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Protective Desmoplasia in Pancreatic Adenocarcinoma: High Vitamin D Receptor Expression and Collagen Content

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Abstract. *Background/Aim: Vitamin D receptor (VDR) has been shown to suppress desmoplasia in pancreatic ductal adenocarcinoma (PDAC). Our aim was to assess the clinical effects of VDR expression and its correlation with collagen content in the desmoplasia of PDAC patients. Patients and Methods: This is a retrospective analysis of 127 patients with peritumoral desmoplasia resected for PDAC. VDR expression and collagen content were assessed by immunohistochemistry and correlated with clinical outcome. Results: Patients were classified into those with high and those with low VDR expression. High VDR expression was associated with improved overall survival (OS) in localized disease (N0) (median= 33; 95%CI=26.4-39.6 vs. 18; 15.5-20.5 months, p=0.01). Patients with high vs. low collagen content had improved OS [34, (range=22.3-45.6 months) vs. 17, (range=14.4-19.6 months), p<0.001]. The number of VDR+ cells was the same for patients with either high or low collagen content. Conclusion: Protective desmoplasia is associated with increased VDR expression and collagen content.*

Pancreatic ductal adenocarcinoma (PDAC) is the most lethal malignancy of the gastrointestinal tract with an overall 5-year survival of 6-8% (1, 2). Although implementation of novel protocols have improved the survival of patients in the metastatic (3, 4) and adjuvant settings (5, 6), there is still a need for further improvement in treatment outcomes. The role of the tumor microenvironment in tumor pathogenesis and treatment resistance are among the novel aspects of PDAC biology that have been discovered over the past few years (7). PDAC is characterized by a dense fibroblastic reaction, which involves activation of pancreatic stellate cells (PSC) that lose their lipid storing properties and acquire myofibroblastic properties. Upon tumor-induced activation, these cells produce stromal collagen and extracellular matrix (ECM), often referred to as a desmoplastic reaction (8). The role that the desmoplastic reaction plays in tumor pathogenesis is under intense investigation. Some experimental evidence suggests that desmoplasia impairs anti-neoplastic drug delivery due to the paucity of blood vessels in this tissue, and increases intra-tumoral pressure due to the stiffness it confers (7). However, clinical data did not show any improvement in overall survival (OS) or progression-free survival (PFS) by the use of desmoplastic inhibitors (9, 10). Furthermore, experimental data have shown that desmoplastic inhibition facilitates metastases formation with shortened OS, and is associated with higher-grade tumors (11). A new concept emerged in an attempt to resolve these and other contradictory data regarding the role of desmoplasia. It is assumed that desmoplasia is a heterogeneous process induced by different populations of cancer-associated fibroblasts (12). Accordingly, different stromal genetic subtypes were discovered, each with a specific molecular phenotype, clinical significance, and possible therapeutic targets (13, 14).

Vitamin D receptor (VDR) is expressed in various PDAC cell lines, and its activation is known to suppress the proliferative activity of cancer cells and induce their differentiation (15, 16). This led to the hypothesis that VDR agonists have a therapeutic potential in PDAC. However, data from epidemiological studies on the association of vitamin D levels and PDAC are inconclusive. Whereas some groups have reported that high vitamin D levels are associated with decreased risk of PDAC development (17) and increased survival of PDAC patients (18), others failed to demonstrate any correlation (19). However, VDR is also present in stromal cells in the tumor microenvironment. Vitamin D agonists were recently found to modulate the inflammatory response of cancer-induced activation of PSC via the VDR (20). Upon VDR activation, PSC returned to

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their quiescent state: the cells acquired their lipid-storing capacities, and halted the production of ECM components, including collagen and pro-inflammatory cytokines. Accordingly, the combination of gemcitabine with calcipotriol, a VDR agonist, decreased tumor desmoplasia, increased survival, and increased chemotherapy availability in a mouse model of PDAC. Thus, experimental data suggest that a combination of VDR agonists, as stroma-modifying agents, with conventional chemotherapy may be a novel strategy against PDAC.

The ability to clinically utilize VDR agonists for stromal reprogramming is dependent upon the expression of VDR in target tissue. It would be important to verify that VDR is still expressed in mature desmoplastic tissue after production of ECM and tissue remodeling had already occurred, as in the case when PDAC is diagnosed. However, the patterns of VDR expression in human desmoplastic tissue and its relation to clinical outcomes are currently unknown.

The aim of this study was to examine the pattern of VDR expression in the desmoplastic tissue of patients with PDAC, and to correlate VDR expression with clinical outcomes. Given that collagen is one of the major proteins produced by activated PSCs, we also aimed to correlate VDR expression with collagen content in human desmoplastic tissue.

Patients and Methods

Patients. The study included 127 adult patients pathologically diagnosed with PDAC and desmoplasia who were operated between January 2009 and December 2017 at the Tel Aviv Sourasky Medical Center. Clinical and pathologic data were extracted from a prospectively maintained institutional database. Tumors were staged according to the 8th edition of the AJCC/UICC TNM staging system. Tumor grade was determined according to the World Health Organization classification (4th) of digestive system tumors. Patients treated by neoadjuvant therapy and those who died in the postoperative course were excluded. Prior to study inclusion, hematoxylin and eosin slides were reviewed by an expert pathologist (A.I.) and confirmed to have a significant desmoplastic peri-tumoral reaction. All kinds of pancreatic resections were included, among them pancreaticoduodenectomy, distal pancreatectomy, and total pancreatectomy. Patient participation and tissue analysis were approved by the Institutional Review Board (TLV-16-0586).

Assessment of vitamin D receptor expression and collagen content in desmoplastic tissue. Immunohistochemistry for VDR (AB8756, ABCAM, Cambridge, UK) was performed on formalin-fixed, paraffin-embedded tissue sections of 4 μM as described before (21). A representative slide of the desmoplastic area (which contains both tumor and desmoplasia) was stained for each patient, and slides were automatically scanned with a digital scanner (Ultra-fast scanner, Philips, Amsterdam, Netherlands). VDR-positive cells in the desmoplastic area were manually counted in 3 representative fields (×20, corresponding to 0.05 mm^2). To determine collagen content, the slides were stained with a Trichrome stain kit (TRM-1, ScyTek, UT, USA), and collagen content was estimated after digital scanning as above according to 3-tier classification (weak, moderate and strong), which was judged by fiber density, content, and intensity. Preliminary survival analysis according to the 3-tier classification had shown that the moderate class had the same clinical effect as the weak class, and consequently, the two classes were combined for further analysis. Histological quantifications were done by 2 independent trained observers blinded to clinical outcomes. Any discrepancy over VDR count or collagen content classification was further discussed with a pathologist.

Statistical analysis. Median and interquartile range (IQR) were used to describe continuous variables, while categorical variables were expressed as absolute and relative frequency and compared by means of the Wilcoxon rank sum test or Pearson Chi-Square test. OS was defined as the time between surgery and death from any cause. Disease-free survival (DFS) was defined as the time from surgery to disease recurrence at any site (local or systemic) by abdominal imaging [time was censored at the date of last follow-up for surviving patients (October 2019)]. Survival curves were estimated with the Kaplan–Meier method and compared by the log-rank test. Multivariable analysis for OS was performed by the Cox proportional hazards regression model. All statistical analyses were performed using SPSS for Windows version 25 (IBM). A p-value <0.05 was considered to be statistically significant.

Results

Patient characteristics. We analyzed 127 patients during the study period. Their clinical and pathological characteristics are described in Table I. The patients were classified into groups of low and high VDR according to the median number of VDR+ cells in the desmoplastic tissue (Figure 1). Similarly, patients were classified into high and low collagen groups. Comparisons of the clinical and pathological characteristics between the various groups revealed there was no statistically significant difference between them (Table I), indicating that the number of VDR+ cells and collagen content are apparently not associated with these clinical and pathological characteristics. In the cohort analyzed, only 17% used vitamin D supplementation.

High VDR expression in desmoplasia is associated with improved outcomes in localized disease. Patients were classified into high and low VDR groups according to the median VDR expression (=16 cells per 0.05 mm^2) as described above (Figure 1). VDR expression did not have a statistically significant effect on OS [median: 22; 95% confidence interval (CI)=17.2-26.8 *vs*. 21; 95%CI=18.3-23.7 months for high *vs*. low VDR expression, p=0.27) or DFS (median: 13; 95%CI=7.9-18.1 *vs*. 11; 95%CI=6.8-15.2 months for high *vs*. low VDR expression, p=0.5) when the entire patient cohort was analyzed (Figure 2A and B). With previous reports having shown that desmoplasia is less abundant in metastases than in primary tumors (22), we further analyzed the effects of VDR expression on prognosis in N0 disease, *i.e*., when disease is localized to the pancreas

Table I. *Patient's clinical and pathological characteristics.*

	All patients (n=127)	Low VDR (n=57)	High VDR (n=69)	*p*-Value	Low collagen (n=65)	High collagen (n=45)	*p*-Value
Age, mean (range), y	69 (61-77)	68 (61-76)	69 (61-77)	0.58	68 (61-76)	69 (61-77)	0.77
Gender F/M, n (%)	58/69 (45.7/54.3)	27/30 (47.4/52.6)	30/39 (43.5/56.5)	0.66	32/33 (49.2/50.8)	20/25 (44.4/55.6)	0.62
Op. type: PD/DP/TP, n (%)	77/48/2 (60.6/37.8/1.6)	33/24/0 (57.9/42.1/0)	43/24/2 (62.3/34.8/2.9)	0.33	35/29/1 (53.8/44.6/1.5)	31/13/1 (68.9/28.9/2.2)	0.62
T stage: 1/2/3/4, n (%)	22/49/49/7 (17.3/38.6/ 38.6/5.5)	9/21/23/4 (15.8/36.8/ 40.4/7)	12/28/26/3 (17.4/40.6/ 37.7/4.3)	0.89	13/22/28/2 (20/33.8/ 43.1/3.1)	5/16/19/5 (11.1/35.6/ 42.2/11.1)	0.26
N stage 0/1/2, n (%)	71/42/14 (55.9/33.1/11)	38/14/5 (66.7/24.6/8.8)	32/28/9 (46.4/40.6/13)	0.07	35/23/7 (53.8/35.4/10.8)	19/19/7 (42.2/42.2/15.6)	0.46
Perineural invasion absent/present, n (%)	45/81 (35.4/63.8)	23/33 (41.1/58.9)	21/48 (30.4/69.6)	0.21	24/41 (36.9/63.1)	13/32 (28.9/71.1)	0.96
Grade 1/2/3, n (%)	28/74/23 (22/58.3/18.1)	12/30/15 (21.1/52.6/26.3)	16/44/8 (23.2/63.8/11.6)	0.15	14/36/15 (21.5/55.4/23.1)	9/29/7 (20/64.4/15.6)	0.56

OP type: Operation type; PD: pancreaticoduodenectomy; DP: distal pancreatectomy; TP: total pancreatectomy.

without lymph node metastases (note that VDR is indeed expressed in lymph node desmoplastic tissue, data not shown). The results showed that patients in the high VDR group had longer OS and DFS compared to those in the low VDR group (median: 33; 95%CI=26.4-39.6 *vs.* 18; 95%CI=15.5-20.5 months, $p=0.01$ and 22; 95%CI=13.7-30.3 *vs.* 8; 95%CI=5-11 months, respectively, $p=0.015$) (Figure 2C and D). Although adjuvant chemotherapy treatment and collagen content showed a trend for improved prognosis, the multivariate analysis revealed that VDR was the only statistically significant parameter to predict OS (HR=0.35, 95%CI=0.16-0.74, $p=0.006$) (Table II).

High collagen content in desmoplastic tissue is associated with improved outcomes. We determined the collagen content in the peri-tumoral desmoplastic tissue (Figure 1) and classified the patients into high and low collagen content groups as described above. Patients with a high collagen content had an improved median OS and DFS compared to those with a low collagen content (median: 34, 95%CI=22.3-45.6 *vs.* 17, 95%CI=14.4-19.6 months, $p<0.001$ and 17, 95%CI=12.6-21.4 *vs.* 9, 95%CI=6.9-11.1 months, $p=0.04$, respectively) (Figure 3). The effect of collagen content was maintained in the multivariate analysis for both OS (HR=0.42, 95%CI=0.27-0.66, $p<0.001$) and DFS (HR=0.42, 95%CI=0.27-0.66, $p<0.001$) (Table III).

Association between VDR expression and collagen content. Activated PSCs produce collagen, which is the main component of the peri-tumoral ECM. We aimed to correlate the VDR expression with collagen content to determine whether the number of VDR+ cells is increased in patients with "dense" (*i.e.*, high collagen content) desmoplasia.

As shown in Figure 4, the number of VDR+ cells did not differ between patients with high *vs.* low collagen content (15.6±5.2 *vs.* 17.2±5.9, $p=0.3$). Similar results were obtained when the number of VDR+ cells in localized disease was compared among patients in the high and low collagen groups (15.6±4.5 *vs.* 14.9±5.7, respectively, $p=0.86$).

Discussion

PDAC stroma has gained much attention as a potential source for novel therapeutic targets. The ability to modulate the PDAC microenvironment may disrupt the pro-tumorigenic cross-talk between tumor cells and their surroundings, and facilitate the penetration of chemotherapy into the tumor. The combination of stroma-modifying agents, such as VDR, agonists and chemotherapy, such as gemcitabine, has shown potential in experimental models (20) and is under intense clinical research (NCT03415854, NCT03331562, NCT03520790, and other trials). However, the presence of VDR in human desmoplasia needs to be demonstrated for the purposes of future clinical application. This study shows that VDR expression persists in PDAC even after desmoplastic stroma has been formed, and this makes VDR agonists a potential therapeutic tool.

The variable levels of expression among patients with desmoplasia, however, may have therapeutic implications. It is currently unknown if the response rate to VDR agonists depends on the numbers of VDR+ cells in the stromal desmoplasia. Hopefully, data will emerge from the various ongoing clinical trials that use VDR agonists. Our data demonstrated that VDR expression may serve as a prognostic marker when the tumor is localized to the pancreas, as opposed to when it has already metastasized to the lymph

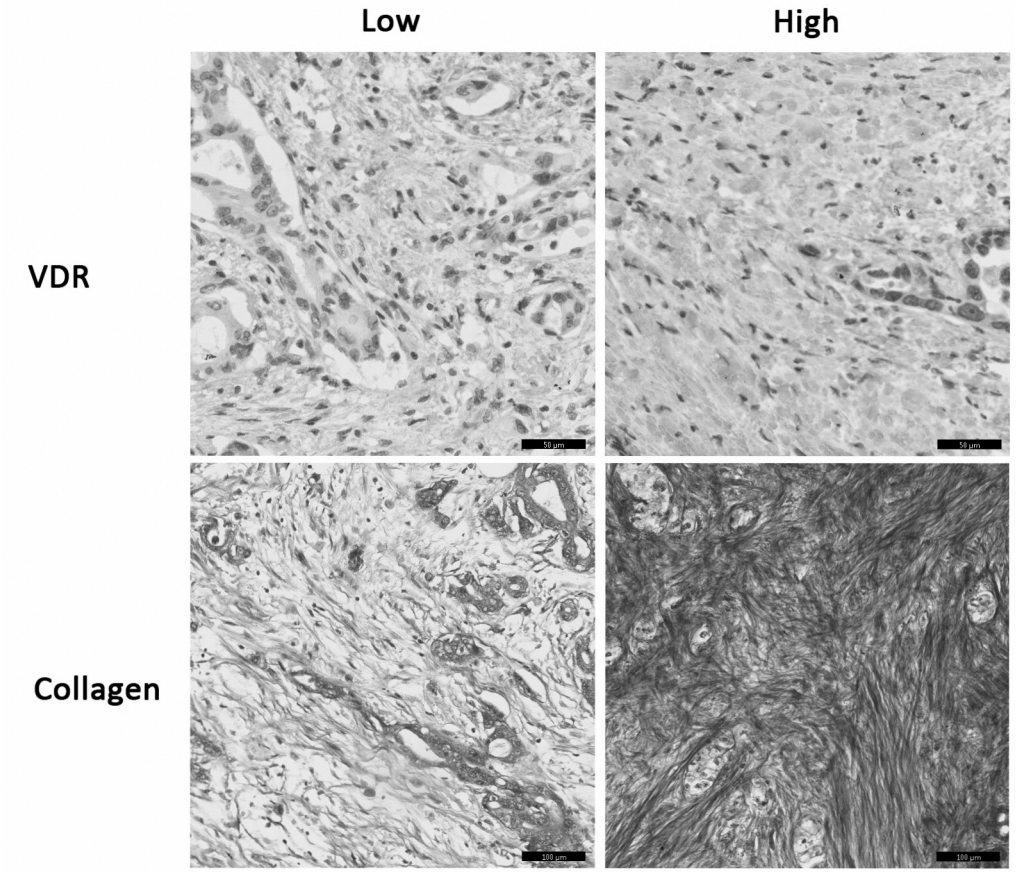

Figure 1. *Patterns of vitamin D receptor (VDR) expression and collagen deposition in peritumoral stroma. Anti-VDR antibody was used to determine VDR expression, and trichrome stain was used to determine collagen content. Note that VDR was also expressed on tumor cells, (not quantified in this study).*

Table II. *Multivariate analysis of risk factors associated with clinical outcomes.*

	Overall survival			Disease-free survival		
	HR	95%CI	*p*-Value	HR	95%CI	*p*-Value
Age[a]	1.03	1.00-1.05	0.006	1.01	0.99-1.03	0.2
T stage	0.88	0.37-2.1	0.78	0.74	0.28-1.94	0.55
N stage	1.7	1.27-2.27	<0.001	1.8	1.28-2.52	0.001
Perineural invasion	0.8	0.5-1.26	0.34	0.94	0.58-1.53	0.81
Grade[b]	1.72	1.18-2.52	0.005	1.77	1.12-2.8	0.01
High collagen content	0.42	0.26-0.66	<0.001	0.58	0.36-0.92	0.02

[a]For each year; [b]*Vs*. low grade. HR: Hazard ratio; CI: confidence interval.

nodes. Although desmoplasia is also present in lymph nodes (23, 24), Torphy *et al*. recently demonstrated that desmoplasia is less prominent in a metastasis compared to a primary tumor (22). The number of VDR+ cells was the only factor significantly associated with prognosis in our multivariate analysis, even when co-analyzed with collagen content. It is important to note that our study took place in an era before Folinic Acid (Leucovorin)-Fluorouracil-Irinotecan-Oxaliplatin (FOLFIRINOX) was used as an adjuvant, and that the adjuvant treatment consisted of

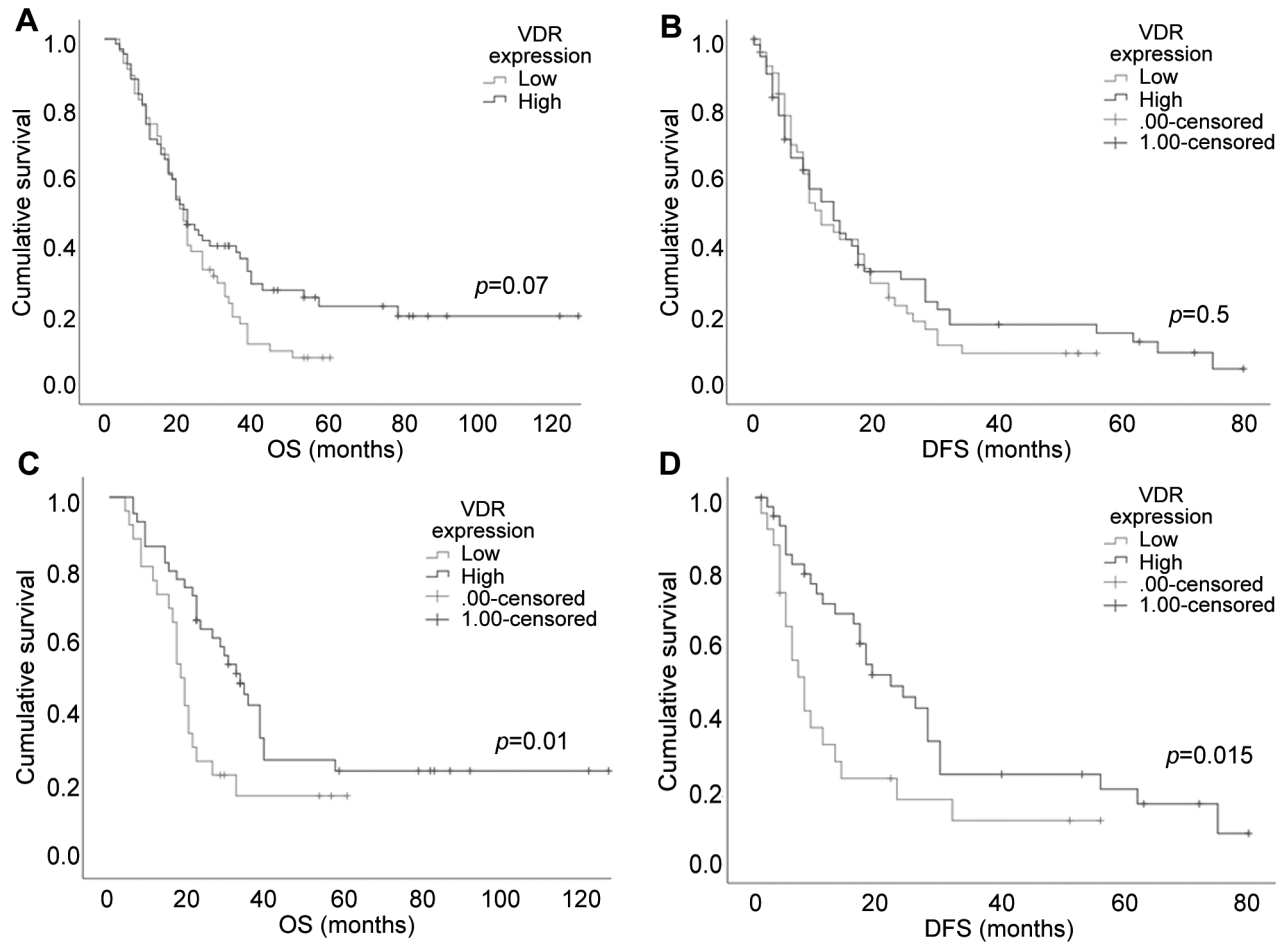

Figure 2. *Effects of vitamin D receptor (VDR) expression on clinical outcomes. Patients were divided into groups of low and high VDRs according to the median number of VDR+ in stromal cells as described in materials and methods. A, B: Analysis of the entire patient cohort. C, D: Analysis of patients without lymph node metastases (n =70). p-Values by log-rank test. OS: Overall survival; DFS: disease-free survival.*

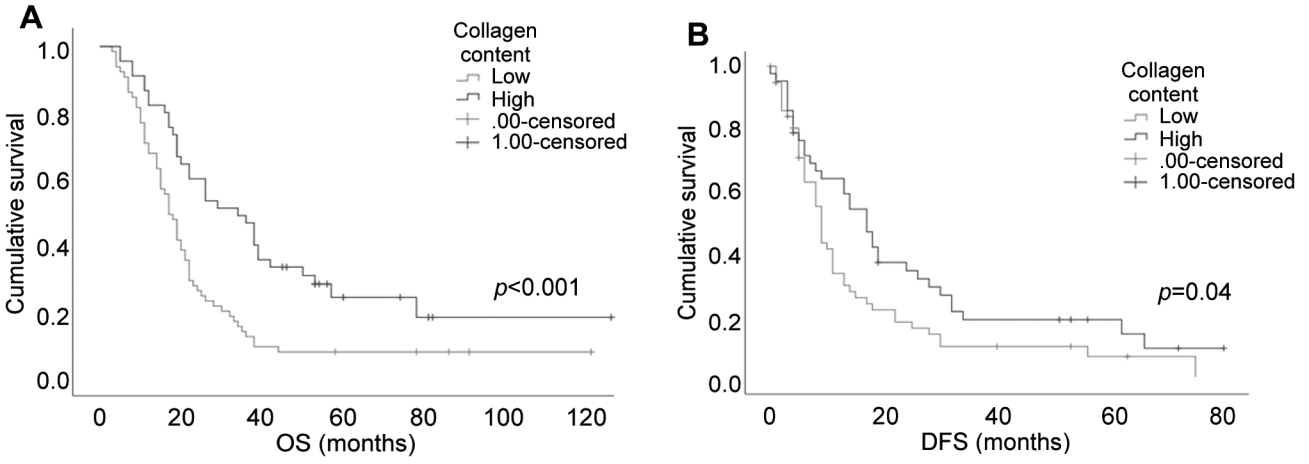

Figure 3. *Clinical outcomes of patients with different collagen contents. Patients were divided into groups of high and low collagen contents after determination of collagen content by trichrome stain (see materials and methods). A. Overall survival (OS) B. Disease-free survival (DFS). p-Values by log-rank test.*

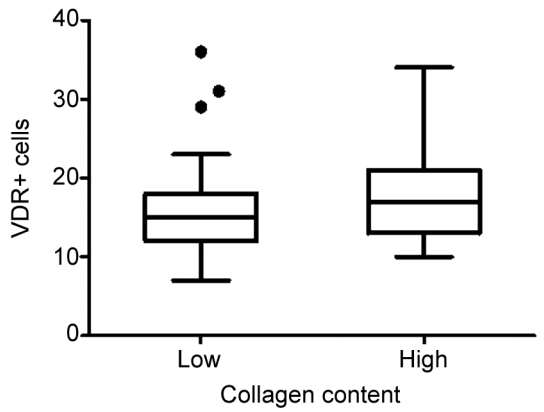

Figure 4. *Comparison of vitamin D receptor (VDR)-positive cells between patients with low and those with high collagen content. p=0.86 by Wilcoxon rank sum test.*

gemcitabine in the vast majority of our patients. This, together with the relatively small number of treated patients, may explain why adjuvant treatment was not associated with a statistically significant effect on outcomes.

The significance of the VDR+ cell number is currently unknown. We hypothesized that VDR expression is correlated with collagen content, and that it may be associated with ECM production of activated of PSCs. However, the number of VDR+ cells did not differ between the patients with high and low collagen levels. It is possible that VDR expression is a marker for a specific PSCs subpopulation, which is not associated with collagen production (12).

Our results show that collagen content in the desmoplastic stroma is significantly associated with improved clinical outcomes. Our data are in line with those of previous studies which reached the same conclusions based on analyses of human tissue (13, 22, 25, 26), and on experimental data on the protective role of desmoplasia (11). Whereas paracrine factors secreted by CAFs can promote tumor progression, and stroma-induced intratumoral pressure can diminish chemotherapy availability (7), collagen may act as a barrier for tumor metastases (11). Rather than select stromal depletion as a therapeutic target, stromal modification with the preservation of beneficial stromal anti-tumor effects might be a more appropriate therapeutic target. Interestingly, tumor enhancement in the portal phase on an abdominal computerized tomographic scan was positively correlated with stromal density (22, 27). This feature can serve to facilitate the preoperative assessment of collagen content, as well as the resultant collagen content in response to stromal modifying agents. In contrast, however, as shown in our study, the number of VDR+ cells is not correlated to collagen content, and therefore cannot be predicted by stroma imaging density.

Our study has several limitations that bear mention, starting with it being a retrospective analysis. Although the beneficial effect of stromal collagen content has been reported by different groups and on several independent patient cohorts, it should be validated in prospective trials. Second, despite our efforts to standardize pathological quantification of both VDR and collagen content, the method is subjective and there were cases of inter-observer discrepancy. The automatic algorithm suggested by Torphy *et al*. (22) may be a potential solution for this drawback.

In summary, a high number of VDR+ cells and an increased collagen content in tumor-induced desmoplasia characterize a protective stromal response. Further studies are warranted to delineate the mechanism by which this type of stroma restricts tumor development. Whether this high VDR expression makes patients more sensitive to VDR agonists remains to be established.

Conflicts of Interest

The Authors report no conflicts of interest pertaining to this work.

Authors' Contributions

A.V.B. and E.N. designed the study, AV.B., S.B.D, A. B., A.I. and O.G. collected data, A.V.B., S.B.D. and E.N. analyzed the data, I.W., J.K, G.L. and E.N. interpreted and discussed the findings, A.V.B., I.W. and E.N. wrote the manuscript. All the Authors revised and commented on the manuscript.

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