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Original Article

Investigation of piwil2 expression as a biomarker in solid tumors of childhood

Yilmaz Secilmis[1], Musa Karakukcu[2], Ozlem Canoz[3]

[1]Department of Pediatric, Division of Pediatric Emergency, Faculty of Medicine, Erciyes University, Kayseri,Turkey
[2]Department of Pediatric, Division of Pediatric Oncology, Faculty of Medicine, Erciyes University, Kayseri,Turkey
[3]Department of Pathology, Faculty of Medicine, Erciyes University, Kayseri,Turkey

Abstract

Aim: PIWIL 2 belongs to a subgroup of argonaute proteins, which has an essential role in germ cell development and repair. Recent studies have demonstrated PIWIL 2 as a stem cell marker in some solid tumors. In the present study, we aimed to evaluate the expression of PIWIL 2 in childhood solid tumors and to show being a potential marker for a therapeutic drug.

Materials and Methods: The study included 150 patients who were diagnosed with pediatric solid tumor and followed between 2000 and 2011. Solid tumor preparations were stained with polyclonal PIWIL 2 antibody by using immunohistochemical methods.

Results: Assessment of the tumor sub-groups showed that, among 30 patients with neuroblastoma, there was a strong staining in 5 (16.70%) (p=0.020). PIWIL 2 was showed strong staining in 3 (21.40%) osteosarcoma patients (p=0.030). Among 34 patients with a germ cell tumor, there was a strong staining in 6 (17.60%) patients (p=0.040).

Conclusion: Significant PIWIL 2 positivity was found in neuroblastoma, osteosarcoma and germ cell tumors and it can be a potential marker for these tumors. Because of non-tumor tissue involvement in Wilms' tumor it can be particularly useful in determining surgical margins.

Keywords: Biomarker; cancer stem cell; PIWIL 2; solid tumors of childhood

INTRODUCTION

Thanks to advances in stem cell technology and molecular biology, stem cell studies have gained importance for understanding cancer etiopathogenesis. According to the latest hypotheses, cancer originates from stem cells that emerge in the tumor tissue (1). Cancer stem cells are thought to be the cells that are responsible for cancer initiation, progression, and treatment resistance, as well as for relapses and metastases after surgery or chemotherapy (1,2).

PIWIL 2 is the abbreviation of the concept "P element induced wimpy testis-like2". PIWIL 2 is a member of the piwi gene family in mice and humans (3, 4). It is specifically expressed in testis tissue (5,6). It has been shown to be expressed most notably in seminomatous tumors, but also prostate, breast, gastrointestinal system, ovarian, and endometrial cancers in humans, and in breast cancer, rhabdomyosarcoma, and medulloblastoma in mice.

PIWIL 2 was first found in Drosophila ovary. It plays an important role in cell autonomy in stem cell. While removal or suspension of production of Piwi protein significantly reduces stem cell division, extreme expression of piwi significantly increases germ cell number and division rate. PIWIL 2 is a key role in signaling pathways in cancer stem cells in proliferation and apoptosis. PIWIL 2, which is effective in oncogenesis, is known to interact and co-express with many genes. Among these, genes that are thought to be most effective on oncogenic mechanisms are PIWIL1 and DDX4 (the human ortholog of Drosophila Vasa) (7-9).

Among childhood solid tumors, neuroblastoma, rhabdomyosarcoma, Wilms' tumor, and germ cell tumors are the most common subtypes (10-12).

Neuroblastoma can originates from anywhere along the sympathetic nervous system. The vast majority of these tumors occur in the retroperitoneal area, arising from the adrenal gland. These children experience abdominal pain and abdominal mass that may cross the midline. The most common soft-tissue sarcoma in children is rhabdomyosarcoma. Rhabdomyosarcoma often originates from mesenchymal cells. Not only committed normally to skeletal muscle formation but can also arise from smooth muscle cells. Wilms' tumor is the most common type of renal tumor in children. Children with Wilms' tumor may have associated anomalies including aniridia, hemihypertrophy, cryptorchidism, and

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Corresponding Author: Yilmaz Secilmis, Department of Pediatric, Division of Pediatric Emergency, Faculty of Medicine, Erciyes University, Kayseri,Turkey **E-mail:** yildosec@hotmail.com

hypospadius. Germ cell tumors are less common than other solid tumors. Teratomas, embryonal carcinoma, choriocarcinoma and endodermal sinus tumor are among the most common germ cell tumors (13-14).

The aim of the present study was to show PIWIL 2 expression in childhood solid tumors using immunohistochemical methods.

This study aimed to determine if PIWIL 2 could be used as a cancer stem cell marker for childhood tumors and to elucidate the relationship between these tumors' clinical and prognostic features using PIWIL 2.

MATERIALS and METHODS

This study was approved by Local Ethics Committee on 04.01.2011 (Number: 2011/14) and completed in accordance with the tenets of the Declaration of Helsinki. We included 150 patients who were followed by our Department of Pediatric Hematology-Oncology and Pathology with childhood solid tumor for a period of 11 years.

The patients with solid tumors were divided into four groups. The first group was called the neuroblastoma group. This group comprised 30 patients of whom 24 were diagnosed with neuroblastoma and 6 with ganglioneuroblastoma. The second group was called the Wilms' tumor group and consisted of 42 patients with Wilms' tumor. The third group was called as the sarcoma group and consisted of 16 rhabdomyosarcoma, 14 osteosarcoma, and 14 Ewing sarcoma patients, making a total of 44 patients. The last group was the Germ cell tumor group, consisting of 11 mature teratomas, 8 immature teratomas, 14 yolk sac tumors, and 1 dysgerminomas, making a total of 34 cases.

The study was originally planned to enroll a total of 209 pediatrics patients who were diagnosed with solid tumors between 2000 and 2011. However, fifty-nine patients were excluded due to having inadequate material on paraffin blocks. The paraffin blocks of the remaining 150 patients were included and stained. A standard protocol provided by the manufacturer was used for PIWIL 2 immunohistochemical staining.

Human normal testis tissue was used as the control tissue. After observing a positive result with the polyclonal PIWIL 2 antibody of rabbit origin, immunohistochemical staining was performed on 10 microscope slides. In each staining cycle control tissue staining was definitely performed, positive staining was observed. Stainings were examined by an expert pathologist and a pediatric hematooncologist. According to the examination result, PIWIL 2 staining patterns in different tumor tissues were determined.

Control Tissue

The antibody PIWIL 2 used in this study was first isolated from germe cells in the adult testis tissue. As the testis tissue was mentioned as the positive control tissue in the antibody's data sheet, we used adult normal testis tissue.

Staining scores;

1. 0-5% PIWIL 2 stained cells, staining score 0 or negative

2. 5-25% PIWIL 2 stained cells, staining score 1 (+) or mildly positive

3. 25-50 % PIWIL 2 stained cells, staining score 2 (++) or moderately positive

4. Above 50% PIWIL 2 stained cells, staining score 3 (+++) or strongly positive.

Histological Parameters

Necrosis: The extent of tumor necrosis was semiquantitatively assessed at low magnification (×40) and recorded as either absent (score 0), focal (score 1; ≤10% of the tumor area), moderate (score 2; 10%-30% of the tumor area), or extensive (score 3; ≥30% of the tumor area) based on the histologic evaluation of all tumor blocks (15). The relationship between extent of necrosis and staining pattern was assessed.

Mitosis: According to the mitotic activity index of the World Health Organization, it is evaluated up to 5 mitoses per 10 fields scores 1 point, 6-10 scores 2 points and more than 10 scores 3 points (16-17). We used a microscope with wide angle eyepieces and x40 objective, this gives a field area of 0.152 mm2. The relationship between mitosis rate and antibody staining was assessed.

Histological Type: As the histological type (favorable vs unfavorable) predicts prognosis particularly in Wilms' tumor, it was also taken into consideration. The relationship between unfavorable histology and strong PIWIL 2 staining was assessed. Wilms' tumor is defined in two groups as favorable and unfavorable histology according to the presence or absence of anaplasia in histopathological evaluation. Presence of anaplasia indicates that the tumor has "poor histology". Anaplasia is seen at a rate of 5%, its frequency increases with age. While it is rarely seen in young babies, the frequency increases to 10% above the age of 5 (18).

Alphafetoprotein: The relationship between PIWIL 2 staining rate and AFP elevation was assessed in 14 patients with yolk sac tumor.

Statistical Analyis

Statistical analyses of the study data was performed using 'SPSS (Statistical Package for the Social Sciences) for Windows, Version 17.0, SPSS Inc, U.S.A' software package. Descriptive statistics was used for demographic properties and findings, and included mean, standard deviation, and frequency. Qualitative data were analyzed using Pearson Chi-Square test or Fisher's Exact Test. The results were reported at a confidence interval of 95% and at a significance level of $p<0.05$.

RESULTS

The enrolled patients were grouped into four groups as neuroblastoma, Wilms' tumor, Sarcomas, and germ cell tumors. Of a total of 150 patients, 30 (20%) had neuroblastoma, 42 (28%) Wilms' tumor, 44 (29.30%) sarcoma, and 34 (22.70%) Germ cell tumor.

Table 1. Patient distribution by tumor groups and subgroups

	Tumor Groups	n (%)	Subgroups	n (%)
1	Neuroblastoma		Neuroblastoma	24(16%)
		30(20%)	Ganglioneuroblastoma	6(4%)
2	Wilms' Tumor	42(28%)		42(28%)
3	Sarcomas		Rhabdomyosarcoma	16(10.7%)
			Osteosarcoma	14(9.3%)
		44(29.3%)	Ewing Sarcoma	14(9.3%)
4	Germ Cell Tumor		Mature Teratoma	11(7.3%)
			Immature Teratoma	8(5.3%)
			Yolk Sac Tumor	14(9.3%)
		34(22.7%)	Dysgerminoma	1(0.7%)
	Total	150(100%)		150(100%)

n: Number of patient

As for the tumor subgroups, 24 (16%) patients in the neuroblastoma group were diagnosed with neuroblastoma and 6 (4%) with ganglioneuroblastoma. All (28%) patients in the Wilms' tumor group had Wilms' tumor. Sixteen (10.70%) patients in the sarcoma group had rhabdomyosarcoma; 14 (9.30%) had osteosarcoma; and 14 (9.30%) had Ewing sarcoma. The germ cell tumor group consisted of 11(7.30%) mature teratomas, 8 (5.30%) immature teratomas, 14 (9.30%) yolk sac tumors, and 1 (0.70%) dysgerminoma (Table 1).

The study population had a mean age of 9.87± 5.30 years. Neuroblastoma group had a mean age of 7.70±4.30 years; Wilms' tumor group 7.50±4 years; Sarcoma group 13.60±5 years; and Germ cell tumor group 9.80±5.20 years. Of 150 patients, 76 (50.70%) were male and 74 (49.30%) were female. Among 30 neuroblastoma cases, 17 (56.70%) were male and 13 (43.30%) were female; among 42 patients with Wilms' tumor, 23 (%54.80%) were male and 19 (%45.20%) were female; among 44 patients with sarcoma, 29 (%65.90%) were male and 15 (%34.10%) were female; and among 34 patients with germ cell tumor, 7 (%20.60%) were male and 27 (%79.40%) were female.

In 77 (50.70%) of 150 patients there was no staining. Among the remainders, 73 (48.70%) had weak, moderately strong, or strong positive staining. In the neuroblastoma group 9 (30%) patients had negative staining and 21 (70%) had positive PIWIL 2 staining; the Wilms' tumor group showed 27 (64.30%) negative staining and 15 (35.70%) positive staining; the sarcoma group had 25 (56.80%) patients with negative staining and 19 (43.20%) patients with positive staining; the germ cell tumor group had 16 (47.10%) patients with negative staining and 18 (52.90%) patients with positive staining. Immunohistochemical stainings with PIWIL 2 in the tumor groups were shown on Table 2.

Positive staining rate in the neuroblastoma and germ cell tumor groups were statistically significant than those of the other groups ($p<0.05$).

In PIWIL 2 immunohistochemical staining studies, a moderate-to-strong staining was considered significant. Negative or weak staining was considered insignificant. Accordingly, a statistically significant staining was similarly observed in the neuroblastoma and germ cell tumor groups ($p=0.020$, $p=0.040$, respectively).

Table 2. PIWIL 2 staining numbers and percentages by tumor groups

	n	Staining rate		
Tumor Type	**(%)**	**Negative**	**Positive**	**P value**
Neuroblastoma	30(20%)	9(30%)	21(70%)	p=0.020*
Wilms' tumor	42(28%)	27(64.30%)	15(35.70%)	p=0.100
Sarcomas	44(29.30%)	25(56.80%)	19(43.20%)	p=0.080
Germ Cell Tumor	34(22.70%)	16(47.10%)	18(52.90%)	p=0.040*
TOTAL	150(100%)	77(51.30%)	73(48.70%)	

*p<0.05 statistically significant n: number of patient

1.Neuroblastoma

A stage-based analysis of the patients indicated that 3 (10%) of 30 patients had a stage 1 tumor; 3 (10%) had a stage 2 tumor; 2 (6.20%) had a stage 3 tumor; and 22 (73.30%) had a stage 4 tumor in neuroblastoma patients. Of 22 patients with a stage 4 tumor, 5 (22.70%) had negative staining; 8(36.40%) had weak staining; 4 (18.20%) had moderately strong staining; and 5 (22.70%) had strong staining. No significant relationship was found between tumor stage and staining intensity.

When the patients were evaluated in terms of prognosis, it was seen that 8 (26.60%) of 30 patients in the neuroblastoma group dead during the follow-up. The follow-up of 22 (73.30%) patients continues. Of the 8 patients who died, 6 were stage 4, and the remaining 2 patients were stage 3. In 8 patients who died, 1 (12.50%) patient had negative staining, 5 (62.50%) patients had weak staining, 2 (25%) had moderate staining, and none had strong staining. Of 22 surviving patients were examined, 8 (36.40%) patients were negative, 6 (27.30%) were weak, 3 (13.60%) were moderate and 5 (22.70%) were stained strongly. No statistically significant correlation was found between PIWIL 2 positivity and poor prognosis (p> 0.05).

2. Wilms' tumor

A tumor stage-based analysis of the patients revealed that 18 (42.90%) of 42 patients were in stage 1; 9 (21.40%) in stage 2; 11 (26.20%) in stage 3; and 4 (9.50%) in stage 4 in Wilms' tumor group. No relationship was evident between tumor stage and PIWIL 2 staining intensity (p>0.05). Patients with Wilms' tumor were grouped into two groups based on histological type, as favorable and unfavorable groups. Twenty-nine (69%) of 42 patients had favorable histology and 14 (31%) patients had unfavorable histology. Eighteen (62.10%) of 29 patients with a favorable histology were negatively stained while 11 (37.90%) were positively stained. None of the patients showed strong staining. In the group with unfavorable histology none of the patients showed strong staining. Favorable and unfavorable histology and PIWIL 2 staining were not significantly correlated (p>0.05).

According to their prognosis, 42 patients were followed-up in 10 years follow-up. Thirty-three (78.60%) patients had a good prognosis and 9 patients (21.40%) died. Six of 9 dead patients (66.70%) had negative staining, while 3 (33.30%) had weak staining. In the group with good prognosis, 21 (63.60%) of 33 patients were negative and 12 (36.40%) were stained weakly. Moderate and strong staining was not seen in both groups. No statistically significant correlation was found in patients who died and survival group in terms of positive staining rates (p> 0.05).

3. Sarcomas

Among the sarcomas included in this study, only one (6.30%) of 16 rhabdomyosarcoma cases showed strong staining. None of the 14 Ewing sarcoma cases showed strong staining. Three (21.40%) of 14 osteosarcoma cases showed strong staining (Figure 1). Strong PIWIL 2 staining seen in the osteosarcoma cases in the sarcoma group was statistically significant (p<0.05) (Table 3).

4. Germ cell tumors

Germ cell tumors were classified into 3 groups as mature teratoma, immature teratoma, yolk sac tumor. The group contained 34 patients. Among the cases, 11 (32.40%) had mature teratoma; 8 (23.50%) had immature teratoma; 14 (14.20%) had yolk sac tumor; and 1 (2.90%) had dysgerminoma. Since the number of dysgerminomas was only 1, no statistical analysis was done in this group.

According to their prognosis 26 (76.40%) patients had a good prognosis and 8 patients (23.60%) had poor prognosis. Four of 8 (50%) in patients with poor prognosis had negative staining, while 4 (50%) had positive. In the group with good prognosis, 12 (46.10%) of 26 patients were negative and 14 (53.90%) were stained positive. No statistically significant correlation was found in patients who poor prognosis and survival group in terms of positive staining rates (p> 0.05).

Yolc sac tumor patients were studied for alphafetoprotein (AFP) levels at the time of diagnosis. AFP level was high in 11 (78%) of 14 patients. The mean AFP level was 11983±16584 ng/mL. A correlation of borderline significance was present between elevated AFP level and PIWIL 2 staining intensity (p=0.050).

Mitosis rate was examined in all tumor groups. Twenty-four (15.80%) patients had a high mitosis rate. There were no significant differences between the groups in terms of PIWIL 2 staining intensity (p>0.05). Severe necrosis was detected in 20 patients (13.20%). Necrosis rate was not different with respect to PIWIL 2 staining intensity (p>0.05).

Table 3. PIWIL 2 staining rates of the tumor subgroups in the sarcomas group

Tumor subtype	n (%)	Negative (%)	Weak staining (%)	Moderately strong staining (%)	Strong staining (%)	p
Rhabdomyosarcom	16 (10.70%)	10 (62.50%)	4 (25%)	1 (6.30%)	1 (6.30%)	
Osteosarcoma	14(9.30%)	6(42.90%)	4(28.60%)	1(7.10%)	3(21.40%)	0.030*
Ewing Tumor	14(9.30%)	9(64.30%)	2(14.30%)	3(21.40%)	0	
Total	44(100%)	25(56.80%)	10(22.70%)	5(11.30%)	4(9%)	

*n: number of patient ; *p<0.05 statistically significant

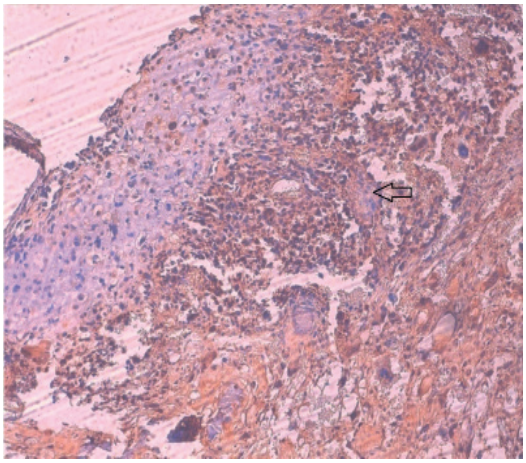

Figure 1. Osteosarcoma Tissue, 3+ (Strong) Staining

In some cases with a negative stained of Wilms' tumor tissue, the normal kidney tissue adjacent to tumor tissue stained positively as normal kidney was also removed during tumor resection. Staining occurred predominantly in renal tubules but not in glomerules (Figure 2).

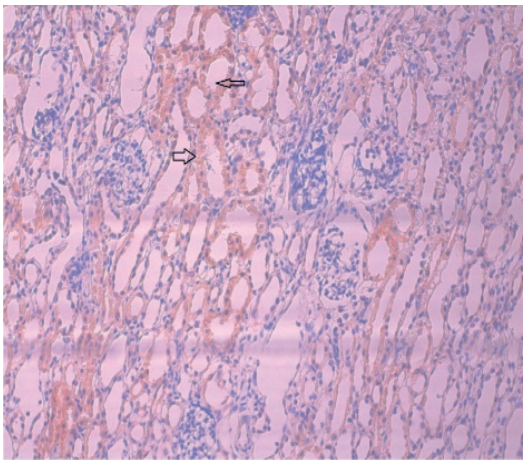

Figure 2. A strongly positively stained (3+) kidney tissue (Tubules were stained but glomerules were not) adjacent to a negatively stained Wilms' tumor tissue

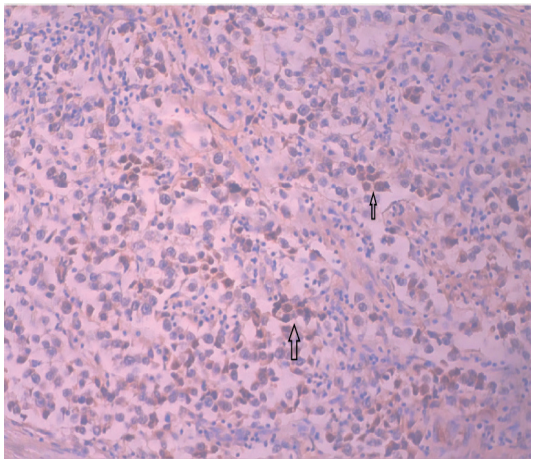

Figure 3. 3+ strong staining and nuclear staining pattern in dysgerminoma tumor

Nuclear Staining Pattern

PIWIL 2 polyclonal antibody was stained both cytoplasmic and nuclear pattern in germ cell tumors. However the other tumors were stained only cytoplasmic pattern. Strong staining pattern was detected especially in dysgerminoma tumor (Figure 3, 4).

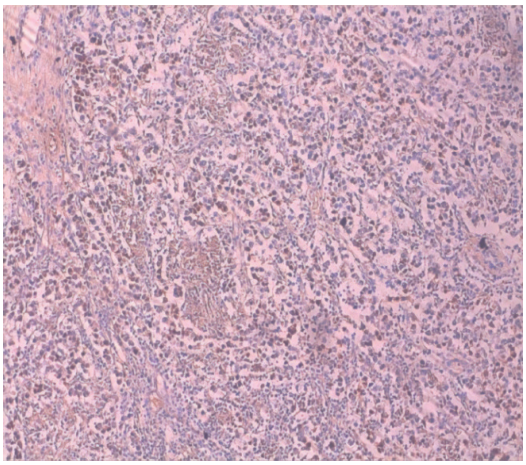

Figure 4. 20x magnification and nuclear staining pattern from different segments in the same patient

DISCUSSION

We have, for the first time showed that PIWIL 2 is positivity in patients with neuroblastoma, germ cell tumors, and osteosarcoma. In a study dated 2010 which investigated PIWIL 2 as a biomarker, Liu et al studied 126 archived paraffin embedded breast cancer tissues, and found weak staining in 51% of samples, moderate staining in 32%, and strong staining in 17%, with none showing negative staining (19). Staining pattern was nuclear, cytoplasmic, or both nuclear and cytoplasmic. In our study, germ cell tumors exhibited both cytoplasmic and nuclear staining while other tumors showed cytolasmic staining only. Furthermore, in our study, 77 of 150 patients were negatively stained; 42 were weakly stained; 16 moderately strongly stained; and 15 strongly stained, all irrespective of the tumor type.

A human and rat tissue study, which was reported by Jae Ho et al in 2005, used 20 different human organs and 9 rat tissues. PIWIL 2 positivity was only shown in the testis tissue of both rats and humans (20). In that study PIWIL 2 expression was shown to increase in germ cell tumors, as was the case in our study. In our study, we used normal human testis tissue as the control tissue. Of the 34 germ cell tumor tissues, 6 showed strong staining and, 6 of them showed moderately strong staining pattern, which were statistically significant ($p<0.05$).

A study reported by Gang et al in 2010 performed immunohistochemical staining in 91 patients with cervical lesions, where highly different tumor groups were enrolled. Among these, all of 36 sections with the diagnosis of invasive squamous carcinoma were PIWIL 2 positive (21). The mean staining score was 2+ (moderately strong staining). Apart from normal cervical

tissue, other precancerous tissues also showed positive staining patterns, with a similar mean staining score of 2+ (moderately strong staining).

We used tumors originating not from a single organ, but from highly different tissues. However, no significant correlation was found between tumor's origin and PIWIL 2 staining for certain tumors. In a study by Lee et al reported in 2010, 149 invasive breast biopsies were stained with PIWIL 2 monoclonal antibody. Ninety percent of 125 invasive ductal carcinoma cases were found to show expression (22). Approximately 50% of tissues with PIWIL 2 expression showed moderately strong (2+) and strong (3+) staining whereas only 25% of carcinoma in situ cases were. Despite highly varied tumor groups being present in our study sample, 20.40% of 150 patients showed moderately strong (2+) and strong (3+) staining. In that study, a cytoplasmic staining was shown in the majority of cases. Although relevant data were not provided, a very slight amount of nuclear staining was detected. In our study we also obtained a high rate of cytoplasmic staining.

In another study, Sun Ju et al immunohistochemically stained 60 colorectal adenocancer tissues with PIWIL 2 to examine clinical and pathological effects of the staining rates (23). Forty-four (73%) patients showed a high expression grade (≥2+). It was shown that a higher expression grade was significantly associated with a deep invasion, a low differentiation grade, and perineural invasion. Five-year survival was greater in those with a lower expression than those with higher expressions and age, sex, distant metastasis, lymph node involvement, tumor stage, and venous invasion were not significantly different between the groups. We could not demonstrate any significant difference in PIWIL 2 expression with respect to disease stage, organ of origin, age, and sex. The depth of invasion was not discussed since there was no data about the depth of invasion depth in our study.

In contrast, in our study, we did not find a statistical significance for the survival rate although majority (70%) of neuroblastoma cases were diagnosed at stage 4 and a high expression was detected as a sign of worse prognosis in this tumor group, the difference did not reach statistical significance ($p>0.05$).

In 2010 Li et al reported that immunohistochemically stained members of the tissue argonaute family in 75 patients with colon cancer and 75 subjects without. Cytoplasmic staining pattern was determined in tumor tissues. Of 75 cancer cases, one was negatively stained with PIWIL 2; 27 patients had strong staining (3+). No significant difference had been shown according to age, sex, distant metastasis, and lymph node involvement. PIWIL 2 was significantly more expressed in tumor tissues (24). Similarly we could not detect any significant difference between staining rates in connection with age, sex, stage, and distant metastases ($p>0.05$).

In another study by Yang et al on 182 patients with gastric cancer, staining was performed with all proteins of the arganaoute family (25). All argonaoute proteins showed significant positive staining in tumor tissues and a significant correlation was found between staining rates and 5-year survival. However, in our study, we could not find any significant relationship between parameters such as age, sex, stage, and metastasis. PIWIL 2 only demonstrated strong staining in the germ cell tumor and neuroblastoma groups. There was marked staining especially in the yolk sac tumors in the germ cell tumor group. Ovary and sympathetic chain were the most commonly involved tissues in the germ cell tumor group, and in the neuroblastoma group respectively. In addition, moderately strong and strong staining were relatively more common in the ovarian tissue and sympathetic chain. The only dysgerminoma was of ovarian origin and had a 3+ staining pattern. In 2018, Erdoğdu et al reported that PIWIL2 expression is a predictive marker in thyroid papillary tumors. Unlike our study, PCR analysis was also included in this study (26). In the study conducted by Tosun et al. on adult patients with prostate cancer in 2019, it was determined that PIWIL2 cannot be used as a suitable diagnostic marker. However, in this study, results were performed only by analysis of serum PIWIL2 level (27).

Our study had some limitations. The limitations of this study are the inability to investigate PIWIL 2 expression in tumor tissues by cell line culture and polymerase chain reaction and the use of therapeutic polyclonal antibodies. In a study conducted by Tian et al. on neuroblastoma in 2015, they reported that the therapeutic use of PIWIL2 could be promising by inhibiting Sodium orthovanadate (28).

In conclusion, in this study, we demonstrated that immunohistochemical staining pattern associated with PIWIL 2 expression in childhood solid tumors. PIWIL 2 was shown to have a statistically significant positivity in neuroblastoma and germ cell tumor. Among sarcomas, osteosarcoma subgroup exhibited a strong PIWIL 2 staining as well. In the other solid tumors, no significant positivity was seen with PIWIL 2 staining. Negative staining was notable particularly in the Wilms' tumor.

CONCLUSION

In this study, we have, for the first time showed that PIWIL 2 is positive in patients with neuroblastoma, germ cell tumors, and osteosarcoma. The anti-PIWIL 2 antibody can be a potential therapeutic agent in tissues which highly PIWIL 2 protein is expressed. Non-tumor tissue involvement in Wilms' tumor can be particularly useful in determining surgical margins.

Competing Interests: The authors declare that they have no competing interest.

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Ethical Approval: This study was approved by Local Ethics Committee on 04.01.2011 (Number:2011/14).

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