Expression of Stem Cell-associated Marker HES77 in Rectal Neuroendocrine Tumors

JUHA JERNMAN[1,5], JAANA HAGSTRÖM[1], HANNA MÄENPÄÄ[8], MATTI J. VÄLIMÄKI[2], HANNU HAAPASALO[5], OLLE NILSSON[6], CHRISTIAN FERMÉR[7], CAJ HAGLUND[3,4*] and JOHANNA AROLA[1*]

[1]Department of Pathology, [2]Division of Endocrinology, Department of Medicine, [3]Department of Surgery, and [4]Research Programs Unit, Translational Cancer Biology, University of Helsinki and Helsinki University Hospital, Helsinki, Finland; [5]Department of Pathology, Fimlab Laboratories, Tampere, Finland; [6]Onson Consulting, Gothenburg, Sweden; [7]Fujirebio Diagnostics AB, Gothenburg, Sweden; [8]Department of Oncology, Helsinki University Central Hospital, Helsinki, Finland

Abstract. *Background: Expression of novel stem cell-associated marker human embryonic stem cell 77 (HES77) was studied in rectal neuroendocrine tumors (NETs), which comprise 10 to 15% of gastroenteropancreatic NETs, some with metastatic potential. Materials and Methods: WHO 2010 classification was applied, and immunohistochemical positivity for HES77 was assessed in 72 primary tumors and 6 metastases. Correlations were calculated between HES77 expression, metastasis and patient survival. Results: Expression of HES77 strongly positively correlated with metastatic potential and poorer prognosis. The proliferative index determined in the metastasis did not correlate with patient survival. Conclusion: Novel stem cell-associated marker HES77 has a strong prognostic value in patients with rectal NETs and may be useful in selecting those who are at-risk for developing metastatic disease, and who may benefit from intensive adjuvant therapy. Proliferative index in the metastasis did not predict for outcome. Characterization of the HES77 epitope would certainly enhance the interest in the antibody.*

Neuroendocrine tumors (NETs) are rare neoplasms occurring in almost any organ, most commonly in the gastrointestinal tract, pancreas and lung.

Rectal NETs originate from endocrine cells of the rectal mucosa, cells that are part of the diffuse endocrine system of the gastrointestinal tract. The rectum harbours mainly serotonin-producing enterochromaffin cells, and L-cells that produce glucagon-like immunoreactants and pancreatic polypeptide-like peptides. Somatostatin-producing D-cells in the colon and rectum are very few in number (1). Most rectal NETs grow slowly and cause symptoms late. They confer a favourable prognosis of 5-year overall survival of 88% to 91%, but some patients have aggressive metastatic disease with much poorer prognosis when compared to patients with localized disease (2-5).

In the novel WHO 2010 classification for neuroendocrine tumors of the pancreas and gastrointestinal tract, all neuroendocrine tumors are considered potentially malignant. These tumors are graded uniformly in every organ of the gastrointestinal tract according to their proliferative index and mitotic frequency (Table I) (6). In our earlier report, we re-classified 73 rectal NETs according to the WHO 2010 classification. During follow-up, 10 tumors had metastasized. None of the G1 tumors showed metastasis, whereas among 11 G2 tumors, 9 had undergone metastasis during follow-up (7).

At histology, prediction of prognosis is difficult. Proliferation is higher in aggressive tumors, but we need better markers of metastatic potential. Stem cell-derived markers represent a new generation of tumor markers. By the hybridoma technique, we produced monoclonal antibodies against a human embryonic stem cell (hESC) line in an attempt to identify new biomarkers. We then studied the immunohistochemical expression of one marker, recognized by monoclonal antibody HES77, in rectal NETs. In this article, the antigen is called HES77, although the exact antigen epitope of this antibody remains to be revealed thus far.

Studies on stem cell marker expression in NETs are not numerous, and many concern NETs of the lung. The protein cluster of differentiation 117 (CD117) has prognostic

*These Authors contributed equally to this study.

Correspondence to: Juha Jernman: Fimlab Laboratories, Teiskontie 35, Tampere PL66, FIN-33101. Tel: +358 331175755, e-mail: juha.jernman@gmail.com

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Table I. *Distribution of grade in a series of 73 consecutive rectal neuroendocrine tumors (NETs) according to the WHO 2010 classification (6).*

WHO 2010 grade	Number	Metastasis	No Metastasis	Ki-67	Mitotic count/ 10 HPF
G1 NET	61	0	61	≤2%	<2
G2 NET	11	9	2	3 to 20%	2 to 20
G3 NEC	1	1	0	>20%	>20
Total	73	10	63		

HPF: High-power fields; NEC: neuroendocrine carcinoma.

Table II. *Correlation between human embryonic stem cell 77 (HES77) and metastases, grade, tumor size, gender, and age by Chi-square test.*

	HES77		
Factor	Negative	Positive	*p*-Value
Metastasis			
No	51	11	<0.001
Yes	1	9	
WHO grade			
G1	50	10	<0.001
G2, G3	2	10	
Size			
≤20 mm	51	16	0.024
>20 mm	1	4	
Gender			
Male	21	7	0.668
Female	31	13	
Age (years)			
<50	19	8	0.373
50-64	20	7	
65-74	8	2	
>75	5	3	

significance in colorectal neuroendocrine carcinoma and mixed adenoneuroendocrine carcinoma (8). Expression of stem cell markers such as ATP-binding cassette sub-family G member 4, CD117, or CD133 provided no prognostic value in NETs of the lung, insulin-like growth factor II mRNA-binding protein 3 is associated with an unfavourable prognosis (10). Immunohistochemical positivity for transcription factors spalt-like transcription factor 4 and sex-determining region Y-box 2 has been detected in pulmonary small cell carcinoma; expression of the latter correlates with adverse prognosis (11, 12).

Materials and Methods

Tumor series and patients. Our tumor series comprised of 73 consecutive rectal NETs from 1980 through 2008 identified from our Pathology Laboratory database (Table I). All tumors were re-classified according to the WHO 2010 classification for gastrointestinal NETs (6). For the 28 men and 45 women, the mean age at diagnosis was 58.3 years. The mean follow-up after surgery was 148 months (median=137 months, range=23 days to 346 months. The last follow-up date was in October 2013.

For all tumors, the mitotic count was very low, with grading based on Ki-67 index. G1 NETs numbered 61, G2 NETs 11, and G3 NEC only one, of large cell type. All tumors stained positive for chromogranin A (CgA), confirming their neuroendocrine nature. Glandular, trabecular, insular or diffuse growth pattern was recorded but failed to correlate with metastatic potential or prognosis. TNM stage we considered unreliable in our tumor series because many tumors were removed in pieces, and invasion of the muscularis propria was difficult to exclude. Lymph node status was available for only two patients, and at the time of diagnosis, very few patients were examined radiologically to exclude metastasis (7).

During follow-up, 10 tumors had metastasized. Tissue material was available for immunohistochemical analysis for six patients with disseminated rectal NET: three of these patients underwent resection of liver metastases, one a liver transplantation, and one a biopsy specimen obtained from a liver metastasis, and one a specimen from a peritoneal metastasis. Proliferative indices were determined in the metastatic tumor tissue as a part of routine diagnostics in each case. Table III shows the proliferative indices.

For the 12 patients with WHO 2010 grade 2 to 3, detailed clinical data came from their charts. Treatment, serological findings (CgA and 5-hydroxyindoleacetic acid) at the time of diagnosis and at occurrence of metastases, and the manner in which the follow-up was carried out were of particular interest (Table IV).

The Ethics Committee of the Helsinki University Central Hospital and The National Authority for Medicolegal Affairs approved the research setting (Approval number 185/E6/06).

HES77 monoclonal antibody. The undifferentiated hESC line SA167 (Cellartis AB, Gothenburg Sweden) served for immunization of the mice. Hybridoma technology was used to create hybridoma cell lines producing monoclonal antibodies against the hESCs. Several monoclonal antibodies were produced against the stem cell line in an identical procedure: the epitope was recognised in only some of them (13). The antibody HES77 showed high specificity for undifferentiated hESCs and loss of reactivity at an early stage of cell differentiation. By immunocytochemistry of hESCs, the target antigen was located in the cell membrane. The antibody stained the cell lysate of the hESC line SA167 only in native conditions, and a conformational-dependent epitope was indicated by western blot data. An attempt to identify the epitope utilized high-affinity peptides selected from a random peptide library displayed on phage for binding to HES77 antigen, but no consensus sequence mimicking the epitope sequence was found. In short: HES77 is a novel antibody, with its antigen epitope and stem cell specificity still undetermined.

Immunohistochemistry. Out of 73 tumors, 72 were available for immunohistochemical analysis, as were six metastases. Tissue sections were de-paraffinised in xylene and rehydrated in an ethanol

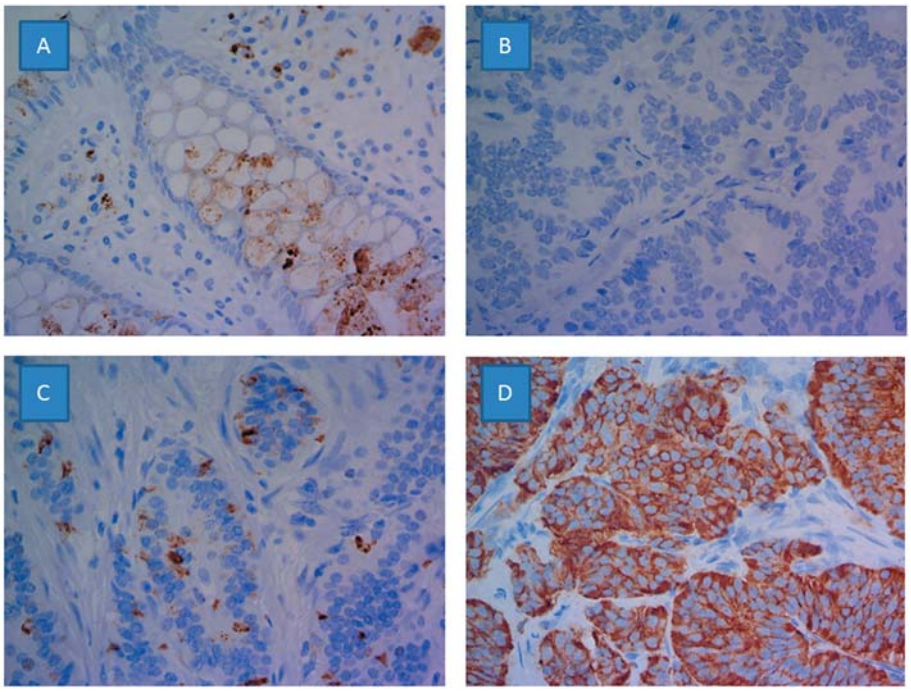

Figure 1. *Expression of HES77 in normal colonic epithelium: Some normal enterocytes as well as white cells stained positively (A). Tumor was negative for HES77 (B). Rectal neuroendocrine tumors with weak (C) and strong positivity (D). Magnification ×400.*

Table III. *Proliferative index and human embryonic stem cell 77 (HES77) for six metastases and their corresponding primary tumors. The prognosis was poor if HES77 expression in metastasis was lower when compared to that of the primary tumor.*

		MIB-1 (% of tumor cells)				HES77	
Sex, age (years)	Tumor grade	Primary tumor	Metastasis	Diagnosis to metastasis (months)	Alive at the end of follow-up	Primary tumor	Metastasis
F, 61	2	5	10	151	Yes	2	4
M, 46	2	3	5	0	No	1	0
M, 42	2	5	10	0	Yes	3	3
F, 23	2	10	10	0	No	3	1
F, 54	2	5	10	51	No	1	0
F, 73	2	5	15	0	No	0	0

series. Antigen retrieval took place in the pretreatment module of an autostainer (Autostainer 480; LabVision UK Ltd., Newmarket, Suffolk, UK). Immunostaining was performed with an automated staining system (Autostainer 480, LabVision Corp., Fremont, CA, USA), using the Dako REAL EnVision detection system (Dako, Glostrup, Denmark). Before the immunostaining procedure, the slides were treated with 0.3% Dako REAL peroxidase-blocking solution to block endogenous peroxidases, followed by primary antibody incubation with HES77 mAb. Primary antibody incubation was followed by a 30-min incubation with Dako REAL EnVision/horseradish peroxidase detection system, with rabbit/mouse (EnVision, Dako) reagent. The slides were finally visualized by Dako REAL DAB+ chromogen for 10 min. The slides were counterstained with Meyer's haematoxylin and mounted in mounting medium (Aquamount; BDH, Poole, Dorset, UK).

Immunohistochemical scoring. Two pathologists (J.J. and J.H.) analyzed the stainings independently while blinded to clinicopathological data. In cases of discordancy, a consensus score served for further analysis. Positive nuclear staining was scored 0 to 4: 0=negative; 1=weak expression (<30% of tumor cells); 2=moderate (30-50%); 3=strong (50-80%); 4=very strong (>80%). For statistical analysis, tumors were divided into two groups: tumors negative for HES77 *vs*. tumors positive for HES77 antigen.

Table IV. *Clinical data on 12 patients with G2 or 3 rectal neuroendocrine tumors. The primary diagnoses are from 1985-2007, and methods of treatment and follow-up were quite diverse, as guidelines were not yet available.*

Patient sex, age (years)	Grade	Metastasis§	At diagnosis-metastasis/ follow-up		Systemic treatment	Method of follow-up	Alive at the end of follow-up
			5-HIAA	CgA			Length of follow-up (months)
F, 57	3	1	–/–	–/–	Chemotherapy	CT, serology	No (22)
F, 61	2	2	Normal/45 (40)	na./Normal	Resection of liver metastasis plus somatostatin analogue	CT, US, chest X-ray, colonoscopy, serology	Yes (195)
M, 46	2	1	Normal/normal	–/Elevated	Chemotherapy, liver transplantation, somatostatin analogue, lutetium octreotate	MRI, CT, serology	No (158)
F, 76	2	1	Normal/normal	–/–	Interferon	US, chest X-ray	No (16)
F, 78	2	2	–/Elevated	–/–	None	Follow-up of clinical symptoms	No (129)
M, 42	2	1	Normal/normal	Normal/ normal	Interferon, somatostatin analogue, chemotherapy, liver resection	CT, PET-CT, serology	Yes (163)
F, 23	2	1	Normal/normal	Normal/normal	Somatostatin analogue, lutetium octreotate, chemotherapy	CT, serology	No (69)
F, 54	2	2	Normal/normal	–/Elevated	Somatostatin analogue, interferon, lutetium octreotate	CT, serology	No (103)
F, 65	2	1	–/–	–/–	None	Follow-up of clinical symptoms	No (25)
F, 73	2	1	na./na.	na./na.	na.	na.	No (1)
F, 30	2	0	Normal/normal	–/na.	None	Colonoscopy	Yes (142)
F, 42	2	0	Normal/normal	na./na.	None	Colonoscopy	Yes (198)

§0: None, 1: concomitant, 2: later. 5-HIAA: 5-hydroxyindoleacetic acid; CgA: chromogranin A; –: not examined; na.: information not available; CT: computed tomography; US: ultrasound; MRI: magnetic resonance imaging; PET: positron-emission tomography.

Statistical analysis. Assessment of the correlation between HES77 expression and clinicopathological variables was carried out with the chi-square test; univariate survival analysis with Kaplan–Meier life-tables and the log rank test, and multivariate analysis with the Cox regression test, with distant metastasis, *i.e.* disease-free survival, and disease-specific survival as the end-points. *p*-Values less than 0.05 were considered statistically significant.

Results

HES77 positivity in normal tissue. Normal enterocytes of the colon, as well as some histiocytes and lymphocytes, exhibited focal, granular cytoplasmic positivity, whereas nuclei were negative (Figure 1).

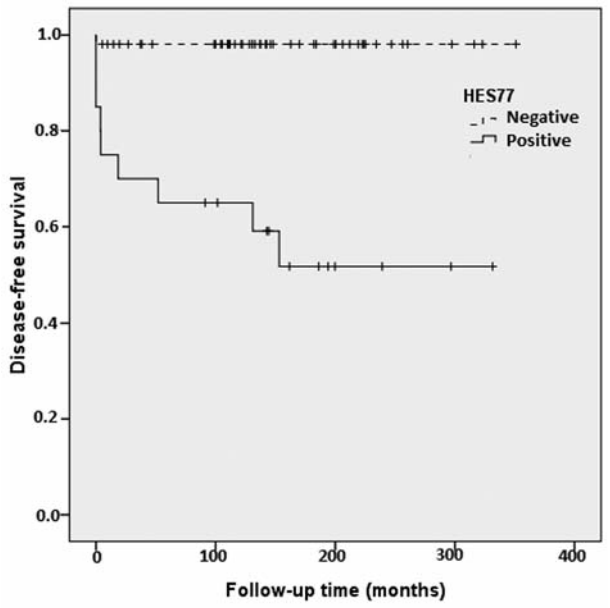

Figure 2. *Kaplan–Meier life-table with disease-free survival as the end-point. HES77 strongly correlated with metastatic potential (p<0.001).*

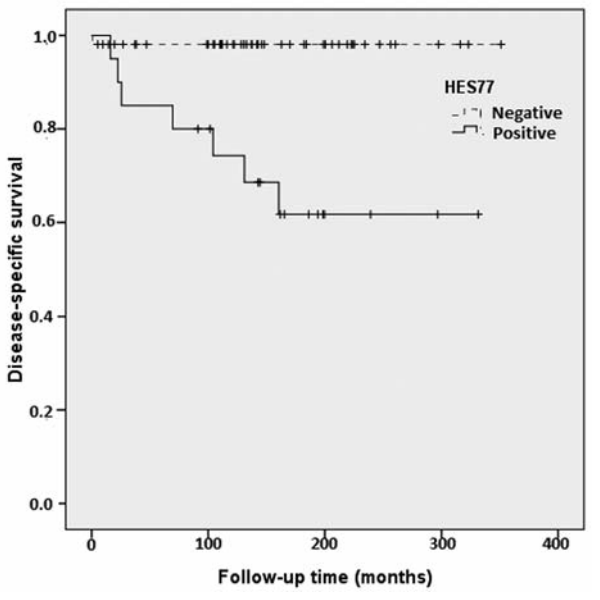

Figure 3. *Kaplan–Meier life table with disease-specific survival as the end-point shows strong correlation between HES77 and poor prognosis (p<0.001).*

HES77 positivity in primary tumors. Cytoplasmic and membranous positivity for HES77 antibody appeared in 20 tumors (28%), whereas 52 tumors (72%) were negative. Out of positive tumors, 12 exhibited weak, three moderate and five strong positivity. In all tumors, the mean HES77 score was 0.46 with a median of 0. In metastatic tumors, the mean HES77 score was 1.9 with a median of 2, and in non-metastatic tumors 0.23 with a median of 0.

Patients were divided into two groups: tumors negative *vs*. positive for HES77. In chi-square univariate analysis, HES77 positivity correlated with metastatic potential ($p<0.001$), grade by WHO 2010 ($p<0.001$), and tumor diameter ($p=0.024$), (Table II). It also correlated with expression of cyclin A ($p<0.001$) (14), and prospero homeobox 1 (PROX1) ($p=0.020$) (unpublished data), which we earlier showed to correlate with poorer prognosis. HES77 correlated neither with age ($p=0.373$) nor sex ($p=0.668$).

Correlation of HES77 with metastatic potential and poorer disease-specific survival is shown in life-table analysis (log-rank test, $p<0.001$) (Figures 2 and 3). In multivariate analysis, however, it did not emerge as an independent prognosticator. Other parameters included in this analysis were the WHO 2010 classification, cyclin A and PROX1 expression, and tumor size. The only independent prognostic factor was the WHO 2010 classification. In patients with disseminated tumors, expression of HES77 antigen in primary tumors showed no correlation with progression-free survival.

HES77 positivity in metastases. Six metastases were available for immunohistochemistry. In two cases, the expression was similar to that of the primary tumor. In one case, the metastasis expressed HES77 more strongly than did the primary lesion, and in three cases, the metastases expressed HES77 less strongly than did their primary tumors. Out of patients with increased or similar expression of HES77 in metastases, one out of three had died of metastatic NET by the end of follow-up, whereas all three patients with lower expression of HES77 in metastases had died.

Proliferative index in metastases. In only one patient were the proliferative indices in primary and metastatic tumor tissue identical (Table III). This patient had been treated with somatostatin analogue, lutetium octreotate and chemotherapy. In five patients, the proliferative index was higher in metastatic tumor tissues than in the primary tumor. Of these, four patients had received systemic treatment including somatostatin analogue, interferon, chemotherapy, and lutetium octreotate (Table IV). Out of six patients with tissue material available from metastasis, four had died of metastatic NET by the end of follow-up: two patients were alive, and in both cases, the metastatic tumor had a higher proliferative index than did the primary tumor; one patient had been treated with resection of liver metastasis and somatostatin analogue, and the other with interferon, somatostatin analogue and chemotherapy.

Discussion

We report that 28% of rectal NETs express the novel stem cell-derived marker HES77. Expression of HES77 antigen correlates with metastatic potential and patient survival. Decreased expression of HES77 in metastasis compared to that for the primary tumor predicted poor survival.

Rectal NETs are quite rare, and in recent years their classification and treatment have undergone substantial changes. The term 'carcinoid' is no longer recommended, since that term is associated with benign behaviour. Most rectal NETs remain local, but some are clinically aggressive. The WHO 2010 classification considers all gastrointestinal NETs to be potentially malignant, with the ability to metastasize. Grading that is based on Ki-67 index and mitotic count correlates well with the metastatic potential of rectal NETs (14). This is helpful for clinicians in determining which patients need the closest monitoring; patients with a G1 NET do not require for intensive follow-up, whereas patients with a G2 NET are prone to developing metastases even very late, making careful monitoring necessary.

Few studies involve the proliferative index in NET metastases. Lindholm *et al*. assessed 13 cases of metastatic midgut NETs: in 10 cases, the Ki-67 values in the primary tumor and metastatic tumor tissue were identical. In one case, the primary tumor was G2, but the lymph node and organ metastases had lower Ki-67 (<2%, G1). In two cases, the primary tumors were G1, and their corresponding organ metastases exhibited a proliferative index consistent with G2 (3-20%). The significance for prognosis was not under study, but the authors state that varying levels of differentiation were present in the primary tumors and metastases (15). In our study, the two patients alive at the end of follow-up had a higher proliferative index in their metastases than in their primary tumors. Thus, the proliferative index determined from the metastatic tumor tissue does not seem to predict outcome.

In conclusion, we report that the expression of a novel stem cell-associated marker HES77 correlates with prognosis in patients with rectal NETs, although less strongly than does grade. When the metastasis exhibits weaker expression of HES77 than the primary tumor, risk of a lethal outcome is high. Characterization of the still unknown HES77 epitope would increase the interest in this novel marker. The proliferative index of the metastatic tumor tissue does not seem to correlate with patient survival. The number of patients with available tissue material from metastatic lesions was very small, only six, which can be considered a weak point of the study, and more extensive studies are necessary to confirm our findings. When assessed in the metastasis, HES77 may be a useful marker in identifying patients that might benefit from intensive treatment protocols.

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