Wnt-5a Protein Expression in Primary Dukes B Colon Cancers Identifies a Subgroup of Patients with Good Prognosis

Janna Dejmek,1 Annika Dejmek,2 Annette Säfholm,1 Anita Sjölander,1 and Tommy Andersson1

1Experimental Pathology and 2Pathology, Department of Laboratory Medicine, Lund University, Malmö University Hospital, Malmö, Sweden

Abstract

Oncogenic Wnt/β-catenin signaling occurs in a majority of colorectal cancers. In contrast, very little is known about the role of the nontransforming Wnt protein family member Wnt-5a in those tumors. In the most common of the three colon cancer stages, Dukes B or lymph node–negative, the outcome is the hardest to predict. We searched for a predictive marker in this group and observed loss of or reduced Wnt-5a expression in 50% of Dukes B tumors. Such Wnt-5a negativity was a strong predictor of adverse outcome, with a relative risk of death of 3.007 (95% confidence interval, 1.336–6.769; P = 0.008) after 5 years in Wnt-5a-negative patients. Furthermore, the median survival time after diagnosis was 109.1 months for patients with Wnt-5a-positive primary tumors but only 58 months for those with Wnt-5a-negative primary tumors. To find a possible biological explanation for these results, we studied the invasive and poorly differentiated human colon cancer cell line, SW480, which does not express Wnt-5a protein and the Wnt-5a-expressing and moderately differentiated Caco2 colon cancer cell line. We found that the addition of recombinant/purified Wnt-5a significantly reduced the migratory capacity of SW480 cells. By comparison, equivalent treatment did not significantly alter migration in the Wnt-5a-expressing Caco2 colon cancer cell line. These findings indicate that the expression of Wnt-5a in primary Dukes B colon cancer tissue constitutes a good prognostic marker for longer survival, which can be explained by the ability of Wnt-5a to impair tumor cell migration and thus reduce invasiveness and metastasis. (Cancer Res 2005; 65(20): 9142-6)

Introduction

Wnt-5a is a nontransforming member of the Wnt family of secreted and cysteine-rich proteins that exert their cellular effects via autocrine or paracrine routes. Wnts influence multiple processes in development and have also been implicated in carcinogenesis (1, 2). Wnt proteins relate to cell surfaces and extracellular matrix and bind to members of the Frizzled family of G protein–coupled receptors (3, 4). Wnt signaling is complex and can display several distinct characteristics, depending on the Wnt and the cell type (4–6).

A major problem encountered with most forms of cancer is the capacity to metastasize. This process requires changes in the cell adhesion, migration, and proliferation that occur due to reciprocal and dynamic interactions between adhesion molecules, extracellular matrix proteins, and soluble factors (7). We have shown that the protein Wnt-5a plays a role in preventing metastatic disease in invasive breast carcinomas (8), presumably through its ability to increase the adhesive capacity of the epithelial cells (9). In agreement with that finding, Wnt-5a has also been reported to augment the adhesive capacity of malignant melanoma cells, but in those cells, it leads to elevated spread of the tumor cells (10). In this context, it is important to realize that breast epithelial cells are normally stationary and adhere strongly to both the basement membrane and each other, whereas melanocytes are single, motile cells that show dynamic adhesion to the interstitial stroma. Breast epithelial cells that undergo tumor transformation become increasingly motile, and that change requires reduced adhesion, such as that seen in those cells when they have lost the ability to express Wnt-5a (9). By comparison, the elevated motility observed in melanoma cells is compatible with amplified adhesion, such as that which occurs when Wnt-5a expression is augmented (10).

In colorectal carcinomas, inactivation of the adenomatous polyposis coli (APC) gene is present in >80% of the cases. The APC gene product is part of a protein complex that targets β-catenin for degradation, counteracting the nuclear accumulation of β-catenin and subsequent activation of Tcf/Lef transcription factors (11). Thus, inactivation of the APC gene is similar to the effect of constant activation of the canonical Wnt signaling cascade. Furthermore, Smith et al. observed that Wnt-5a mRNA was expressed to the same extent in both normal colorectal tissue and colorectal carcinomas (human samples), but not in colorectal cancer cell lines (12). Any studies of Wnt-5a expression in correlation to APC mutations in colorectal carcinoma or adenoma tissue have, to our knowledge, never been done. However, other investigators used in situ hybridization and found strong expression of Wnt-5a mRNA in normal mucosa and in colorectal cancer cell lines (13). It might, however, well be that this discrepancy does not reflect the expression of Wnt-5a protein in these cells and tissues. In support of that suggestion, we recently noted that breast carcinomas lacking Wnt-5a protein expression still contained detectable, normal, or elevated mRNA levels, indicating that expression of this protein is, at least in breast epithelial cells, regulated at the translational level (14).

The aim of the current study was to assess a possible role for Wnt-5a as a predictor of prognosis in the most common stage of colon cancer, Dukes B, which is locally invasive but lymph node–negative. The incentive for this work was that patients diagnosed with Dukes B colon tumors have the most variable prognosis, and, even more importantly, they are also the group that would benefit most from the discovery of a prognostic factor that can identify individuals for whom adjuvant treatment would be most advantageous (15).
Materials and Methods

**Tumor samples.** Samples were collected from primary tumors from 55 consecutive patients with Dukes B colon cancer who underwent curative surgery in 1990 at Malmö University Hospital. None of the patients had received radiation treatment or chemotherapy before surgery. All tissue samples were fixed in formalin, embedded in paraffin, and used for routine morphologic examination (grading and immunostaining) and construction of tumor tissue arrays, as previously described (14).

**Cell culture.** The human colon cancer cell lines SW480 and Caco2 (American Type Culture Collection, Rockville, MD) were grown as suggested by the supplier. These cell lines were selected based on their degree of differentiation and invasiveness. SW480 is a moderately differentiated, highly tumorigenic, invasive colon carcinoma cell line derived from the primary tumor of a patient with Dukes B adenocarcinoma (16). Caco2 is an adherent cell line derived from a moderately well-differentiated primary colon adenocarcinoma that is less tumorigenic and less invasive (16).

**Antibodies.** A polyclonal rabbit IgG raised against Wnt-5a (amino acids 275-290) that exhibited only 65% homology with Wnt-5b in the corresponding sequence was developed in our laboratory (9). The biotinylated anti-rabbit IgG used as secondary antibody in immunohistochemistry was included in a DAKO ChemMate kit purchased from BioTek Solutions (Winooski, VT). The monoclonal mouse anti-actin C4 antibody was from MP Biomedicals (Irvine, CA).

**Western blotting and immunohistochemistry.** Cell lysis and preparation and analysis of the samples were carried out as previously described (14). Immunohistochemistry was done using a DAKO ChemMate kit purchased from BioTek Solutions (Winooski, VT). The monoclonal mouse anti-actin C4 antibody was from MP Biomedicals (Irvine, CA).

**Statistical analysis.** SPSS 10.1 statistical software was used for all calculations. All stained samples were divided into two subgroups (designated +/+ and +/-) based on expression of Wnt-5a protein, and the subgroups were subsequently compared with regard to survival and different clinicopathologic variables. \( \chi^2 \) and Student's \( t \) tests were done to evaluate statistical differences between the subgroups. All tests were two-tailed and \( P < 0.05 \) was considered to be statistically significant. Death was used as an end point, univariate analysis of survival was computed by the Kaplan-Meier method, and log rank testing was applied to determine the relative risk [with a 95% confidence interval (CI)] of death from colon cancer in the Wnt-5a+/− subgroup as compared with the Wnt-5a++/+ group. A Cox's regression model was used for multivariate analysis of survival.

**Migration assay.** Cell migration was assessed in a modified transwell Boyden chamber (Costar, Cambridge, MA), in which the two chambers were separated by a polycarbonate membrane (pore diameter, 8.0 \( \mu \)m). Caco2 or SW480 cells were grown to subconfluence in tissue culture plates and then detached by treatment with Versene, after which they were centrifuged and resuspended as single cells in serum-free culture medium supplemented with 0.5% bovine serum albumin, with or without the addition of 0.4 or 0.8 \( \mu \)g/mL recombinant Wnt-5a as indicated. The cell suspensions were added to wells with a membrane placed in the bottom. Medium that was or was not supplemented with 1 ng/mL of insulin-like growth factor (IGF) was added to the lower compartment of the Boyden chamber, IGF was chosen as a chemoattractant because both cell lines we used express functional IGF receptors. The cells were allowed to migrate for 18 hours at 37°C in this assay. Thereafter, the medium was discarded, nonmigrated cells removed with cotton-tipped applicator and the membranes were cut out of the chamber and then stained with 0.5% crystal violet. The response was evaluated in a light microscope by counting the number of cells that had migrated into the membrane.

**Figure 1.** A, immunostaining of Wnt-5a protein in normal colon tissue and representative sections of Dukes B colon carcinomas. Staining was done with a polyclonal rabbit antibody against human Wnt-5a (diluted 1:200). Magnification, \( \times 400 \). Kaplan-Meier analysis of overall 5-year (A) and 10-year (B) survival of patients diagnosed with Dukes B colon cancer stratified by expression of Wnt-5a protein.
Wound-healing assay. SW480 cells were grown to confluence on coverslips, after which a wound was inflicted in the culture with the tip of a sterile pipette, and cell growth was allowed to continue in the absence or presence of 0.4 μg/ml recombinant Wnt-5a for 18 hours. The width of the wound was estimated under a phase contrast microscope at the start and the end of each experiment. Thereafter, the cells were fixed in 4% paraformaldehyde for 10 minutes, permeabilized with 0.3% Triton X-100 for 5 minutes, and then blocked in 3% BSA-0.3% Triton X-100 for 45 minutes. Next, the cells were incubated with 1:500 AlexaFluor 488 phalloidin (Molecular Probes, Eugene, OR) in 1% BSA and subsequently washed several times, and finally mounted. The experiments were repeated five times, and triplicate samples were used in each experiment. The samples were examined and photographed in a Nikon Eclipse 800 microscope.

Results and Discussion

The present immunohistochemistry data reveal that Wnt-5a protein is expressed in normal colon epithelium (Fig. 1A). The highest level of expression was clearly seen at the base of the crypts, which agrees well with previous results showing that the peak in Wnt-5a mRNA also occurs at this site (13). We observed that, compared with normal colon epithelium, roughly 50% of the Dukes B colon cancers exhibited reduced expression of Wnt-5a (Fig. 1A; Table 1), and, interestingly, this is similar to the situation seen in invasive ductal breast carcinomas (8). Considering these results, it should be noted that, in the context of treatment, Dukes A and C colon cancers constitute a more homogeneous group, whereas this is not as apparent for corresponding Dukes B cancers. Consequently, we decided to compare Wnt-5a staining with survival in patients with Dukes B colon cancer. We found that loss of Wnt-5a expression in the tumors we studied was associated with a significantly shorter overall survival after 5 and 10 years (Fig. 1B and C). The mean total survival time measured from the date of the primary surgery for the patients with Dukes B tumors was 109.1 months (95% CI, 86.9-131.2) in the Wnt-5a-positive group, but only 58 months (95% CI, 37.5-78.6) in the Wnt-5a-negative group. The relative risk of death in the patients with Wnt-5a-negative tumors was 3.007 (95% CI, 1.336-6.769; \( P = 0.008 \)) and 2.534 (95% CI, 1.285-4.999; \( P = 0.007 \)) after 5 and 10 years, respectively. The patients with Wnt-5a-positive and -negative Dukes B colon cancer did not differ in other aspects that might have influenced prognosis, such as age, tumor location, histologic tumor type, or grade of tumor differentiation (Table 1). Nevertheless, we did a multivariate survival analysis to exclude the possibility of a confounder causing the difference in survival between the two Wnt-5a groups. When adjusted for the factors displayed in Table 1, we found Wnt-5a-negativity to be an independent risk factor with a relative risk of death of 2.331 (95% CI, 1.133-4.796; \( P = 0.021 \)).

Table 1. Clinicopathologic features in relation to Wnt-5a immunoreactivity in Dukes B colon tumors

<table>
<thead>
<tr>
<th>Variable</th>
<th>n</th>
<th>Wnt-5a+++/++</th>
<th>Wnt-5a+/-</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>55</td>
<td>72.7</td>
<td>74.3</td>
<td>0.762</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>27</td>
<td>12</td>
<td>15</td>
<td>0.180</td>
</tr>
<tr>
<td>Female</td>
<td>28</td>
<td>18</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Tumor localization</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Right colon</td>
<td>31</td>
<td>19</td>
<td>12</td>
<td>0.286</td>
</tr>
<tr>
<td>Left colon</td>
<td>24</td>
<td>13</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>Histologic type</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonmucinous</td>
<td>42</td>
<td>24</td>
<td>18</td>
<td>0.537</td>
</tr>
<tr>
<td>Mucinous</td>
<td>13</td>
<td>6</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Differentiation grade</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>10</td>
<td>3</td>
<td>7</td>
<td>0.216</td>
</tr>
<tr>
<td>Moderate</td>
<td>42</td>
<td>25</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Tumor, node, and metastasis classification</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \text{T3N0M0} )</td>
<td>10</td>
<td>6</td>
<td>4</td>
<td>0.457</td>
</tr>
<tr>
<td>( \text{T3N4M0} )</td>
<td>41</td>
<td>23</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>( \text{T4N0M0} )</td>
<td>4</td>
<td>1</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>

To identify the mechanism underlying our clinical findings, we studied the two human colon cancer cell lines, Caco2 and SW480. We found that the moderately differentiated, less tumorigenic Caco2 cells expressed significant levels of Wnt-5a protein (Fig. 2A), whereas Wnt-5a was barely detectable in the poorly differentiated, highly tumorigenic, and metastatic SW480 cells (Fig. 2A; ref. 16). The inactivating APC mutation in SW480 cells, equivalent to constant canonical Wnt signaling, and the concurrent absence of Wnt-5a protein expression in SW480 cells, suggests that there might be some sort of antagonism between Wnt-5a and canonical Wnt signaling. It has been reported that Wnt-5a increases the adhesion of breast cancer cells to collagen-coated surfaces, which implies that the presence of this protein would also impair tumor cell motility (9, 17). Accordingly, we wanted to test the hypothesis that the presence of Wnt-5a can prolong survival of patients with Dukes B colon cancer by impairing the ability of the tumor cells to migrate and thereby decreasing their capacity for invasion and metastasis. In support of that hypothesis, we found that exposure to exogenous Wnt-5a significantly decreased migration of the non-Wnt-5a-expressing SW480 cells (Fig. 2B) but had no effect on migration of the Wnt-5a-expressing Caco2 cells (Fig. 2B). Furthermore, we observed that in the wound-healing assay, the addition of exogenous Wnt-5a to a wounded SW480 cell monolayer caused a statistically significant reduction in the ability of the cells to heal, indicating that Wnt-5a decreased the motility of the cells (Fig. 2C, left). The wound in untreated cells was almost completely closed after 18 hours, whereas essentially no migration had occurred in the Wnt-5a-treated cells (Fig. 2C, right). Notably, staining to determine the content of filamentous actin in the cells clearly showed that the added Wnt-5a had altered the balance between globular and filamentous actin in favor of the latter form (Fig. 2C, right). Such modulation of the actin network could increase the stiffness of the cells (18), which might at least partly explain why Wnt-5a reduces the motility of colon cancer cells.

Traditionally, Wnt-1 signals via the β-catenin pathway also referred to as the canonical pathway (4–6). Wnt-5a, on the other hand, predominantly signals via noncanonical signaling pathways (4–6). Interestingly enough, a recent study has indicated that Wnt-5a can inhibit the canonical Wnt/β-catenin pathway by promoting the degradation of β-catenin (19). Because a majority of colon carcinomas exhibit activation of the canonical Wnt pathway due to APC mutations, this clearly is not determining the patients’ prognosis. However, it is possible that Wnt-5a counteracts the transforming effects of canonical Wnt/β-catenin signaling. Indeed, transfection of cells with
antisense Wnt-5a causes cell transformation equal to that induced by activation of the Wnt/\beta\)-catenin pathway (20). Consequently, we cannot exclude that loss of Wnt-5a, in addition to its effect on cell migration, might indirectly increase the canonical Wnt signal. Such an effect would adversely affect the prognosis of the patient.

Taken together, our results support the notion that expression of Wnt-5a protein is a strong predictor of good prognosis in Dukes B/lymph node–negative colon cancer. To obtain evidence sustaining that concept, and to explain our clinical results, we also conducted experiments to determine how Wnt-5a affects the aggressiveness of the tumor cells. We found that Wnt-5a impaired the ability of human colon cancer cells to migrate, a function that is necessary for tumor invasion and metastasis. Our findings also suggest that activation of the Wnt-5a signaling pathway might be a useful means of adjuvant treatment of patients with Dukes B colon cancer.

Acknowledgments

Received 5/18/2005; revised 7/25/2005; accepted 8/26/2005.

Grant support: Swedish Cancer Foundation (T. Andersson), the U-MAS Research Foundations (T. Andersson and J. Dejmek), Apotekare Hedberg Foundation (T. Andersson), Gunnar Nilsson’s Cancer Foundation (T. Andersson), the Royal Physiographic Society in Lund (J. Dejmek), and the SverigeConsortium for their support of a local tumor tissue array center at Malmö University Hospital. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The authors are grateful to E. Nilsson for expert technical assistance with immunochemistry staining and P. Odnan for linguistic revision of the manuscript.

References

Wnt-5a Protein Expression in Primary Dukes B Colon Cancers Identifies a Subgroup of Patients with Good Prognosis

Janna Dejmek, Annika Dejmek, Annette Säfholm, et al.


Updated version  Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/65/20/9142

Cited articles  This article cites 16 articles, 4 of which you can access for free at:
http://cancerres.aacrjournals.org/content/65/20/9142.full#ref-list-1

Citing articles  This article has been cited by 19 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/65/20/9142.full#related-urls

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.