Increased neuroendocrine cells in resected metastases compared to primary colorectal adenocarcinomas.

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ORIGINAL ARTICLE

Increased neuroendocrine cells in resected metastases compared to primary colorectal adenocarcinomas

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Abstract Neuroendocrine differentiation has been described in rectal adenocarcinomas receiving neoadjuvant therapy prior to radical surgery, but its clinical relevance is controversial and no data are currently available in colorectal carcinoma metastases as compared to primary tumors. The presence of chromogranin A positive tumor cells was investigated by means of immunohistochemistry on surgical specimens from 54 primary colorectal carcinomas and their corresponding metastases, resected at diagnosis or during tumor progression. In 47 patients, tumor metastases were resected 1 month to 12 years after chemotherapy and/or radiotherapy, while in the remaining seven patients no additional therapy after primary surgery was performed. In primary tumors, neuroendocrine differentiation was found in 12/54 cases (22.2%) as compared to 25/54 metastatic lesions (46.3%; $p=0.01$). The presence of neuroendocrine phenotype was not correlated with any clinical pathological parameter nor with a different proliferation index. However, patients having neuroendocrine cells in the primary tumor had a significantly shorter survival from the time of metastatic spread than those having not (33.3 vs. 55.5 months; $p=0.04$). In summary, our data show that colorectal carcinoma metastases contain a higher percentage of neuroendocrine differentiated cells as compared to their corresponding primaries, a finding possibly related to the influence of chemotherapy in neuroendocrine differentiation during colorectal carcinoma progression.

Keywords Colorectal carcinoma · Metastases · Neuroendocrine differentiation · Chemotherapy

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Introduction

Neuroendocrine differentiation has been described in human adenocarcinomas of the prostate, breast, stomach, lung, and colorectum, among others [1–8]. The clinical significance of this finding is debated and in any case differs among the various organs. In the prostate, neuroendocrine differentiation has been demonstrated in therapy naïve cases but it has been specifically associated to hormone refractory cancers and indeed found associated to tumor progression and a worse prognosis [5]. At variance, in breast cancer neuroendocrine differentiation has been consistently demonstrated to lack any prognostic significance [9], whereas its association with a more aggressive disease is still controversial in lung [7, 10] and stomach carcinomas [6].

In colorectal carcinoma, neuroendocrine cells were identified in up to 77% of cases, largely depending on the method used to assess the neuroendocrine cell population [1, 11, 12]. Classically, chromogranin A immunohisto-

chemistry is the most common technique, although it is generally considered that a panel of neuroendocrine markers, including synaptophysin, CD56 and others, has to be preferred in order to increase the sensitivity. Nevertheless, the clinical relevance of neuroendocrine differentiation in colorectal carcinoma is still debated being either associated to poor survival [13] or not [11].

The mechanisms leading to neuroendocrine differentiation in colorectal carcinoma are incompletely understood and are currently accepted as belonging to divergent differentiation processes that might be modulated by therapeutic interventions. In fact, Shia et al. [14] described a significant increase of chromogranin A positive cells in resected rectal adenocarcinomas after neo-adjuvant chemotherapy alone or combined to radiotherapy; comparing endoscopic biopsies with the corresponding rectal surgical specimen, it was shown that 68% of resected rectal carcinomas after chemotherapy had chromogranin A positive tumor cells, as compared to 30% of cases in pre-treatment endoscopic biopsies and to 17% of cases in a control series of conventional, untreated cases. This observation suggests that pharmacological intervention may have potential effects on the selection of specific cell types that are not sensitive (or not as sensitive) to conventional treatment strategies. This phenomenon is well known in prostatic adenocarcinoma treated with androgen deprivation therapy: an increase of chromogranin A immunoreactive cells in tumor tissue (in association to raised chromogranin A serum levels) has been correlated to the onset of hormonal refractory disease. In addition, the presence of a neuroendocrine cell component in therapy naïve prostate cancer patients was found predictive of androgen deprivation treatment failure [5].

When re-evaluating the morphological features of a rectal adenocarcinoma that occurred in a young woman that is following a long disease free survival after complete resection and medical treatment of a local pelvic recurrence, we observed a significant increase in the amount of chromogranin A positive cells in the recurrent pelvic tumor compared to the primary carcinoma specimen. In between the two operations, the patient received several courses of chemotherapy, as described elsewhere [15]. This observation, in agreement with the data reported by Shia et al. [14], prompted us to investigate if chemotherapy can also affect the extent of neuroendocrine differentiation in resected metastases of relapsing colorectal carcinomas, compared to their primary tumor. We here show that neuroendocrine differentiation, as detected by chromogranin A immunoreactivity, is significantly higher in resected pulmonary, liver, or abdominal metastases of colorectal cancer compared to the corresponding primaries. In addition, median survival was shorter for colorectal carcinomas with a neuroendocrine differentiated population in the primary tumor, indicating that the neuroendocrine cell population may respond differently to current medical treatment for colorectal cancer and may possibly affect survival.

Materials and methods

Patients From the colorectal carcinoma database established at the Oncology Unit of the San Luigi Hospital and University of Turin, 54 patients were retrospectively collected in the period from 1996 to 2008, having a radically resected colorectal adenocarcinoma and also a resection of a synchronous (21 cases) or metachronous (33 cases) metastasis of the same tumor, from the lung, liver, or peritoneum. All but seven patients received adjuvant chemotherapy after resection of the primary, and eight rectal carcinomas had also radiotherapy administrated. The chemotherapy protocols administrated to the 47 patients included oxaliplatin-based chemotherapy (FOLFOX or XELOX or chronomodulated FFL 4/10 regimens) in 39, irinotecan-based chemotherapy (FOLFIRI) in 2, and 5-FluoroUracil based chemotherapy (De Gramont's schema or protracted continuous infusion of 5FloroUracil) in six of them.

Tissues Representative paraffin blocks from both primary and metastatic tumors as well as complete follow-up information were available for all cases. Metastatic lymph nodes were also analyzed in 20 cases, as a control group. All pathological material was anonymized by a pathology staff member not involved in the current project and tissues from primary and metastatic tumors of the same patient were coded to allow the comparison in each anonymized case.

Immunohistochemistry Chromogranin A was selected as a reliable marker of neuroendocrine differentiation based on extensive literature data [14], and the immunohistochemical reactions were performed on sections collected onto charged slides serial to those stained with conventional hematoxylin and eosin. A standard immunohistochemical technique was used, including microwave oven antigen retrieval (15 min in 10 mmol/L citrate buffer solution at pH 6.0) and incubation with the primary chromogranin A antibody (clone LK2H10, diluted 1:800; Novocastra laboratories, Newcastle Upon Tyne, UK). The immunoreactions were revealed by a dextran-chain (biotin-free) detection system (EnVysion, DakoCytomation, Glostrup, Denmark), using 3,3′-diaminobenzidine (DAB, Dako) as a chromogen. The slides were then counterstained with hematoxylin, dehydrated, and mounted. Neuroendocrine cells of the surrounding colorectal mucosa in primary tumors served as positive internal control. Sections of pancreatic islet cells served as external positive controls for chromogranin A in each immunohistochemical run. Chromogranin A reactivity was scored as reported by Shia [14] in a

three-tie system, including score 0: no staining in tumor cells; score 1+: 1–20% of tumor cells stained, and score 2+: >20% of cells stained. In all cases, the proliferative activity was assessed by means of Ki-67 immunostaining (clone MIB1, Dako, diluted 1:300), using the above described procedure. The proliferative fraction was expressed as percentage of Ki-67 reactive nuclei, after counting positive cells out of a thousand. In all cases with a chromogranin A-positive population, both in primary and metastatic samples, Ki-67 and chromogranin A were investigated by double immunohistochemical reactions, using immunoperoxidase procedure for Ki-67, followed by immunoalkaline phosphatase method Envision-AP (Dako) with Vector blue alkaline phosphatase substrate kit III (from Vector Laboratories, CA, USA) for chromogranin A, at the same conditions reported above.

Statistical analysis Differences between chromogranin A expression and clinical pathological variables were analyzed by the chi-square test with Yates' correction, when appropriate, whereas mean Ki-67 indexes within different groups were compared using Student's *t* test. Survival curves were plotted using the Kaplan–Meier method and were statistically evaluated using the log-rank test. These statistical computations were performed using the SPSS for Windows and STATISTICA for Windows software. The significance level was set at $p<0.05$.

Results

Patients The present case series of 54 cases (Table 1) included 23 females and 31 males having a median age of 60.5 years (range 28–79). The primary tumors were located in the right colon (12 cases), left colon (25 cases), and rectum (17 cases). Conventional adenocarcinoma was observed in 48 cases, while the remaining six cases were mucinous carcinomas. With regard to tumor grade, three cases were well differentiated, 40 moderately and 11 poorly differentiated. According to the 2010 TNM 7th edition staging system, 48 cases were staged pT3–T4, being the remaining six tumors staged pT1–T2. Most cases (43/54, 79.6%) had lymph node metastases at the time of diagnosis. The site of distant metastasis examined in the present study was liver in 33 patients, peritoneum in 11 patients, lung in nine patients, and ovary in one patient.

Neuroendocrine differentiation Positive chromogranin A immunostaining, including both scores 1 and 2, was observed in 12 of the 54 primary colorectal carcinomas (22.2%) and in 25/54 metastases (46.3%). This difference

Table 1 Clinico-pathological data of 54 colorectal carcinomas that underwent surgical resection of a metastasis at diagnosis or along tumor progression

Number of patients	54	
Median age (years) (range)	60.5 (28–79)	
Male/Female ratio	31/23	
Primary tumor location	R/colon: 12, L/colon: 25, Rectum: 17	
Tumor grade	G1: 3, G2: 40, G3: 11	
Tumor stage	T1: 1, T2: 5, T3: 37, T4: 11; N0: 11, N1-2: 43	
Surgically resected metastasis	Liver: 33, Lung: 9, Peritoneal: 11, Ovarian: 1	
Chemotherapy administrated	47	
No chemotherapy administrated	7 (5 at diagnosis and 2 during tumor progression)	
Chromogranin A expression	12/54 (22.2%) in primary CRC	
	25/54 (46.3%) in CRC metastases	
	Chi-square $p=0.01$	
Ki-67 proliferation index	67.1% (primary CRC) and 62.4% (metastases)	
Median survival from first diagnosis	CgA negative in primary CRC	59.9 months (42 pats)
	CgA positive in primary CRC	35.9 months (12 pats)
	Log rank $p=0.08$	
Median survival from metastatic spread	CgA negative in primary CRC	55.5 months (42 pats)
	CgA positive in primary CRC	33.3 months (12 pats)
	Log rank $p=0.04$	
	CgA negative in CRC metastasis	53.8 months (29 pats)
	CgA positive in CRC metastasis	38.3 months (25 pats)
	Log rank $p=0.18$	

R right, *L* left, *CRC* colorectal carcinoma, *CgA* chromogranin A

was statistically significant (p=0.01 by chi-square test). In primary colorectal carcinomas, chromogranin A-positive cases were equally distributed between those with synchronous metastases (six cases, 26.3%) and those that developed metastases at follow up (the other six, 20%).

When comparing chromogranin A staining between primary tumor and metastasis of the same patient, in 37 cases (68.5%) we failed to show any change (27 double negative and ten double positive, without any increase in the extent of positivity in the latter group), whereas an increase was evident in 15/54 (27.8%), including 13/47 chemotherapy-treated, and 2/7 chemo-naïve patients. In ten of these 15 cases, the chromogranin A-positive population was detected in the metastatic tissue only (Fig. 1a–b), whereas in the remaining five it was already focally present in the primary tumor (immunohistochemical score 1) but was increased in the metastasis (immunohistochemical score 2; Fig. 1c–d). The remaining two cases could not be analyzed due to the presence of extensive mucin lakes with very few neoplastic cells in the metastases. Synchronous metastatic lymph nodes were analyzed in 20 cases, from one to three metastatic lymph nodes for each case. Among these, ten cases corresponded to chromogranin A positive and ten cases to chromogranin A negative primary tumors. All lymph node samples showed no chromogranin A immunoreactivity, except for two cases (both having a positive chromogranin A in the primary tumor, score 1) with rare scattered positive cells.

The presence of neuroendocrine-differentiated primary colorectal cancers was associated to shorter overall survival (35.9 versus 59.9 months) and shorter survival from the time of metastatic spread (33.3 versus 55.5 months), being the latter statistically significant in univariate analysis (log rank p= 0.04; Fig. 2); a similar trend was observed considering those cases having chromogranin A positive cells in the metastases as opposed to negative cases (38.3 versus 53.8 months from the time of metastatic spread; log rank p=0.18).

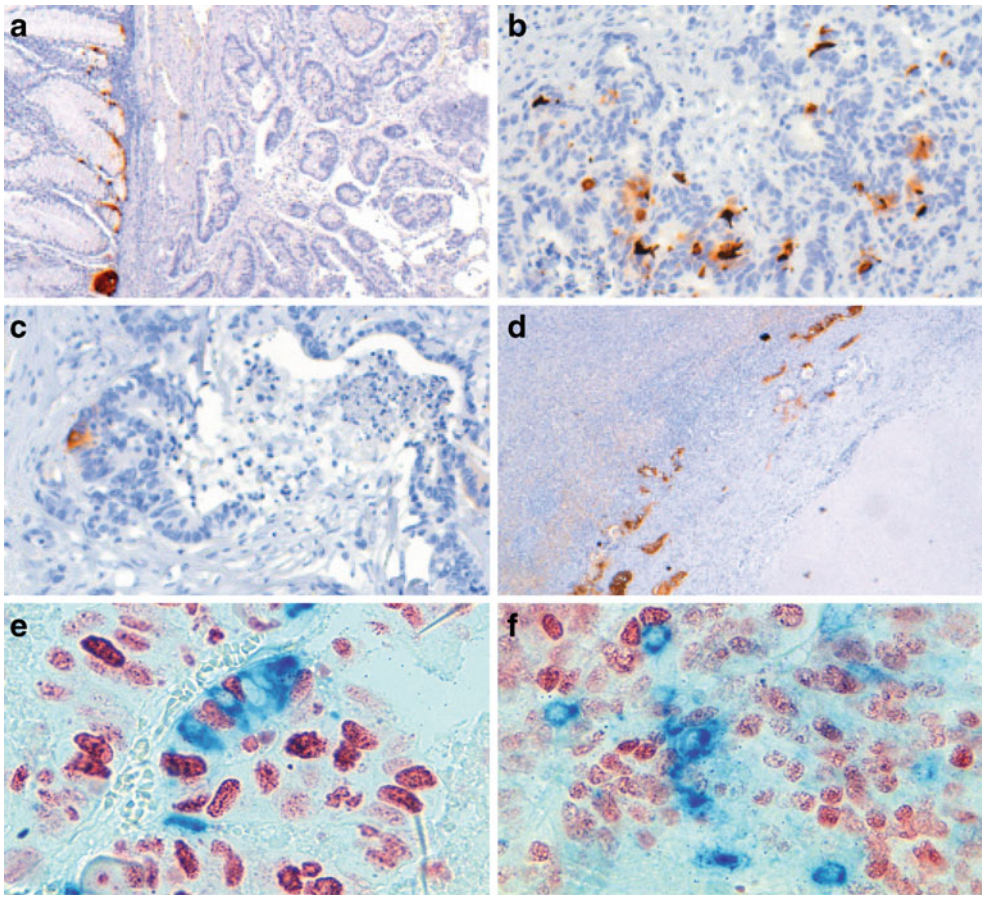

Fig. 1 Primary rectal adenocarcinoma (**a**, case 4) with no evidence of neuroendocrine differentiation (normal colonic mucosa in the left). After 18 months and following 5-FU treatment, a liver metastasis eventually developed (**b**), showing numerous scattered neuroendocrine cells (score 2). Primary colonic adenocarcinoma (**c**, case 18), with focal neuroendocrine differentiation (score 1), with subsequent (9 months later) extensively necrotic liver metastasis (**d**), following 5-FU and oxaliplatin treatment, showing diffuse neuroendocrine differentiation (score 2; normal liver in the upper left corner). Double immunostaining for chromogranin A (*blue*) and Ki-67 (*brown*) in a primary colonic adenocarcinoma (**e**) and in a liver metastasis (**f**) showing numerous proliferating tumor cells and few neuroendocrine cells in a resting phase (**a**–**d** chromogranin A staining, immunoperoxidase; **e** and **f** double sequential immunoperoxidase for Ki-67 and immunoalkaline phosphatase for chromogranin A; original magnifications **a** and **d** 100×; **b** and **c** 200×; **e** and **f** 400×)

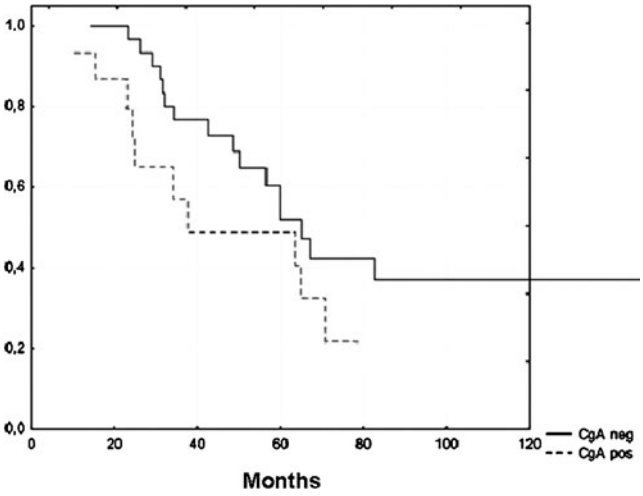

Fig. 2 Survival of colorectal carcinoma patients from the time of metastatic progression, grouped according to chromogranin A reactivity (positivity including immunohistochemical scores 1 and 2) in the primary tumors (log rank test, $p=0.04$)

The proliferative activity was assessed by means of Ki-67 immunostaining in all cases of colorectal carcinoma, both in the primary and in the metastatic locations (means 67.1% and 62.4%, respectively). No statistically significant difference was observed between the mean Ki-67 proliferative index of colorectal carcinomas with neuroendocrine differentiation and negative cancers (78.5% versus 64%, respectively). The proliferative activity of metastatic tissues was also similar in neuroendocrine and non- neuroendocrine differentiated tumor groups (Ki-67 index of 59.1% and 65.3%, respectively). Double immunostainings combining chromogranin A and Ki-67 showed that the neuroendocrine cell population generally was not actively proliferating, both in primary tumors and metastases (Fig. 1e–f).

When the presence of neuroendocrine differentiation was compared with primary tumor location (right and left colon and rectum), tumor histotype (conventional versus mucinous), tumor stage and grade of differentiation, site of metastasis (liver versus peritoneum and lung), and response to chemotherapy (stable disease versus partial or complete response), no significant correlation was observed between any of the above considered parameters and chromogranin A expression, neither in primary nor in metastatic specimens.

Discussion

In this study, we have shown that distant metastases of colorectal carcinoma contain a significantly higher amount of neuroendocrine cells compared to the corresponding primary tumors, as detected by chromogranin A immunostaining in surgical specimens. This may be part of the natural history of colorectal cancer progression or be related to the effects of chemotherapy, which generally preceded the surgical removal of distant metastases.

Since it is virtually impossible to analyze a control group of surgically resected colorectal carcinomas with no subsequent chemotherapy (only seven cases were recruited in the current series), the demonstration of a direct effect of chemotherapy on the increase of neuroendocrine cells is not possible. However, this was already demonstrated in a study on rectal adenocarcinoma preoperatively treated by chemo and/or radiotherapy [14]. The authors restricted their analysis to primary tumors, comparing the neuroendocrine cell density in pre-treatment biopsies versus the corresponding surgical specimen of rectal cancer, and found a significantly higher amount of neuroendocrine cells in treated rectal carcinomas compared to the control group of adenocarcinomas that did not undergo neoadjuvant therapy.

Based on this report, we therefore expanded the analysis to colonic and rectal carcinomas and to their metastatic locations, which are now increasingly being resected especially from lung and liver, to assess the phenotypic profile of cancer cell populations in the setting of metastatic and progressing disease. Indeed, distant metastases were found to contain a higher amount of neuroendocrine cells, as compared to their primaries, and this may be the result of relative selection of tumor clones not responsive to conventional chemotherapy protocols, or to direct effects of chemotherapy agents on a fraction of neoplastic cells differentiated along the neuroendocrine lineage. Moreover, we analyzed synchronous metastatic lymph nodes in 20 cases, both from chromogranin A negative and positive primaries, and failed to demonstrate any significant chromogranin A immunoreactivity. Although the limited bulk of tumor tissue in lymph nodes as compared to primaries or distant metastases might be a bias, this finding seems to suggest that the selection of neuroendocrine tumor cell clones is more likely related to anti-neoplastic treatments rather than to metastatic progression.

The role of chemotherapy in favoring the increase and the selection of neuroendocrine differentiated neoplastic cell clones is controversial. Shia et al. [14] demonstrated a role of neoadjuvant treatments in inducing a significant increase of the neuroendocrine cell population in primary colorectal carcinoma specimens. The same may well be the case in metastatic locations, although a statistical validation of this event is currently not possible. The very few cases (seven) that did not (or could not) undergo chemotherapy had a variable amount of neuroendocrine cells in their metastases, but the extremely limited number of cases hampers any statistical analysis and definite conclusion. In addition, in some cases, the development of distant metastases occurred many years after surgery and subsequent chemotherapy, suggesting that the different tumor cell populations, exocrine (mucinous), and neuroendocrine may

play complex reciprocal interactions. In this respect, the expression of transcription factors known to drive neuroendocrine differentiation in the embryonic life, such as human achaete-scute-homologue type 1 (hASH1) may be involved in the occurrence of neuroendocrine differentiation [16]. Unfortunately, in the present series, no hASH1 expression was observed (data not shown) suggesting that at least in the intestinal area, the development of neuroendocrine differentiated cells may follow alternative pathways, compared to other models such as the prostate [17].

In this context, it is of interest that the neuroendocrine population both in primary tumors and metastases showed a very low proliferative activity, in agreement with earlier observations in gastric carcinoma [18] that pointed out that neuroendocrine cells are terminally differentiated cells, devoid of any proliferative potential. Moreover, in our cases, the mean Ki-67 proliferation index was similar in primary and metastatic cancers, as well as in colorectal carcinomas with or without neuroendocrine features.

A second issue is related to the prognostic influence of neuroendocrine differentiation in colorectal carcinoma. In the present series, cases having a neuroendocrine differentiated component in the primary tumors had a slightly shorter survival both from the time of diagnosis and the time from metastatic spread but statistical significance was reached for the latter case only, possible due to the relatively small sample size. Other literature data are in line with this observation [13, 19, 20], although some authors found no prognostic differences between neuroendocrine differentiated and conventional colorectal carcinoma [21]. No correlation was also observed with any clinical pathological parameter. In conclusion, we herein reported an increased detection on neuroendocrine tumor cells in metastatic tissues of chemotherapy-treated colorectal cancer patients, as compared to the corresponding primary tumors, associated to reduced patients' survival; the biological and predictive meaning of this observation would deserve further elucidation but is possibly related to the positive selection by chemotherapy of neuroendocrine tumors clones that are characterized by a very low proliferative activity.

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Conflict of interest statement We declare that we have no conflict of interest.

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