1p									
Yes	1	2	0.63	1	2	0.09	0	2	0.75
No	7	14		1	20		1	21	
Metastasis									
Yes	5	2	0.01	0	7	0.34	0	7	0.49
No	3	14		2	15		1	16	
Relapse									
Yes	6	1	> 0.001	0	7	0.34	0	7	0.49
No	2	15		2	15		1	16	
Survival									
Demise	3	0	0.008	0	3	0.57	0	3	0.69
Survived	5	16		2	19		1	20	

Table 4
Correlation Between Treatment Protocol and NCAM + ALDH1 + Expression in Wilms' Tumor.

	Blastemal NCAM + ALDH1 +			Epithelial NCAM + ALDH1 +			Stromal NCAM + ALDH1 +		
	Positive $n=8$	Negative $n=16$	p value	Positive $n=2$	Negative $n=22$	p value	Positive $n=1$	Negative $n=23$	p value
DD4A									
Yes	4	8	1.00	0	12	0.13	0	12	0.30
No	4	8		2	10		1	11	
EE4A									
Yes	2	7	0.37	1	8	0.70	1	8	0.18
No	6	9		1	14		0	15	
M_CE									
Yes	2	3	0.72	1	4	0.28	0	5	0.60
No	6	13		1	18		1	18	
Radiotherapy									
Yes	5	8	0.56	1	12	0.90	0	13	0.26
No	3	8		1	10		1	10	
HUI (ICE)									
Yes	4	1	0.01	0	5	0.44	1	4	0.04
No	4	15		2	17		0	19	
Glivec									
Yes	1	0	0.14	0	1	0.75	0	1	0.83
No	7	16		2	21		1	22	
Neoadjuvant									
Yes	5	4	0.07	0	9	0.25	1	8	0.18
No	3	12		2	13		0	15	

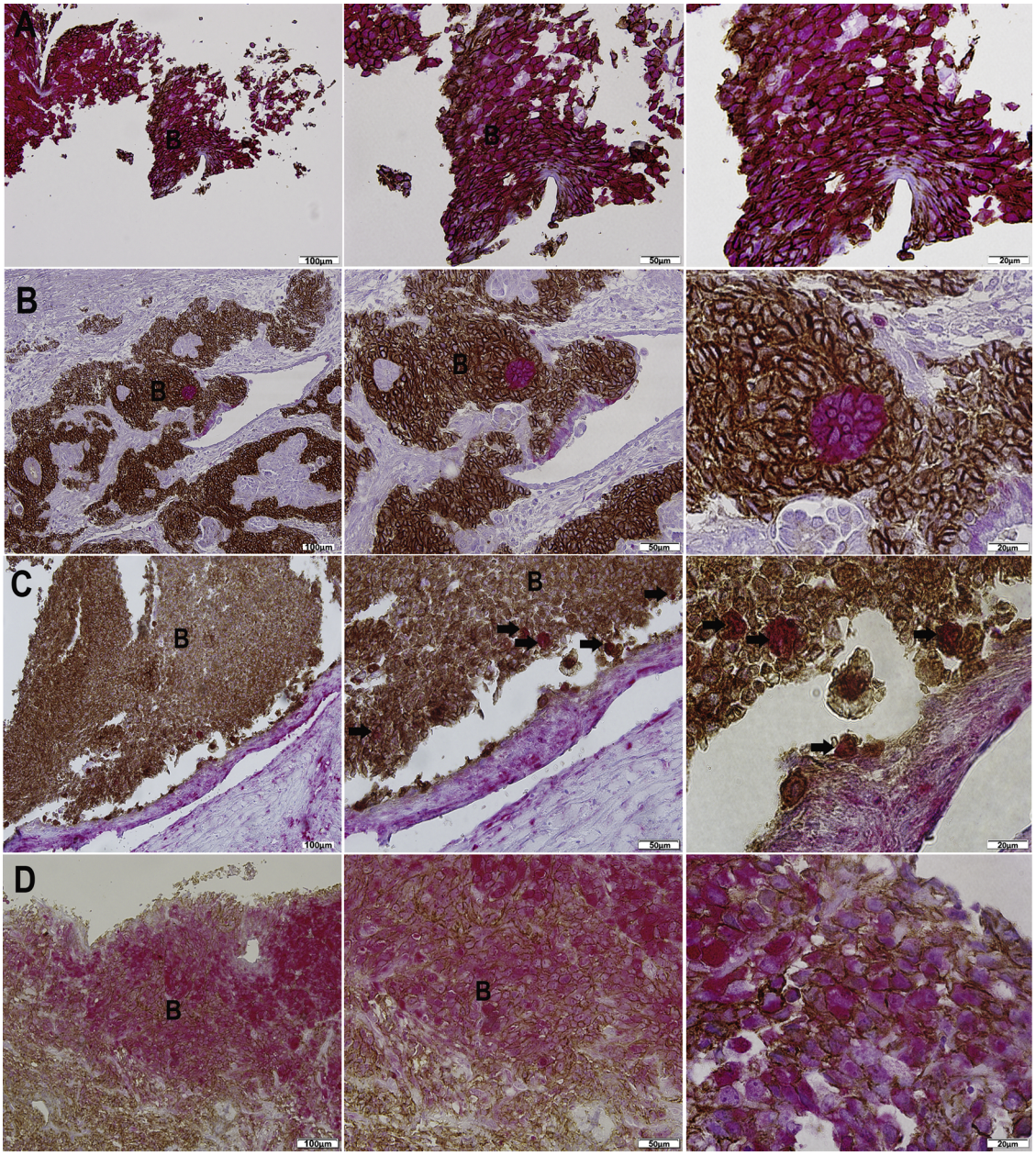

Fig. 3. Representative expression patterns of blastemal NCAM + ALDH1 + staining in WTs that recurred. Immunohistochemical staining for NCAM (brown) and ALDH1 (red) in representative primary WTs that had recurred showing co-localization of NCAM and ALDH1 in WT blastema (A–D); Arrows indicate representative cells with co-localization of NCAM and ALDH1; B-Blastema. Scale bars are indicated in the images. In each panel magnifications are x20, x40 and x100 (from left to right) of the same area in the tumor.

patients in our cohort (Tables 3 and 4, Fig. 3 and supplemental Figure S5). Immunopositivity of blastemal NCAM + ALDH1 + significantly correlated with distance metastasis ($p = 0.01$), unfavorable histology ($p = 0.03$), disease relapse ($p < 0.001$) and patient demise (0.008). No significant correlation was seen between blastemal NCAM + ALDH1 + positivity and patient age ($p = 1$), gender ($p = 0.24$), origin ($p = 0.55$), presentation ($p = 0.19$), stage ($p = 0.69$), renal failure ($p = 0.95$) or LOH-16q_1p ($p = 0.63$) (Table 3). Epithelial or stromal NCAM + ALDH1 + staining showed no significant correlation with clinicopathologic parameters.

Analysis of the DP staining with regards to specific treatment protocol used showed significant correlation between blastemal DP staining and the use of ICE chemotherapy protocol ($p = 0.01$) (Table 4).

It should be noted that neoadjuvant treatment as a whole did not significantly correlated with NCAM + ALDH1 + DP staining ($p = 0.07$). However, blastemal predominance following such treatment showed significant correlation with DP staining ($p = 0.02$) as well as with tumor relapse and patient demise ($p = 0.004$ and $p = 0.02$, respectively) as previously shown.

3.4. Blastemal NCAM + ALDH1 + cells correlate with clinical outcome of WT patients

OS and DFS curves according to NCAM and ALDH1 staining are shown in Fig. 4. In order to determine the correlation between blastemal NCAM + ALDH1 + immunoreactivity and DFS and OS, Kaplan-Meirs curves were used. As shown in Fig. 4, blastemal DP staining of NCAM and ALDH1 significantly affected the DFS and OS of WT patients. Patient with blastemal DP immunostaining had a shorter DFS and OS compared with those whose WTs were negative for blastemal

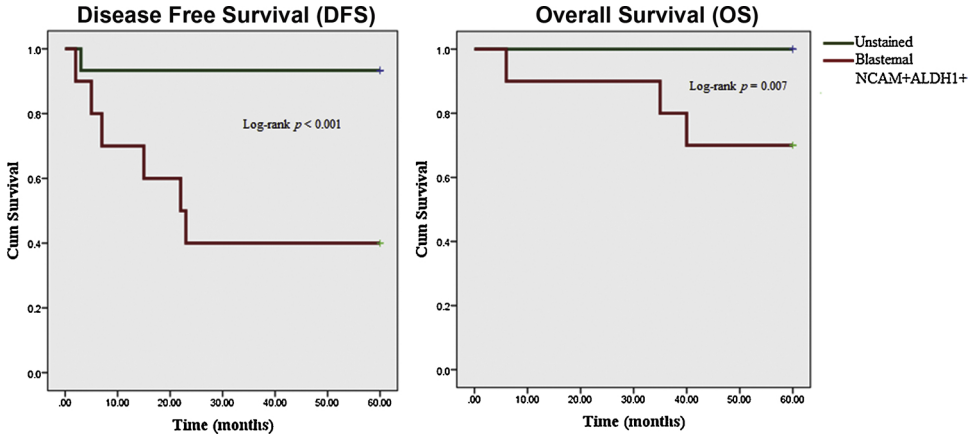

Fig. 4. Blastemal NCAM + ALDH1 + expression correlates with DFS and OS.
Kaplan-meier curves showing significantly inverse correlation between co-localization of NCAM and ALDH1 in WT blastemal cells and (A) DFS or (B) OS. $p < 0.001$ and $p = 0.007$ respectively.

NCAM + ALDH1 + ($p = < 0.001$ and 0.007, respectively). No significant association was observed between epithelial or stromal DP NCAM + ALDH1 + staining and DFS or OS.

4. Discussion

CSCs have been put forward as the driving force behind tumor initiation and progression. Their resistance to conventional anti-neoplasm treatments (i.e. chemotherapy and radiation) suggests a role in disease relapse [20–22,33,34].

Previous work in search of additional immunohistochemical markers for risk stratification in WT were largely based on markers or pathways suspected to be associated with cell proliferation or survival in malignancies in general or on global gene/protein expression analyses of WTs [9,35,36].

Since the WT CSCs were previously identified as NCAM + ALDH1 + cells, localized to WT blastemal element, we aimed in this pilot study to investigate whether there is an association between the WT NCAM + ALDH1 + CSCs and disease prognosis.

First, we show that NCAM + ALDH1 + double positive (DP) staining can be found in either element of the tumor: epithelial, stromal or blastemal. In the hFK (the non-malignant WT counterpart), no overlap of NCAM and ALDH1 staining was observed as ALDH1 was completely absent from the cap mesenchyme containing the normal embryonic renal stem cells and from the s/comma-shaped bodies (Fig. 2). There was no DP staining in the human adult kidney since NCAM is not expressed postnatally by mature kidney structures (Figure S4) [37]. These findings support the previous observation of WT CSCs as distinct from their normal fetal equivalent stem cells residing in the Cap-Mesenchyme as opposed to mirroring each other [32]. Moreover, the absence of such DP cells in the normal hAK puts forward the WT CSCs as a highly specific therapeutic target. Second, we evaluated the correlation between NCAM + ALDH1 + cells with several clinicopathological parameters. A significant association was found between blastemal NCAM + ALDH1 + cells and metastasis, treatment with ICE chemotherapy protocol (used for treatment of an aggressive disease and recurrent cases), blastemal predominance following neoadjuvant treatment, disease recurrence and patient demise (Tables 3 and 4). An inverse correlation was found between blastemal DP cells and either DFS or OS (Fig. 4). Thus, this analysis showed that blastemal NCAM + ALDH1 + cells correlate with adverse clinicopathologic parameters.

Consistent with our findings, in a recent work, Trink et al. divided Wilms' tumors according to their gene expression, into three major architypes: blastemal, epithelial and stromal [38]. This analysis demonstrated that Wilms' tumors with higher stages tend to localize closer to the blastemal archetype. These findings are also in line with our previous results in Wilms' tumor patient-derived xenografts (PDXs) [39] in which propagation in immuno-deficient mice resulted in a selection for the subset of blastemal cells, increased NCAM + ALDH1 + CSC number and loss of the more differentiated epithelial tubular structures with increasing passage. This was in parallel to shorter time to WT growth in mice and lower cell number required for establishment of PDXs suggesting increased aggressiveness. These late passage xenografts mimic high-risk Wilms' tumors (i.e blastemal predominance following preoperative chemotherapy). These findings support the correlation between the localization of WT CSCs to the blastema and a more aggressive disease course.

This association is exemplified through the illustrative case of a patient in this cohort (see "Results" section). This 1.5-year-old boy presented with bilateral disease and pulmonary metastases. Tumor histological features included blastemal predominance following preoperative treatment and diffused anaplasia (either one an independent poor prognostic factor). Disease course was highly aggressive with early relapse during adjuvant chemotherapy treatment. Immunohistochemical staining showed diffused blastemal NCAM + ALDH1 + cells comprising over 50% of the tumor (Fig. 3D). Hence, the aggressive course of his illness stands in line with the diffused blastemal DP staining.

CSC resistance to conventional anti-neoplastic treatment protocols, and their persistence after such treatments, has put forward their role in disease relapse [19,40,41]. It had been shown that WT blastemal component increases after in vitro treatment with chemotherapy and radiation [42,43]. Furthermore, predominant blastemal histology following preoperative chemotherapeutic treatment was shown to be a significant factor for poor outcome and recurrent disease [8,14,15]. Moreover, we have previously shown that the number of NCAM + WT cells significantly increases following in vitro treatment with first line chemotherapeutic protocols (that include vincristine and dactinomycin) suggesting resistance of the WT blastema/NCAM + CSC containing compartment to these protocols. However, treatment with second line chemotherapy protocols (i.e. ICE) resulted in a significant decrease in NCAM + cells supporting their use in relapse cases [44].

As risk stratification in WT is partially based on tumor histological features (i.e. blastemal predominance following neoadjuvant therapy, anaplasia, etc.) we suggest that addition of NCAM and ALDH1 staining to the initial histologic diagnosis might assist in achieving an even more accurate stratification prior to treatment initiation. Furthermore, this information could guide treatment as these cells have been shown to be more sensitive to specific chemotherapy protocols than others [44].

The limitation of this pilot study is the relatively small sample of patients and thus we suggest that based on our results further studies on

the prognostic relevance of the WT CSCs will need to be performed in order to validate our findings in a larger cohort.

5. Conclusions

We have found a significant correlation between WT blastemal NCAM + ALDH1 + CSCs and adverse clinicopathologic parameters including: metastases, tumor relapse, patient demise, high risk treatment protocol (HUI) and blastemal predominance following neoadjuvant treatment. Blastemal NCAM + ALDH1 + staining inversely correlated with DFS and OS. These results prompt the consideration of NCAM and ALDH1 blastemal co-localization as an additional histological prognostic marker for initial risk stratification in WT patients.

Ethics statement

This study was conducted according to the principles expressed in the Declaration of Helsinki and was approved by the Institutional Review Boards of Sheba Medical Center, Hadassah-Ein Kerem and Asaf Harofeh Medical Centers.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Author contributions

D.R., I.T.-L. and N.P.-S. designed the experiments. L.A., D.R. I.T.-L. and N.P.-S., performed the experiments. D.R., I.T.-L. N.P.-S., B.D., and I.B., analyzed the data. B.D., D.R. and N.P.-S. wrote the manuscript.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.prp.2019.152491.

References

[1] H. Golan, R. Shukrun, R. Caspi, E. Vax, N. Pode-Shakked, S. Goldberg, O. Pleniceanu, D.D. Bar-Lev, M. Mark-Danieli, S. Pri-Chen, et al., In vivo expansion of Cancer stemness affords novel Cancer stem cell targets: malignant rhabdoid tumor as an example, Stem Cell Reports 11 (3) (2018) 795–810.
[2] N. Pode-Shakked, R. Shukrun, M. Mark-Danieli, P. Tsvetkov, S. Bahar, S. Pri-Chen, R.S. Goldstein, E. Rom-Gross, Y. Mor, E. Fridman, et al., The isolation and characterization of renal cancer initiating cells from human Wilms' tumour xenografts unveils new therapeutic targets, EMBO Mol. Med. 5 (1) (2013) 18–37.
[3] D. Herzlinger, C. Koseki, T. Mikawa, Q. al-Awqati, Metanephric mesenchyme contains multipotent stem cells whose fate is restricted after induction, Development 114 (3) (1992) 565–572.
[4] J.A. Oliver, J. Barasch, J. Yang, D. Herzlinger, Q. Al-Awqati, Metanephric mesenchyme contains embryonic renal stem cells, Am. J. Physiol. Renal Physiol. 283 (4) (2002) F799–809.
[5] N. Pode-Shakked, B. Dekel, Wilms tumor–a renal stem cell malignancy? Pediatr. Nephrol. 26 (9) (2011) 1535–1543.
[6] M.N. Rivera, D.A. Haber, Wilms' tumour: connecting tumorigenesis and organ development in the kidney, Nat. Rev. Cancer 5 (9) (2005) 699–712.
[7] G.G. Re, D.J. Hazen-Martin, D.A. Sens, A.J. Garvin, Nephroblastoma (Wilms' tumor): a model system of aberrant renal development, Semin. Diagn. Pathol. 11 (2) (1994) 126–135.
[8] E. Szychot, J. Apps, K. Pritchard-Jones, Wilms' tumor: biology, diagnosis and treatment, Transl. Pediatr. 3 (1) (2014) 12–24.
[9] M.A. Ghanem, G.J. van Steenbrugge, R.J. Nijman, T.H. van der Kwast, Prognostic markers in nephroblastoma (Wilms' tumor), Urology 65 (6) (2005) 1047–1054.
[10] M.M. Khine, W. Aung, P.D. Sibbons, C.V. Howard, E. Clapham, F. McGill, D. Van Velzen, Analysis of relative proliferation rates of Wilms' tumor components using proliferating cell nuclear antigen and MIB-1 (Ki-67 equivalent antigen) immunostaining and assessment of mitotic index, Lab. Invest. 70 (1) (1994) 125–129.
[11] E.H. van Leeuwen, A. Postma, J.W. Oosterhuis, A. Meiring, C.J. Cornelisse, J. Koudstaal, W.M. Molenaar, An analysis of histology and DNA-ploidy in primary wilms tumors and their metastases and a study of the morphological effects of therapy, Virchows Arch. A Pathol. Anat. Histopathol. 410 (6) (1987) 487–494.
[12] M.J.W.J. Li, J.Q. Mao, Q. Shu, Pediatric Malignant Renal Tumors: Current Progress in Treatment, Clin. Oncol. Res. 1 (1) (2018).
[13] N. Breslow, A. Olshan, J.B. Beckwith, D.M. Green, Epidemiology of wilms tumor, Med. Pediatr. Oncol. 21 (3) (1993) 172–181.
[14] J.I. Geller, Current standards of care and future directions for "high-risk" pediatric renal tumors: anaplastic Wilms tumor and Rhabdoid tumor, Urol. Oncol. 34 (1) (2016) 50–56.
[15] J. Brok, T.D. Treger, S.L. Gooskens, M.M. van den Heuvel-Eibrink, K. Pritchard-Jones, Biology and treatment of renal tumours in childhood, Eur. J. Cancer 68 (2016) 179–195.
[16] A. Weirich, R. Ludwig, N. Graf, U. Abel, I. Leuschner, G.M. Vujanic, O. Mehls, J. Boos, J. Beck, B. Royer-Pokora, et al., Survival in nephroblastoma treated according to the trial and study SIOP-9/GPOH with respect to relapse and morbidity, Ann. Oncol. 15 (5) (2004) 808–820.
[17] J.S. Dome, E.J. Perlman, N. Graf, Risk stratification for wilms tumor: current approach and future directions, Am. Soc. Clin. Oncol. Educ. Book (2014) 215–223.
[18] E. Markovsky, E. Vax, D. Ben-Shushan, A. Eldar-Boock, R. Shukrun, E. Yeini, I. Barshack, R. Caspi, O. Harari-Steinberg, N. Pode-Shakked, et al., Wilms tumor NCAM-Expressing Cancer stem cells as potential therapeutic target for polymeric nanomedicine, Mol. Cancer Ther. 16 (11) (2017) 2462–2472.
[19] M.F. Clarke, J.E. Dick, P.B. Dirks, C.J. Eaves, C.H. Jamieson, D.L. Jones, J. Visvader, I.L. Weissman, G.M. Wahl, Cancer stem cells–perspectives on current status and future directions: AACR Workshop on cancer stem cells, Cancer Res. 66 (19) (2006) 9339–9344.
[20] C.W. Chang, Y.S. Chen, C.C. Chen, I.O. Chan, C.C. Chen, S.J. Sheu, T.W. Lin, S.H. Chou, C.J. Liu, T.C. Lee, et al., Targeting cancer initiating cells by promoting cell differentiation and restoring chemosensitivity via dual inactivation of STAT3 and src activity using an active component of antrodia cinnamomea mycelia, Oncotarget 7 (45) (2016) 73016–73031.
[21] A. Alama, A.M. Orengo, S. Ferrini, R. Gangemi, Targeting cancer-initiating cell drug-resistance: a roadmap to a new-generation of cancer therapies? Drug Discov. Today 17 (9-10) (2012) 435–442.
[22] J.A. McCubrey, L.S. Steelman, S.L. Abrams, N. Misaghian, W.H. Chappell, J. Basecke, F. Nicoletti, M. Libra, G. Ligresti, F. Stivala, et al., Targeting the cancer initiating cell: the ultimate target for cancer therapy, Curr. Pharm. Des. 18 (13) (2012) 1784–1795.
[23] S. Bao, Q. Wu, R.E. McLendon, Y. Hao, Q. Shi, A.B. Hjelmeland, M.W. Dewhirst, D.D. Bigner, J.N. Rich, Glioma stem cells promote radioresistance by preferential activation of the DNA damage response, Nature 444 (7120) (2006) 756–760.
[24] F. Collina, M. Di Bonito, V. Li Bergolis, M. De Laurentiis, C. Vitagliano, M. Cerrone, F. Nuzzo, M. Cantile, G. Botti, Prognostic value of Cancer stem cells markers in triple-negative breast Cancer, Biomed Res. Int. 2015 (2015) 158682.
[25] C. Ginestier, M.H. Hur, E. Charafe-Jauffret, F. Monville, J. Dutcher, M. Brown, J. Jacquemier, P. Viens, C.G. Kleer, S. Liu, et al., ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome, Cell Stem Cell 1 (5) (2007) 555–567.
[26] F. Zeppernick, R. Ahmadi, B. Campos, C. Dictus, B.M. Helmke, N. Becker, P. Lichter, A. Unterberg, B. Radlwimmer, C.C. Herold-Mende, Stem cell marker CD133 affects clinical outcome in glioma patients, Clin. Cancer Res. 14 (1) (2008) 123–129.
[27] L. da Silva Meirelles, P.C. Chagastelles, N.B. Nardi, Chymal stem cells reside in virtually all post-natal organs and tissues, J. Cell. Sci. 119 (Pt 11) (2006) 2204–2213.
[28] S. Gattenlohner, T. Stuhmer, E. Leich, M. Reinhard, B. Etschmann, H.U. Volker, A. Rosenwald, E. Serfling, R.C. Bargou, G. Ertl, et al., Specific detection of CD56 (NCAM) isoforms for the identification of aggressive malignant neoplasms with progressive development, Am. J. Pathol. 174 (4) (2009) 1160–1171.
[29] P. Garin-Chesa, E.J. Fellinger, A.G. Huvos, H.R. Beresford, M.R. Melamed, T.J. Triche, W.J. Rettig, Immunohistochemical analysis of neural cell adhesion molecules. Differential expression in small round cell tumors of childhood and adolescence, Am. J. Pathol. 139 (2) (1991) 275–286.
[30] C. Bouvier, F. Bertucci, P. Metellus, P. Finetti, A. Maues de Paula, F. Forest, K. Mokhtari, C. Miquel, D. Birnbaum, A. Vasiljevic, et al., ALDH1 is an immunohistochemical diagnostic marker for solitary fibrous tumours and haemangiopericytomas of the meninges emerging from gene profiling study, Acta Neuropathol. Commun. 1 (2013) 10.
[31] e J. carcinoma Peotvegfiappmipt, A.J. Murphy, A. Panzer, C. de Caestecker, G.D. Ayers, D. Neblett, K. Saito-Diaz, M. de Caestecker, H.N. Lovvorn 3rd, SIX2 effects on wilms tumor biology, Transl. Oncol. 7 (6) (2014) 800–811.
[32] R. Shukrun, N. Pode-Shakked, O. Pleniceanu, D. Omer, E. Vax, E. Peer, S. Pri-Chen, J. Jacob, Q. Hu, O. Harari-Steinberg, et al., Wilms' tumor blastemal stem cells dedifferentiate to propagate the tumor bulk, Stem Cell Reports 3 (1) (2014) 24–33.
[33] M.F. Clarke, What can we learn about breast cancer from stem cells? Adv. Exp. Med. Biol. 617 (2008) 17–22.

[34] M.F. Clarke, M. Fuller, Stem cells and cancer: two faces of eve, Cell 124 (6) (2006) 1111–1115.
[35] M.A. Ghanem, T.H. van der Kwast, W.M. Molenaar, M.A. Safan, R.J. Nijman, G.J. van Steenbrugge, The predictive value of immunohistochemical markers in untreated Wilms' tumour: are they useful? World J. Urol. 31 (4) (2013) 811–816.
[36] S. Wittmann, C. Wunder, B. Zirn, R. Furtwangler, J. Wegert, N. Graf, M. Gessler, New prognostic markers revealed by evaluation of genes correlated with clinical parameters in Wilms tumors, Genes Chromosomes Cancer 47 (5) (2008) 386–395.
[37] M. Abbate, D. Brown, J.V. Bonventre, Expression of NCAM recapitulates tubulogenic development in kidneys recovering from acute ischemia, Am. J. Physiol. 277 (3) (1999) F454–463.
[38] A. Trink, I. Kanter, N. Pode-Shakked, A. Urbach, B. Dekel, T. Kalisky, Geometry of gene expression space of wilms' tumors from human patients, Neoplasia 20 (8) (2018) 871–881.
[39] B. Dekel, S. Metsuyanim, K.M. Schmidt-Ott, E. Fridman, J. Jacob-Hirsch, A. Simon, J. Pinthus, Y. Mor, J. Barasch, N. Amariglio, et al., Multiple imprinted and stemness genes provide a link between normal and tumor progenitor cells of the developing human kidney, Cancer Res. 66 (12) (2006) 6040–6049.
[40] M. Diehn, M.F. Clarke, Cancer stem cells and radiotherapy: new insights into tumor radioresistance, J. Natl. Cancer Inst. 98 (24) (2006) 1755–1757.
[41] R.W. Cho, Clarke MF: recent advances in cancer stem cells, Curr. Opin. Genet. Dev. 18 (1) (2008) 48–53.
[42] A.J. Garvin, J.L. Sullivan, D.D. Bennett, W.S. Stanley, T. Inabnett, D.A. Sens, The in vitro growth, heterotransplantation, and immunohistochemical characterization of the blastemal component of Wilms' tumor, Am. J. Pathol. 129 (2) (1987) 353–363.
[43] J.G. Wen, G.J. van Steenbrugge, R.M. Egeler, R.M. Nijman, Progress of fundamental research in Wilms' tumor, Urol. Res. 25 (4) (1997) 223–230.
[44] N. Pode-Shakked, S. Metsuyanim, E. Rom-Gross, Y. Mor, E. Fridman, I. Goldstein, N. Amariglio, G. Rechavi, G. Keshet, B. Dekel, Developmental tumourigenesis: NCAM as a putative marker for the malignant renal stem/progenitor cell population, J. Cell. Mol. Med. 13 (8B) (2009) 1792–1808.