Tumor Cell-associated Hyaluronan as an Unfavorable Prognostic Factor in Colorectal Cancer

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ABSTRACT

Hyaluronan (HA) is a linear high molecular weight extracellular polysaccharide. It is thought to be involved in mitosis and the enhancement of wound healing, tumor invasion, and metastasis. Because its clinical relevance in cancer has not been explored, we scored HA in colorectal adenocarcinoma and studied its relationship with patient survival. A specific probe prepared from cartilage proteoglycan aggregates was used to stain paraffin-embedded tumor samples from 202 colorectal adenocarcinoma patients treated in Kuopio University Hospital and followed up for a mean of 14 years. The hypothesis that the percentage of HA-positive carcinoma cells (HA%) and HA intensity in cancer cells correlated with survival was tested with the log-rank test, hazard ratios, and their confidence intervals. Ninety-three % of tumors had at least a proportion of carcinoma cells positive for HA. HA intensity in tumor epithelium was stronger in Dukes' stages C and D tumors and in high-grade tumors. The cancer-related survival rate was lower among patients with strong HA intensity in tumor epithelium (P < 0.001) and high HA% (P < 0.001). Recurrence-free survival was also shorter in patients with an intense signal for HA (P = 0.001) and high HA% in tumor epithelium (P = 0.04). HA intensity in tumor epithelium independently predicted survival and recurrence-free survival (Cox's analysis). We conclude that a high proportion of HA-positive cancer cells and high intensity of the HA-signal predicts a poor survival rate. The abnormal expression of HA in the neoplastic colon epithelial cells is suggested to provide a distinct advantage for invasive growth and metastasis.

INTRODUCTION

HA (also known as hyaluronic acid and hyaluronate) is an unbranched extracellular and cell surface polysaccharide expressed in connective, neural, and epithelial tissues (1-3). Elevated concentrations of HA are associated with tissue remodeling and rapid cell proliferation, e.g., in embryonic tissues and healing wounds (4). Some tumors contain more HA than the corresponding normal tissue (5). Elevated levels of HA are most evident in human mesotheliomas (6) and Wilm's tumor (7) but occur in more common malignancies such as breast cancer (8). HA forms a matrix and presumably provides a favorable environment for mitotic cell rounding and cell movements by allowing low-affinity adhesion and specific signals for cell migration (9). The increased HA expression correlates to invasiveness of carcinoma cells in vitro (10). Cells bind to HA through cell-surface receptor proteins, of which two have been cloned and characterized (11, 12). Interestingly, CD44, the most abundant of the receptors, is up-regulated in colon cancers; in particular, one of its high molecular weight variants is related to poor prognosis (13, 14).

Although there has been considerable interest in the possible role of CD44 in malignant growth, few studies exist on the presence of HA, its major ligand, on tumor cells. To the best of our knowledge, no studies have related the expression of HA in carcinoma cells to prognosis. This prompted us to study colorectal cancer, which is a common malignant tumor in the Western world and the second leading cause of cancer mortality in the United States (15). A series of 202 colorectal cancer patients followed-up for 14 years on the average were evaluated for the expression of HA and its independent prognostic value. Stronger than expected association between HA and patient survival can give us important additional prognostic information for cancer treatment decisions, e.g., in selecting patient groups for more intensive therapies, and provides new aspects for the pathogenesis of the spread of cancer.

PATIENTS AND METHODS

Patients. The present study consists of 202 patients treated for colorectal adenocarcinoma between 1976 and 1986 and subsequently followed-up for a mean of 14 years (Table 1). These patients were selected from the original cohort of 308 patients, from which 106 patients were excluded because adequate histological material for HA staining was not available. The samples contained either a rather small number of tumor tissues, or there was no tumor left in the section. The clinical staging of all tumors was completed according to UICC (16) and modified Dukes' classification by Turnbull et al. (17). Staging was based on the results of abdominal ultrasonography, bone and chest radiographs, bone scans, computed tomography, colography, endoscopy (recto-sigmoidoscopy and colonoscopy), and laboratory tests suggesting possible metastasis. All patients underwent operations. Of these, 24 were treated with chemotherapy and 19 with radiotherapy (9 were treated by both chemotherapy and radiotherapy and 4 patients received adjuvant therapy). The follow-up was done according to a standard practice of our clinic, usually by the same team of gastroenterologists. The pertinent data of the patients are summarized in Table 1.

Tumor Samples. The tumor samples obtained at time of operation were fixed in 10% buffered formalin (pH 7.0), embedded in paraffin, and sectioned at 5 μm. All histological slides were examined by two observers unaware of the clinical data or the disease outcome. Tumors were graded as well, moderate, or poorly differentiated (WHO grade). The histopathological data of patients are shown in Table 1.

Preparation of the Probe and Staining of HA. bHABC, used as a HA-specific probe, was prepared from bovine articular cartilage as described (18) and contained the biotinylated HA-binding G1 fragment of aggrecan and link protein, which stabilizes the ternary complex. PAGE of the probe showed only bands corresponding to the HA-binding region of aggrecan and link protein.

The sections were incubated with 0.3% H2O2 in 30% methanol for 10 min to block tissue peroxidases, washed with 0.1 M sodium phosphate buffer (pH 7.4), and incubated in 1% (w/v) BSA in the phosphate buffer for 30 min to block nonspecific binding. The sections were incubated with the bHABC (5 μg/ml, diluted in 1% BSA) overnight at 4°C, washed thoroughly with the buffer, and treated with avidin-biotin-peroxidase (Vector Laboratories, Irvine, CA: 1:200 dilution) for 1 h at room temperature. The sections were washed with the buffer and incubated in 0.05% 3,3'-diaminobenzidine (Sigma Chemical Co., St. Louis, MO) and 0.03% H2O2 in the phosphate buffer at room temperature. After washes, the sections were counterstained with Mayer's hematoxylin and mounted in Depex.

Table 1.

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The abbreviations used are: HA, hyaluronan; bHABC, biotinylated hyaluronan binding region of aggrecan and link protein complex; TIL, tumor-infiltrating lymphocytes; HPF, high-power field; RFS, recurrence-free survival.
Staining specificity was controlled by predigesting sections with Streptomyces hyaluronidase [100 turbidity-reducing units/ml 0.1 m sodium acetate buffer (pH 5) for 3 h at 37°C] in the presence of protease inhibitors (19). The digestion experiment also included controls incubated under otherwise similar conditions but lacking the enzyme. Other control sections were stained using a probe preincubated with HA-oligosaccharides (with a length of 11–12 disaccharide units, 3 µg oligosaccharide per 1 µg probe) to block the HA-binding site and reveal possible nonspecific attachment to the sections (20). No staining was observed in the hyaluronidase or oligosaccharide controls.

### Analysis of HA Staining

In tumor epithelium, the signal intensity of HA was categorized into four grades: 0 (HA absent); 1 (weak); 2 (moderate); and 3 (strong). In stroma, three grades were used for the intensity of HA staining: 1 (weak); 2 (moderate); and 3 (strong). The percentage of tumor cells positive for HA (HA%) in tumor epithelium was categorized as seen in Fig. 1: 0—25%; (weak); 2 (moderate); and 3 (strong). In stroma, three grades were used for the intensity of HA signal: 1 (weak); 2 (moderate); and 3 (strong). The large invasive Dukes’ C and D tumors had a higher mean HA intensity value compared to Dukes’ 0, A, and B tumors (Table 2). HA% was also higher in Dukes’ C and D tumors (P = 0.001; \( \chi^2 \), 10.6). A highly significant association existed between Dukes’ classification and HA intensity in tumor epithelium, and there was also positive correlation between Dukes’ classification and HA% in tumor epithelium. There was an inverse relationship between the number of TILs and HA intensity in tumor epithelium: a moderate or strong HA signal was present in TIL grades 0 and 1 (P = 0.002; \( \chi^2 \), 9.3).

### Survival

### Univariate Analysis

There was a significant association of HA intensity and HA% in tumor epithelium with cancer-related survival in 187 patients available for analysis. Patients with strong HA intensity in tumor epithelium had a low survival rate (15% survived 14 years; Fig. 2A; Table 3). Patients with low HA% (0—25%) survived longer (60% survived 14 years) than patients with high HA% (75–100%; 30% survived 12 years; Fig. 2B; Table 3).

### Expression of HA in Cancers

Normal intestinal epithelium adjacent to tumors was devoid of HA, whereas normal stroma was positive. Seven % of tumors were completely negative for epithelial HA, but the rest showed variable signal intensity and percentage of positive cells. The HA in tumor epithelium was detected pericellularly and/or in the cytoplasm of carcinoma cells (Fig. 1).

### HA Related to Other Prognostic Factors

In general, high-grade tumors had moderate or strong HA intensity in tumor epithelium, and there was a significant but not very strong association between HA intensity in tumor epithelium and tumor stage (Table 2). The large invasive Dukes’ C and D tumors had a higher mean HA intensity value compared to Dukes’ 0, A, and B tumors (Table 2). HA% was also higher in Dukes’ C and D tumors (P = 0.001; \( \chi^2 \), 10.6). A highly significant association existed between Dukes’ classification and HA intensity in tumor epithelium, and there was also positive correlation between Dukes’ classification and HA% in tumor epithelium. There was an inverse relationship between the number of TILs and HA intensity in tumor epithelium: a moderate or strong HA signal was present in TIL grades 0 and 1 (P = 0.002; \( \chi^2 \), 9.3).
epithelium also predicted survival ($P = 0.04$; relative risk, 1.68; confidence interval, 1.02–2.78).

In patients with no distant metastases diagnosed at the time of operation ($T_1-4N_0-3M_0$, $n = 141$), N category, sex (longer RFS in males), TILs, and HA intensity in tumor epithelium independently predicted RFS, whereas in patients without spread to local lymph nodes ($T_1-3N_0M_0$, $n = 104$), only HA intensity in tumor epithelium independently predicted RFS (Table 4). Accordingly, in Dukes’ B tumors, HA intensity in tumor epithelium was the only independent predictor of RFS ($P < 0.001$; relative risk, 2.10; confidence interval, 1.36–3.24).

To address the roles of HA, we analyzed the patients with distant metastases both in univariate and multivariate survival statistics. In the univariate analyses, there were no statistically significant correlations between patient survival and HA intensity in stroma or in tumor epithelium and HA% in tumor epithelium. These factors were not significant independent predictors of survival in the multivariate analyses either.

**DISCUSSION**

Cancer cells are dependent on their interactions with the extracellular matrix in every phase of their growth. Matrix molecules like decorin have direct influences on cell cycle (23), certain types of matrix receptors are enriched on cancer cells (24), and proteinases modulating cell-matrix interactions are intimately involved in malignant growth (25, 26).

HA is an ubiquitous matrix molecule, present in variable concentrations in all connective tissues and many epithelia (1, 3). We found recently that normal colon epithelium and some well-differentiated adenocarcinoma cells are negative for HA, but some colon cancer

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**Table 2** Association between HA intensity in tumor epithelium and the staging of tumors according to grade and Dukes' classification

<table>
<thead>
<tr>
<th>HA intensity</th>
<th>Grade</th>
<th></th>
<th></th>
<th>Dukes</th>
<th></th>
<th></th>
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<td></td>
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<td>2</td>
<td>3</td>
<td>0</td>
<td>A</td>
<td>B</td>
<td>C</td>
<td>D</td>
</tr>
<tr>
<td>0</td>
<td>1</td>
<td>4 (7)</td>
<td>0</td>
<td>1 (33)</td>
<td>1</td>
<td>3</td>
<td>3</td>
<td>33</td>
<td>51</td>
</tr>
<tr>
<td>1</td>
<td>25 (44)</td>
<td>49 (41)</td>
<td>1 (4)</td>
<td>0</td>
<td>21 (64)</td>
<td>35 (43)</td>
<td>9 (27)</td>
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</tr>
<tr>
<td>2</td>
<td>19 (33)</td>
<td>40 (34)</td>
<td>14 (54)</td>
<td>1</td>
<td>33 (15)</td>
<td>31 (38)</td>
<td>18 (55)</td>
<td>18 (35)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>9 (16)</td>
<td>22 (18)</td>
<td>9 (34)</td>
<td>1</td>
<td>33 (2)</td>
<td>2 (6)</td>
<td>10 (12)</td>
<td>6 (18)</td>
<td>21 (41)</td>
</tr>
<tr>
<td>Total</td>
<td>57</td>
<td>119</td>
<td>26</td>
<td>3</td>
<td>33</td>
<td>82</td>
<td>33</td>
<td>51</td>
<td></td>
</tr>
</tbody>
</table>

* Mantel-Haenszel test for linear association.

* $X^2$, 5.7; $P = 0.02$

* $X^2$, 29.1; $P < 0.001$. 

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**Fig. 1.** Affinity histochemical staining for HA in human colon carcinoma. Sections from paraffin-embedded tumor biopsies were incubated with the bHABC probe, visualized with the avidin-peroxidase technique, and counterstained with hematoxylin as described in "Patients and Methods." A, a sample with 0–25%; B, 25–50%; C, 50–75%; and D, >75% of tumor cells HA positive, respectively. Note the intracellular (+, B) and pericellular (arrow, C) localization of HA in tumor cells. Stroma (S; A) is positive in all cases. Bar, 40 μm.
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malignant tumor. In colon adenocarcinoma, HA is associated with cell surface receptors, such as CD44. CD44 is also present on a few crypt epithelial cells in normal colorectal mucosa (29), but its expression is markedly increased in neoplastic carcinoma cells and their precursor adenomas (29). Furthermore, CD44, the variant with exon v6 in particular, is associated with a poor survival rate in colon cancer (13, 14). It is thus possible that CD44 contributes to the surface presentation of HA on colon adenocarcinoma. The intracellular signal of HA in the colon adenocarcinoma cells may also be related to CD44, which has been shown to mediate uptake of HA in other cell types (30–32).

Nevertheless, cell surface HA may remain bound to HA synthase (33, 34), implying that CD44 is not required for the expression of HA on the cell surface. The presence of CD44 does not exclude activation of HA synthase either, and further work is under way to distinguish the contribution of endogenous HA synthesis in these colon carcinomas. In advanced malignancies, the elevated levels of HA may also result from blockage of lymphatic drainage, the physiological route of clearance of tissue HA.

How Could HA Enhance Cancer Growth and Spreading? The present and previous studies show that tumor stroma is invariably enriched in HA as compared with the supporting connective tissue of normal parenchyma. Some malignant cells secrete or present membrane-bound activities stimulating HA synthesis in adjacent fibroblasts (35). Stimulated HA synthesis by cancer cells or stromal fibroblasts may force gaps through connective tissue, creating space for the invading cancer cells (10). Increased concentrations of HA stimulate cancer cell motility (11). Another mechanism of HA involvement is angiogenesis, which is vital to tumor growth and strongly stimulated

Table 4 Independent predictors of survival and recurrence-free survival in Cox’s analysis

<table>
<thead>
<tr>
<th>Survival</th>
<th>All cases (n = 187)</th>
<th>Dukes</th>
<th>HA intensity in tumor epithelium</th>
<th>Sex</th>
<th>TILs</th>
<th>HA intensity in tumor epithelium</th>
<th>HA% in tumor epithelium</th>
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<tr>
<td>β (SE)</td>
<td>Hazard rate (95% CI)</td>
<td>0.738 (0.109)</td>
<td>0.001</td>
<td>2.1 (1.69—2.59)</td>
<td>0.419 (0.208)</td>
<td>0.04</td>
<td>1.52 (1.01—2.86)</td>
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</table>

Table 3 HA intensity in stroma and tumor epithelium and HA% in tumor epithelium related to recurrence (M0 cases) and survival (all cases)

<table>
<thead>
<tr>
<th>RFS at 14 years</th>
<th>No. of patients</th>
<th>%</th>
<th>χ²</th>
<th>p</th>
<th>No. of patients</th>
<th>%</th>
<th>χ²</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA intensity in stroma</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>1</td>
<td>21</td>
<td>60</td>
<td>2.7</td>
<td>0.26</td>
<td>24</td>
<td>55</td>
<td>4.9</td>
<td>0.08</td>
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<tr>
<td>2</td>
<td>63</td>
<td>55</td>
<td>0.001</td>
<td>0.96</td>
<td>83</td>
<td>45</td>
<td>1.86</td>
<td>0.17</td>
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<tr>
<td>3</td>
<td>57</td>
<td>45</td>
<td>0.75</td>
<td>0.38</td>
<td>80</td>
<td>35</td>
<td>2.16</td>
<td>0.14</td>
</tr>
<tr>
<td>HA intensity in tumor epithelium</td>
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<td>16.0</td>
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<td>55</td>
<td>27.1</td>
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<tr>
<td>1</td>
<td>62</td>
<td>75</td>
<td>0.001</td>
<td>0.97</td>
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<td>2.15</td>
<td>0.14</td>
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<tr>
<td>2</td>
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<td>40</td>
<td>0.001</td>
<td>0.97</td>
<td>67</td>
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<td>0.14</td>
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<tr>
<td>3</td>
<td>16</td>
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<td>0.001</td>
<td>0.97</td>
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<td>15</td>
<td>2.15</td>
<td>0.14</td>
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<tr>
<td>HA % in tumor epithelium</td>
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<td>70</td>
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<td>0.97</td>
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<td>75–100</td>
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<td>15</td>
<td>30</td>
<td>2.15</td>
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</table>

* Log-rank analysis.
by low molecular weight HA fragments (36). Some tumors, including human colon carcinoma (37), express hyaluronidase activity, which may generate these oligosaccharides (38). Thus, both synthesis and degradation of HA presumably support tumor invasion and growth.

Cancer cells invested in a coat of HA may be protected from the attack of cytotoxic cells (39). In the present study, a highly significant inverse relationship was found between the number of TILs and HA expression on cancer cells (40). However, the number of TILs and HA expression in individual tumors is not correlated, but there is a significant inverse relationship between the number of TILs and HA expression in all tumors. The present staining method offers a simple way of improving the detection of HA expression in cancer tissues.

Enhanced metastasis could be one of the factors behind the lower mean survival rate in the cases with high HA expression. Comparison of two cancer cell lines otherwise identical, but differing in the expression and content of cell surface HA, indicated that the cells with high HA expression generated 50-fold more lung metastases when injected i.v. in mice (43). This may be due to entrapment of the large HA-coated cells, or cell clusters, aggregated through blood leukocyte CD44 receptors binding to the HA on cancer cells (43), or formation of emboli through HA to fibrin/fibrinogen (44), or through direct binding of HA to CD44 receptors on endothelial cells (37).

The metastatic ability of pancreatic adenocarcinoma cells did not correlate with the HA affinity of the CD44 variant transfected into these cells (45), suggesting that HA represents an additional malignant growth-promoting effect independent of CD44. This view is supported by the recent findings that there are other HA-binding proteins associated with invasive growth (46). The HA receptor RHAMM, when overexpressed, is the sole requirement to transform a fibroblast cell line and induce its spontaneous metastasis in mice (47). Moreover, a targeted mutation in RHAMM that reduces its affinity for HA totally prevents ras transformation (47). There are limited data presently available on RHAMM expression in patient material, but it is interesting that ras is involved in colon cancer (48). It is quite apparent that HA is one of the key extracellular matrix molecules that control cellular events associated with malignant growth.

Clinical Importance of the Unfavorable Prognosis Related to HA.

The results indicate that expression of HA is a novel prognostic marker in colorectal cancer. Patients with absent or weak HA intensity and HA% in tumor epithelium survived longer than patients with moderate or strong HA intensity. HA intensity and HA% in tumor epithelium also significantly predicted RFS. In a multivariate Cox's analysis, HA intensity in tumor epithelium was an independent predictor of survival and RFS in all subgroups analyzed. In fact, HA intensity in tumor epithelium was the only significant predictor of survival and RFS in local (T1-3,NM0) tumors as well as in Dukes' B tumors. These results strongly support the hypothesis that the patients with strongly HA-positive tumors do have a worse clinical outcome and should be treated using as radical a therapy as possible. Also, carcinomas presently regarded as inoperable should perhaps be reconsidered for surgical treatment if biopsy shows them to be negative for HA. A prospective study with alternate treatment protocols will answer these questions.

The present staining method offers a simple way of improving the diagnostic accuracy of the diagnosis and may be useful in selecting treatment for an individual patient with colon carcinoma. The findings should encourage more work to define the molecular pathways involved in the putative malignant growth promotion by HA. New therapeutic approaches can be studied based on the present finding that an abnormally located and excessive HA may offer a growth advantage for colon carcinoma cells.

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