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RAPID COMMUNICATION

Gene expression in midgut carcinoid tumors: Potential targets for immunotherapy

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Abstract

Classical midgut carcinoids are serotonin-secreting tumors derived from enterochromaffin cells in the gut. Metastatic disease represents a therapeutic challenge and immunotherapy implies a novel approach for treatment. In order to define antigens suitable for T-cell therapy with a preferential expression in midgut carcinoid tissue a broad screening of genes with preferential neuroendocrine restriction, genes described as over-expressed in various malignancies, and genes encoding cancer-testis associated antigens was performed. The expression of 32 genes was analyzed by reverse transcription polymerase chain reaction (RT-PCR) in 28 midgut carcinoid specimens, in the cell line BON and in normal tissues. Immunohistochemistry (IHC) was used to evaluate protein expression. Expression is shown of genes that have previously not been observed in midgut carcinoid tumors, such as Survivin and GAGEs. Also the expression is confirmed of genes that encode pivotal proteins in enterochromaffin cells, such as TPH1 and VMAT1, and their tissue-restricted expression is indicated. In addition, gene expression of IA-2 and CDX-2 in normal gastrointestinal (GI) tract and in tumor is shown. Protein expression of TPH, VMAT1, and Survivin was detected in tumor tissue. This study elucidates that TPH1, VMAT1, and Survivin should be further investigated as potential target antigens for T cell-mediated immunotherapy of midgut carcinoids.

Neuroendocrine tumors of the gut, by tradition known as carcinoids, are rare tumors originating from the dispersed neuroendocrine cell system. The incidence rate is 2.5 in 100 000 people per year. Classical midgut carcinoids arise from the enterochromaffin cells in the ileum, cecum, and ascending colon. They are characterized by the secretion of serotonin and other hormones that often cause clinical symptoms in patients, denoting carcinoid syndrome. Most patients have developed metastatic disease at the time of diagnosis due to the lack of sensitive and specific methods for early detection of primary tumors [1–4]. Curative treatment is limited in these patients and development of new therapies, such as immunotherapy, is therefore highly warranted. Appropriate candidate antigens for T-cell therapy should, besides immunogenicity, demonstrate high-level expression in the tumor, and restricted expression in normal tissues. Several studies have led to the identification of tumor-specific and tumor-associated antigens. Tumor vaccines based on cancer-testis associated antigens, melanoma-associated antigens and prostate-specific differentiation antigens have been used in clinical trials [5–8].

In this study we analyzed the expression of 32 target genes in order to define potential antigens suitable for cytotoxic T-cell (CTL) -mediated immunotherapy. Amphiphysin [9,10], Chromogranin A (CGA) [11], IA-2 [12,13], Protein gene product 9.5 (PGP9.5) [14], Synaptophysin [15], Tryptophan hydroxylase (TPH) 1 [16], and Vesicular monoamine transporters 1 and 2 (VMAT1, VMAT2) [17,18] represent proteins that are considered essential for enterochromaffin cell function, i.e. tissue-restricted differentiation antigens. CDX-2 [19], Mac 2 binding protein (M2BP) [20,21], Mucin1 (MUC1) [22,23], RAGE-1 [24,25], SART-1 [26], Survivin

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[27,28] and TROP-2 [29] represent proteins that are over-expressed in many solid tumors. BAGE-1, CAGE, GAGE-1,2,8, GAGE-3-7b, HAGE, MAGE-1, MAGE-3, NY-ESO-1, PAGE-1/GAGE-9, SAGE, and XAGE-1 [5,30–33] are shared-tumor antigens, proteins that are expressed in many different malignancies but not in normal tissue with the exception of the testis. We investigated the expression pattern of these genes by RT-PCR in a panel of up to 28 midgut carcinoid specimens; 14 primary tumors and 14 metastases from both treated and untreated patients, in the human endocrine pancreatic tumor cell line BON and in normal tissue. We also investigated protein expression by IHC.

Material and methods

Tumor specimens

Tumor specimens from 25 patients diagnosed with midgut carcinoid tumor at the Department of Endocrine Oncology, University Hospital, Uppsala, Sweden were included in the study. In all, 14 patients were untreated before surgery while the remaining 11 had been treated with interferon and/or somatostatin analogs prior to operation. A total of 28 tumor specimens, 14 primary tumors and 14 metastases, were analyzed by RT-PCR. Protein expression was investigated by IHC in 12 specimens, 7 primary tumors and 5 metastases (see Table I for further details). The study was approved by the local ethical committee of Uppsala University, Uppsala, Sweden.

Cell culture

The human endocrine pancreatic tumor cell line BON, a generous gift from Prof. J.C. Thompson, Galveston, TX, was cultured in DMEM (Dulbecco's mem with Glutamax-I) and F12 K Nutrient Mixture (Kaighn's modification) at a 1:1 ratio, supplemented with 10% of fetal bovine serum (FBS), 1% penicillin/streptomycin (PeSt) and 1 mM sodium-pyruvate. All cell culture reagents were from Invitrogen, Carlsbad, CA, USA, except sodium-pyruvate (Sigma, St Louis, MO, USA).

RNA isolation and RT-PCR analysis

Total RNA was isolated from snap-frozen midgut carcinoid specimens and BON cells using TRIzol reagent (Invitrogen) according to the manufacturer's

Table I. Midgut carcinoid specimens and analyses.

Patient	Sex/age[1]	Treatment	Tumor specimen	Tissue	Analyses
1	F/68	–	Primary	Ileum	RT-PCR, IHC
2	M/80	–	Primary	Ileocecum	RT-PCR
3	M/50	–	Primary	Ileum	RT-PCR, IHC
4	F/76	–	Primary	Ileum	RT-PCR, IHC
5	M/63	–	Primary	Ileum	RT-PCR
6	M/54	–	Primary	Ileum	RT-PCR
7	F/63	–	Primary	Ileum	RT-PCR, IHC
8	F/67	–	Primary	Ileum	RT-PCR
9	F/43	–	Primary	Ileum	RT-PCR, IHC
10	F/56	–	Primary	Ileum	RT-PCR
11a	M/70	–	Primary	Ileum	RT-PCR, IHC
11b	″	–	Metastasis	Mesentery	RT-PCR, IHC
12a	F/64	–	Primary	Ileum	RT-PCR
12b	″	–	Metastasis	Omentum	RT-PCR
13	F/50	–	Metastasis	Mesentery	RT-PCR
14	M/56	–	Metastasis	Liver	RT-PCR
15	F/57	+	Primary	Ileum	RT-PCR
16a	F/52	+	Primary	Ileum	RT-PCR, IHC
16b	″	+	Metastasis	Mesentery	RT-PCR, IHC
17	M/54	+	Metastasis	Mesentery	RT-PCR, IHC
18	M/51	+	Metastasis	Mesentery	RT-PCR
19	M/54	+	Metastasis	Mesentery	RT-PCR
20	F/44	+	Metastasis	Mesentery	RT-PCR
21	M/41	+	Metastasis	Mesentery	RT-PCR
22	F/40	+	Metastasis	Liver	RT-PCR, IHC
23	M/52	+	Metastasis	Liver	RT-PCR
24	M/52	+	Metastasis	Liver	RT-PCR, IHC
25	F/64	+	Metastasis	Liver	RT-PCR

RT-PCR, reverse transcription-PCR, IHC, immunohistochemistry.
[1]Age at the time of operation.

protocol. The RNA concentration was determined by spectrophotometer absorbance measurement (BioPhotometer, Eppendorf, Germany) and RNA quality was analyzed by electrophoresis on 1% agarose gel. Two μg of each RNA sample were subjected to cDNA synthesis using SUPERSCRIPT™ RNase H⁻ Reverse Transcriptase (Invitrogen) according to the manufacturer's protocol. PCR was performed to analyze expression of 32 genes using the primers described in Table II and GAPDH expression was used as a positive control. To amplify cDNA only and not trace amounts of genomic DNA, primer pairs were designed to generate PCR products spanning either two or more exons, with a singular exception for the TROP-2 gene, which consists of only one exon. All primers were designed de novo except for primers previously published for BAGE-1[34], GAGE-1,2,8, [30], GAGE-3-7b [30] and MAGE-3 [31]. PCR amplification was performed for 1 min at 94°C, 1 min at 58°C (except for GAGEs where the annealing temperature was 55°C) and 1 min at 72°C for 35 cycles, followed by a 7 min elongation step at 72°C. The expected sizes of the PCR products are indicated in Table II. The PCR reactions were analyzed on agarose gels and specific products were cloned into TA vectors (Invitrogen) and subsequently sequenced. PCR analyses using commercially available cDNA (Clontech Laboratories, Palo Alto, CA, USA) were performed to investigate gene expression in normal tissues. The PCR products were quantified by densitometry using the NIH Image 1.62 software.

Immunohistochemistry

Formalin-fixed paraffin-embedded tumor material was obtained from the Department of Pathology, Uppsala University Hospital, Sweden. Tissue sections were deparaffinized and subjected to microwave treatment in 10 mM citrate buffer, pH 6. Endogenous peroxidase activity was blocked in a 0.6% hydrogen peroxide solution. The sections were incubated at 4°C overnight with a mouse monoclonal antibody to TPH (clone WH-3, Sigma) and rabbit polyclonal antibodies to VMAT1 (H100, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and Survivin (ab469, Abcam Ltd., Cambridge, UK). All antibodies were diluted 1:100 in ChemMate Antibody Diluent (DAKO Cytomation, Glostrup, Denmark). Incubation with diluent only served as negative controls. Bound antibody was visualized using mouse or rabbit DAKO EnVision+™ Peroxidase (DAKO) followed by Ready-to-use AEC Substrate-Chromogen (DAKO) according to the manufacturer's instructions. Appropriate washing with PBS containing 0.05% tween was performed between each step. The sections were finally counterstained in Mayer's hematoxylin (HistoLab Products AB, Gothenburg, Sweden), mounted, and evaluated. A pathologist (MT) examined the sections microscopically and based on the percentage of positively stained tumor cells estimated protein expression as: (a) negative, $\leq$10% positive staining; (b) moderate, 10–50% positive staining; (c) extensive, $\geq$50% positive staining; (d) universal, $\geq$90% positive staining.

Results

Gene expression in midgut carcinoids, BON cells and normal tissue

Expression of 32 potential tumor antigens was investigated by RT-PCR in a panel of 16 untreated tumor specimens and BON cells. CGA and Synaptophysin, which are used in the diagnosis of midgut carcinoids, showed intense PCR amplicons, thus confirming a significant amount of tumor RNA in the sample. In addition, the potential differentiation antigens IA-2, PGP 9.5, TPH1, VMAT1, and VMAT2 yielded intense PCR amplicons in all specimens and BON. A majority of specimens also showed strong expression of all antigens described as over-expressed in tumors, except RAGE-1. The GAGE genes were the only shared tumor antigens to be expressed in a majority of carcinoid specimens (15/16) and BON. BON cells were also positive for PAGE-1/GAGE-9. A minority of specimens showed faint PCR amplicons of NY-ESO (1/16), MAGE-1 (5/16) and XAGE (3/16). The complete results of this screening are given in Table III.

Genes that were expressed by a majority of carcinoid specimens from untreated patients were further investigated for their expression in normal tissue. RT-PCR was performed on commercial cDNA from a number of vital organs and the GI tract. Based on this screening a number of antigens described as differentiation antigens or antigens over-expressed in tumors were excluded as carcinoid tumor antigens owing to their ubiquitous and intense expression in normal tissue. The complete results of this screening are given in Tables IV and V.

Genes that showed a more restricted expression in normal tissue or showed an expression thought to be derived from normal neuroendocrine cells such as IA-2, TPH1, VMAT1, CDX-2, Survivin, and GAGEs were further screened for in a panel of 12 carcinoid specimens from treated patients. The antigens of interest were expressed in a vast majority of these specimens as well, as shown in Table III. Expression of potential antigens by a representative carcinoid specimen is shown in Figure 1.

Table II. Oligonucleotide primers for RT-PCR analyses.

Target	Primers (5′ to 3′)	Product size
Amphiphysin	TGGAAACACTGCATGATTTTGAGG (S) CATTAAGAGCATAATAACAAAAGTG (AS)	302 bp
Chromogranin A (CGA)	CGAGGCTACCCCGAGGAGAAGAAA (S) CAGAAGTTATTGCAGTTGTGCCC (AS)	454 bp
IA-2	GAGCTTCTACCTGAAGAACGTGCA (S) ACTGGGGCAGGGCCTTGAGGATG (AS)	382 bp
Protein Gene Product 9.5 (PGP 9.5)	CTATGAACTTGATGGACGAATGCC (S) TACAGACAGAAACCAAAGTAGCCA (AS)	443 bp
Synaptophysin	CAGGCTGCACCAAGTGTACTTTGAT (S) GAACACAGCCGTGGCCAGAAAGTC (AS)	224 bp
Tryptophan hydroxylase 1 (TPH1)	TGCACTTTCTGGACATGCCAAAGTA (S) ATACTCGGCTTCCTGCTGACCTTA (AS)	303 bp
Vesicular Monoamine Transporter 1 (VMAT1)	TATGCTCCACTCTGCTACTACCTGC (S) TGCGGCACCAAGGCATAGAGGAAGA (AS)	372 bp
Vesicular Monoamine Transporter 2 (VMAT2)	TCCTTCTGCTGGTGGTGCTATTGCA (S) GGGATGGATGGTATGACTAAGACAG (AS)	337 bp
CDX-2	AGTGAAAACCAGGACGAAAGACAAA (S) CACTGAGGCTTGCAGGGAAGACAC (AS)	337 bp
Mac-2-binding protein (M2BP)	GTGTGCCCATGGTCAGGGACCTTC (S) GAAGGGCACAGTGTGGAATTCCAG (AS)	539 bp
Mucin1 (MUC1)	GATACCTACCATCCTATGAGCGAG (S) GTTTTATTCAGTCCAGTTCAGGATC (AS)	461 bp
RAGE-1	TGAAGTGCATCCCTGCGAGCAAGAA (S) ACCTTCCAACCACGCCCAACACATCA (AS)	441 bp
SART-1	GACTTCAAGGAGAAGGACGGCTACA (S) TAATATGAAGGTTGAGGCAGGGCCG (AS)	356 bp
Survivin	AACCCTTGGTGAATTTTTGAAACTG (S) AAGCAGCCACTGTTACCAGCAGCA (AS)	363 bp
TROP-2	GGATCGTTTGCAAGTAACTGAATC (S) CATTACCAATATCAGTGGCAGTAAG (AS)	338 bp
BAGE-1	GCTGGAGCCTGTAACACCGTGGC (S) GTATATGTCAGAAATACTGCACAGTCC (AS)	840 bp
CAGE	GAAGATCCTACATCCCTGATGAAAA (S) ACTCTTGAAAGTAGCTCTATTGGCT (AS)	302 bp
GAGE-1,2,8	GACCAAGACGCTACGTAG (S) CCATCAGGACCATCTTCA (AS)	244 bp
GAGE-3-7b	GACCAAGGCGCTATGTAC (S) CCATCAGGACCATCTTCA (AS)	244 bp
HAGE	GGAGGACTGGTGTTTCCATTACAACT (S) TTAAAGAAAGTATAAAACACTGGATT (AS)	366 bp
NY-ESO-1	AGAGCCGCCTGCTTGAGTTCTACCT (S) TAAGCCGTCCTCCTCCAGCGACAAA (AS)	408 bp
MAGE-1	CAGAGGAGCACCAAGGAGAAGATC (S) CTCTGGGGAGGATCTGTTGACCCA (AS)	264 bp
MAGE- 3	TGGAGGACCAGAGGCCCCC (S) GGACGATTATCAGGAGGCCTGC (AS)	680 bp
PAGE-1 (GAGE-9)	AGCCTGAAGCTGATAGCCAGGAACT (S) GCAGAAGGCTGTAAAGCTTTATTGG (AS)	357 bp
SAGE	GAAAGAATTTTCATTTTGCTTGAAG (S) GCAGCTTTTACTAACAGAGATTTAT (AS)	352 bp
XAGE-1 (1a, 1b, 1c)	AAACACAGAACCACACAGCCAGTCC (S) CAGCTTGCGTTGTTTCAGCTTGTCT (AS)	313 bp
XAGE-1 (1d)	AAACACAGAACCACACAGCCAGTCC (S) TTGTGGTTGCTCTTCACCTGCTTCT (AS)	294 bp
Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH)	CTTTGGTATCGTGGAAGGACTCATG (S) GTCATACCAGGAAATGAGCTTGACA (AS)	445 bp

Table III. Gene expression in midgut carcinoid specimens and BON cells.

	Untreated		Treated			
Antigen	Primary	Metastases	Primary	Metastases	Total	BON
Amphiphysin	10/12 +	2/4 +			12/16	−
CGA	12/12 ++	4/4 ++			16/16	++
IA-2	12/12 ++	4/4 ++	2/2 ++	10/10 ++	28/28	++
PGP 9.5	12/12 ++	4/4 ++			16/16	++
Synaptophysin	12/12 ++	4/4 ++			16/16	++
TPH1	12/12 ++	4/4 ++	2/2 ++	10/10 ++	28/28	++
VMAT1	12/12 ++	4/4 ++	2/2 ++	9/10 ++	27/28	++
VMAT2	12/12 ++	4/4 ++			16/16	++
CDX-2	11/12 ++	4/4 ++	2/2 ++	10/10 ++	27/28	++
M2BP	12/12 ++	4/4 ++			16/16	++
MUC1	12/12 ++	4/4 ++			16/16	++
RAGE-1	0/12 −	0/4 −			0/16	−
SART-1	12/12 ++	4/4 ++			16/16	++
Survivin	10/12 ++	4/4 ++	2/2 ++	10/10 ++	26/28	++
TROP-2	12/12 ++	4/4 ++			16/16	++
BAGE-1	0/12 −	0/4 −			0/16	−
CAGE	0/12 −	0/4 −			0/16	−
GAGE-1,2,8	11/12 +	4/4 +	2/2 +	6/10 +	23/28	+
GAGE-3-7b	11/12 +	4/4 +	2/2 +	10/10 +	27/28	+
HAGE	0/12 −	0/4 −			0/16	−
NY-ESO	0/12 −	1/4 +			1/16	−
MAGE-1	2/12 +	3/4 +			5/16	−
MAGE-3	0/12 −	0/4 −			0/16	−
PAGE-1 (GAGE-9)	0/12 −	0/4 −			0/16	+
SAGE	0/12 −	0/4 −			0/16	−
XAGE-1 (1a, 1b, 1c)	2/12 +	1/4 +			3/16	−
XAGE-1 (1d)	0/12 −	0/4 −			0/16	−

−absent, +faint, ++intense.

Protein expression of TPH, VMAT1, and Survivin in midgut carcinoids

Protein expression of TPH, VMAT1, and Survivin was evaluated with immunohistochemistry on 12 carcinoid specimens, 7 primary tumors, and 5 metastases (see Table I). All antibodies stained carcinoid tumor cells specifically. In addition, TPH and VMAT1 antibodies also stained cells at the base

Table IV. Gene expression in normal tissues.

	Placenta	Testis	Brain	Heart	Lung	Skeletal Muscle	Kidney	Adrenal Gland	Liver	Pancreas
Amphiphysin	−	+	+	−	−	−	−	n.d	−	+
CGA	−	+	+	−	+	−	−	+	−	+
IA-2	−	+	+	−	−	−	−	−	−	+
PGP 9.5	+	+	+	+	+	−	+	+	+	+
Synaptophysin	−	+	+	+	+	−	+	−	+	+
TPH1	−	+	+	+	+	−	−	−	−	+
VMAT1	−	−	−	−	−	−	−	+	−	−
VMAT2	+	+	+	+	+	−	+	+	+	+
CDX-2	−	−	−	−	−	−	−	−	−	+
M2BP	+	+	+	+	+	+	+	+	+	+
MUC1	+	+	−	−	+	−	+	+	−	+
SART-1	+	+	+	+	+	+	+	+	+	
Survivin	+	+	−	−	−	−	−	−	−	−
TROP-2	+	+	−	−	+	−	+	+	+	+
GAGE-1,2,8	−	+	−	−	−	−	−	−	−	+
GAGE-3-7b	−	+	−	−	−	−	−	−	−	+

n.d. =not done.

Table V. Gene expression in normal gastrointestinal tract.

	Esophagus	Stomach	Duodenum	Jejunum	Ileum	Ileocecum	Cecum	Asc. colon	Trans.colon	Desc.colon	Rectum
Amphiphysin	−	+	+	+	+	+	+	+	+	−	−
CGA	+	+	+	+	+	+	+	+	+	+	+
IA-2	−	+	+	+	+	+	+	+	+	+	+
PGP 9.5	+	+	+	+	+	+	+	+	+	+	+
Synaptophysin	+	+	+	+	+	+	+	+	+	+	+
TPH1	−	−	+	+	+	+	+	+	+	+	+
VMAT1	−	+	+	+	+	+	+	+	+	+	+
VMAT2	+	+	+	+	+	+	+	+	+	+	+
CDX-2	−	−	+	+	+	+	+	+	+	+	+
M2BP	+	+	+	+	+	+	+	+	+	+	+
MUC1	+	+	+	+	+	+	+	+	+	+	+
SART-1	+	+	+	+	+	+	+	+	+	+	+
Survivin	+	+	+	+	+	+	+	+	+	+	+
TROP-2	+	+	+	−	+	+	+	+	+	−	+
GAGE-1,2,8	−	+	−	−	−	−	−	−	−	−	−
GAGE-3-7b	−	+	−	−	−	−	−	−	−	−	−

of the ileal crypts, which constitute normal enterochromaffin cells. TPH was regarded to have an extensive expression in all sections except for 2 metastases that showed moderate expression. The VMAT1 antibody yielded the highest intensity of all IHC stainings performed. All primaries and metastases showed extensive expression of VMAT1 and a majority of sections were universally stained. The Survivin antibody gave a more heterogeneous result. The intensity of the stainings was in general faint; however, scattered cells within the tumor mass revealed stronger intensity. Four primary tumors showed extensive Survivin expression, while the remaining 3 showed moderate expression. One metastasis showed extensive Survivin expression, 2 metastases showed moderate expression, and 2 metastases were regarded as negative. A summary of the results is given in Table VI and representative stainings are shown in Figure 2.

Discussion

The gene expression profile of midgut carcinoid tumors is unknown. This is partly due to the fact that midgut carcinoids are extremely rare and that it is difficult to obtain sufficient amounts of normal neuroendocrine cells from the GI tract as controls for construction of cDNA libraries that could be used for serological identification of antigens by recombinant expression cloning (SEREX) [35] and microarray analysis. If genes with antigenic properties and preferential expression in carcinoids can be identified, the gene products may be used as targets for novel therapy such as dendritic cell vaccines. Dendritic cells can be modified to present cancer-associated antigens in various ways and used to evoke an anti-tumor CTL response [36–38]. In this study, we investigated the expression of 32 potential antigens by RT-PCR in a broad panel of classical midgut carcinoid tumors. We also investigated the expression of potential antigens in panels of normal tissues. The aim was to identify genes with a preferential expression in carcinoids whose gene products could be evaluated for use in T-cell-mediated therapy.

As expected, the genes encoding pivotal proteins in enterochromaffin cells such as CGA [11], PGP9.5 [14], Synaptophysin [15], TPH1 [16], VMAT1, and VMAT2 [17,18] were robustly expressed in midgut carcinoid tissue. We also confirmed the expression of IA-2 [12,13] and CDX-2 [19]. In addition, our analyses revealed expression of several genes in the

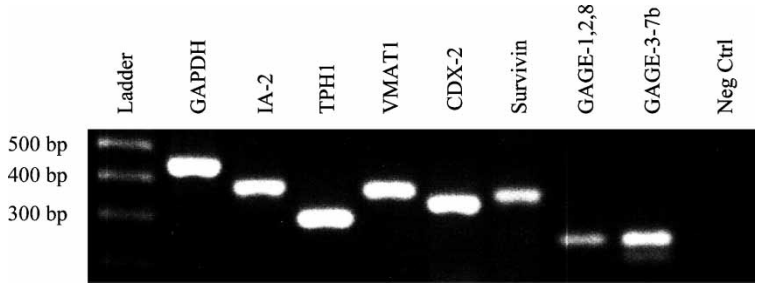

Figure 1. Gene expression of potential antigens for immunotherapy in a representative midgut carcinoid liver metastasis from patient 14.

Table VI. IHC on midgut carcinoid specimens.

Antibody	Primary	Metastases	Total positive staining
TPH (WH-3)	7/7 ++	3/5 ++	12/12
		2/5 +	
VMAT1 (H100)	7/7 ++	5/5 ++	12/12
Survivin (ab469)	4/7 ++	1/5 ++	10/12
	3/7 +	2/5 +	
		2/5 −	

++extensive, +moderate, −negative.

tumor area that have previously not been described in midgut carcinoid tumors, such as: Amphiphysin [9,10], M2BP [20,21], MUC1 [22,23], SART-1 [26], Survivin [27,28], TROP-2 [29], GAGE-1,2,8 and GAGE-3-7b [30]. We analyzed the expression of these genes in normal tissue and excluded the genes that show broad expression in normal tissues from further protein expression analyses, including MUC1 and M2BP, although they are already under investigation in immunotherapeutic protocols [21,23]. We focused our attention on TPH1, VMAT1, Survivin, and GAGEs to verify the possibility to use their gene products as target antigens for T-cell-mediated therapy. TPH is the first-step and rate-limiting enzyme in the process of serotonin synthesis. We have shown the expression of this enzyme in carcinoid tissue and normal enterochromaffin cells by IHC. A new isoform of TPH, now referred to as TPH2, has recently been described [16]. This isoform is expressed exclusively within the central nervous system (CNS) and its expression within the brain is far more abundant than the previously known TPH1. TPH1 is expressed in the pineal gland but mainly by neuroendocrine cells in the gut, and targeting the TPH1 isoform selectively might thus not affect brain serotonin levels. A group of patients with the rare autoimmune disorder APS1 exhibit symptoms of intestinal dysfunction, and biopsies from some of these patients reveal a complete lack of serotonin-producing cells in the GI tract. The patients exhibit IgG autoantibodies against TPH, thus proving that

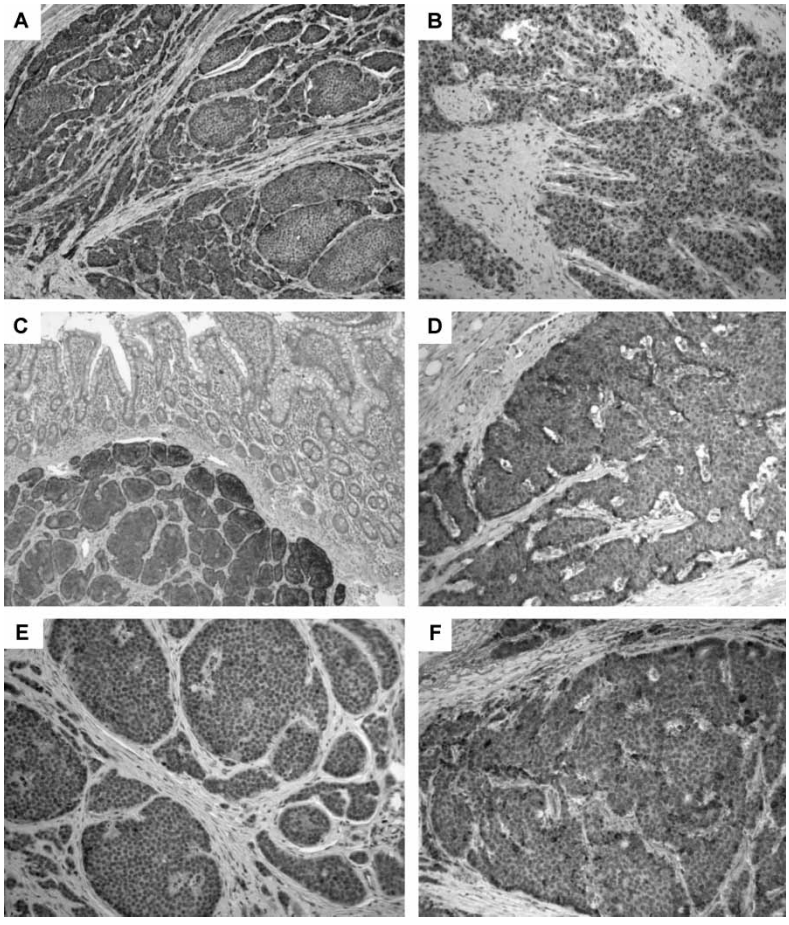

Figure 2. IHC on midgut carcinoid tissue. Representative stainings are shown; (A) TPH, primary tumor from patient 7, (B) TPH, metastasis from patient 24, (C) VMAT1, primary tumor from patient 9, (D) VMAT1, metastasis from patient 16, (E) Survivin, primary from patient 11, (F) Survivin, metastasis from patient 17. (A, B, D, E, F at 10 ×magnification, C at 5 ×magnification).

tolerance has been broken against T-helper epitopes of this protein [39].

VMATs mediate the transport of monoamines like dopamine, adrenaline, noradrenaline, serotonin and histamine from the cytoplasm into secretory vesicles of neurons and neuroendocrine cells. Expression of VMAT1 has been described mainly in enterochromaffin cells of the gut and the adrenal medulla [17,40,41]. Our gene expression analysis confirms that VMAT1 RNA expression in normal tissues is restricted to cells from the GI tract and adrenal gland. Total brain did not show VMAT1 expression although detailed analysis revealed a faint band in substantia nigra (data not shown). We have shown by IHC that the VMAT1 protein is strongly expressed in primary as well as metastasized carcinoid tumors.

Survivin is a member of the inhibitor of apoptosis protein (IAP) family and is highly expressed in a variety of human cancers. Recently its immunogenicity has been proved with CTL recognition of several HLA-restricted antigenic peptides [27,28]. We have shown mRNA expression in a majority of carcinoids and expression of the protein by IHC.

Previous analyses have shown that the GAGE genes are expressed during ontogenesis, in normal adult germ-line cells of the testis as well as in a number of solid tumors. Two antigenic peptides derived from GAGE proteins have been identified [30]. Although GAGE amplicons have been detected in the examined tumors, GAGE protein is not detectable in the same material by Western blot analyses (data not shown). Therefore, the feasibility of using GAGE antigens as potential targets for T-cell-mediated immunotherapy of midgut carcinoid tumors needs further investigation.

Protein expression of IA-2 and CDX-2 has been shown by other authors in midgut carcinoid tissue [12,19], and the expression has been further validated by our RNA analysis. We believe these proteins may be used as potential target antigens as well. Several type I diabetes patients show IgG autoantibodies against IA-2, thus indicating immunogenicity of this protein. The use of IA-2 in an immunotherapeutic protocol must however consider the risk of causing autoimmunity against the insulin-producing β cells. [42]. Regarding CDX-2, our gene expression analysis confirms that the expression of CDX-2 is restricted to the GI tract and it might therefore constitute a suitable target antigen.

We also included the BON cell line in our analyses. BON is a human neuroendocrine cell line derived from a pancreatic carcinoid tumor. It secretes serotonin and expresses a common set of genes also expressed by midgut carcinoid tumors [43,44]. Our RT-PCR analyses revealed that IA-2, TPH-1, VMAT1, CDX-2, Survivin, and GAGEs are also expressed in BON cells. Therefore, BON cells may be useful for development of an experimental carcinoid immunotherapy model.

In conclusion, our analyses indicate that TPH1, VMAT1, and Survivin represent potential target antigens for T-cell-mediated therapy. Further characterization of HLA-restricted antigenic peptides of these proteins will determine whether these antigens can be used in T cell-mediated immunotherapy of midgut carcinoid tumors.

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