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

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Running title: CART in small bowel carcinoid tumors

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Beside the recently introduced TNM stage and proliferation index which together give some indication of the disease course, prognostic markers of small bowel carcinoid are scarce. Cocaine- and amphetamine-regulated transcript (CART) peptide functions as a neurotransmitter but also as a hormone and is produced by enteroendocrine cells, among others. We recently demonstrated that CART is expressed in several types of neuroendocrine tumors. The significance of tumor CART expression is previously unknown. In this study we present evidence for worse survival in patients with CART-producing small bowel carcinoid tumors. In support of this, we also show that cell viability of two intestinal tumor cell lines is increased in the presence of CART peptide. These findings suggest CART as a putative prognostic biomarker, but also as a new potential target for anti-tumor treatment.

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 small bowel carcinoid patients. Secondary aims were to assess if 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 based on the proportion of CART immunoreactive cells. On inclusion, specimens were examined by routine histopathological 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 hazard ratio (HR) 5.47, 95% confidence interval (CI) 0.71 to 42.46; and High CART HR 9.44, 95% CI 1.14 to 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.
**Introduction**

Cocaine- and amphetamine-regulated transcript (CART) was discovered as an mRNA that was up-regulated in rat striatum in response to psychostimulant administration (1). CART peptides have since been found in the central, peripheral, and enteric nervous systems, and also in endocrine cells in the pancreatic islets (2-4), the gastrointestinal (GI) tract mucosa (5, 6), the thyroid (7), and the adrenal medulla (7, 8). Thus it appears that CART peptide is a so-called *brain-gut peptide*, acting both as a neurotransmitter and as a hormone. Within the brain, the spatial distribution of CART peptides together with various experimental studies suggest a role of CART peptides in regulating food intake and body weight (9, 10) with an overall anorexigenic effect via largely unknown mechanisms (10, 11). This is also supported by observations that CART prepropeptide-encoding gene *CARTPT* null mice and humans carrying a mutated *CARTPT* gene develop obesity and signs of type 2 diabetes (12-14). In addition, there is evidence for CART involvement in other brain processes such as mechanisms of reward and stress response (9, 10).

Hormonal expression of CART in pancreatic islets occurs mainly in the somatostatin-producing δ-cells (2, 3), and the CART peptides participate in the regulation of insulin, glucagon, and somatostatin secretion (3, 15). Within the GI tract mucosa, CART expression has been identified mainly in gastrin-producing G-cells, but also in enterochromaffin (EC) cells of the small bowel (5, 6). The physiological function of CART in the enteric neurons and enteroendocrine cells remains poorly elucidated (16).

We recently showed that CART is expressed in tumor cells in human neuroendocrine tumors (NETs) of various origin, including EC cell-derived small bowel carcinoids (17). Expression of CART was found in a similar proportion of tumors regardless of the anatomical site of
origin. The frequency of CART immunoreactive (IR) cells varied from none in some tumors to a majority of the tumor cells in others. In small bowel carcinoids, CART was consistently co-expressed with the established EC 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 raised 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 small bowel carcinoid patients. A second aim was to investigate if CART expression is associated with other tumor characteristics such as disease stage or histopathological 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 ileocecal valve, from 1960 to 2005, were identified in two 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 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 to the eligible (28% versus 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, Sweden.

**Patient data and follow-up**

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

**Routine histopathological examinations**

Before inclusion, sections from all specimens were histopathologically re-examined with routine staining procedures, including immunohistochemistry (IHC) to confirm presence of serotonin. In order 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 WHO 2010 histopathological grading system, tumors were divided into G1 with Ki67 proliferation index equal to or less than two per cent, G2 with an index between three and 20 per cent, and G3 with an index of more than 20 per cent (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 three groups correspond to stages I-IIB, IIIA-IIIB, and IV, respectively, in the recently introduced 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 five 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, Måløv, Denmark). The antibody has been used previously for IHC 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 six 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. Prior to immunostaining, antigen retrieval was performed by boiling sections in 0.01M citrate buffer (pH 6.0) in a microwave oven for 2x7
min at 650 W. Sections were incubated with the primary antibody, diluted in PBS with 0.25% bovine serum albumin and 0.25% Triton-X100, overnight at 4°C. After rinsing 2x10 min with PBS containing 0.25% Triton-X100, sections were incubated with a secondary antibody with specificity for rabbit IgG and conjugated with Cy2 (Jackson, West Grove, PA) for 1h 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 IR by at least two independent observers in the microscope using the same visual field (25x objective), and a grading (0-4) was introduced based on the proportion of CART IR cells. In our previous study it was noted that CART IR frequently was heterogeneous between different areas of the same tumor (17). Therefore, one grade was assigned to the most common pattern of CART IR, and a second grade to the next most common pattern. If the pattern was homogenous, both values were the same. Areas with no CART IR cells were designated 0; areas with 1-10 CART IR cells per visual field were designated 1; areas with 10-100 CART IR cells per visual field were designated 2; areas with 100-1,000 CART IR cells were designated 3; and areas with more than 1,000 CART IR cells were designated 4 (Fig. 1). For example, a tumor predominated by areas with a few scattered CART IR cells but also containing a smaller part with a high frequency of CART IR cells could be designated as 1 + 4.

These grades were then used to divide the tumors into three 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 + 1-3, 1 + 0-3, and 2 + 0-2; and *High CART* contained 2 + 3, 3 + 0-3, and 4 in either position. This classification was referred to as CART score.
All statistical analyses were performed using the CART score, and in addition comparing tumors with no CART IR cells to those with any level of CART IR, this division was referred to as CART 0/+.

**Imaging**

Immunofluorescence was examined in an epi-fluorescence 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, 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, 2mM 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, 2mM 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 h. Thereafter medium was replaced by new medium with or without 10 or 100nM CART 54-102 peptide (kindly provided by Dr. Lars Thim, Novo Nordisk, Måløv, Denmark), or 10nM glucagon-like peptide-1 (GLP-1; Sigma-Aldrich, St. Louis, MO) used as positive control, and cells were cultured for another 48 h. Then 10 μL of WST-1 reagent (Roche Applied Science, Rotkreuz, Switzerland) 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 h. Thereafter cells were incubated with 10nM CART 54-102 peptide for another 48 h. After the treatment, cells were washed with PBS and lysed in Lysis-M reagent supplemented with Complete Mini Protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). The protein concentration in the samples was determined using the Bio-Rad protein assay (Bio-Rad, Hercules, CA). Proteins were separated by SDS PAGE electrophoresis and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). The membranes were probed with primary antibodies followed by horseradish peroxidase-conjugated secondary antibodies and visualized using the SuperSignal Femto Chemiluminescent Substrate (Pierce Biotechnology, Rockford, IL). Antibodies used were against cyclin D1 (1:500; Cell signaling Technology, Beverly, MA) and β-tubulin (1:1,000; Cell signaling Technology, Beverly, MA). 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’s rank correlation coefficient (τ, tau) was used to determine the strength of relationship between variables on ordinal scales. Fisher’s 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 death patients who died from reasons other than small bowel carcinoid tumor or when the cause of death could not be established. 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 histopathological grade. Differences in *in vitro* cell viability and protein expression were tested using one-way ANOVA, followed by Dunnet’s multiple comparison test. All statistical analyses were performed using Statistica 9.1 (StatSoft, Tulsa, OK). All tests were two-tailed and *P*-values < 0.05 were considered statistically significant.

**Results**

**Tumor specimens**

One-hundred and thirty-one specimens from the 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 IR 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 IR levels in the primary tumors and the corresponding metastases, Kendall’s τ was 0.453 (P = 0.013) indicating a fair correlation. Consequently, the proportion of CART IR cells in the metastases was used for those 16 patients where specimens from the primary tumor were unavailable.

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

**Association between CART and symptoms**

Since 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 0/+ (P = 1.000) or CART score (P = 0.923) 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 three CART score tiers using the Kaplan-Meier method, increasing CART score was associated with worse survival ($P = 0.033$) (Fig. 3A). Comparing tumors with and tumors without CART IR (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 the 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 five patients that 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 histopathological grade in multivariable Cox proportional hazards regression, the hazard ratio (HR) compared to *No CART* was 5.47 (95% confidence interval (CI) 0.71 to 42.46) for *Low CART*, and HR 9.44 (95% CI 1.14 to 78.14) for *High CART* (Table 2).

**CART enhances tumor cell viability in vitro**

Since our clinical data revealed that presence of CART in tumors is associated with increased mortality, we hypothesized that CART promotes tumor cell viability. To test this we cultured GLUTag cells (murine enteroendocrine cancer cell line) in the presence of 10 and 100nM of CART 54-102 peptide, and found that both doses of CART significantly increased cell
viability (163% and 167% compared to control, respectively; $P < 0.01$; data from six experiments run in quadruplicate) (Fig. 4A). In fact, the effect of CART was even stronger than that of 10nM GLP-1 used as positive control (135% compared to control; $P < 0.05$; $n = 5$). The effect of CART on cell proliferation was confirmed by Western blot for cyclin D1. There was a trend for increased cyclin D1 protein expression in CART-treated GLUTag cells (mean 184%; range 116-327%; $P = 0.06$; $n = 5$) (Fig. 4C, D). Next, we repeated the viability experiments in the human colon cancer cell line HCT-116 using the lowest effective dose 10nM of CART peptide. In line with the data obtained in GLUTag cells, CART provoked a moderate, but significant augmentation of viability also in HCT-116 cells (114% compared to control; $P < 0.05$; $n = 8$) (Fig. 4B).

**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 IR was used. A main finding of the present study was that tumors with any level of CART expression were associated with worse disease-specific survival. In addition, we found that increasing levels of CART IR was associated with worse survival.
Patients with localized small bowel carcinoid tumors have a far better prognosis than those with metastases, as previously established (20, 22). It was therefore intriguing that the majority of localized tumors contained CART-expressing cells when CART tumor expression is 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, since they were diagnosed ante mortem – ten of them because of the symptoms they caused (six GI hemorrhage and four bowel obstruction), and eight 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 performed 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 histopathological grade. No such association was evident. Adjusting for these established prognostic factors in multivariable analysis, presence of CART was still associated with an increased hazard ratio.

The third aim was to assess whether presence of CART was associated with clinical symptoms. Of particular interest were hormonal symptoms such as flush, diarrhea, and above all weight loss, bearing in mind the physiological 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 \textit{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 crucial for regulation of pancreatic islet β-cell viability, both by reducing apoptosis and by increasing proliferation (31, 32).

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 potential treatment target in NETs. Interesting in this context is that Bech \textit{et al.} found higher levels of circulating CART in patients with progressive NET disease (18).

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

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References


<table>
<thead>
<tr>
<th>CART score</th>
<th>No.</th>
<th>Age (years) median (IQR)</th>
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<tr>
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<td>Regional metastases (IIIA-IIIB)</td>
<td>Distant metastases (IV)</td>
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<td>16  (16)</td>
<td>72 (58, 81)</td>
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<td>11</td>
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<td>61  (63)</td>
<td>67 (59, 75)</td>
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<td>31</td>
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<tr>
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<td>20  (21)</td>
<td>74 (68, 81)</td>
<td>5</td>
<td>10</td>
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<tr>
<td>All</td>
<td>97  (100)</td>
<td>69 (59, 77)</td>
<td>18 (19)</td>
<td>52 (54)</td>
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Values in parentheses are percent except for age.
Table 2. Uni- and multivariable Cox proportional hazards regression of disease-specific survival

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<td>&gt;75 (n = 32)</td>
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<td>18.47 (2.47, 137.94)</td>
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<td>20.62 (2.71, 156.64)</td>
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<td>+ (n = 81)</td>
<td>8.12 (1.11, 59.25)</td>
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<td>High CART (n = 20)</td>
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<td>9.44 (1.14, 78.14)</td>
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Values in parentheses are 95% confidence interval.
**Fig 1.** Illustration of CART immunoreactivity grades used to establish the CART score, grade 0 not shown. Areas with 1-10 cells per visual field in 25x objective were grade 1 (A), areas with 10-100 cells were grade 2 (B), areas with 100-1000 cells were grade 3 (C), and areas with more than 1000 cells were grade 4 (D). Scale bar = 100 μm.

**Fig 2.** Illustration of abundant CART immunoreactivity (IR) and distinct tumor growth patterns. A: Insular growth with rounded nests and peripheral palisading, frequently with more abundant CART IR close to the mucosa. B: CART IR was often high in areas of deep local invasion, where carcinoid tumor cells typically are oriented into chords and files, exemplified in connective tissue. C: Liver metastasis with solid growth pattern and CART IR tumor cells to the left, normal hepatocytes to the right. D: CART IR tumor cells invading muscle layers. E: Negative control staining without primary CART antibodies of the same area as in D in a consecutive section. Scale bar = 100 μm.

**Fig 3.** Disease-specific survival by A CART score, \( P = 0.033 \) (logrank); and B CART 0/+, \( P = 0.011 \) (logrank).

**Fig 4.** Effect of CART on tumor cell line viability and proliferation. A: GLUTag cell viability presented as % of viability in control medium (C). GLP-1 was used as positive control. B: HCT-116 cell viability as % of viability in control medium (C). C: Representative western blot showing that CART treatment increases cell cycle regulator cyclin D1. D: Quantification of immunoblots from 5 separate experiments. ** \( P < 0.01 \) vs. control, * \( P < 0.05 \) vs. control.
A

Specific survival

Disease-specific survival

No CART
Low CART
High CART

Time after diagnosis (years)

B

No. at risk

CART 0  CART +

16  81
12  46
7   26

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

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