VITAMIN D- STATUS AND RECEPTOR EXPRESSION PATTERNS IN PEDIATRIC SOLID TUMORS
A POTENTIAL PROGNOSTIC BIOMARKER

PhD Thesis

Orsolya Juhász

Pathological Sciences Doctoral School
Semmelweis University

Supervisor: Miklós Garami, MD. Ph.D.
Official reviewers: András Arató MD. D.Sc.
Ágnes Vojcek MD. Ph.D.
Complex Examination Committee:
Chairman: György Reusz, MD. D.Sc.
Members: Gergely Kriván MD. Ph.D.
Attila Marcell Szász MD. Ph.D.

Budapest
2023

1. Introduction

Increasing data demonstrates the beneficial impact of vitamin D supplementation on the reduction of cancer-related mortality and, recently, the correlation between vitamin D receptor (VDR) polymorphisms, protein expression pattern and cancer risk and progression, prompting the hypothesis that vitamin D and its metabolites could play a role in cancer treatment and prevention.

On the correlation between vitamin D and childhood cancer, there are only a limited amount of data from a small population of children. The evaluation of vitamin D status and VDR profile as potential biomarkers in pediatric solid tumors is therefore an area requiring further study.

According to a multinational assessment, the incidence of most forms of childhood and adolescent cancer has significantly increased in the last decades. Despite outstanding survival rates, cancer

is the major cause of non-accidental death in children beyond infancy in high-income nations, responsible for more than 5000 potentially avoidable deaths annually in people below 15 years of age.

The literature on the potential pathomechanisms and precise etiology of childhood tumor development is expanding, and simultaneously, diagnostic, and therapeutic biomarkers are being discovered. These could be beneficial in the future to evaluate and enhance the prognosis more accurately, and to treat more effectively pediatric solid tumors that currently have a poor prognosis. In parallel, countless studies have been published in the literature on the anti-tumor effects of vitamin D via VDR, but the childhood aspect of these is very limited. Due to these facts, we decided to investigate the role of vitamin D and VDR receptor patterns in pediatric solid tumors as potential prognostic biomarkers.

2. Objectives

1. The first goal was to assess whether the initial serum vitamin D level of children with solid tumors is lower than in the average population, to compare retrospectively the serum vitamin D level of our patients with the average childhood vitamin D level calculated for the given period.

2. We aimed to investigate whether higher initial vitamin D levels are statistically associated with a more favorable prognosis and whether the initial vitamin D levels are statistically distinct between the favorable and unfavorable prognostic groups.

3. The next goal was to assess the efficacy of vitamin D supplementation given to the children diagnosed with solid malignancies, i.e., whether vitamin D supplementation given along with the oncological treatment had increased serum vitamin D levels.

4. In addition, we investigated whether the higher vitamin D level measured after vitamin D supplementation was associated with a more favorable prognosis and whether the vitamin D levels after supplementation are statistically distinct between the favorable and unfavorable prognostic groups.

5. In the second phase of the research, we aimed to detect the potential presence of VDR in samples of solid childhood tumors and to determine whether an association between higher VDR expression and better prognosis can be detected.

6. We aimed to investigate whether there is a difference in VDR expression patterns between childhood solid tumor types.

7. We intended to record any additional observations seen during the pathological evaluation.

3. Methods

Patient and Sample Selection

1. In the retrospective phase of our study we included 173 children (96 male, 77 female) aged 2 weeks to 19 years (mean = 6.11, SD = 5.28) with solid tumors treated at Semmelweis University (2009-2013), whose serum vitamin D was measured at the time of diagnosis. The cancer patients' group contained neuroblastoma (26.6%), medulloblastoma (13.9%), Ewing's sarcoma (12.1%), ganglioneuroma (5.8%) and other solid tumors (represented fewer than 5%). The first blood sampling was performed before the initiation of oncological treatment and parallel vitamin D supplementation (500-2000 IU based on bodyweight), the second sampling took place after 4-6 weeks. Patients whose serum vitamin D levels were not measured within four to six weeks after

supplementation were excluded (n = 87) from further examination.

As the reference average vitamin D level, we used the mean of serum vitamin D values measured in the 0–19 age group at Semmelweis University between 2009–2010 and published in the literature.

A serum vitamin D level under 30 ng/ml was considered a vitamin D deficiency. Prognosis of the disease was grouped into two categories: complete response (n=116), partial response (n=11) and stable disease (n=15) were classified as favorable while progressive disease (n=5) and death (exitus, n=26) were classified as unfavorable.

2. For the immunohistochemistry examination, 177 children with solid tumors were selected whose biopsies and tumor tissue formalin-fixed, paraffin-embedded tissue blocks were available. Survival datasets of 110 children were available (62 male, 48 female). The mean age was 5.6 years (SD = 4.67).

The analysis included ependymoma, Ewing's sarcoma, ganglioneuroma, hepatoblastoma, medulloblastoma, neuroblastoma, papillary thyroid carcinoma, PNET, rhabdoid renal tumor, teratoma, Wilms' tumor samples.

Methodology

1. The 25-(OH)-D3 vitamin levels were measured by immunoanalytical methods.
We performed the statistical calculations on the retrospective dataset using Microsoft Excel 2013 and Stata 18 statistical software at significance level of 5%.

2. Tissue microarrays (TMA) were created from representative tumor areas of the hematoxylin-eosin-stained slides and labeled with VDR antibody for immunohistochemistry.

The evaluation was carried out by two independent pathologists. The following scores were assigned to the expression level of the VDR protein: negative (none) and low (< 10%) were considered as minor-, intermediate (11-50%) and high (51-100%) were considered as major VDR expression in the samples. During this phase of the research, the analyses were conducted using R version 4.1.0., significance level was set to 5%.

4. Results

1. Based on the relevant literature the mean serum vitamin D value of the average children population was 28.4 ng/ml (SD = 6.07), while the patient group had a mean value of 24.3 ng/ml (SD = 11.17). The mean of the initial vitamin D levels of children with solid tumors was significantly lower than the mean value of the non-cancerous, average population ($t(172) = -4.97$, $p < 0.001$).

2. We found that the odds of a favorable prognosis occurring increased by 95 % (OR= 1.95 95% CI (1.21-3.13) p=0.006) for a ten ng/ml increase in the initial serum vitamin D level. The initial vitamin D level showed a significant difference in the favorable and unfavorable prognostic groups (ANOVA-1 $p = 0.009$).

3. Vitamin D supplementation in fact increased the serum vitamin D levels of children; the difference between initial (mean= 24.44, SD = 10.7) and

supplemented values (mean= 30.23, SD = 12.2) was significant (t(85)= -4.13, p <0.001).

4. There was no significant difference in the odds of a favorable prognosis for ten ng/ml increase in the serum vitamin D level by supplementation (OR = 1.48 95 % CI (0.91-2,41) p = 0.114). Vitamin D levels measured after supplementation showed no significant difference in the favorable and favorable prognostic groups (ANOVA-1 p= 0.09).

5. Minor VDR expression was associated significantly with a less favorable prognosis (*p*=0.0061) in the examined childhood solid tumors.

6. The rate of VDR expression differed significantly between various tumor types (p<0.001).

7. Wilms-tumor was generally characterized by major VDR expression, which was seen mainly on the epithelial cells of the tubules.
Minor VDR expression was characteristic for ependymoma and medulloblastoma. Paranuclear

dot-like positivity was observed in some ependymoma samples. Major VDR expression was observed in PNET.

Hepatoblastoma samples revealed major, diffuse VDR expression of the tumor cells.

In neuroblastoma samples the mature ganglion cells in the tumor showed mainly major VDR expression.

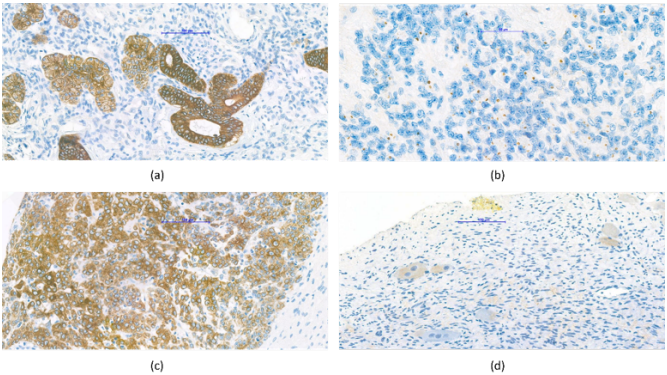

Figure 1 *VDR protein expression patterns in specific tumor subtypes.* ***(a)*** *Wilms tumor: strong membrane/cytoplasmic expression, seen predominantly in the epithelial component.* ***(b)*** *Ependymoma: paranuclear dot-like positivity.* ***(c)*** *Hepatoblastoma: strong, diffuse membrane/cytoplasmic VDR expression.* ***(d)*** *Ganglioneuroma: positive ganglion cells.*

5. Conclusions

1. The mean value of the initial vitamin D levels of children with cancer were significantly lower than the average population mean. Although there is conflicting literature on the association between cancer incidence and vitamin D deficiency in adult cancers, and insufficient data is available in the childhood aspect, overall preventive vitamin D supplementation in the pediatric population is recommended.

2. Our result indicated that higher initial serum vitamin D levels were associated with a favorable prognosis and patients in the favorable prognostic group had a significantly higher initial vitamin D levels. The extensive literature and our data agree on the argument for vitamin D supplementation in the currently healthy child population. Based on the most recent literature, daily doses of 400–1000 IU

recommended, and for children aged 0–18 years, with daily doses of 600–1000 IU.

3. Higher VDR expression in childhood solid tumor cells is associated with a favorable prognosis, which raises the potential role of the VDR profile as a prognostic biomarker.

4. VDR expression differed significantly by tumor type. Although the number of elements is small for some entities, further investigation of these may be useful. For Wilms' tumor and hepatoblastoma, VDR profiling with immunohistochemistry is recommended as an adjunct to histopathology. In case of neuroblastoma, it would be beneficial to investigate the role of vitamin D analogues in further maturation through the presence of VDR.

5. All in all, VDR profiling of individual pediatric solid tumors may have a potential prognostic biomarker role. On this basis, it may be worthwhile to identify groups of patients for whom its

incorporation into a personalized, accurate histological diagnosis may also act as a therapeutic biomarker through the maturation-promoting and proliferation-inhibiting effects of vitamin D and analogues through VDR.

Implication for practice and research

In the future, our work may contribute to complementing the histological diagnostic methodology with this new prognostic and therapeutic biomarker, the VDR expression profiling, in certain priority patient groups.
A tumor specific VDR expression profiling study on a larger number of subgroups of elements would be advisable. Furthermore, based on the literature trends, a study investigating VDR gene polymorphisms will be explored to clarify the prognostic biomarker association of VDR polymorphisms in specific tumor types.

New statements

1. This study is the first in Hungary to investigate subclinical vitamin D deficiency as a potential risk factor for childhood cancers and VDR expression patterns in solid tumor samples from multiple pediatric entities by immunohistochemistry.
2. The mean value of initial vitamin D levels of children with cancer were significantly lower than the mean of the average children population.
3. Higher initial serum vitamin D levels are associated with a favorable prognosis.
4. Elevated VDR expression in childhood solid tumor cells is correlated with a favorable prognosis, suggesting that the VDR profile may serve as a potential prognostic biomarker.
5. Previously unreported dot-like VDR expression has been detected in ependymoma samples.

6. Bibliography of my publications

Original articles written in English that serve as the basis for the thesis

1. Juhász O, Jakab Z, Szabó A, Garami M. Examining the vitamin D status of children with solid tumors. Journal of the American College of Nutrition. 2020;39(2):128-34.
2. Juhász O, Jákob N, Rajnai H, Imrei M, Garami M. Immunohistochemical Detection of the Presence of Vitamin D Receptor in Childhood Solid Tumors. Cancers. 2022;14(14):3295.

Original articles written in Hungarian that serve as the basis for the thesis

3. Juhász O, Erdélyi Z, Garami M. D-vitamin-státusz vizsgálata szolid tumoros gyermekekben. Gyermekgyógyászat. 2016.
4. Juhász O, Jákob N, Rajnai H, Imrei M, Brückner E, Garami M. D-vitamin jelentősége a gyermekonkológiai megbetegedésekben (etiológia, terápia). Gyermekorvos továbbképzés. 2022.

Publications in Hungarian not directly related to the theses

6. Juhász O, Bense T, Tory K, Sallai Á. AVPR2 gén teljes deléciója okozta nephrogén diabetes insipidus – Esettanulmány. Gyermekgyógyászat. 2019.
7. Brückner E, Juhász O, Hegedüs P, Sági V, Pfeffer A, Fekete Gy, Garami M. Az I-es típusú neurofibromatosishoz társuló idegrendszeri daganatok diagnózisa és korszerű kezelése. Gyermekgyógyászati Továbbképző szemle. 2021.
8. Pfeffer A, Beniczky N, Juhász O, Török D, Garami M. A gyermekkori mellékvesekéreg-tumorok ellátása: út a diagnózistól a gyógyulásig. Gyermekgyógyászat. 2021.
9. Brückner E, Juhász O, Fekete Gy, Kovács Á, Garami M. Klinikai ajánlások az NF I. betegek kezeléséhez: új terápiás lehetőségek. Gyermekorvos továbbképzés. 2022.