



High expression of insulinoma-associated protein 1 (INSM1) distinguishes colorectal mixed and pure neuroendocrine carcinomas from conventional adenocarcinomas with diffuse expression of synaptophysin

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Abstract

Complementary to synaptophysin and chromogranin A, insulinoma-associated protein 1 (INSM1) has emerged as a sensitive marker for the diagnosis of neuroendocrine neoplasms. Since there are no comparative data regarding INSM1 expression in conventional colorectal adenocarcinomas (CRCs) and colorectal mixed adenoneuroendocrine carcinomas/neuroendocrine carcinomas (MANECs/NECs), we examined INSM1 in a large cohort of conventional CRCs and MANECs/NECs. In conventional CRC, we put a special focus on conventional CRC with diffuse expression of synaptophysin, which carry the risk of being misinterpreted as a MANEC or a NEC. We investigated INSM1 according to the immunoreactive score in our main cohort of 1,033 conventional CRCs and 21 MANECs/NECs in comparison to the expression of synaptophysin and chromogranin A and correlated the results with clinicopathological parameters and patient survival. All MANECs/NECs expressed INSM1, usually showing high or moderate expression (57% high, 34% moderate, and 9% low), which distinguished them from conventional CRCs, which were usually INSM1 negative or low, even if they diffusely expressed synaptophysin. High expression of INSM1 was not observed in conventional CRCs. Chromogranin A was negative/low in most conventional CRCs (99%), but also in most MANECs/NECs (66%). Comparable results were observed in our independent validation cohorts of conventional CRC ($n = 274$) and MANEC/NEC ($n = 19$). Similar to synaptophysin, INSM1 expression had no prognostic relevance in conventional CRCs, while true MANEC/NEC showed a highly impaired survival in univariate and multivariate analyses (e.g. disease-specific survival: $p < 0.001$). MANECs/NECs are a highly aggressive variant of colorectal cancer, which must be reliably identified. High expression of INSM1 distinguishes MANEC/NEC from conventional CRCs with diffuse expression of the standard neuroendocrine marker synaptophysin, which do not share the same dismal prognosis. Therefore, high INSM1 expression is a highly specific/sensitive marker that is supportive for the diagnosis of true colorectal MANEC/NEC.

Keywords: INSM1; colorectal carcinoma; neuroendocrine carcinoma; conventional adenocarcinoma; synaptophysin

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Introduction

Colorectal mixed adenoneuroendocrine carcinomas (MANECs) and neuroendocrine carcinomas (NECs) are rare histological subtypes of colorectal carcinoma with distinctive morphology and prognosis [1–3]. Although genetically related [4], they behave more aggressively than conventional colorectal adenocarcinomas (CRCs) [5] and are treated differently [6]. A safe distinction of conventional CRCs from MANECs/NECs is therefore of great importance.

Despite their glandular, non-neuroendocrine morphology, positivity for the neuroendocrine marker synaptophysin is often found in conventional CRCs, usually in the form of scattered positive cells [7]. However, in a small fraction of conventional CRCs, synaptophysin is diffusely expressed. Adenocarcinomas with a neuroendocrine component that accounts for more than 30% of the tumor theoretically fulfill the immunohistochemical criteria of a MANEC according to the current WHO classification. Therefore, conventional CRCs in which the number of synaptophysin positive cells reaches or even exceeds this 30% threshold may be highly problematic regarding their classification [3,7], especially if they have an ambiguous morphology.

In a previous study, we were able to demonstrate that conventional CRCs with diffuse expression of synaptophysin do not have the same poor outcome as true MANECs/NECs, which reveal their inherent neuroendocrine nature by the presence of a large cell or small cell carcinoma component, which is already appreciable in hematoxylin–eosin (H&E) stained sections [3,7,8].

In addition to the traditional neuroendocrine markers synaptophysin and chromogranin A, insulinoma-associated protein 1 (INSM1), a zinc-finger transcription factor that regulates neuroendocrine cell differentiation [9], has been established as a complementary marker for the diagnosis of neuroendocrine neoplasms [10]. While high sensitivity and specificity [10–19] of INSM1 expression have been reported for neuroendocrine neoplasms from various anatomic sites, a specific evaluation of INSM1 expression in colorectal MANECs/NECs is still lacking. Furthermore, there are no data regarding the frequency and prognostic relevance of INSM1 expression in conventional CRCs and it is also not known whether INSM1 expression in conventional CRCs and MANECs/NECs is fully comparable to the expression of the traditional neuroendocrine markers synaptophysin/chromogranin A.

To answer these questions, we investigated the frequency and the extent of INSM1 expression according to the immunoreactive score (IRS) [20] in our main

cohort of 1,033 conventional colorectal carcinomas and 21 colorectal MANECs/NECs, and in two separate validation collectives (validation cohort 1: conventional CRC, validation cohort 2: MANEC/NEC). In the large main cohort of 1,054 cancers, we then correlated the results with clinicopathological parameters, expression of synaptophysin/chromogranin A, and patient survival. We put special emphasis on the expression of INSM1 in conventional CRCs with diffuse expression of synaptophysin, in order to find out whether INSM1 could be diagnostically useful for the distinction of these neoplasms from MANEC/NEC.

Materials and methods

Main cohort

The main cohort included 1,054 colorectal carcinomas from the University Hospital rechts der Isar of the Technical University Munich ($n = 1,044$, conventional CRC and MANEC/NEC) and the University Hospital Marburg ($n = 10$, MANEC/NEC), which were surgically resected from 1997 to 2022. Patients with other neoplasms of the colorectal system (e.g. well-differentiated neuroendocrine tumors, nonepithelial tumors, etc.), appendiceal neoplasms, incomplete clinicopathological/survival data, or insufficient tissue on the constructed tissue microarray (TMA) were excluded. Survival data as well as clinicopathological characteristics from all patients were extracted from the Munich Cancer Registry or from hospital records. All recorded patient deaths were noted for overall survival (OS), and only tumor-associated deaths were recorded as events for disease-specific survival (DSS). An event for disease-free survival (DFS) was defined as either locoregional or distant recurrence. For all survival comparisons, the endpoints were either events or a loss of follow-up. In this case, the patients were censored at the time of the last available entry regarding the specific patient or after a maximum follow-up of 120 months. The treatment concepts of included patients followed internal policies, which were based on the given German guidelines at the time of diagnosis, generally meaning that all patients were intended to receive stage-adapted treatment.

Our cohort comprised 1,033 conventional CRC, which we defined as colorectal adenocarcinomas [adenocarcinomas, not otherwise specified (NOS) and variants according to the WHO classification] [3] without histological features suggestive of a NEC on H&E staining. In addition, we investigated 21 colorectal tumors which were diagnosed as MANECs/NECs, in

which more than 30% of the tumor cell population showed the typical histology of a large or small cell NEC on H&E staining, which was immunohistochemically confirmed by the expression of synaptophysin (and/or chromogranin A). In accordance with the criteria given by the WHO classification, small cell NECs were composed of small to medium sized cells with scant basophilic cytoplasm, elongated hyperchromatic nuclei without distinctive nucleoli, and high mitotic activity. Large cell NECs were diagnosed if the neoplasms were composed of solid sheets of medium to large sized tumor cells with rounded vesicular nuclei harboring prominent nucleoli [3,7,21,22]. Colorectal MANECs were also diagnosed according to the WHO classification guidelines; specifically, if one of the described, clearly morphologically recognizable NEC components were admixed with an adenocarcinoma component, either in a mosaic pattern or closely intermingled in a composite pattern.

A TMA from all cases comprising cores from the tumor center and the invasive front was constructed using the TMA grand master (Sysmex/3DHitech, Budapest, Hungary) as previously described [7]. The majority of tumors were already investigated in previous studies from our group [5,7,21,22] and were characterized using morphological parameters such as grade and histological subtype according to the current WHO classification [3], expression of synaptophysin, or microsatellite instability (MSI) status. All tumors that had not been included in our previous studies were reclassified using the above parameters.

Our study was approved by the local ethics committees of the Technical University of Munich (reference numbers: 252/16 s and 2022/565-S-KK), the University Hospital Mainz (reference number: 837.075.16), and of the University Hospital Marburg (reference number: AZ 43/21).

Immunohistochemical analyses of the main cohort

A TMA containing two separate cores from 1,054 colorectal carcinomas from the main cohort was stained with an INSM1 antibody (clone BSB-123, dilution: 1:50, Medac GmbH, Wedel, Germany). INSM1 expression was evaluated manually by an experienced gastrointestinal pathologist (MJ). Only nuclear staining of INSM1 was considered specific. The staining in normal pancreatic islets served as control. The number of positive carcinoma cells was assessed for each individual patient, counting a minimum of 500 tumor cells, resulting in a cumulative percentage score for both cores that was assigned for each CRC (range: 0–100%). The expression intensity was graded as strong (comparable to normal islet cells),

moderate (clearly visible staining but notably weaker than in normal islet cells), weak (barely perceptible and only notable in high magnifications), and negative (no staining reaction). Afterwards, all carcinomas were assigned to different INSM1 expression groups according to their IRS [20], which is derived from a sum score of the percentage of expressing cells (score 0–4) and the maximum staining intensity (score 0–3), which are then multiplied by each other. According to the IRS, four INSM1 expression groups were defined (INSM1 negative, IRS 0–1; INSM1 low, IRS 2–3; INSM1 moderate, IRS 4–8; INSM1 high, IRS 9–12) [23,24]. The algorithm to determine the IRS as well as the resulting INSM1 expression groups is shown in detail in Table 1.

To correlate the results obtained from the analysis on the TMA with whole tissue sections, INSM1 expression was investigated on whole slides of 35 carcinomas from the main cohort (including all 21 MANECs/NECs).

Synaptophysin expression was evaluated as previously described [7]. Conventional CRCs were assigned to four synaptophysin expression groups: synaptophysin negative, synaptophysin low (>0–9% positive tumor cells), and synaptophysin moderate (10–29% positive tumor cells). Conventional CRCs that showed expression of synaptophysin in 30–100% of the tumor cells were classified as synaptophysin high/diffuse, as they reached or exceeded the 30% threshold of neuroendocrine differentiation given by the WHO classification for the diagnosis of a MANEC [3].

Cytoplasmic expression of chromogranin A (clone SP-12, dilution: 1:200, ZytoMed, Berlin, Germany) was also evaluated for all tumors on the TMA. The expression groups were also allocated in accordance with the IRS described above for INSM1.

Table 1. Algorithm to determine the IRS and respective INSM1 expression groups

Immunoreactive score (IRS)		
Score	Staining intensity	Percentage of positive cells
0	No staining reaction	0
1	Weak staining reaction	<10
2	Moderate staining reaction	10–50
3	Strong staining reaction	51–80
4		>80
IRS = score (staining intensity) × score (percentage of positive cells)		
INSM1 expression groups		
IRS 0–1	INSM1 negative	
IRS 2–3	INSM1 low	
IRS 4–8	INSM1 moderate	
IRS 9–12	INSM1 high	

Validation cohort of conventional CRC

Our validation cohort of conventional CRC consisted of 274 resected cases collected from the archives of the University Hospital Mainz as well as from the University Hospital Marburg. Mean age at diagnosis was 69 years. Fifty-seven percent of patients were male. After resection, the histopathological workup revealed 51 UICC stage I (19%), 81 stage UICC II (29%), 82 stage UICC III (3%), and 60 stage UICC IV patients (22%). All of these cases were investigated for INSM1/synaptophysin/chromogranin A as described above on a TMA carrying a minimum of two and up to three cores per case.

Validation cohort for colorectal MANEC/NEC

To further validate the expression of INSM1/synaptophysin/chromogranin A in colorectal MANEC and NEC, we also included a separate MANEC/NEC validation cohort consisting of 19 additional cases. These cases were diagnosed at the Institute of Pathology ÜGP/MVZ Mittelhessen between 2016 and 2021 and were reclassified before inclusion in our study as described above. Fifteen cases were classified as NEC and four were classified as MANEC. The cohort consisted of 10 male and 9 female patients, with a median age at diagnosis of 75 years. No clinical follow-up data were available for these tumors. Because of the rarity of these tumors, our validation cohort included resection specimens and biopsies. From all cases of the MANEC/NEC validation cohort, *whole tissue sections* were examined for their immunohistochemical expression of INSM1, synaptophysin, and chromogranin A using the same methodology as described earlier.

Statistics

Statistical analyses were performed with IBM SPSS Statistics, version 28 (IBM Corp, Armonk, NY, USA). Hypothesis tests of associations were performed by χ^2 test and Fisher's exact test (two-sided). Univariable survival probabilities were estimated with the Kaplan–Meier method and log-rank tests were used to probe their statistical significance, p values ≤ 0.05 were considered statistically significant. Multivariable analyses were performed with the Cox proportional hazards model.

Results

Clinicopathological features and survival in the main cohort

The detailed characteristics of the main cohort, including age, sex, TNM, UICC stage, resection status, MSI

status, WHO grade, and tumor localization are shown in Table 2. As expected, the pTNM categories, resulting UICC stages, WHO grade, resection status, and MSI status profoundly impacted patient survival.

INSM1 expression in conventional CRCs and MANECs/NECs in the main cohort

INSM1 expression was found in 145/1,033 (14%) conventional CRCs and in all MANECs/NECs (21/21, 100%). Most conventional CRCs were INSM1 negative (888/1,033, 86%). Conventional CRCs with expression of INSM1 mostly showed low (133/145, 92%) and rarely moderate (12/145, 8%) positivity. In contrast, most MANECs/NECs fell into the INSM1 high (12/21, 57%) or the INSM1 moderate subgroup (7/21, 34%), with only 2/21 (9%) of these neoplasms in the INSM1 low subgroup (Table 3, $p < 0.001$). INSM1 expression in conventional CRC did not correlate with pTNM, UICC-stage, resection status, MSI status, age, or gender. In 75% of the MANEC, the adenocarcinoma component was completely INSM1 negative; 25% showed weak INSM1 expression.

Comparison of the INSM1 scores from the TMA and whole slides of 35 tumors including all MANECs/NECs showed almost perfect concordance ($\kappa = 0.96$, $p < 0.001$). Figures 1 and 2 illustrate and compare INSM1 expression in conventional CRCs (Figure 1) and in MANECs/NECs (Figure 2).

Synaptophysin expression in conventional CRCs and MANECs/NECs in the main cohort

Synaptophysin expression was found in 243/1,033 conventional CRCs (24%). In 201/243 (83%) tumors, there were only scattered positive cells accounting for up to 9% of the tumor cell population, while in 15/243 (6%) tumors synaptophysin expression was moderate. Synaptophysin expression in 30–100% of the tumor cells was observed in 27/243 (11%) conventional CRCs with any expression of synaptophysin; this represents 3% of all conventional CRCs (27/1,033) in the main cohort. By definition, in MANECs and NECs of the main cohort, all neuroendocrine solid components suggestive of neuroendocrine differentiation expressed synaptophysin [7].

Chromogranin A expression in conventional CRCs and MANECs/NECs in the main cohort

Expression of chromogranin A was found in 116/1033 conventional CRCs (11%). Most of these conventional CRCs (106/116, 91%) had only few chromogranin A positive cells (IRS 2–3, chromogranin A low). Only 10/116 (9%) conventional CRCs showed

Table 2. Distribution and survival associations of clinicopathological characteristics of the main cohort

	Overall n (%)	Mean OS (SE) [months]	p value	Mean DSS (SE) [months]	p value	Mean DFS (SE) [months]	p value
Age			<0.001		0.02		0.945
Below median	511 (48.5%)	86.10 (2.2)		90.88 (2.1)		82.03 (2.3)	
Median and above	543 (51.5%)	71.61 (2.2)		83.63 (2.3)		82.13 (2.4)	
Sex			0.26		0.94		0.69
Male	608 (57.7%)	77.52 (2.1)		87.33 (2.1)		82.83 (2.2)	
Female	446 (42.3%)	80.64 (2.4)		87.24 (2.4)		80.96 (2.6)	
pT			<0.001		<0.001		<0.001
1	80 (7.6%)	96.57 (4.9)		114.12 (3.2)		108.49 (3.8)	
2	188 (17.8%)	92.35 (3.2)		103.32 (2.8)		99.37 (3.2)	
3	588 (55.8%)	79.44 (2.1)		88.03 (2.1)		81.83 (2.2)	
4	198 (18.8%)	56.92 (3.7)		60.15 (3.8)		54.83 (4.0)	
pN			<0.001		<0.001		<0.001
0	581 (55.1%)	89.00 (1.9)		101.20 (1.7)		99.08 (1.8)	
1	296 (28.1%)	75.01 (3.0)		80.23 (3.0)		72.49 (3.2)	
2	177 (16.8%)	51.65 (3.9)		54.73 (4.1)		43.21 (4.0)	
pM			<0.001		<0.001		<0.001
0	897 (85.1%)	86.21 (1.6)		95.98 (1.5)		90.77 (1.7)	
1	157 (14.9%)	39.60 (3.4)		42.15 (3.7)		33.72 (3.6)	
UICC stage			<0.001		<0.001		<0.001
1	213 (20.2%)	96.64 (2.9)		111.11 (2.1)		107.84 (2.4)	
2	351 (33.3%)	86.01 (2.6)		97.00 (2.4)		95.76 (2.6)	
3	327 (31.0%)	80.60 (2.8)		86.44 (2.8)		76.13 (3.0)	
4	163 (15.5%)	38.80 (3.3)		41.27 (3.6)		32.69 (3.4)	
WHO grade			<0.001		<0.001		<0.001
Low grade	708 (67.2%)	86.00 (1.8)		95.24 (1.7)		89.62 (1.9)	
High grade	346 (32.8%)	64.74 (2.8)		71.72 (2.9)		66.83 (3.0)	
Resection margin			<0.001		<0.001		<0.001
R0	974 (92.4%)	82.53 (1.6)		91.81 (1.5)		86.78 (1.7)	
R1	50 (4.7%)	41.00 (7.2)		42.53 (7.5)		29.47 (6.1)	
R2	30 (2.8%)	24.99 (4.5)		25.00 (4.5)		21.52 (3.7)	
Tumor localization			0.14		0.34		0.16
Cecum	150 (14.2%)	78.24 (4.1)		88.30 (4.1)		87.20 (4.2)	
Ascending colon	276 (26.1%)	77.62 (3.2)		86.24 (3.2)		78.61 (3.4)	
Transverse colon	83 (7.9%)	70.26 (6.0)		83.40 (5.9)		83.82 (6.1)	
Descending colon	94 (8.9%)	74.69 (5.1)		81.15 (5.1)		77.02 (5.5)	
Sigmoid colon	332 (31.4%)	84.60 (2.7)		91.67 (2.6)		86.41 (2.9)	
Rectum	119 (11.3%)	75.80 (4.6)		83.88 (5.9)		74.48 (5.1)	
Tumor histology			<0.001		<0.001		<0.001
Conventional adenocarcinoma	1,033 (98.0%)	79.44 (1.6)		88.05 (1.6)		82.81 (1.7)	
MANEC/NEC	21 (2.0%)	26.51 (6.2)		26.51 (6.2)		22.99 (6.1)	
MSI status			0.014		<0.001		<0.001
Microsatellite stable	889 (84.3%)	77.21 (1.7)		85.04 (1.7)		79.46 (1.8)	
Microsatellite unstable	165 (15.7%)	88.36 (3.8)		100.88 (3.4)		97.29 (3.7)	

Table 3. Distribution of INSM1 expression subgroups among synaptophysin expression groups of conventional CRCs and colorectal MANEC/NEC in the main cohort

	INSM1 negative (IRS 0–1)	INSM1 weak (IRS 2–3)	INSM1 moderate (IRS 4–8)	INSM1 strong (IRS 9–12)	Total number	p value
Adenocarcinoma, synaptophysin negative	733	54	3	0	790	
Adenocarcinoma, synaptophysin low (1–9%)	132	67	2	0	201	
Adenocarcinoma, synaptophysin moderate (10–29%)	7	4	4	0	15	
Adenocarcinoma, synaptophysin high (30–100%)	16	8	3	0	27	
MANEC/NEC	0	2	7	12	21	
Total number	888	135	19	12	1,054	<0.001

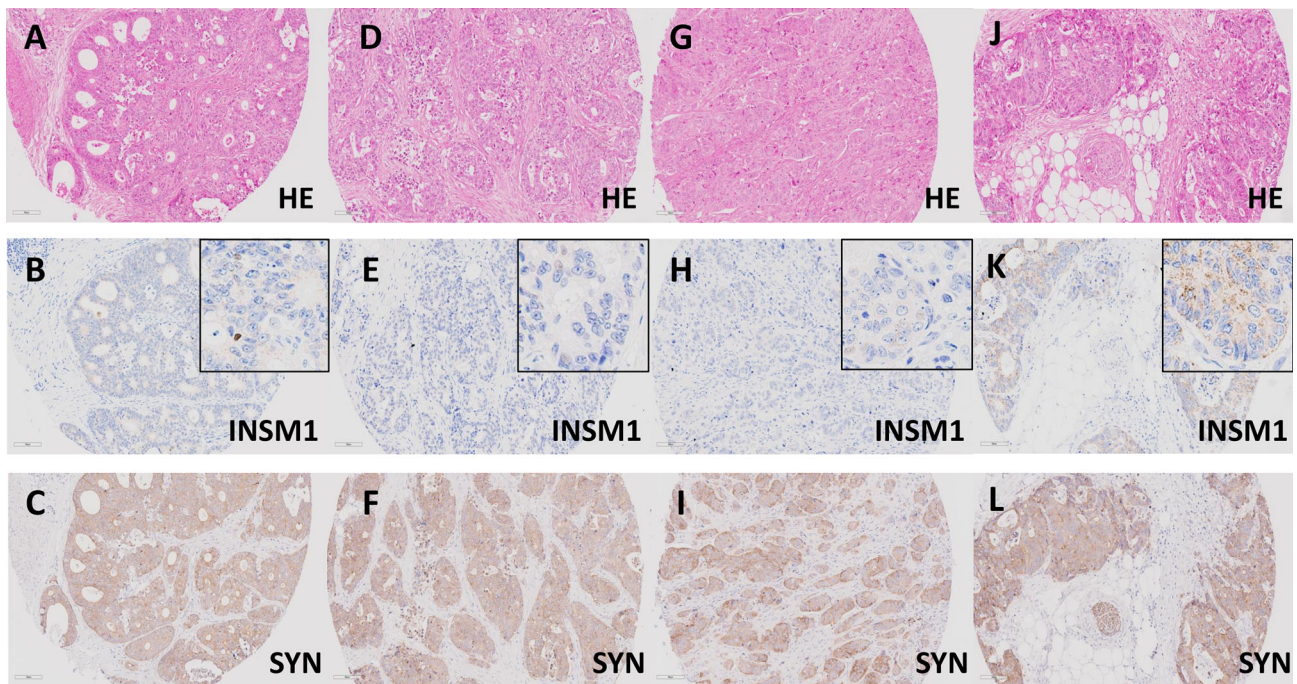


Figure 1. INSM1 expression groups in conventional CRCs with diffuse expression of synaptophysin. (A–C) Conventional CRC (A, H&E, $\times 20$) with diffuse expression of synaptophysin (C, $\times 20$). The adenocarcinoma shows only scattered INSM1 positive cells ($<10\%$), some showing strong nuclear staining (B, $\times 20$, inset $\times 40$), resulting in an IRS of 3 (INSM1 low). (D–F) Conventional CRC (D, H&E, $\times 20$) with diffuse expression of synaptophysin (F, $\times 20$). The adenocarcinoma shows scattered INSM1 positive cells ($<10\%$) with very weak, barely perceptible nuclear staining (E, $\times 20$, inset $\times 40$), resulting in an IRS of 1 (INSM1 negative). (G–I) Further examples of two conventional CRCs with diffuse expression of synaptophysin (G and J, H&E, $\times 20$). Both adenocarcinomas show diffuse expression of synaptophysin in all tumor cells (I and L, $\times 20$). Nuclear expression of INSM1 was not observed, resulting in an IRS 0 (INSM1 negative). Note the nonspecific cytoplasmic staining, that was not considered specific (H and K, $\times 20$, inset $\times 40$).

moderate chromogranin A expression, equaling 1% of all conventional CRCs (10/1,033) in the main cohort. Colorectal MANECs/NECs expressed chromogranin A in 13/21 cases (62%); 8 MANECs/NECs (38%) were completely chromogranin A negative. Of the 13 chromogranin A positive MANECs/NECs, 5 showed high chromogranin A expression, 2 cases showed moderate, and 6 neoplasms showed weak expression.

Correlation of INSM1 expression in conventional CRCs and in MANECs/NECs with expression of synaptophysin in the main cohort

Table 3 shows that the majority of conventional CRCs with diffuse synaptophysin positivity were INSM1 negative (16/27, 59%). The remaining conventional CRCs with diffuse expression of synaptophysin mostly belonged to the subgroups with low (8/27, 29.6%) or rarely moderate INSM1 (3/27, 11%) expression.

In contrast, all MANECs/NECs were INSM1 positive and mostly showed high or at least moderate

INSM1 expression (19/21, 91%), thus differing significantly ($p < 0.001$) from conventional CRCs with diffuse synaptophysin positivity. Very few synaptophysin negative conventional CRCs showed low (54/790, 7%) or moderate (3/790, $<1\%$) INSM1 expression.

Correlation of INSM1 expression with chromogranin A expression in conventional CRCs and in MANECs/NECs in the main cohort

Conventional CRCs with chromogranin A expression were mostly INSM1 negative (72/116, 62%) or weakly positive (41/116, 35%). Among the 918 chromogranin A negative conventional CRCs, weak or moderate expression of INSM1 was noted in 92/917 (10%) and 9/917 (1%) cases, respectively. The remaining chromogranin A negative conventional CRCs were also INSM1 negative ($p < 0.001$, supplementary material, Table S1).

In MANECs/NECs, there was no significant correlation between chromogranin A and INSM1 expression ($p = 0.70$). Seven of the 12 MANECs/NECs with

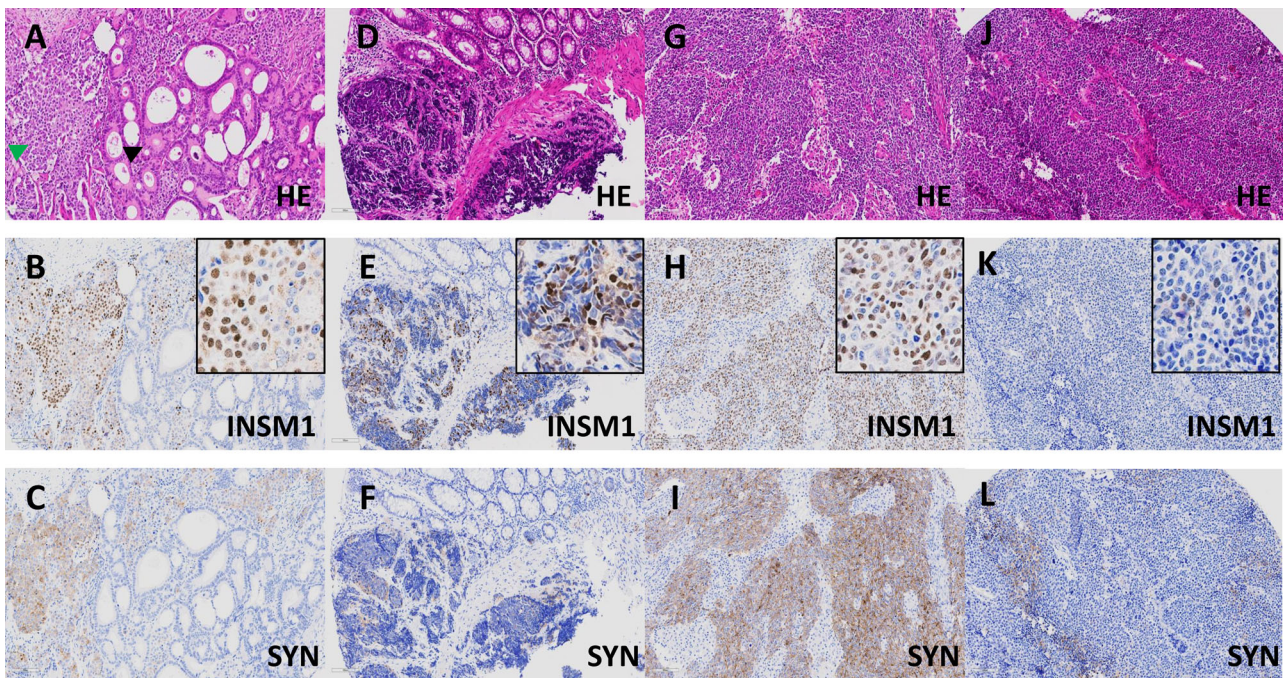


Figure 2. INSM1 expression groups in colorectal MANEC and NEC. (A–C) Colorectal MANEC (A, H&E, $\times 20$) consisting of an adenocarcinoma NOS (black arrow) intermingled with a large cell NEC (green arrow), which shows diffuse expression of synaptophysin (C, $\times 20$). The NEC shows a substantial number of positive cells (60%) with variable staining intensities with some showing strong nuclear staining (B, $\times 20$, inset $\times 40$), resulting in an IRS of 9 (INSM1 high). The adenocarcinoma component is INSM1 negative. (D–F) Colorectal small cell NEC (D, H&E, $\times 20$) showing weak to moderate expression of synaptophysin (F, $\times 20$). The NEC shows a substantial number of positive cells (55%) with variable staining intensities ranging from very weak to strong (E, $\times 20$, inset $\times 40$), resulting in an IRS of 9 (INSM1 high). (G–I) Colorectal large cell NEC (G, H&E, $\times 20$) with diffuse expression of synaptophysin (I, $\times 20$) and diffuse and strong (90%) expression of INSM1 (H, $\times 20$, inset $\times 40$), resulting in an IRS of 12 (INSM1 high). (J–L) Colorectal large cell NEC (J, H&E, $\times 20$) with patchy expression of synaptophysin (I, $\times 20$) and scattered (10%) expression of INSM1, with most positive nuclei showing weak but some showing moderate staining intensity (K, $\times 20$, inset $\times 40$), resulting in an IRS of 4 (INSM1 moderate).

strong INSM1 expression (58%) were either chromogranin A negative or only weakly positive, while in 7/8 (88%) chromogranin A negative NECs INSM1 expression was either moderate or strong (supplementary material, Table S2).

Patient survival in INSM1-expressing conventional CRCs compared with MANECs/NECs in the main cohort

We observed no survival differences between conventional CRCs regardless of their expression of INSM1 (OS: $p = 0.849$; DSS: $p = 0.273$; DFS: $p = 0.884$, Figure 3). In contrast, MANECs/NECs showed significantly poorer survival for OS, DSS, or DFS ($p < 0.001$, respectively) compared with all INSM1 expression subgroups of conventional CRC. For example, mean DSS for conventional CRCs without INSM1 expression was 87.1 months, compared

with 95.0 and 89.0 months for conventional CRCs with low or moderate INSM1 expression, while MANEC/NEC showed a mean DSS of 26.5 months. Figure 3 depicts the respective Kaplan–Meier curves. The detailed mean survival times of the different groups for all survival comparisons are shown in supplementary material, Tables S3 and S4.

The stage-independent impact on patient survival of true MANEC/NEC but not of conventional CRC with INSM1 expression was confirmed by multivariate analyses including UICC stage, resection status, MSI, gender, sex, and WHO grade (DSS: $p = 0.005$, hazard ratio: 2.435, Table 4; OS: $p = 0.014$; hazard ratio: 2.167, supplementary material, Table S5; DFS: $p = 0.03$, hazard ratio: 1.936, supplementary material, Table S6).

As described previously [7], synaptophysin expression in conventional CRCs did not impact OS ($p = 0.67$) or DSS ($p = 0.80$), but showed a prognostic impact on DFS ($p = 0.011$) in univariate analysis, which

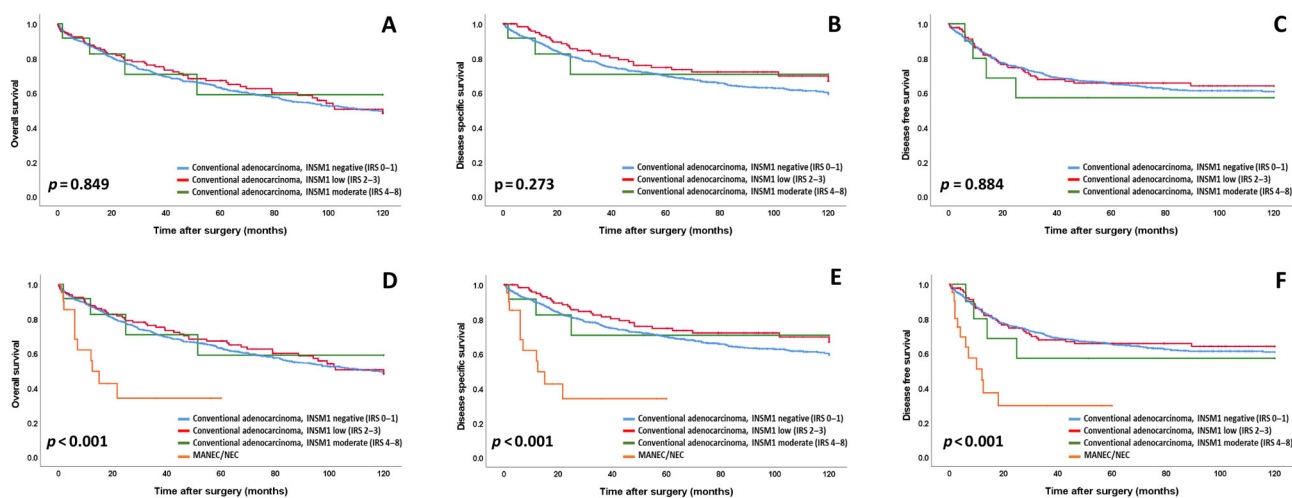


Figure 3. OS, DSS, and DFS for (A–C) INSM1 expression groups in conventional adenocarcinomas with a non-neuroendocrine morphology and for (D–F) INSM1 expression groups in conventional adenocarcinomas compared with MANEC/NEC.

was not confirmed in multivariate analysis ($p = 0.38$). Expression of chromogranin A in conventional CRCs had no impact on survival ($p = \text{n.s.}$, data not shown).

Expression of INSM1/synaptophysin/chromogranin A in the conventional CRC validation cohort

Of the 274 cases in the conventional CRC validation cohort, 20 (7%) showed weak expression of INSM1 and 27 (10%) showed a weak expression of chromogranin A. Synaptophysin was expressed in 38 conventional CRC (14%), of which 5 (2%) showed diffuse expression of synaptophysin exceeding 30%. Four of these diffuse synaptophysin expressing conventional CRC were INSM1 negative (4/5, 80%), while one tumor showed low INSM1 expression. The clinicopathological characteristics and the expression of INSM1/synaptophysin/chromogranin A in the conventional CRC validation cohort are summarized in supplementary material, Table S7.

Expression of INSM1/synaptophysin/chromogranin A in the MANEC/NEC validation cohort

In the MANEC/NEC validation cohort, all cases expressed synaptophysin. INSM1 was expressed in all tumors, with 9/19 tumors showing strong (47%) and 6/19 tumors showing moderate INSM1 positivity (32%), 4/19 tumors showed weak expression (21%). Chromogranin A was negative in 12/19 tumors (63%), one tumor showed weak expression (5%), while moderate positivity was observed in 5/19 cases (26%).

In the four MANECs, INSM1 expression was not detected in the adenocarcinoma components. The clinicopathological characteristics and the expression of INSM1/synaptophysin/chromogranin A in the MANEC/NEC validation cohort are summarized in supplementary material, Table S8.

Discussion

INSM1 is a highly conserved transcription factor that regulates the development of neuroendocrine cells throughout the body [9,25–28]. INSM1 has emerged as a sensitive immunohistochemical marker for the diagnosis of neuroendocrine neoplasms, with several studies showing higher specificity and sensitivity compared with the traditional neuroendocrine markers synaptophysin and chromogranin A [10,12,13].

Our study investigated the nuclear immunoreactivity of INSM1 according to the IRS [20] in more than 1,050 colorectal carcinomas and demonstrates that, besides morphology, high expression of INSM1 distinguishes true colorectal MANEC/NEC from conventional CRC with diffuse expression of synaptophysin (>30% positive tumor cells) [10]. MANEC/NEC usually showed high or at least moderate expression of INSM1 (91%, 9% with low INSM1 expression), while almost 90% of conventional CRC with diffuse synaptophysin expression were either INSM1 negative or INSM1 low. These general observations from our main cohort were then validated in two independent collectives of conventional CRC and MANEC/NEC.

Table 4. Multivariate analysis for DSS including INSM1 expression groups in conventional adenocarcinomas compared with MANECs including UICC stage, WHO grade, MSI, resection status, age, and gender in the main cohort

	Hazard ratio (DSS)	Lower CI (95%)	Upper CI (95%)	p value
INSM1 expression groups in conventional adenocarcinoma versus MANEC/NEC				0.005
Conventional adenocarcinoma INSM1 negative (IRS 0–1)	1			
Conventional adenocarcinoma INSM1 low (IRS 2–3)	0.785	0.536	1.149	
Conventional adenocarcinoma INSM1 moderate (IRS 4–8)	0.588	0.187	1.849	
MANEC/NEC	2.435	1.303	4.549	
UICC stage				<0.001
I	1			
II	2.504	1.521	4.122	
III	3.457	2.128	5.616	
IV	9.430	5.671	15.681	
Resection status				<0.001
R0	1			
R1	2.243	1.517	3.316	
R2	2.072	1.330	3.231	
MSI				0.014
Microsatellite stable	1			
Microsatellite unstable	0.592	0.391	0.899	
WHO grade				0.001
Low grade	1			
High grade	1.486	1.167	1.891	
Gender				0.173
Male	1			
Female	0.850	0.673	1.074	
Age				<0.001
Below median	1			
Median and above	1.585	1.263	1.990	

In line with our previous observations for synaptophysin [7], the survival of conventional CRC patients was not influenced by the expression of INSM1, regardless of its intensity, while patients suffering from true MANEC/NEC showed a stage independent, highly decreased survival on all comparisons. These findings strengthen the assumption that the expression of any neuroendocrine marker in colorectal carcinomas has to be correlated with the underlying morphology and is not automatically equal to a NEC. Interestingly, a recent study from Fassin *et al* [29] revealed synaptophysin-expressing conventional CRC to be a prognostic subgroup in *BRAF*-mutated CRC, indicating that the molecular context may also be of importance when the clinical relevance of endocrine markers in conventional CRC is considered.

The spectrum of neuroendocrine differentiation in colorectal and extra-colorectal carcinomas with non-neuroendocrine morphology varies from scattered cells to neoplasms with diffuse expression of (traditional) neuroendocrine markers, especially synaptophysin. Although most previous studies including our own [7,30–40] considered this phenomenon to be of minor prognostic relevance, these cases frequently cause

diagnostic and clinical confusion over whether to classify those tumors as MANECs or NECs and to treat them accordingly [1,2,41]. Although the WHO understandably requires NEC morphology for the diagnosis of MANEC or NEC, some conventional CRCs with diffuse expression of synaptophysin that exceeds the 30% threshold may be misinterpreted as MANEC or NEC [42,43]; this should be avoided at all costs, because of the vast prognostic differences and the diverging treatment modalities between conventional CRC and NECs. The data from our study may be helpful in diagnostically challenging cases. For example, in carcinomas that are morphologically ambiguous regarding possible neuroendocrine differentiation (e.g. medullary CRC, poorly differentiated adenocarcinoma NOS) and show weak but diffuse immunoreactivity for synaptophysin, negative staining for INSM1 can increase confidence for the diagnosis of a conventional CRC. Conversely, in carcinomas that are morphologically consistent with a poorly differentiated NEC, which is the most important requirement for the diagnosis, but only show weak or focal expression of traditional neuroendocrine markers, high expression of INSM1 can greatly increase the certainty of the diagnosis of a true MANEC/NEC.

The results of our study are concordant with data on gynecological carcinomas by Zou *et al* [44], who also found no or only weak INSM1 expression in conventional ovarian or uterine carcinomas (e.g. high-grade serous carcinoma or poorly differentiated endometrioid carcinoma) with diffuse expression of neuroendocrine markers, but non-neuroendocrine morphology. In addition to INSM1, our study also evaluated the expression of chromogranin A immunoreactivity in conventional CRCs and MANECs/NECs. High or moderate chromogranin A expression was quite specific for MANEC/NEC, but not very sensitive, as the majority of MANEC/NEC showed weak expression of chromogranin A or were entirely negative.

Our study has some limitations. First, it is of retrospective nature. However, to minimize this disadvantage, we performed our analyses on a very large cohort of more than 1,054 colorectal carcinomas including 21 MANECs/NECs, which is close to the incidence of these tumors described in the literature [3,6], and added two substantial validation cohorts comprising conventional CRC as well as MANEC/NEC. Another shortcoming of our work is that our analyses were performed on TMAs because of the large size of our cohort. However, comparing our TMA data with full block slides revealed an almost perfect concordance, thus supporting the robustness of our approach.

In conclusion, our study shows that high INSM1 expression in conventional colorectal carcinoma has no prognostic significance, but is helpful for distinguishing colorectal MANEC/NEC from conventional colorectal carcinoma with diffuse synaptophysin expression. Furthermore, our data suggest that INSM1 has a higher specificity for colorectal MANEC/NEC than synaptophysin and a higher sensitivity than chromogranin A, further strengthening the role of INSM1 as a complementary marker for the diagnosis of poorly differentiated neuroendocrine neoplasms. Further studies are needed to investigate the molecular basis of diffuse synaptophysin expression in conventional colorectal carcinomas and to clarify whether this phenomenon is due to the recognition of synaptophysin-like proteins or whether it is another, more benign form of neuroendocrine differentiation that is not associated with a worse clinical outcome.

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Author contributions statement

MJ and BK designed this study. MJ and ASL wrote the manuscript with assistance from GK, AK, CD and WW. SF, MS, FK, TG, BL, DKB, AR and PJ collected clinical or pathological data. ASL, MJ, BK, AK and BK performed histopathological or immunohistochemical analyses.

Data availability statement

Data from this study are available from the corresponding author upon reasonable request.

References

1. Yamaguchi T, Machida N, Morizane C, *et al*. Multicenter retrospective analysis of systemic chemotherapy for advanced neuroendocrine carcinoma of the digestive system. *Cancer Sci* 2014; **105**: 1176–1181.
2. Tanaka T, Kaneko M, Nozawa H, *et al*. Diagnosis, assessment, and therapeutic strategy for colorectal mixed adenoneuroendocrine carcinoma. *Neuroendocrinology* 2017; **105**: 426–434.
3. WHO Classification of Tumours Editorial Board. Digestive system tumours. In *WHO Classification of Tumours Series, Volume 1* (5th edn). International Agency for Research on Cancer: Lyon, France, 2019; 157–162. [Accessed 10 August 2023]. Available from: <http://publications.iarc.fr/579>
4. Jesinghaus M, Konukiewitz B, Keller G, *et al*. Colorectal mixed adenoneuroendocrine carcinomas and neuroendocrine carcinomas are genetically closely related to colorectal adenocarcinomas. *Mod Pathol* 2017; **30**: 610–619.
5. Jesinghaus M, Schmitt M, Lang C, *et al*. Morphology matters: a critical reappraisal of the clinical relevance of morphologic criteria from the 2019 WHO classification in a large colorectal cancer cohort comprising 1004 cases. *Am J Surg Pathol* 2021; **45**: 969–978.
6. Ilett EE, Langer SW, Olsen IH, *et al*. Neuroendocrine carcinomas of the gastroenteropancreatic system: a comprehensive review. *Diagnostics (Basel)* 2015; **5**: 119–176.
7. Konukiewitz B, Kasajima A, Schmitt M, *et al*. Neuroendocrine differentiation in conventional colorectal adenocarcinomas: incidental finding or prognostic biomarker? *Cancers (Basel)* 2021; **13**: 5111.
8. Nagtegaal ID, Odze RD, Klimstra D, *et al*. The 2019 WHO classification of tumours of the digestive system. *Histopathology* 2020; **76**: 182–188.

9. Mellitzer G, Bonn  S, Luco RF, et al. IA1 is NGN3-dependent and essential for differentiation of the endocrine pancreas. *EMBO J* 2006; **25**: 1344–1352.
10. Bellizzi AM. Immunohistochemistry in the diagnosis and classification of neuroendocrine neoplasms: what can brown do for you? *Hum Pathol* 2020; **96**: 8–33.
11. Kuji S, Watanabe R, Sato Y, et al. A new marker, insulinoma-associated protein 1 (INSM1), for high-grade neuroendocrine carcinoma of the uterine cervix: analysis of 37 cases. *Gynecol Oncol* 2017; **144**: 384–390.
12. Rooper LM, Sharma R, Li QK, et al. INSM1 demonstrates superior performance to the individual and combined use of synaptophysin, chromogranin and CD56 for diagnosing neuroendocrine tumors of the thoracic cavity. *Am J Surg Pathol* 2017; **41**: 1561–1569.
13. Rosenbaum JN, Guo Z, Baus RM, et al. INSM1: a novel immunohistochemical and molecular marker for neuroendocrine and neuroepithelial neoplasms. *Am J Clin Pathol* 2015; **144**: 579–591.
14. Gonz lez I, Lu HC, Sninsky J, et al. Insulinoma-associated protein 1 expression in primary and metastatic neuroendocrine neoplasms of the gastrointestinal and pancreaticobiliary tracts. *Histopathology* 2019; **75**: 568–577.
15. Tanigawa M, Nakayama M, Taira T, et al. Insulinoma-associated protein 1 (INSM1) is a useful marker for pancreatic neuroendocrine tumor. *Med Mol Morphol* 2018; **51**: 32–40.
16. Mukhopadhyay S, Dermawan JK, Lanigan CP, et al. Insulinoma-associated protein 1 (INSM1) is a sensitive and highly specific marker of neuroendocrine differentiation in primary lung neoplasms: an immunohistochemical study of 345 cases, including 292 whole-tissue sections. *Mod Pathol* 2019; **32**: 100–109.
17. McHugh KE, Mukhopadhyay S, Doxtader EE, et al. INSM1 is a highly specific marker of neuroendocrine differentiation in primary neoplasms of the gastrointestinal tract, appendix, and pancreas. *Am J Clin Pathol* 2020; **153**: 811–820.
18. Couvelard A, Cros J. An update on the development of concepts, diagnostic criteria, and challenging issues for neuroendocrine neoplasms across different digestive organs. *Virchows Arch* 2022; **480**: 1129–1148.
19. Zhang Q, Huang J, He Y, et al. Insulinoma-associated protein 1 (INSM1) is a superior marker for the diagnosis of gastroenteropancreatic neuroendocrine neoplasms: a meta-analysis. *Endocrine* 2021; **74**: 61–71.
20. Remmele W, Stegner HE. Recommendation for uniform definition of an immunoreactive score (IRS) for immunohistochemical estrogen receptor detection (ER-ICA) in breast cancer tissue. *Pathologie* 1987; **8**: 138–140.
21. Konukiewicz B, Schmitt M, Silva M, et al. Loss of CDX2 in colorectal cancer is associated with histopathologic subtypes and microsatellite instability but is prognostically inferior to hematoxylin–eosin-based morphologic parameters from the WHO classification. *Br J Cancer* 2021; **125**: 1632–1646.
22. Schmitt M, Silva M, Konukiewicz B, et al. Loss of SATB2 occurs more frequently than CDX2 loss in colorectal carcinoma and identifies particularly aggressive cancers in high-risk subgroups. *Cancers (Basel)* 2021; **13**: 6177.
23. Kaemmerer D, Peter L, Lupp A, et al. Comparing of IRS and Her2 as immunohistochemical scoring schemes in gastroenteropancreatic neuroendocrine tumors. *Int J Clin Exp Pathol* 2012; **5**: 187–194.
24. Regierer AC, Wolters R, Kurzeder C, et al. High estrogen receptor expression in early breast cancer: chemotherapy needed to improve RFS? *Breast Cancer Res Treat* 2011; **128**: 273–281.
25. Wang HW, Muguira M, Liu WD, et al. Identification of an INSM1-binding site in the insulin promoter: negative regulation of the insulin gene transcription. *J Endocrinol* 2008; **198**: 29–39.
26. Zhang T, Chen C, Breslin MB, et al. Extra-nuclear activity of INSM1 transcription factor enhances insulin receptor signaling pathway and *Nkx6.1* expression through RACK1 interaction. *Cell Signal* 2014; **26**: 740–747.
27. Zhang T, Wang H, Saunee NA, et al. Insulinoma-associated antigen-1 zinc-finger transcription factor promotes pancreatic duct cell trans-differentiation. *Endocrinology* 2010; **151**: 2030–2039.
28. Gierl MS, Karoulias N, Wende H, et al. The zinc-finger factor *Insm1* (IA-1) is essential for the development of pancreatic beta cells and intestinal endocrine cells. *Genes Dev* 2006; **20**: 2465–2478.
29. Fassan M, Milione M, Maddalena G, et al. Synaptophysin expression in (V600EBRAF)-mutated advanced colorectal cancers identifies a new subgroup of tumours with worse prognosis. *Eur J Cancer* 2021; **146**: 145–154.
30. Chavez-Blanco A, Taja-Chayeb L, Cetina L, et al. Neuroendocrine marker expression in cervical carcinomas of non-small cell type. *Int J Gynecol Pathol* 2002; **21**: 368–374.
31. Chen Y, Liu F, Meng Q, et al. Is neuroendocrine differentiation a prognostic factor in poorly differentiated colorectal cancer? *World J Surg Oncol* 2017; **15**: 71.
32. Shinji S, Naito Z, Ishiwata T, et al. Neuroendocrine cell differentiation of poorly differentiated colorectal adenocarcinoma correlates with liver metastasis. *Int J Oncol* 2006; **29**: 357–364.
33. Lloyd RV, Schroeder G, Bauman MD, et al. Prevalence and prognostic significance of neuroendocrine differentiation in colorectal carcinomas. *Endocr Pathol* 1998; **9**: 35–42.
34. Foley EF, Gaffey MJ, Frierson HF Jr. The frequency and clinical significance of neuroendocrine cells within stage III adenocarcinomas of the colon. *Arch Pathol Lab Med* 1998; **122**: 912–914.
35. Yao GY, Zhou JL, Lai MD, et al. Neuroendocrine markers in adenocarcinomas: an investigation of 356 cases. *World J Gastroenterol* 2003; **9**: 858–861.
36. Suresh PK, Sahu KK, Pai RR, et al. The prognostic significance of neuroendocrine differentiation in colorectal carcinomas: our experience. *J Clin Diagn Res* 2015; **9**: EC01–EC04.
37. Ogimi T, Sadahiro S, Kamei Y, et al. Distribution of neuroendocrine marker-positive cells in colorectal cancer tissue and normal mucosal tissue: consideration of histogenesis of neuroendocrine cancer. *Oncology* 2019; **97**: 294–300.
38. Sterlacci W, Fiegl M, Hilbe W, et al. Clinical relevance of neuroendocrine differentiation in non-small cell lung cancer assessed by immunohistochemistry: a retrospective study on 405 surgically resected cases. *Virchows Arch* 2009; **455**: 125–132.

39. Howe MC, Chapman A, Kerr K, *et al.* Neuroendocrine differentiation in non-small cell lung cancer and its relation to prognosis and therapy. *Histopathology* 2005; **46**: 195–201.
40. Ionescu DN, Treaba D, Gilks CB, *et al.* Nonsmall cell lung carcinoma with neuroendocrine differentiation – an entity of no clinical or prognostic significance. *Am J Surg Pathol* 2007; **31**: 26–32.
41. Mityr E, Baudin E, Ducreux M, *et al.* Treatment of poorly differentiated neuroendocrine tumours with etoposide and cisplatin. *Br J Cancer* 1999; **81**: 1351–1355.
42. Smith JD, Reidy DL, Goodman KA, *et al.* A retrospective review of 126 high-grade neuroendocrine carcinomas of the colon and rectum. *Ann Surg Oncol* 2014; **21**: 2956–2962.
43. Ramage JK, De Herder WW, Delle Fave G, *et al.* ENETS consensus guidelines update for colorectal neuroendocrine neoplasms. *Neuroendocrinology* 2016; **103**: 139–143.
44. Zou Q, Zhang L, Cheng Z, *et al.* INSM1 is less sensitive but more specific than synaptophysin in gynecologic high-grade neuroendocrine carcinomas: an immunohistochemical study of 75 cases with specificity test and literature review. *Am J Surg Pathol* 2021; **45**: 147–159.

SUPPLEMENTARY MATERIAL ONLINE

Table S1. Correlation between INSM1 expression and expression of chromogranin A in conventional adenocarcinomas

Table S2. Correlation between INSM1 expression and expression of chromogranin A in MANEC/NEC

Table S3. Survival associations of INSM1 expression groups among conventional adenocarcinomas

Table S4. Survival associations of INSM1 expression groups among conventional adenocarcinomas compared with colorectal MANEC/NEC

Table S5. Multivariate analysis for OS including INSM1 expression groups in conventional adenocarcinomas compared with MANECs

Table S6. Multivariate analysis for DFS including INSM1 expression groups in conventional adenocarcinomas compared with MANECs

Table S7. Cohort characteristics and expression of synaptophysin, chromogranin A and INSM1 in the conventional CRC validation cohort

Table S8. Cohort characteristics and expression of synaptophysin, chromogranin A and INSM1 in the MANEC/NEC validation cohort