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SHORT COMMUNICATION

No loss of sst receptors gene expression in advanced stages of colorectal cancer

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Abstract

As demonstrated by several studies, the pan-inhibitory peptide somatostatin (SS) is implicated in a large variety of physiological processes in the gastrointestinal tractus. SS inhibits hormonal and gastric acid secretions, and decreases gastric and intestinal motility, mesenteric blood flow and intestinal absorption. *In vitro* and *in vivo* studies showed also that the antiproliferative potency of SS analogs may be a target to improve the prognosis of colorectal cancer. Here we report the expression profile of the five SS receptor subtypes (hsst1–5) mRNAs in a large set of tumoral and normal colon. Using reverse transcription-PCR, we showed that hsst5, hsst1 and hsst2 mRNA subtypes were the most frequently expressed hsst mRNA subtypes in normal and pathological colon. Interestingly, we found that the frequency of hsst5 mRNA expression in the left colon was significantly higher in tumors than in normal samples: 81.2% (13/16) and 36.4% (4/11) respectively ($0.025 > P > 0.01$, χ^2 test with Yates' correction). We did not find any influence of Dukes' stage on hsst mRNAs expression. Of interest, no loss of hsst2 and hsst5 mRNA expression in advanced stages was noted. Some differences in the frequency of expression of hsst mRNAs according to the origin of the tissue (left or right colon) were evident. The expression of hsst5 and hsst2 mRNA in advanced colorectal carcinoma associated with the development of new SS analogs boost the relevance of colorectal cancer treatment by somatostatin analogs.

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Introduction

Despite progress in cancer treatment, colorectal cancer remains one of the most important public health problems. For the advanced stages, the failure of conventional therapies to improve the prognosis of patients needs urgent alternative approaches. A new treatment modality may involve the use of analogs of the ubiquitous pan-inhibitory peptide somatostatin (SS). In the gastrointestinal tract, SS inhibits hormone secretion and gastric acid secretion, and decreases gastric and intestinal motility, mesenteric blood flow and intestinal absorption (1). In addition, SS can inhibit cell proliferation in a wide variety of cell types. The antiproliferative effects of SS are mediated both indirectly through inhibition of the release of various hormones and growth factors which promote cell growth, through inhibition of angiogenesis, and directly through SS receptors located on tumor tissue inducing an inactivation of growth factors or an activation of programmed cell death (2, 3). SS and its analogs inhibit *in vivo* and *in vitro* tumoral colon cell proliferation and metastatic development (4, 5). However, patients with advanced colorectal cancer treated with SS analogs did not present significant benefits although their tumoral markers decreased (6, 7).

The actions of SS are mediated via membrane-bound receptors coupled to GTP binding protein. Five SS receptors of seven transmembrane domains termed hsst1–5 have been cloned (3). They are all negatively coupled to adenylate cyclase and can also modulate guanidylate cyclase, calcium or potassium channels, activate protein phosphatase, transmodulate tyrosine kinase type receptors and act on the mitogen activated protein kinase (MAP kinase) signaling pathway and even induce apoptosis (3, 8).

Binding assays and autoradiographic studies have shown the presence of hsst in most hormone-producing tumors as well as in lymphomas and adenocarcinomas originating from breast, prostate, ovary, kidney or colon.

In the case of colic tissue, hsst mRNA subtypes have been found in normal and tumoral mucosa, nerve plexus, lymphoid tissue and peritumoral veins (9–12). A few recent studies have attempted to characterize the pattern of expression of the different hsst mRNA subtypes in colic tumors and have given controversial results. Using reverse transcriptase-polymerase chain reaction (RT-PCR), Buscail *et al.* (13) found an heterogeneous expression of hsst1-hsst5 mRNA in colorectal cancer with a loss of hsst2 mRNA expression in advanced stages, which could explain partially the lack of effect of SS analogs in clinical trials. Using both RT-PCR and *in situ* hybridization, Laws *et al.* (14) found a retained expression of hsst2 mRNA but a decreased expression of hsst5 mRNA in late stage tumors.

In order to bring further insight into the pathophysiological basis of the use of SS analogs in colorectal cancer treatment, we reassessed, using RT-PCR, hsst mRNA subtypes expression in a large set of tumoral samples and normal tissues.

Materials and methods

Materials

Fifty-three samples of colorectal cancers (primary tumor) and forty-six morphologically normal mucosae (taken from the same patient at a minimal distance of 5 cm from the diseased area) were obtained during surgery and frozen in liquid nitrogen. All patients gave their written consent to participate in the study which was approved by the local ethics committee (Marseilles I). Colon carcinomas were staged according to the Dukes' classification system (15) (stage A, 12 patients; stage B, 10; stage C, 19; stage D, 12); staging, tumor grade (1 hyperdifferentiated, 42 well differentiated, 7 moderately differentiated, 3 undifferentiated) and tumor site (23 rectum, 16 left colon, 14 right colon) are shown in Table 1. Immunohistological study of epidermal growth factor receptors (EGF-R) was performed (ab-2, Calbiochem, La Jolla, CA, USA): ++ corresponds to a high level of EGF-R + corresponds to a moderate level, and ± corresponds to a low level of EGF-R. Ki67 immuno-detection (Dako, Copenhagen, Denmark) is given as a percentage of positive nuclei. Mutations of the apoptotic protein P53 were detected with 3 monoclonal anti-P53 antibodies (MAB 1801, Calbiochem; MAB 240, Novocastra (Newcastle, UK); DO7, ab-1, Novocastra) (Table 1).

RNA extraction and reverse transcription

Total RNA was isolated as described by Chomczynsky and Sacchi (16). Since the somatostatin receptor genes lack intronic sequences, the RNA (10 μg) was treated with 10 U RNase free DNase I-RQI (Promega, Madison, WI, USA) for 2 h at 37 °C. RT reaction was performed as described by the manufacturer (Life Technologies, Gaithersburg, MD, USA). Briefly, each sample was split in one positive reaction with reverse transcriptase and one control reaction without reverse transcriptase to check for DNA contamination.

Polymerase chain reaction

RNA integrity was verified by co-amplification of the hsst with the constitutively expressed cyclophilin mRNA. Each primer couple was used at 1 μmol final concentration, with 200 μmol of each dNTP and 200 U Expand long template PCR system polymerase (Boehringer, Mannheim, Germany) in 50 μl 1 × manufacturer's buffer. Optimal temperature and cycling conditions were as follows.

hsst1: denaturing at 95 °C for 5 min followed by 35 cycles of annealing at 65 °C for 90 s and extension at 72 °C for 120 s; sense: 129–150; antisense: 1122–1100; PCR product: 993 bp. hsst2: denaturing at 95 °C for 5 min followed by 35 cycles of annealing at 62 °C for 90 s and extension at 72 °C for 120 s; sense: 480–498; antisense: 1372–1355; PCR product: 892 bp. hsst3: denaturing at 95 °C for 5 min followed by 45 cycles of annealing at 70 °C for 90 s and extension at 72 °C for 120 s; sense: 308–329; antisense: 961–940; PCR product: 653 bp. hsst4: denaturing at 95 °C for 5 min followed by 45 cycles of annealing at 64 °C for 90 s and extension at 72 °C for 120 s; sense: 139–156; antisense: 1173–1155; PCR product: 1034 bp. hsst5: denaturing at 95 °C for 5 min followed by 35 cycles of annealing at 70 °C for 60 s and extension at 72 °C for 120 s; sense: 647–668; antisense: 1228–1204; PCR product: 581 bp.

PCR products were resolved on 1% agarose gels, stained with ethidium bromide and visualized by UV transillumination (Fig. 1). Specificity of the PCR product was confirmed by Southern blotting and hybridization with the corresponding cDNAs as previously described (17). All samples were tested for DNA contamination. PCR samples positive without reverse transcriptase treatment were systematically discarded from the study.

Statistical analysis was performed using the χ^2 test with Yates' correction.

Results

We found that 85% of normal tissues and 97% of tumors expressed at least one hsst mRNA subtype. The subtypes hsst5, hsst2 and hsst1 were the more frequently expressed, respectively at 68.6%, 54.7% and 56.6% in the tumoral samples and at 48.8%, 60.8% and 41.3% in the normal tissues (Table 2). hsst3 and hsst4 mRNA subtypes were less frequently expressed both in tumors, at 15.2%, and 16.9% respectively and in normal tissues at 17.9% and 8.7% respectively. We did not find any influence of Dukes' stages on the hsst mRNAs expression profile. Furthermore, there was no loss of hsst2 mRNA expression in the advanced

Table 1 Histopathological and immunohistological study of the tumors.

Tumor number	Age (years)	Sex	Stage*	Grade†	Site	EGF-R ‡	P53§	Ki67(%) #
1	84	F	A	WD	Rec	+	–/ – /–	25
2	60	F	A	WD	Rec	+	+/ + /+	nd
3	78	F	A	WD	Rec	nd	nd	nd
4	76	M	A	MD	Rec	+	–/ – /–	25
5	75	F	A	WD	Rec	–	–/ – /–	< 5
6	70	M	A	HD	Rec	+	+/ + /+	< 5
7	72	M	A	WD	LC	–	–/ – /–	5
8	66	F	A	WD	LC	nd	+/ + /+	< 5
9	73	M	A	WD	RC	++	–/ – /–	5
10	60	F	A	WD	RC	++	–/ – /–	10
11	60	F	A	WD	RC	++	+/ + /+	nd
12	62	M	A	WD	RC	+	–/ – /–	5
13	45	F	B	WD	Rec	–	+/nd/+	10
14	73	M	B	WD	Rec	nd	nd	nd
15	60	M	B	WD	Rec	–	+/ + /+	5
16	86	F	B	MD	Rec	+	+/ – /+	< 5
17	67	M	B	WD	LC	+	–/ – /–	25
18	65	F	B	WD	LC	+	–/ – /–	< 5
19	70	M	B	WD	RC	+	–/ – /–	25
20	62	F	B	WD	RC	+	–/ – /–	10
21	78	F	B	WD	RC	±	+/ + /+	25
22	73	M	B	WD	RC	+	+/ + /+	10
23	54	M	C	WD	Rec	+	+/ – /+	10
24	72	F	C	WD	Rec	–	+/ – /+	5
25	64	F	C	WD	Rec	+	–/ – /–	< 5
26	65	F	C	UD	Rec	+	+/ – /+	5
27	55	M	C	WD	Rec	+	+/ + /+	5
28	71	F	C	WD	LC	–	+/ + /+	10
29	59	F	C	WD	LC	+	+/ + /+	5
30	74	F	C	WD	LC	nd	nd	nd
31	77	F	C	WD	LC	+	+/ + /+	10
32	48	F	C	WD	LC	+	–/ – /–	< 5
33	66	F	C	WD	LC	–	–/ – /–	25
34	59	F	C	WD	LC	+	+/nd/+	50
35	70	M	C	MD	LC	+	+/ + /+	5
36	28	M	C	WD	RC	+	+/ + /+	10
37	84	F	C	UD	RC	–	–/ – /–	5
38	56	M	C	UD	RC	++	+/ + /+	5
39	71	M	C	WD	RC	±	nd	25
40	75	F	C	WD	RC	+	–/ – /–	5
41	65	F	C	WD	RC	+	–/ – /–	< 5
42	68	M	D	WD	Rec	+	+/ – /+	5
43	77	F	D	WD	Rec	±	+/ + /+	25
44	53	F	D	MD	Rec	+	+/ – /+	< 5
45	71	F	D	MD	Rec	nd	nd	nd
46	76	M	D	MD	Rec	nd	nd	nd
47	74	F	D	WD	Rec	–	–/ – /+	10
48	75	M	D	WD	Rec	nd	nd	nd
49	75	F	D	WD	Rec	–	+/ + /+	5
50	74	M	D	WD	LC	nd	nd	nd
51	71	F	D	MD	LC	–	+/ + /–	nd
52	69	M	D	WD	LC	–	+/ + /+	5
53	62	M	D	WD	LC	+	–/ – /–	< 5

F, female; M, male; nd, not determined; Rec, Rectum; LC, left colon; RC, right colon.
* Stage according to Dukes' classification. † Grade is degree of differentiation: HD, hyperdifferentiated; WD, well differentiated; MD, moderately differentiated; UD, undifferentiated. ‡ EGF-R: semiquantitative immunohistochemical detection (ab-2): ++, high levels; +, moderate levels; ±, low levels; –, negative, § P53: positive (+) or negative (–) immunohistochemical detection of the apoptotic protein P53 mutations with 3 monoclonal anti-P53 antibodies (MAB 1801; MAB 240; DO7, ab-1). # Ki67: percentage of positive nuclei.

colorectal cancers. Interestingly, the high frequency of hsst5 expression was retained in advanced tumors.

No correlation was found between tumor characteristics and immunohistological markers except for P53 mutation which was more frequent in the rectum and the left colon than in the right colon. Moreover, no correlation could be observed between the tumor markers and the hsst mRNAs expression. Some

Table 2 hsst genes expression by Dukes' stage and tumor location.

	Normal	Tumor	Dukes' stage A	B	C	D	Right colon Normal	Tumor	Left colon Normal	Tumor	Rectum Normal	Tumor
hsst1	19/46 (41/3%)	30/53 (56.6%)	7/12 (58.3%)	5/10 (50%)	12/19 (63.1%)	6/12 (50%)	8/12 (66.6%)	7/14 (50%)	5/13 (38.5%)	10/16 (62.5%)	6/21 (28.6%)	13/23 (56.5%)
hsst2	28/46 (60.8%)	29/53 (54.7%)	5/12 (41.6%)	4/10 (40%)	11/19 (57.8%)	9/12 (75%)	8/12 (66.6%)	7/14 (50%)	7/13 (53.8%)	9/16 (56.2%)	13/21 (61.9%)	13/23 (56.5%)
hsst3	7/39 (17.9%)	7/46 (15.2%)	1/10 (10%)	2/10 (20%)	2/16 (12.5%)	2/10 (20%)	1/8 (12.5%)	1/10 (10%)	2/11 (18.2%)	3/15 (20%)	4/20 (20%)	3/21 (14.3%)
hsst4	4/46 (8.7%)	9/53 (16.9%)	2/12 (16.6%)	1/10 (10%)	4/19 (21%)	2/12 (16.6%)	0/12 (0%)	0/14 (0%)	2/13 (15.4%)	5/16 (31.2%)	2/21 (9.5%)	4/23 (17.4%)
hsst5	21/43 (48.8%)	35/51 (68.6%)	9/12 (75%)	6/10 (60%)	12/18 (66.6%)	8/11 (72.7%)	5/12 (41.6%)	7/14 (50%)	4/11 (36.4%)	13/16 (81.2%)	12/20 (60%)	15/21 (71.4%)

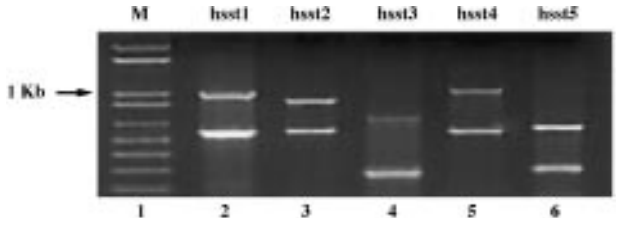

Figure 1 Ethidium bromide stained gel of a representative RT-PCR assay of the five sst receptors from colon carcinoma mRNA samples. RT-PCR products were separated in 1% agarose gel. Lane 1 corresponds to 1 μg DNA marker: 1 Kb plus DNA ladder (Life Technologies). Lanes 2, 3 and 5 correspond to hsst1 (993 bp), hsst2 (892 bp) and hsst4 (1035 bp) respectively, co-amplified with a 549 bp fragment of the cyclophilin gene. Lanes 4 and 6 correspond to hsst3 (653 bp) and hsst5 (581 bp) respectively, co-amplified with a 349 bp fragment of the cyclophilin gene.

differences in the expression of hsst mRNAs were seen according to the origin of the tissue. We found that the frequency of hsst5 mRNA expression was significantly higher in tumors than in normal samples in the left colon: 81.2% (13/16) and 36.4% (4/11) respectively ($0.025 > P > 0.01$). In normal colon, we found that hsst1 mRNA expression decreased throughout the right colon, left colon and rectum and was significantly higher in right colon than in rectum: 66.6% (8/12) and 28.6% (6/21) respectively ($0.05 > P > 0.025$). The frequency of hsst4 mRNA detection was low in left colon and rectum. hsst4 mRNA was below the level of detection in right colon. Larger series will be useful to confirm these results.

Discussion

Looking for the pathophysiological bases for treatment of colorectal cancer with SS analogs, we found that the five hsst mRNAs were expressed in normal and pathological colon, with hsst5, hsst2 and hsst1 mRNAs being the most frequently expressed. The mRNA coding for hsst5, the receptor displaying a better binding selectivity for SS-28, was frequently found in colic tissue, in which SS-28 is predominantly synthesized. Using RT-PCR, Buscail *et al.* (13) have previously noted this very frequent expression of hsst5 mRNA in tumors (71% of tumors whatever the stage). Laws *et al.* (14) also found an increased frequency of hsst5 mRNA expression in early stage tumors as compared with normal mucosa (75% vs 45%). This increase in frequency was lacking in late stage tumors. Our findings showing that hsst5 mRNA was more frequently expressed in tumors, even those in Dukes' stages C and D, than in normal tissues underlines the importance of hsst5 in pathological colonic processes. These observations are interesting in view of the results of Cordelier *et al.* (18) who have demonstrated that hsst5 expressed in Chinese hamster ovary cells mediates the antiproliferative effects of SS analogs by inhibition of

soluble guanidylate cyclase, protein kinase G, and p42 MAP kinase activities. Moreover, these antiproliferative effects of hsst5 were described using cholecystokinin (CCK)-stimulated cells. Interestingly, we have previously shown the existence of CCK-B long and short isoforms and CCK-C receptors in the tumors analyzed in the present study (19).

Previously, Buscail *et al.* (13) have shown that hsst2 frequency of expression decreased with the tumor stage (3/6, 1/5, or 0/3 in Duke's stages B, C, and D respectively). Using RT-PCR and *in situ* hybridization, Laws *et al.* (14) have found that hsst2 mRNA was widely distributed in normal mucosa and stroma in 90% of samples. We found that hsst2 mRNA was expressed at a high frequency throughout all tumor stages without any decrease of expression in advanced stage tumors.

Our results suggest that the recently described SS analogs, particularly those specific to hsst5, could be considered as possible treatments even in advanced stages of colorectal cancer. However, immunohistological studies with specific antibodies are needed to confirm the presence of sst proteins.

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References

1 Lewin MJM. The somatostatin receptor in the GI tract. *Annual Review of Physiology* 1992 **54** 455–468.
2 Lamberts SWJ, Krenning EP & Reubi JC. The role of somatostatin receptors and its analogs in the diagnosis and treatment of tumors. *Endocrine Reviews* 1991 **12** 450–482.
3 Patel YC. Molecular pharmacology of somatostatin receptor subtypes. *Journal of Endocrinological Investigation* 1997 **20** 348–367.
4 Gouyon BA, Reyl-Desmars F, Le Romancer M, Pigeon C, Lewin MJM & Lehy T. *In vivo* effect of somatostatin analogue, lanreotide, and/or GRP antagonist, BIM 26226, on the growth of colon cancer peritoneal carcinomatosis in the rat. *International Journal of Oncology* 1995 **7** 1167–1173.
5 Qin Y, Shally AV & Willems G. Treatment of liver metastases of human colon cancers in nude mice with somatostatin analogue RC-160. *International Journal of Cancer* 1992 **52** 791–796.
6 Goldberg GM, Moertel CG, Wiehand HS, Krook JE, Schutt AJ, Veeder MH *et al.* A phase III evaluation of a somatostatin analogue (octreotide) in the treatment of patients with asymptomatic advanced colon carcinoma. *Cancer* 1995 **76** 962–966.
7 Cascinus S, Del Ferro E, Grianti C, Ligi M, Ghiselli R, Foglietti G *et al.* Inhibition of tumor cell kinetics and serum insulin growth factor I by octreotide in colorectal patients. *Gastroenterology* 1997 **113** 767–772.
8 Florio T & Schettini G. Multiple intracellular effectors modulate physiological function of the cloned somatostatin receptors. *Journal of Molecular Endocrinology* 1996 **17** 89–100.
9 Radulovic SS, Milovanovic SR, Cai RZ & Shally AV. The binding of bombesin and somatostatin and their analogs to human colon cancers. *Proceedings of the Society for Experimental Biology and Medicine* 1992 **200** 394–401.
10 Reubi JC, Horisberger U & Laissue J. High density of somatostatin receptors in veins surrounding human cancer tissue: role in tumor–host interaction. *International Journal of Cancer* 1994 **56** 681–688.
11 Iftikhar SY, Thomas WM, Rooney PS & Morris DL. Somatostatin receptors in human colorectal cancer. *European Journal of Surgical Oncology* 1992 **18** 27–30.
12 Reubi JC, Horisberger U, Waser B, Gebber JO & Laissue J. Preferential location of somatostatin receptors in the germinal centers of human gut lymphoid tissue. *Gastroenterology* 1992 **103** 1207–1214.
13 Buscail L, Saint-Laurent N, Chastre E, Vaillant J-C, Gespach C, Capellà G *et al.* Loss of sst2 receptor gene expression in pancreatic and colorectal cancer. *Cancer Research* 1996 **56** 1823–1827.
14 Laws S, Gough AC, Evans AA, Bains MA & Primerose JN. Somatostatin receptor subtype mRNA expression in human colorectal cancer and normal colonic mucosa. *British Journal of Cancer* 1997 **75** 360–366.
15 Dukes CE & Bussey HJR. The spread of rectal cancer and its effect on prognosis. *British Journal of Cancer* 1958 **12** 309–320.
16 Chomczynski P & Sacchi N. Single-step methods of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry* 1987 **162** 156–159.
17 Dutour A, Kumar U, Panetta R, Ouafik L, Fina F, Sasi R *et al.* Expression of somatostatin receptor subtypes in human brain tumors. *International Journal of Cancer* 1998 **76** 620–627.
18 Cordelier P, Estève J-P, Bousquet C, Delesque N, O'Carroll A-M, Shally AV *et al.* Characterization of the antiproliferative signal mediated by the somatostatin receptor subtype sst5. *Proceedings of the National Academy of Sciences of the USA* 1997 **94** 9343–9348.
19 Biagini P, Monges G, Vuaroqueaux V, Parriaux D, Cantaloube F & De Micco P. The human gastrin/cholecystokinin receptors: type B and type C expression in colonic tumors and cell lines. *Life Sciences* 1997 **61** 1009–1018.

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