Expression of Cocaine- and Amphetamine-Regulated Transcript Is Associated with Worse Survival in Small Bowel Carcinoid Tumors

Kalle Landerholm1,3, Liliya Shcherbina4, Sture E. Falkmer2, Johannes Järhult1,3, and Nils Wierup4

Abstract

Purpose: Cocaine- and amphetamine-regulated transcript (CART) peptide exerts several regulatory functions acting both as neurotransmitter and hormone. We recently showed that CART is expressed in various neuroendocrine tumors, including small bowel carcinoids. The main objective of the present study was to examine whether CART expression is associated with survival in patients with small bowel carcinoid. Secondary aims were to assess whether CART expression is associated with other tumor characteristics or clinical symptoms.

Experimental Design: Specimens from 97 patients with small bowel carcinoids were examined for CART expression using immunohistochemistry. A CART score was introduced on the basis of the proportion of CART immunoreactive cells. On inclusion, specimens were examined by routine histopathologic methods and detailed clinical patient data were retrieved. The effect of CART on cell viability was assessed in vitro using two intestinal tumor cell lines.

Results: Expression of CART (P = 0.011) and increasing CART score (P = 0.033) were associated with worse disease-specific survival. Adjusting for age, disease stage, and tumor grade in multivariable analysis, CART expression was still associated with worse survival [Low CART HR, 5.47; 95% confidence interval (CI), 0.71–42.46; and High CART HR, 9.44; 95% CI, 1.14–78.14]. CART expression was not associated with patient age, disease stage, tumor grade, or any presenting symptom. Supporting our clinical data, we found that CART promoted tumor cell viability in vitro in two different tumor cell lines.

Conclusion: Expression of CART in small bowel carcinoid tumors is associated with worse survival.

Clin Cancer Res; 1–9. ©2012 AACR.
coexpressed with the established enterochromaffin cell markers serotonin and chromogranin A within the same tumor cell, and sometimes also with neuropeptide K. Our data gain support from a study showing increased levels of circulating CART peptides in patients with a wide range of NETs (18).

The purpose of the present study was to determine whether tumor CART expression is associated with survival in patients with small bowel carcinoid. A second aim was to investigate whether CART expression is associated with other tumor characteristics such as disease stage or histopathologic grade. A third aim was to examine whether tumor CART expression is associated with pronounced weight loss or other symptoms. In addition, the effect of CART on cell viability was assessed in a murine intestinal NET cell line and a human colon cancer cell line.

Materials and Methods

Patient cohort

All patients in Jönköping county (population 338,000) diagnosed with carcinoid tumor in the jejunum or ileum, including the ilocecral valve, from 1960 to 2005, were identified in 2 previous studies (19, 20). In brief, patients were initially found through the Swedish Cancer Registry and the local cancer registry. Patients diagnosed at autopsy were excluded. Another 22 patients were excluded because the diagnosis proved to be incorrect or because the medical records could not be found, leaving 145 patients in the preceding studies. These patients were all eligible for inclusion in the present investigation, under the condition that paraffin-embedded tumor material could be retrieved for analysis. Eventually, 97 patients with adequate specimens were included. Patients with distant metastases were somewhat underrepresented among the included patients compared with the eligible (28% vs. 36%), owing to the fact that some patients with distant metastases never underwent surgical resection.

The study was approved by the Regional Ethical Review Board at Linköping University, Linköping, Sweden.

Patient data and follow-up

All relevant patient data were collected from medical records from the primary health care centers and hospital departments. Patients were followed up until death or until February 1, 2011. The median follow-up time was 6.1 (interquartile range, 3.2–11.5) years.

Routine histopathologic examinations

Before inclusion, sections from all specimens were histopathologically reexamined with routine staining procedures, including immunohistochemistry to confirm presence of serotonin. To calculate Ki67 proliferation index, sections of all included tumors were examined after incubation with the conventional MIB-1 antiserum.

Staging and cause of death

Following the World Health Organization 2010 histopathologic grading system, tumors were divided into G1 with Ki67 proliferation index equal to or less than 2%, G2 with an index between 3% and 20%, and G3 with an index of more than 20% (21). The highest Ki67 index in any primary tumor or metastasis within the first year of diagnosis was applied for each patient.

In accordance with previous studies (20, 22, 23), the disease stage was defined as localized when the tumor was confined to the bowel wall, regional with either local tumor invasion of the adjacent mesentery or regional lymph node metastases, and distant with metastases elsewhere, including the peritoneum other than that covering the adjacent mesentery. These 3 groups correspond to stages I–IIB, IIIA and IIIB, and IV, respectively, in the recently introduced tumor-node-metastasis (TNM) classification (24, 25).

At the end of the observation period, 20 patients were alive and 77 patients were deceased. The cause of death was individually assessed for each patient from medical records and autopsy reports, when available. Death could convincingly be attributed to the carcinoid disease in 38 patients, 34 patients died of other causes, whereas the cause of death was not evident in 5 patients.

Immunohistochemistry

Indirect immunofluorescence was used. The primary antibody was a rabbit polyclonal anti-CART (code 2059A; dilution 1:5,000; kindly provided by Dr. Jes T. Clausen, Novo Nordisk, Maløv, Denmark). The antibody has been used previously for immunohistochemistry and tested for possible cross-reactivity with chemically related substances, including preabsorption tests with CART 54-102 (17). Importantly, the same staining pattern was seen with 6 other CART antisera, and the specificity of all CART antibodies was verified by lack of staining in the GI tract of CARTPT-null mutant mice (17).
Briefly, sections (5-μm thickness) were cut from paraffin-embedded specimens, mounted on slides, deparaffinized, and rehydrated. Before immunostaining, antigen retrieval was conducted by boiling sections in 10 mmol/L citrate buffer (pH 6.0) in a microwave oven 2 times 7 minutes at 650 W. Sections were incubated with the primary antibody, diluted in PBS with 0.25% bovine serum albumin, and 0.25% Triton X-100, overnight at 4°C. After rinsing 2 times 10 minutes with PBS containing 0.25% Triton X-100, sections were incubated with a secondary antibody with specificity for rabbit IgG and conjugated with Cy2 (Jackson ImmunoResearch) for 1 hour at room temperature. Sections were again rinsed and then mounted in 1:1 PBS and glycerol.

**Classification of CART immunoreactivity**

Specimens were examined for CART immunoreactivity by at least 2 independent observers in the microscope using the same visual field (×25 objective), and a grading (0–4) was introduced on the basis of the proportion of CART immunoreactive cells. In our previous study, it was noted that CART immunoreactivity frequently was heterogeneous between different areas of the same tumor (17). Therefore, one grade was assigned to the most common pattern of CART immunoreactivity, and a second grade to the next most common pattern. If the pattern was homogeneous, both values were the same. Areas with no CART immunoreactive cells were designated 0, areas with 1–10 CART immunoreactive cells per visual field were designated I, areas with 10–100 CART immunoreactive cells per visual field were designated II, areas with 100–1,000 CART immunoreactive cells were designated III, and areas with more than 1,000 CART immunoreactive cells were designated IV (Fig. 1). For example, a tumor predominated by areas with a few scattered CART immunoreactive cells but also containing a smaller part with a high frequency of CART immunoreactive cells could be designated as I + IV.

These grades were then used to divide the tumors into 3 groups, with the dominating pattern given more weight than the second pattern. The group No CART contained only grades 0 + 0; Low CART contained 0 + I–III, 1 + 0–III, and II + 0–II; and High CART contained I + III, III + 0–III, and IV in either position. This classification was referred to as CART score.

All statistical analyses were conducted using the CART score, and in addition to comparing tumors with no CART immunoreactive cells with those with any level of CART immunoreactivity, this division was referred to as CART 0/+. 

**Imaging**

Immunofluorescence was examined in an epifluorescence microscope (Olympus BX60). Images were taken with a digital camera (Nikon DS-2Mv).

**Culture of GLUTag and HCT-116 cells**

The GLUTag cell line (kindly provided by Dr. Daniel J. Drucker, Mount Sinai Hospital, Toronto, ON, Canada) was originally isolated from a glucagon-producing enteroendocrine tumor in mice (26). GLUTag cells were routinely cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% FBS, 2 mmol/L glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. The human colon cancer HCT-116 cell line
(AACT, Bethesda, MD) was grown in McCoy’s 5A medium supplemented with 10% FBS, 2 mmol/L glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Cell viability assay
GLUTag and HCT-116 cells were seeded in 96-well plates at a density of 40,000 and 20,000 cells per well, respectively, and cultured for 24 hours. Thereafter, medium was replaced by new medium with or without 10 or 100 nmol/L CART 54–102 peptide (kindly provided by Dr. Lars Thim, Novo Nordisk), or 10 nmol/L glucagon-like peptide-1 (GLP-1; Sigma-Aldrich) used as positive control, and cells were cultured for another 48 hours. Then, 10 μL of WST-1 reagent (Roche Applied Science) was added to each well and absorbance at 450 nm with reference at 690 nm was read after 1 hour.

Western blot
GLUTag cells were seeded in 6-well plates and cultured in full DMEM for 24 hours. Thereafter, cells were incubated with 10 nmol/L CART 54–102 peptide for another 48 hours. After the treatment, cells were washed with PBS and lysed in Lysis-M reagent supplemented with Complete Mini Protease Inhibitor Cocktail (Roche Diagnostics). The protein concentration in the samples was determined using the Bio-Rad protein assay (Bio-Rad). Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad). The membranes were probed with primary antibodies followed by horse-radish peroxidase–conjugated secondary antibodies and visualized using the SuperSignal Femto Chemiluminescent Substrate (Pierce Biotechnology). Antibodies used were against cyclin D1 (1:500; Cell Signaling Technology) and β-tubulin (1:1,000; Cell Signaling Technology). Protein quantification was assessed by band densitometry measurement using ImageJ software (Research Services Branch, National Institute of Mental Health, Bethesda, MD) and presented as cyclin D1 expression relative to that of β-tubulin.

Statistical analysis
Kendall rank correlation coefficient (τ, tau) was used to determine the strength of relationship between variables on ordinal scales. Fisher exact test was used to test differences in proportions, and Mann–Whitney U test for comparisons of ordinal variables. Disease-specific survival was calculated according to the Kaplan–Meier method, censoring at the time of diagnosis. For comparisons of survival curves of subgroups, the log-rank test was used to test differences between the survival curves of subgroups. After confirming the proportional hazards assumption, multivariable Cox proportional hazards regression was used to assess the independent influence of CART score on disease-specific survival adjusted for patient age, disease stage, and histopathologic grade. Differences in in vitro cell viability and protein expression were tested using one-way ANOVA, followed by Dunnett multiple comparison test. All statistical analyses were conducted using Statistica 9.1 (StatSoft). All tests were 2-tailed and P values <0.05 were considered statistically significant.

Results
Tumor specimens
One hundred and thirty-one specimens from 97 patients were analyzed; 79 of them were from primary tumors at the time of diagnosis. In 54 patients, specimens were available only from the primary tumors, in 20 patients from both primary tumors and mesenteric metastases, in 5 patients from both primary tumors and distant metastases, and in 2 patients from primary tumors, mesenteric, as well as distant metastases. In 10 patients, specimens were available only from mesenteric metastases, in 3 patients only from distant metastases, and in 3 patients from both mesenteric and distant metastases. Specimens retrieved within the first year after diagnosis were available from all but 4 patients.

CART immunoreactivity and association with prognostic factors
CART immunoreactive cells were found in the majority of specimens, but with wide variations between different areas of the same tumor, in accordance with our previous observations (17). As expected in small bowel carcinoids, the predominating tumor growth pattern was the insular with rounded nests of densely packed tumor cells and typical peripheral palisading (27). CART immunoreactivity was often higher in these peripheral cells of the insular nests and also higher in nests closer to the mucosa (Fig. 2A). Areas with trabecular, glandular, and solid (Fig. 2C) growth patterns were seen in some tumors (28, 29). Typical cord-like arrangement of tumor cells was seen in areas of deeper local invasion, and CART was often abundant in such chords (Fig. 2B).

Specimens from both primary tumors and metastases from the time of diagnosis were available in 27 patients. With the null hypothesis of no relationship between CART immunoreactive levels in the primary tumors and the corresponding metastases, Kendall τ was 0.453 (P = 0.013) indicating a fair correlation. Consequently, the proportion of CART immunoreactive cells in the metastases was used for those 16 patients where specimens from the primary tumor were unavailable.

Some level of CART immunoreactivity was detected in 81 of 97 patients (84%). Table 1 shows the distribution of CART immunoreactivity, patient age, disease stage, and tumor grade. There was no statistically significant association between the presence of CART (CART 0/+) on one hand, and patient age, disease stage, or histopathologic grade on the other. Neither was CART score associated with any of the other tumor characteristics.

Association between CART and symptoms
Because CART is a regulator of food intake and body weight (10, 11), we next addressed whether the degree of
CART expression was associated with cachexia, observed in 18 patients at diagnosis. However, no association was found between CART expression and pronounced weight loss at presentation. Neither was CART expression associated with any other presenting symptom including flush (n = 7), diarrhea (n = 19), bowel obstruction (n = 31), abdominal pain (n = 44), or GI hemorrhage (n = 12).

**Survival**
Comparing disease-specific survival between patients of the 3 CART score tiers using the Kaplan–Meier method,

<table>
<thead>
<tr>
<th>CART score</th>
<th>No.</th>
<th>Age, median (IQR), y</th>
<th>Localized (I–IIB)</th>
<th>Regional metastases (IIIA–IIIB)</th>
<th>Distant metastases (IV)</th>
<th>Stage</th>
<th>Grade</th>
</tr>
</thead>
<tbody>
<tr>
<td>No CART</td>
<td>16 (16)</td>
<td>72 (58–81)</td>
<td>3</td>
<td>11</td>
<td>2</td>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td>Low CART</td>
<td>61 (63)</td>
<td>67 (59–75)</td>
<td>10</td>
<td>31</td>
<td>20</td>
<td>51</td>
<td>10</td>
</tr>
<tr>
<td>High CART</td>
<td>20 (21)</td>
<td>74 (68–81)</td>
<td>5</td>
<td>10</td>
<td>5</td>
<td>17</td>
<td>3</td>
</tr>
<tr>
<td>All</td>
<td>97 (100)</td>
<td>69 (59–77)</td>
<td>18 (19)</td>
<td>52 (54)</td>
<td>27 (28)</td>
<td>84 (87)</td>
<td>13 (13)</td>
</tr>
</tbody>
</table>

**NOTE:** Values in parentheses are percent except for age. Abbreviation: IQR, interquartile range.
increasing CART score was associated with worse survival ($P = 0.033$; Fig. 3A). Comparing tumors with and tumors without CART immunoreactivity (CART 0+/−) gave a similar result; CART-containing tumors were associated with worse survival ($P = 0.011$; Fig. 3B).

When the analysis was confined to 79 patients with specimens available from the primary tumors at the time of diagnosis, the result was similar with worse survival for patients with CART-containing tumors ($P = 0.039$). Excluding the 5 patients who died within 30 days after diagnosis, the difference was still statistically significant ($P = 0.020$). Excluding the 16 patients, in whom the tumors were detected en passant, the difference was also significant ($P = 0.023$). Limiting the analysis to the 79 patients with regional or distant metastases, those with CART present in the tumor similarly had a shorter survival ($P = 0.020$).

Adjusting for age, disease stage, and histopathologic grade in multivariable Cox proportional hazards regression, the HR compared with No CART was 5.47 (95% confidence interval (CI), 0.71–42.46) for Low CART, and 9.44 (95% CI, 1.14–78.14) for High CART (Table 2).

### Discussion

This study is the first to report worse survival for patients with CART-expressing tumors, as examined in small bowel carcinoid tumors. In support of this, we also found that CART increases tumor cell viability in vitro.

The present results confirm our recent finding that CART is expressed in small bowel carcinoid tumor cells (17). The distribution of the CART score was similar to this previous study, although a somewhat modified classification of CART immunoreactivity was used. A main finding of the present study was that tumors with any level of CART expression were associated with worse survival. One tentative explanation could be that the localized tumors were removed before they had become metastatic. It is known from an autopsy study that most small bowel carcinoids remain localized and asymptomatic throughout the patients’ lives, thereby escaping detection (30). The localized tumors in the present study were likely biologically different, as they were diagnosed ante mortem—10 of them because of the symptoms they caused (6 GI hemorrhage and 4 bowel obstruction), and 8 incidentally during surgery for other reasons. Thus, it is possible that the localized CART-expressing carcinoid tumors of the present study were intrinsically malignant but removed in time because they were, at the same time, more prone to cause symptoms. However, a separate survival analysis was

---

**Figure 3.** Disease-specific survival rates by (A) CART score, $P = 0.033$ (log-rank); and (B) CART 0/−, $P = 0.011$ (log-rank).
conducted for patients with metastases, also showing worse survival in patients with CART-containing tumors.

The second aim of the study was to assess associations between CART and patient age, disease stage, and histopathologic grade. No such association was evident. Adjusting for these established prognostic factors in multivariable analysis; presence of CART was still associated with an increased HR.

The third aim was to assess whether presence of CART was associated with clinical symptoms. Of particular interest

Figure 4. Effect of CART on tumor cell line viability and proliferation. A, GLUTag cell viability presented as percentage of viability in control medium. GLP-1 was used as positive control. B, HCT-116 cell viability as percentage of viability in control medium. C, representative Western blotting showing that CART treatment increases cell cycle regulator cyclin D1. D, quantification of immunoblots from 5 separate experiments. ** P < 0.01 versus control; *, P < 0.05 versus control.

Table 2. Uni- and multivariable Cox proportional hazards regression of disease-specific survival

<table>
<thead>
<tr>
<th></th>
<th>Univariable analysis</th>
<th>Multivariable analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR</td>
<td>P</td>
</tr>
<tr>
<td><strong>Age, y</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤60 (n = 29)</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>61–75 (n = 36)</td>
<td>2.23 (1.01–4.92)</td>
<td>0.046</td>
</tr>
<tr>
<td>&gt;75 (n = 32)</td>
<td>2.45 (1.00–6.03)</td>
<td>0.051</td>
</tr>
<tr>
<td><strong>Stage</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Localized (n = 18)</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>Regional metastases (n = 52)</td>
<td>4.36 (0.58–33.02)</td>
<td>0.154</td>
</tr>
<tr>
<td>Distant metastases (n = 27)</td>
<td>18.47 (2.47–137.94)</td>
<td>0.004</td>
</tr>
<tr>
<td><strong>Ki67</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1 (n = 84)</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>G2 (n = 13)</td>
<td>2.36 (1.07–5.22)</td>
<td>0.033</td>
</tr>
<tr>
<td><strong>CART</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 (n = 16)</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>+ (n = 81)</td>
<td>8.12 (1.11–59.25)</td>
<td>0.039</td>
</tr>
<tr>
<td>No CART (n = 16)</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>Low CART (n = 61)</td>
<td>7.30 (0.99–53.84)</td>
<td>0.051</td>
</tr>
<tr>
<td>High CART (n = 20)</td>
<td>11.34 (1.45–88.71)</td>
<td>0.021</td>
</tr>
</tbody>
</table>

NOTE: Values in parentheses are 95% CI.
were hormonal symptoms such as flush, diarrhea, and above all weight loss, bearing in mind the physiologic functions of CART-regulating hormone secretion and inhibiting appetite. However, there were no tendencies for any associations between CART expression and any hormonal or other symptom.

We also found that CART peptide in vitro caused a significant increase in viability of GLUTag and HCT-116 cells. These data are in agreement with our previous observations that CART is important for regulation of pancreatic islet β-cell viability, both by reducing apoptosis and by increasing proliferation (Sathanoori, Wierup, and colleagues, manuscript in preparation; ref. 31).

Supported by our present clinical data, the increased GLUTag and HCT-116 cell viability suggests that CART expressed in tumors promotes cell survival via enhanced proliferation. The mechanisms leading to increased viability remains to be established, and further studies are needed to evaluate the potential for CART as a treatment target in NETs. Interesting in this context is that Bech and colleagues found higher levels of circulating CART in patients with progressive NET disease (18).

In conclusion, the present study shows that CART expression in small bowel carcinoid tumors is associated with worse survival.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References

Authors’ Contributions
Conception and design: S.E. Falkmer, J. Järhult, N. Wierup
Development of methodology: K. Landerholm, L. Scherhult, N. Wierup
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K. Landerholm, S.E. Falkmer, N. Wierup
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K. Landerholm, L. Scherhult, S.E. Falkmer, J. Järhult, N. Wierup
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K. Landerholm, S.E. Falkmer, N. Wierup
Study supervision: N. Wierup

Acknowledgments
The authors thank Linda Johansson, Jeanette Karlsson, Barbro Nilsson, Doris Persson, and Ann-Helen Thorén-Fischer for excellent technical assistance.

Grant Support
This work was supported by Futurum—Academy of Healthcare at Jönköping County Council, the Foundation for Clinical Cancer Research in Jönköping, the Swedish Research Council (Projects No. 522-2008-4216, K2009-55X-21111-01-4, K2007-55X-04499-33-3), Faculty of Medicine at Lund University, the Novo Nordisk, Gyllenstierna Krapperup, Fredrik and Ingrid Thuring, Magnus Bergwall, Crafoord, and Albert Påhlsson Foundations.

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.

Received September 29, 2011; revised April 13, 2012; accepted April 22, 2012; published OnlineFirst May 2, 2012.
Expression of Cocaine- and Amphetamine-Regulated Transcript Is Associated with Worse Survival in Small Bowel Carcinoid Tumors

Kalle Landerholm, Liliya Shcherbina, Sture E. Falkmer, et al.

Clin Cancer Res  Published OnlineFirst May 2, 2012.

Updated version  Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-11-2513

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.