Vitamin D Receptor Expression as a Predictive Marker of Biological Behavior in Human Colorectal Cancer

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Abstract

To date, none of the potential biological markers in colorectal cancer attempts to link the epidemiological data with the molecular biology of the disease. In an attempt to link dietary and epidemiological factors and to obtain a better understanding of the molecular biology of colorectal cancer, we measured vitamin D receptor (VDR) expression in 75 human colorectal cancers as a potential predictive marker of the biological behavior of the disease.

Our results showed that a high level of VDR expression was associated with a favorable prognosis. The results of the studies reinforce the potential role that VDR may play in the development of the pathogenesis of colorectal cancer. Larger studies looking exclusively at stage I and stage II disease will hopefully lead to the development of a sensitive hormonal marker that can be used to predict the biological behavior of colorectal cancer, identifying at-risk patients in need of adjuvant treatment.

Introduction

Colon cancer, with 150,000 new cases and 60,000 deaths each year, is the third leading cause of death from cancer in the United States and most industrialized countries (1). Colon carcinogenesis is the result of a multistep process in which an increasing number of alterations, including specific gene mutations, occur as cells progress from the normal to the precancerous states of dysplasia and, finally, to invasive cancer. Research to identify biochemical or molecular markers that correlate with the biological behavior of colorectal cancer lags far behind that of other solid epithelial tumors, such as breast cancer. Currently, the use of aneuploidy, the dCC gene, and the measurement of p53 in colorectal cancers have all shown some promise as predictive markers of biological behavior (2-5). To date, however, none of these potential markers attempts to link the epidemiological data in colorectal cancer with the molecular biology of the disease (6).

Several epidemiological studies currently exist that are relevant to the relationship of colorectal cancer and vitamin D. There appears to be an inverse correlation between the level of dietary intake or sunlight exposure and the incidence of colorectal cancer. In an 8-year prospective study of 25,620 volunteers, assessing the relationship of serum 25-hydroxyvitamin D levels with the subsequent risk of colorectal cancer, Garland et al. (7) demonstrated a 3-fold reduction in the incidence of the disease in patients with serum levels of >20 ng/ml (P < 0.05). Additionally, in a 19-year prospective study of 1954 men, a daily intake of >3.75 µg of vitamin D was associated with a 50% reduction in the incidence of colorectal cancer (8). Most of the currently known effects of vitamin D are mediated through a genomic pathway via the VDR, a member of the steroid hormone receptor superfamily.

VDR was first isolated from the nuclei of chicken small intestinal cells in 1969, and DNA coding for the receptor was cloned, sequenced, and expressed in COS-1 monkey kidney cells (9, 10). VDR has been expressed in many solid epithelial tumors, including colon, breast, and ovarian cancers, melanoma, and sarcoma (11-15). In vitro studies measuring VDR in several colorectal carcinoma cell lines strongly suggest that VDR correlates closely with the degree of differentiation of the cell lines, specifically, well-differentiated, biologically favorable cell lines have high expression of VDR and biologically aggressive cell lines with high metastatic potential have low VDR expression (16). In an attempt to link dietary and epidemiological factors and to obtain a better understanding of the molecular biology of colorectal cancer, we proceeded to measure VDR expression in human colorectal cancers as a potential predictive marker of the biological behavior of the disease.

Materials and Methods

Patient Population. Between the years 1990 and 1995, specimens of human colorectal cancers were obtained at the time of surgical resection at Georgetown University Hospital. All of the patients were submitted to the same treatment protocol, in relation to the stage of the tumor. In each case, tissue from the tumor and adjacent normal tissue (10-12 cm away from the original tumor) were obtained from surgical pathology prior to any irrigation or fixation. Tumors were all histologically confirmed to be adenocarcinoma, and the adjacent tissue was confirmed to be normal in each case. Tissue was obtained under
institutional review board approval through Georgetown University Hospital. Clinical follow-up was obtained through the Lombardi Cancer Research Center tumor registry, in addition to chart reviews of deceased patients. Study end points were recurrence of disease or death related to tumor recurrence, whichever occurred first. Medical records were reviewed through August 1997. Median follow-up time was 23 months.

**RNAse Protection Assay.** RNAse protection assay was done on tissue samples from 75 patients who underwent resection of malignant colorectal cancers between 1990 and 1995. Of the 75 patients, 46 had at least 2 years of postoperative surgical follow-up, in addition to available tissue from both tumor and normal mucosa.

RNAse protection assay was performed as we described previously (16). We have created a 350-bp riboprobe of VDR by deleting the 849-bp 3' terminal segment of the cDNA and ligating the vector back to itself. The probe corresponds to a 319-bp protected fragment at the 3' end of the human VDR coding sequence. The 5' and 3' termini of the construct were sequenced to confirm correct restriction enzyme digestion and orientation. At the time of the assay, frozen tissue samples were pulverized using a mortar and pestle. Specimens were homogenized using a stainless steel Dounce homogenizer. RNA was then isolated using the cesium chloride gradient purification protocol. The probe was radiolabeled with ^32^P and incubated with 30 μg of purified RNA. A radiolabeled probe (36B4) that detects a constitutively expressed human acidic ribosomal phosphoprotein was added to the same samples to control for uniformity of loading. The samples were incubated with RNAse A to degrade the nonprotected sequences. The product was run on a 6.5% polyacrylamide gel with a size marker (MspI-digested pBR322). Autoradiography was then performed overnight at room temperature. The densities of the protected fragment bands were measured using a densitometer. The density was expressed as a fraction of that of the band from the adjacent normal tissue. Densitometry was performed for all tumor and normal mucosa samples in 75 patients for VDR expression, and the data were then standardized based on the 36B4 loading control. All of these data were analyzed as it relates to patient characteristics in Table 1 and various prognostic factors in Table 2.

**Statistical Methods.** Plots of the data indicated that the T/N value was best analyzed on the natural logarithm scale to normalize the data and stabilize the variance. T/N ratio is much more accurate and correlates much better with the other prognostic factors related to colorectal cancer. We had attempted to use the tumor value only for analysis but found that the correlation was much looser. The comparison of VDR expression in tumor and nearby normal tissue was determined by a one-sample t test of the null hypothesis that the log T/N value was zero. The association between log T/N value and other patient characteristics was investigated using the ANOVA method for categorical factors (Dukes' stage, differentiation, and sex) and the Pearson correlation coefficient for continuous variables (age).

The association between VDR expression and clinical events was investigated in univariate and multivariate analyses. In a univariate analysis, log T/N values were compared between patients who experienced an event and patients who were event free, using a two-sample t test. In a univariate analysis that adjusted for time on study, patients were split into two groups according to their T/N value (above versus below median), and their EFS times were plotted using the Kaplan-Meier method (17) and compared using a log-rank test (18). To adjust for other prognostic factors, a multivariate analysis was conducted using Cox proportional hazards regression (19). Time was measured from the day of operation. The proportional hazards assumption was checked using the diagnostic procedures of Grambsch and Therneau (20). Diagnostic plots using the Grambsch-Therneau method were used to identify prognostic factors with effects that varied over time (20). All tests were two-sided, and Ps of <0.05 were considered significant.

**Results**

Patient characteristics are listed in Table 1. VDR expression in the tumor specimens was significantly lower than in nearby normal tissue (P < 0.0001). The mean VDR T/N value was 0.45 (median = 0.34; SD = 0.35; range, 0.05–1.49). When converted to the natural logarithm scale to normalize the data, the mean T/N value was −1.11 (median = −1.10; SD = 0.83; range, −2.96–0.40). The only patient characteristic that was related to T/N value was differentiation. Patients with well-differentiated tumors tended to also have high T/N values (P = 0.01; Table 1). There were 13 recurrences observed (ranging from 5 to 29 months postoperation) and 10 deaths without documented recurrence (ranging from 2 to 32 months), for a total of 23 events. The actuarial 2-year EFS rate was 47% (95% confidence interval, 33–68%).

Univariate analyses provided mild evidence that higher T/N values were associated with favorable prognosis. The mean log T/N values among patients who experienced and did not experience an event were −1.33 (SD = 0.87) and −0.88 (SD = 0.74), respectively. This difference between groups approached

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Patient characteristics and their relationship to VDR T/N value</th>
</tr>
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<tbody>
<tr>
<td>Baseline factor</td>
<td>n</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>18</td>
</tr>
<tr>
<td>Female</td>
<td>28</td>
</tr>
<tr>
<td>Dukes' stage</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>19</td>
</tr>
<tr>
<td>C</td>
<td>15</td>
</tr>
<tr>
<td>D</td>
<td>10</td>
</tr>
<tr>
<td>Unknown</td>
<td>2</td>
</tr>
<tr>
<td>Differentiation</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td>3</td>
<td>32</td>
</tr>
<tr>
<td>Age at operation (yr)</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>66.5</td>
</tr>
<tr>
<td>Median</td>
<td>51.0</td>
</tr>
<tr>
<td>SD</td>
<td>13.5</td>
</tr>
<tr>
<td>Range</td>
<td>36–88</td>
</tr>
</tbody>
</table>

* Test of no association between log T/N value and patient characteristic.

b Pearson correlation coefficient: r = −0.05.
Fig. 1 EFS experience among patients with high VDR expression in tumor (T/N > 0.35) compared to low VDR expression (T/N < 0.35). Analyses adjusted for different prognostic factors indicate that VDR expression in colon cancer is associated with a favorable prognosis.

Table 2 Prognostic factors associated with recurrence or death estimated by proportional hazards regression model

<table>
<thead>
<tr>
<th>Prognostic factor</th>
<th>P</th>
<th>Hazard ratio (95% confidence interval)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dukes' stage (D vs. non-D)</td>
<td>&lt;0.001</td>
<td>9.78 (2.58–37.0)</td>
</tr>
<tr>
<td>Age</td>
<td>0.81</td>
<td>1.00 (0.96–1.03)</td>
</tr>
<tr>
<td>T/N value</td>
<td>0.04</td>
<td>0.22 (0.06–0.90)</td>
</tr>
<tr>
<td>Sex (male vs. female)*</td>
<td>0.01</td>
<td>0.05 (0.005–0.47)</td>
</tr>
<tr>
<td>11 days after surgery</td>
<td></td>
<td>0.28 (0.07–1.09)</td>
</tr>
<tr>
<td>1 yr after surgery</td>
<td></td>
<td>1.62 (0.33–8.01)</td>
</tr>
<tr>
<td>2 yr after surgery</td>
<td></td>
<td>1.62 (0.33–8.01)</td>
</tr>
</tbody>
</table>

* Sex was included in the model as a time-dependent covariate with an effect that was continuous in time. The hazard ratio for males vs. females, at \( t \) years after surgery, was estimated to be: \( 0.05 \times (5.70^t) \). The 95% confidence interval for the hazard ratio at time \( t \) was: \( 0.05 \times (5.70^t) \times \exp(\pm 1.96 \sqrt{[1.31 - 1.361 + 0.52/9]}) \).

but did not attain statistical significance (\( P = 0.06 \)). To adjust for time on study, we split patients into two groups according to whether or not their T/N values (on the original scale) exceeded 0.35 and plotted their EFS times (Fig. 1). The difference in EFS times between high and low VDR groups was not significant in Dukes' stage D patients (\( P = 0.15 \)) but approached significance in patients of lesser stage (\( P = 0.08 \)).

Multivariate analyses confirmed that higher T/N values were associated with favorable prognosis. We fit a series of proportional hazards regression models that included T/N as a prognostic factor in addition to Dukes' stage, age at operation, sex, and differentiation. Initially, we fit a regression model in which the factor for Dukes' stage had three levels (C, D, and other) but found that simply dichotomizing the factor (D versus non-D) gave a fit that was just as good. Also, T/N values gave a better fit as a continuous variable than as a dichotomous factor (high versus low). Differentiation score (> 1 versus ≤1) was found to be a nonsignificant factor (\( P = 0.68 \)); moreover, its presence in the regression model only slightly altered the estimates for the other prognostic factors but inflated the SE of the T/N effect. For this reason, differentiation was excluded from the best model fit (Table 2). As shown in Table 2, after adjustment for other baseline factors, T/N was a significant prognostic factor (\( P = 0.04 \)). Each unit increase in T/N (on the original scale) was associated with a 78% reduction (95% confidence interval, 10–94% reduction) in risk of experiencing an event. There was no evidence that the effect of T/N on prognosis differed by Duke's stage (\( P = 0.35 \); not shown in Table 2).

Surprisingly, there was clear evidence that the effect of sex on risk of recurrence/death was not constant but changed over time (test of proportional hazards assumption: \( P = 0.02 \)). In the first few weeks after surgery, males had a much lower risk of experiencing an event than did females of the same age (hazard ratio = 0.05; 95% confidence interval, 0.005–0.47), but the relative risk for males increased by ~5.7-fold each year thereafter. After the first year, the hazard ratio for males versus females grew to 0.28 (95% confidence interval, 0.07–1.09), and after the second year, it increased to 1.62 (95% confidence interval, 0.33–8.01).

Discussion

Previously published data from our laboratory showed a wide range of expression of VDR in a panel of established human colon adenoma and carcinoma cell lines (16). All colon cancer cell lines expressed some VDR, although in the most met-
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astatic and poorly differentiated cell lines, it was quite low. General correlation seems to exist between the level of differentiation and the expression of VDR. Receptor level function was then measured in these cell lines using a radiolabeled ligand binding assay with [3H]1,25-dihydroxyvitamin D3. These results parallel those of the RNase protection assay and confirm functional integrity of the hormone-binding domain of the VDR.

The results of these studies support the hypothesis that the expression of VDR in human colorectal carcinoma, much like the expression of estrogen receptor in human breast carcinoma, is a predictive marker of biological behavior. Identifying clinically useful biological markers becomes extremely important in colorectal cancer in the subset of patients with stage I and stage II disease who are currently not being treated with adjuvant therapy but, despite their prognostically favorable stage of disease, can still recur because they have inherently biologically aggressively tumors (21).

The measurement of VDR in other solid epithelial tumors has been well described (11–15). In 1991, Berger et al. (22), measured VDR in human breast cancers and found that patients who had immunocytochemically detectable VDR had a longer disease-free interval than did those patients with negative VDR. The technique used here of RNase protection assay to assess expression has some drawbacks, in that the heterogeneity of the tumor is poorly detected and excessive stromal tissue may give somewhat misleading results. Despite these limits, however, RNase protection assays are a very sensitive method when tissue samples are isolated to the epithelium, as was performed here. Because VDR is ubiquitously expressed, not only in epithelial cells but also in lymphocytes and stromal cells, the interpretation of VDR immunohistochemistry can become rather difficult in terms of quantitative analysis (23).

Other potential markers of biological behavior currently used on a research basis in colorectal cancer include ploidy and the presence or absence of the ddc gene (18q deletion) and p53 (17p deletion; Refs. 2–5). None of these markers, however, attempt to link any of the epidemiological or dietary risk factors for colorectal cancer to the underlying molecular biology of the disease. VDR may be a useful marker in patients with early-stage disease. Very low VDR expression in early-stage disease may suggest a biologically aggressive tumor with a high risk for recurrence, independent of stage. A similar correlation can be drawn in breast cancer, in early-stage disease, in which the biological prognostic factors, such as estrogen receptor and progesterone receptor, have very low expression, putting the patient at very high risk for recurrence of the disease, independent of the stage of the disease. Although VDR receptor certainly functionally works in a different fashion than estrogen receptor, the ability to biologically define the aggressive nature of a tumor has not been clearly demonstrated in colorectal cancer. VDR expression may be one of the first such prognostic markers to allow this definition to be carried out.

Vitamin D and several of its analogues have been used in chemoprevention animal models to inhibit the growth of both colorectal and breast cancer (24, 25). Although vitamin D can work through both nongenomic and genomic pathways, the majority of its antiproliferative effects appear to be directly linked to its binding to VDR. Therefore, assessing VDR expression in colorectal cancer may help predict therapeutic response to potentially potent vitamin D analogues, just as estrogen receptor expression predicts response to tamoxifen in breast cancer. There is currently no hormonally available treatment for colorectal cancer. As is well known, hormonal treatments currently exist for prostate and breast cancer, and in fact, vitamin D and, more specifically, some highly potent vitamin D analogues are currently being used in Phase I and Phase II trials in Europe in breast cancer. In addition, there are plans to begin Phase I and Phase II trials using potent vitamin D analogues in prostate cancer. Although many of the vitamin D analogues are quite different in their mechanism, as compared to tamoxifen, they all share a common ability to bind with specificity to their parent hormone receptor and to down-regulate tumor growth in colon and breast, respectively.

Vitamin D and its synthetic analogues may have an important role as adjuvant treatment in colon cancer patients with VDR-positive tumors in prevention and/or treatment of patients with stage III or stage IV disease, with lymph node involvement or metastatic disease to solid organs. Additional studies will be necessary to assess VDR expression in metastatic colorectal cancer to lymph nodes and in metastatic deposits in solid organs such as the liver. This may prove to be a very promising area for future investigation.

The results of the above studies reinforce the potential role that VDR may play in the development of the pathogenesis of colorectal cancer. Larger studies are now necessary to confirm and substantiate the above results. This will hopefully lead to the development of a sensitive hormonal marker that can be used to predict the biological behavior of colorectal cancer, identifying at-risk patients in need of adjuvant treatment.

References


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