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RESEARCH PAPER

The role of HMGA1 protein in gastroenteropancreatic neuroendocrine tumors

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ABSTRACT

Neuroendocrine tumors (NETs) are neoplasms derived from neuroendocrine cells. One of their main features is to often remain asymptomatic and clinically undetectable. High Mobility Group A (HMGA) proteins belong to a family of non-histone chromatinic proteins able to modulate gene expression through the interaction with DNA and transcription factors. They are overexpressed in most of the human malignancies, playing a critical role in carcinogenesis. However, their expression levels and their role in neuroendocrine carcinogenesis has not been exhaustively evaluated until now. Therefore, in this study, we have addressed the validity of using the expression of HMGA1 as a diagnostic marker and have investigated its role in NET carcinogenesis. The expression of HMGA1 has been evaluated by qRT-PCR and immunohistochemistry, using NET tissue microarrays, in a cohort of gastroenteropancreatic (GEP)-NET samples. The expression levels of HMGA1 have been then correlated with the main clinical features of NET samples. Finally, the contribution of HMGA1 overexpression to NET development has been addressed as far as the modulation of proliferation and migration abilities of NET cells is concerned. Here, we report that HMGA1 is overexpressed in GEP-NET samples, at both mRNA and protein levels, and that the silencing of HMGA1 protein expression interferes with the ability of NET cells to proliferate and migrate through the downregulation of Cyclin E, Cyclin B1 and EZH2. These results propose the HMGA proteins as new diagnostic and prognostic markers.

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Neuroendocrine tumors; HMGA1; gastroenteropatic tumors

1. Introduction

Neuroendocrine tumors (NETs), known as carcinoid tumors in the past, are neoplasms of enterochromaffin cell origin. According to Berge and Linell [1], the annual incidence of patients affected by NET is 8.4 per 100,000 inhabitants, although many NETs are asymptomatic and often remain undetected. NETs generally affect the gastrointestinal (67%) and bronchopulmonary (25%) system [2]. They are usually classified on the basis of their topological origin: foregut tumors, 25% of cases (from thymus, lung, stomach and proximal duodenum); midgut tumors, 50% of the cases (from small intestine, appendix and proximal colon); hindgut tumors, 15% of the cases (from distal colon and rectum) [3]. However, even the pancreas, liver, kidney, gallbladder, ovary and testis can be site of NETs [2–4].

It is worth to note that NETs of the gastrointestinal tract often retain their secretory capacity, being able to produce various hormones such as serotonin and substance P. The symptoms generated by this condition are collectively called as the carcinoid syndrome [4]. Pancreatic NETs, even if they represent only the 3% of all pancreatic neoplasia (about 95% are adenocarcinomas of exocrine pancreas), account for more than 30% of GEP-NETs [5]. Interestingly, it is not yet clear if they originate from islets of Langerhans or from diffuse neuroendocrine pluripotent cells. Finally, about 60% of the pancreatic NETs are non-secretory or nonfunctional [5].

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Following the classification of the World Health Organization (WHO), NETs can be grouped into three most significant categories [6,7]: (a) well-differentiated (including tumors with benign and/or uncertain behavior), (b) well-differentiated low-grade (with low-grade malignant behavior) and (c) poorly differentiated high-grade (including large cell- and small cell-neuroendocrine tumors) NETs. From the molecular point of view, a list of potential markers has been identified in NETs. Secretory tumors are mainly characterized by their produced hormones such as urine 5-hydroxyindoleacetic acid (5-HIAA), chromogranin A (CgA), synaptophysin (P38), neuron-specific enolase (NSE, gamma–gamma dimer) [8]. Pancreatic NETs express CDX2, the N-terminally truncated variant of Hsp70 and the protein-55 [5]. Gene mutations have been frequently found in pancreatic NETs (ATRX and DAXX mutations in the 40% of the cases) and more in general in NETs (MEN1, TSC2, PTEN and PIK3CA) [9]. However, no prognostic validity has been assigned to these markers, hence the identification of new prognostic markers capable of predicting the metastatic capacity of the tumor, the susceptibility of tumor cells to therapeutic treatments and the survival of patients represents a critical aim of the scientific community.

High Mobility Group A (HMGA) proteins are the product of two genes (*HMGA1* and *HMGA2*) that collectively produce three proteins: HMGA1a and HMGA1b deriving from the alternative splicing of the *HMGA1* gene, and HMGA2 deriving from the *HMGA2* gene [10]. They are non-histone chromatinic proteins able to modulate gene transcription by directly interacting with DNA and several transcription factors [10]. Interestingly, they have been found strongly overexpressed during embryogenesis and in human carcinomas (even in pancreatic carcinomas), whereas they are expressed at very low levels in the normal adult tissues [10,11]. The HMGA proteins have a crucial role in the biological processes, and they are overexpressed in human malignancies to whose onset they have been causally linked. Indeed, the knockdown of HMGA1 expression inhibited thyroid cell transformation, leading cancer cells to undergo apoptotic death [10]. Moreover, their role in carcinogenesis has been extensively validated also by the generation and characterization of transgenic mouse models overexpressing the HMGA proteins [12–15].

Therefore, based on all these observations, the objective of our investigation has been to evaluate the use of HMGA proteins as diagnostic and prognostic markers of NET, possibly characterizing their role during the onset and progression of NETs.

Here, we report that HMGA1 protein is frequently overexpressed in GEP-NETs and that it plays a functional role during GEP-NET carcinogenesis, since its expression levels are able to modulate proliferation and migration of GEP-NET derived cell lines.

2. Materials and methods

2.1. Tissue samples

The expression of HMGA1 has been evaluated in a series of NET samples including GEP-NETs and Merkel cell carcinomas. In this study were included n = 20 fresh-frozen and n = 63 formalin-fixed and paraffin-embedded (FFPE) GEP-NET samples retrieved from the biobank of the Institute of Pathology (Basel, Switzerland), and n = 57 Merkel cell carcinomas, available at Istituto Ortopedico Rizzoli (Bologna, Italy). A comprehensive list of samples used in this study is accurately reported in Table 1.

2.2. Immunohistochemical analysis

A tissue microarray (TMA) comprising n = 63 GEP-NET samples was used to evaluate the expression of HMGA1 protein. The TMA slide comprised n = 39 (n = 25 evaluable) intestinal NET samples, n = 20 (n = 14 evaluable) pancreatic NET samples and n = 4 (n = 4 evaluable) gastric NET samples (Table 1). The TMA was constructed as described elsewhere [16].

Table 1. Main clinico-pathological characteristics of intestinal and pancreatic NET samples included in the tissue microarray.

Bowel (n = 39)		
Site (n = 39)	Ileus	n = 8
	Cecum	n = 1
	Rectum	n = 7
	Appendix	n = 12
	Jejunum	n = 3
	Colon	n = 2
	Duodenum	n = 4
	Omentum	n = 1
	Mesentery	n = 1
Age (n = 34), years	0–25	n = 3
	26–50	n = 8
	51–75	n = 19
	76–100	n = 4
Size (n = 34), cm	0–1.5	n = 23
	1.6–3.0	n = 7
	3.1–4.5	n = 4
Grade (n = 20)	G1	n = 18
	G3	n = 2
Stage (n = 15)	T2	n = 2
	T3	n = 6
	T4	n = 7
Nodal metastasis (n = 21)	0[a]	n = 2
	1[b]	n = 19
Angioinvasiveness (n = 37)	0	n = 20
	1	n = 17
Pancreas (n = 20)		
Age (n = 17), years	0–25	n = 1
	26–50	n = 7
	51–75	n = 8
	76–100	n = 1
Size (n = 19), cm	0–2.5	n = 8
	2.6–5.0	n = 10
	>5.1	n = 1
Grade (n = 19)	G1	n = 16
	G3	n = 3
Nodal metastasis (n = 13)	0[a]	n = 5
	1[b]	n = 8
Angioinvasiveness (n = 20)	0	n = 11
	1	n = 9
Insulin (n = 16)	0	n = 7
	1	n = 9
Glucagon (n = 15)	0	n = 11
	1	n = 4
Somatostatin (n = 11)	0	n = 4
	1	n = 7
Synaptophysin (n = 12)	0	n = 2
	1	n = 10
Chromogranin (n = 11)	0	n = 1
	1	n = 10

[a]absence, [b] presence.

One slide of 4–5 μm thickness was cut from the TMA block. The slide was then stained with specific polyclonal antibodies directed against HMGA1 [17] as previously reported [11]. After the immunohistochemical staining of the TMA, two pathologists independently evaluated the slide. For the evaluation, percentage of positive cells was considered and 50% of the positive cells was set as cutoff for positivity. Then, percentage values were classified in three class: = 100%, <100% and ≥50%, <50%. A statistical analysis and a correlation with clinico-pathological data was also performed; p-value <0.05 was considered statistically significant.

Additionally, n = 57 whole sections (n = 49 evaluable) deriving from Merkel cell carcinoma blocks (Table 1) were also evaluated for the expression of HMGA1, following criteria reported above.

2.3. Quantitative real-time PCR

Quantitative (q)RT-PCR analysis was employed for the evaluation of *HMGA1* in GEP-NET samples. Briefly, total RNA was extracted from n = 20 (n = 19 evaluable) fresh-frozen GEP-NET samples by using Trizol reagent (Life Technologies, Grand Island, NY), according to the manufacturer's instructions. RNA samples were checked for abundance and integrity by using Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and gel electrophoresis, respectively. Subsequently, 1 μg of each sample was reverse-transcribed to obtain cDNA, by using First-Strand cDNA Synthesis Kit (GE Healthcare, Chicago, IL). Amplification reactions were carried-out on a ViiA 7 Real-Time thermocycler (Life Technologies). To perform qRT-PCR experiments on fresh-frozen GEP-NET samples, the well consolidated TaqMan Assay methodology (Thermo Fischer Scientific) was employed, by using primer pairs and TaqMan probes specific for human HMGA1 and 18S rRNA Endogenous Control (Thermo Fischer Scientific). For GEP-NET cell lines mRNA detection, we used QuantiTect Reverse Transcription Kit (Qiagen, Germantown, MD) to reverse transcribe total RNA, and then we performed qRT-PCR by using Power SYBR Green PCR Master Mix (Thermo Fisher Scientific) and the following primers:

HMGA1-Fw 5'-aaggggcagacccaaaaa-3' *HMGA1*-Rev 5'-tccagtcccagaaggaagc-3'

G6PD-Fw 5'-acagagtgagcccttcttcaa-3' *G6PD*-Rev 5'-ataggagttgcgggcaaag-3'

EZH2-Fw 5'-gtctcccctacagcagaa-3', *EZH2*-Rev 5'-cctttgctccctccaaa-3'

CCNB1-Fw 5'- cttagacaaattctgaactagtgtaca-3', *CCNB1*-Rev 5'- attcttgacaacggtgaat-3'

CCNE-Fw 5'-ggccaaaatcgacaggac-3', *CCNE*-Rev 5'-gggtctgcacagactgcat-3'

The ΔΔCt method [18], was used to calculate expression levels.

2.4. Cell cultures and transfections

The functional role of HMGA1 in the modulation of cell growth and migration, has been carried out in neuroendocrine tumor-derived cell lines BON1 (pancreatic neuroendocrine tumor) and QGP1 (pancreatic somatostatinoma). They were maintained in DMEM-F12 (Sigma-Aldrich, St. Louis, MO) supplemented with 1% glutamine, 1% antibiotic and 10% fetal bovine serum; growth was achieved in a humidified incubator containing 5% CO_2 at 37°C.

Cells were transfected with Lipofectamine 2000 reagent (Life Technologies), and shRNA against *HMGA1* (Sigma-Aldrich) was employed to silence the expression of the protein. Cells transfected were selected with 2 μg/ml puromycin for 15 days, after that, several clones for each type were picked and expanded for further analysis. After silencing experiments, total protein lysates were obtained, and Western blotting procedures were performed to confirm the silencing of the HMGA1 protein expression. GAPDH protein was evaluated as protein loading control. Protein lysates and Western blotting procedures have been performed as reported elsewhere [19]. Antibodies used for Western blotting are: anti-EZH2 #3147 (Cell Signaling Technology, Danvers, MA), anti-Cyclin E #4129 (Cell Signaling Technology), anti-Cyclin B1 #12231, anti-GAPDH sc-47724 (Santa Cruz Biotechnology, Dallas, TX). Antibody against HMGA1 protein is described elsewhere [20]

2.5. Growth curve and migration analyses

For the evaluation of cell growth, after the silencing of HMGA1 expression by shRNA transfection, proliferation rate was evaluated by using Burker hemocytometer chamber. Briefly, 3×10^4 cells were plated in a 60 mm plate. Cells were counted in triplicate for 5 days with Burker hemocytometer chamber to evaluate cell growth rate.

For colony assays, BON1 and QGP1 cells at 80% confluency were transfected with shA1 and scrambled in a 100 mm plate. Twenty-four hours after plating, the medium was refreshed and 2 μg/ml puromycin was added. Refreshing of a medium containing puromycin was performed each 48 hours for 15 days, after that, plates were fixed with a solution containing 0.05% crystal violet and 20% methanol.

For the evaluation of migration, 4×10^4 transfected cells were plated in the upper chamber of a transwell (Corning Incorporated, Corning, NY), in a growth medium deprived of bovine serum. Twenty-four hours later, cell migrated toward the lower chamber containing 10% FBS were evaluated. Briefly, transwells were then fixed with a solution containing crystal violet 0.05%, and several images were acquired. Lastly, crystal violet was destained with a solution containing PBS 0.1% SDS, and the lysates obtained were read at 590 nm through a plate reader (LX800, Universal Microplate Reader, BioTek Instruments, Inc., Winooski, VT). Experimental data were normalized versus cell transfected with non-silencing shRNA and fold of induction values were reported in the graph.

The wound healing assay was performed in BON1-shA1 with the relative control in a 60 mm plate and allowing the cellular monolayer to reach full confluence. A P200 pipette tip was used to cause the wound on the monolayer and, in particular, multiple wounds were inflicted in the plates to minimize the possibility of errors. The culture medium was then changed, and each individual plate was observed and acquired every 24 hours to evaluate the wound closure rate and then the migration rate.

2.6. Statistical analysis

GraphPad Prism software (version 6.0) was used to perform statistical analysis. The Mann-Whitney

t-test was applied when required and $p < 0.05$ was considered as cutoff for statistical significance.

3. Results

3.1. Analysis of HMGA1 protein expression in GEP-NETs and its correlation with clinico-pathological data

To evaluate the involvement of the HMGA proteins in NETs, we analyzed the expression of HMGA1 in GEP-NET specimens by immunohistochemistry. We took advantage of a tissue microarray (TMA) [21] containing GEP-NET samples available at the Institute of Pathology (Basel, Switzerland). Using this approach, we were able to analyze a cohort of n = 63 (n = 43 evaluable cases) GEP-NET cases distributed among three different organs, according to Table 1. For the samples contained in the TMA, a wide range of clinico-pathological information was accessible.

After the staining of the TMA, the positivity for HMGA1 expression was given by a clear strong nuclear signal as previously described [22] (Figure 1). HMGA1 was moderately to highly expressed in the vast majority of GEP-NET samples analyzed (Table 1, Figure 1). In particular, HMGA1 resulted overexpressed in 13 out of 14 pancreatic NETs analyzed (93%), in 4 out of 4 gastric NETs analyzed (100%) and in 21 out of 25 intestinal NETs analyzed (84%) (Table 2). It is worth noting that in the vast percentage of cases analyzed, 100% of the tumor cells present in the different TMA spots were positive for HMGA1 expression (Figure 1). Then, the HMGA1 expression results were correlated with clinico-pathological data. However, probably due to the overexpression of the protein in the great majority of the analyzed cases, it was not possible to carry out a significant correlation analysis. Thus, in the case of GEP-NET, it appears that the overexpression of HMGA1 is rather an early event and not associated with tumor progression.

Subsequently, to understand whether HMGA1 protein overexpression also reflected a deregulation of gene transcription, we evaluated the expression of *HMGA1* mRNA in GEP-NETs through qRT-PCR. We retrieved archives of a small series of n = 20 fresh-frozen GEP-NET cases (14 pancreatic NETs, 2 gastric NETs and 4 intestinal NETs) from the Institute of Pathology (Basel, Switzerland). Interestingly, we observed that *HMGA1* was increased in the 50% (7 out of 14 cases analyzed) of pancreatic NET cases

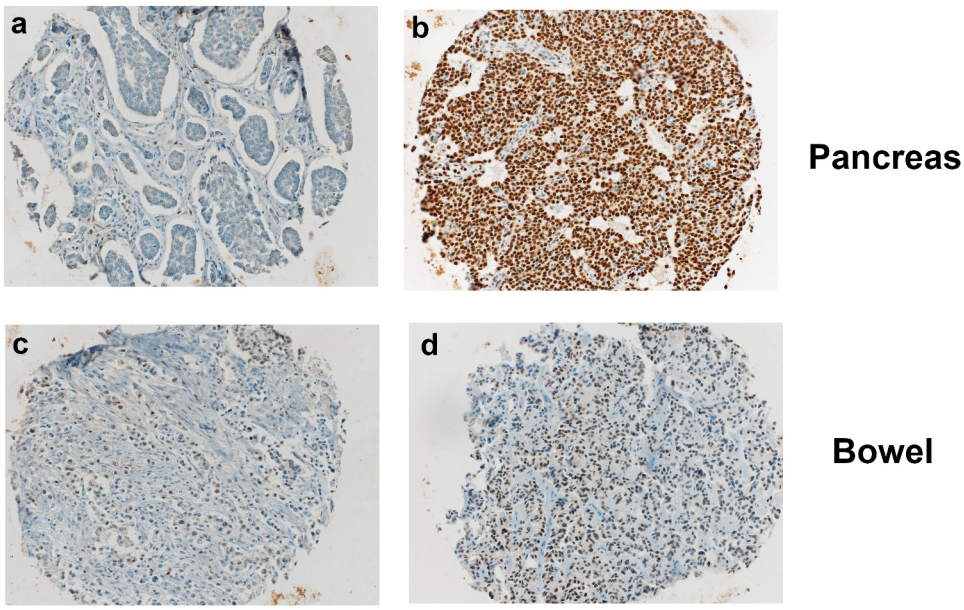

Figure 1. Representative immunohistochemical images showing different degree of HMGA1 expression in GEP-NET samples. (a) Pancreatic NET negative for HMGA1 expression. (b) Pancreatic NET overexpressing HMGA1 at high intensity in the 100% of tumor cells. (c) Intestinal NET negative for HMGA1 expression. (d) Intestinal NET showing intense staining of HMGA1 protein in at least 80% of tumor cells.

Table 2. GEP-NET and Merkel cell carcinoma samples analyzed for the expression of HMGA1 by qRT-PCR and immunohistochemistry.

qRT-PCR	Cases retrieved	Cases evaluable	Overexpression
GEP-NET	***(n = 20)***	***(HMGA1) (n = 19)***	***n = 9***
Pancreatic NET	n = 14	n = 14	n = 7 (50%)
Gastric NET	n = 2	n = 2	n = 0 (0%)
Intestinal NET	n = 4	n = 3	n = 2 (67%)
Immunohistochemistry	**Cases retrieved**	**Cases evaluable**	**Overexpression**
GEP-NET TMA	***(n = 63)***	***(HMGA1) (n = 43)***	***n = 38***
Pancreatic NET	n = 20	n = 14	n = 13 (93%)
Gastric NET	n = 4	n = 4	n = 4 (100%)
Intestinal NET	n = 39	n = 25	n = 21 (84%)
Merkel cell carcinoma	***n = 57***	***n = 49***	***n = 46 (94%)***

analyzed (Figure 2). In addition, HMGA1 was increased in 2 out of 3 intestinal NET cases evaluable (Figure 2).

Therefore, we can conclude that the overexpression of *HMGA1* in GEP-NETs also occurs at mRNA level.

3.2. Analysis of the functional role of HMGA1 in GEP-NET cells

HMGA1 acts on the regulation of critical cancer-related genes and this is immediately reflected in the diverse modulation of cell growth and transformation. Therefore, we analyzed the effects of HMGA1 silencing on cellular functions, such as cell proliferation and migration property. First of all, we analyzed by qRT-PCR and Western blot the expression levels of HMGA1 in a small panel of NET-derived cancer cell lines. We found that HMGA1 protein was abundantly expressed in BON1 and QGP1 cell lines (data not shown).

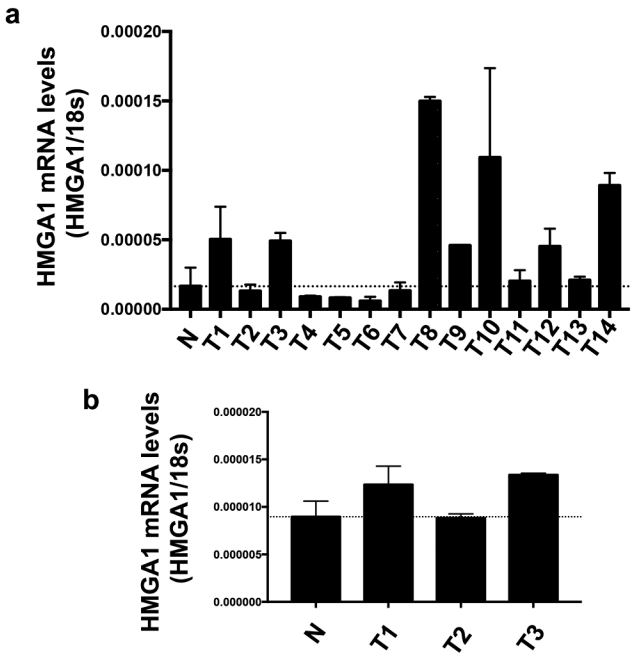

Figure 2. Histograms depicting the overexpression of HMGA1 in the cohort of samples analyzed by qRT-PCR. Expression of HMGA1 in pancreatic and intestinal NETs. 18S rRNA expression was used to normalize values. The horizontal line indicates the $2^{-\Delta Ct}$ mean value corresponding to a pool of normal samples (pancreas, normal controls n = 4; bowel, normal control n = 1).

Subsequently, we generated GEP-NET cell clones stably silenced for the expression of HMGA1 starting from pancreatic QGP1 and BON1 cell lines, then confirmed the reduced expression of HMGA1 by qRT-PCR and Western blot analyses (Figure 3(a)). Interestingly, the growth rate of QGP1 and BON1 cell lines silenced for HMGA1 was significantly reduced, indicating the positive role played by HMGA1 in cell and tumor growth (Figure 3(b)). A similar result was obtained in both these cell lines after performing colony assay experiments: indeed, a reduced number of cell colonies was detected in cell clones silenced for HMGA1, either in BON1 and QGP1 cells (Figure 3(c)). Next, we investigated the mechanisms by which HMGA1 is able to deregulate GEP-NET cell growth. Several papers have pointed out that HMGA1, as an architectural factor of DNA, is able to bind to minor groove of DNA, regulating the expression of several cancer-related genes [10]. In particular, HMGA1 is able to regulate the expression of some genes deeply involved in cell cycle and cell growth regulation, as cyclins [23,24]. As shown in Figure 4, we found a downregulation of both the mRNA and protein levels of Cyclin E and Cyclin B1 in BON1 and QGP1 cells silenced for HMGA1. These two

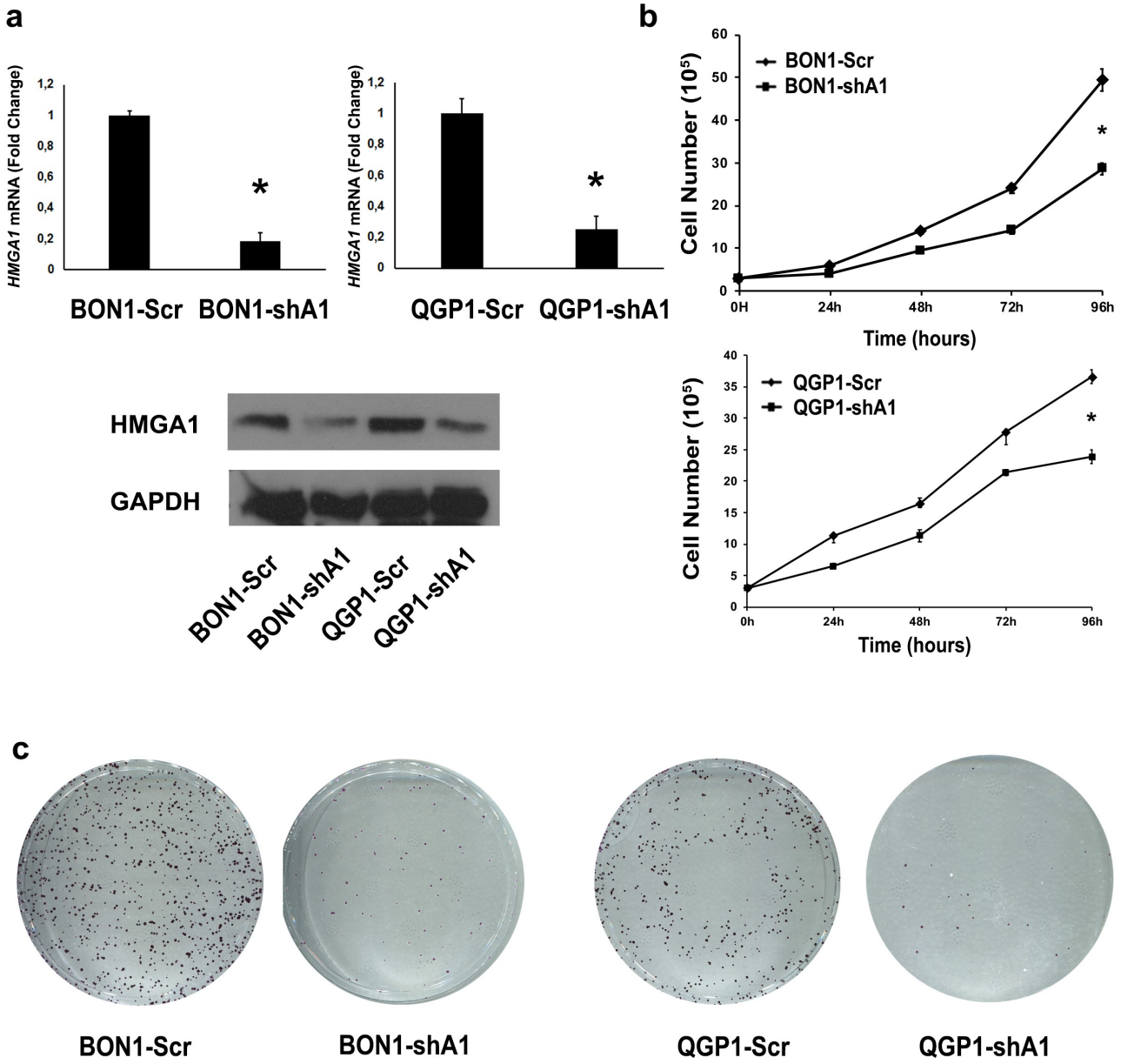

Figure 3. Growth analyses performed after the silencing of HMGA1 protein expression. (a) qRT-PCR (upper panel) and Western blot (lower panel) analyses showing the reduction of HMGA1 expression in pancreatic neuroendocrine cells BON1 and QGP1 following shRNA transfection and selection. (b) Growth curve analysis showing the reduced proliferation rate of BON1 and QGP1 cell clones transfected with shRNA targeting HMGA1 versus the corresponding cell clones transfected with the control vector. (c) Colony assay experiments showing the reduced number of colonies in BON1 and QGP1 cells after the silencing of HMGA1 expression compared with the corresponding cells expressing HMGA1. * $p < 0.05$.

genes, key regulators of cell cycle progression and cell growth, have already been reported to be regulated by HMGA1 [23,24]. Thus, we can conclude that HMGA1 is able to regulate GEP-NET cell cycle and growth by controlling the expression of these genes.

Additionally, we evaluated the migration properties of these two cell clones and observed that the silencing of HMGA1 negatively affected the migratory ability of BON1 and QGP1 cells, as evaluated by Transwell assay (Figure 5(a)). Migratory ability of BON1 cell clones was also

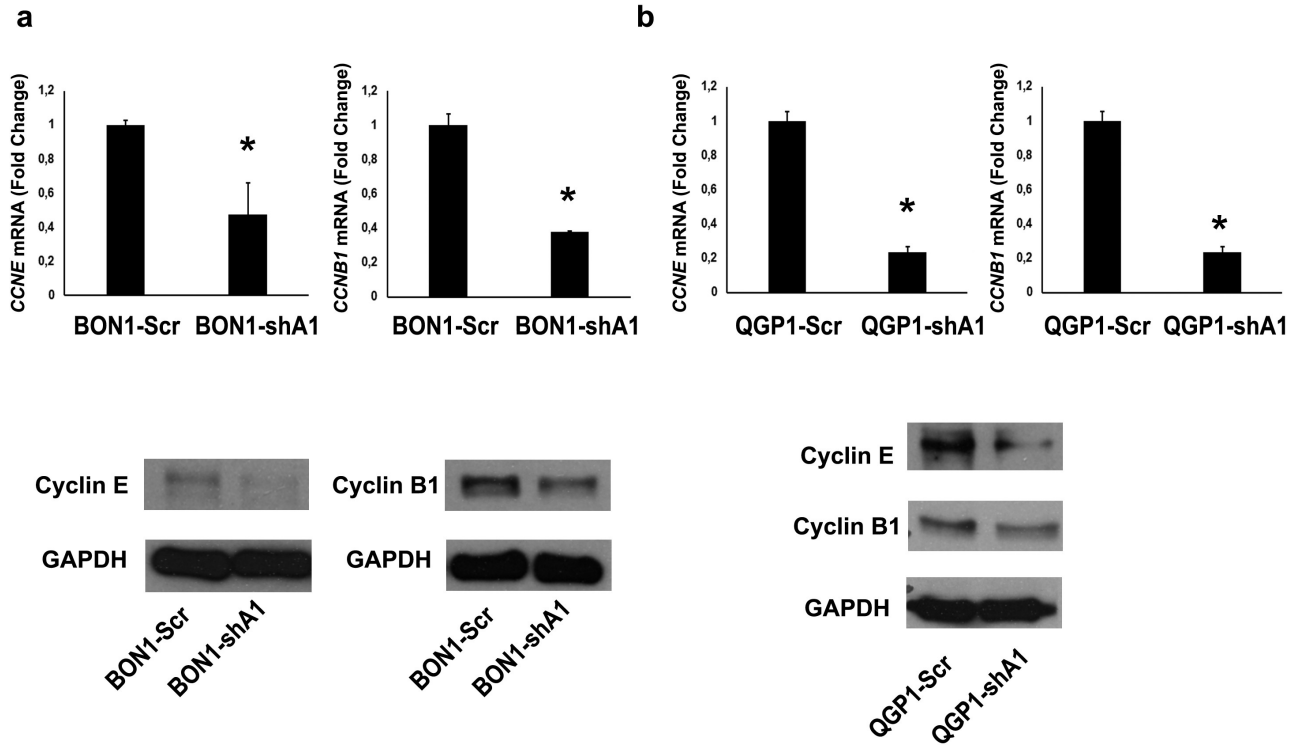

Figure 4. Cyclin E and Cyclin B1 are drastically downregulated in GEP-NETs-derived cell lines silenced for HMGA1. (a) mRNA (upper panel) and protein (lower panel) levels of Cyclin E and Cyclin B1 in BON1 cell clones transfected with shRNA targeting HMGA1 (BON1-shA1) versus the corresponding cell clones transfected with the control vector (BON1-Scr). (b) mRNA (upper panel) and protein (lower panel) levels of Cyclin E and Cyclin B1 in QGP1 cell clones transfected with shRNA targeting HMGA1 (QGP1-shA1) versus the corresponding cell clones transfected with the control vector (QGP1-Scr). * $p < 0.05$.

evaluated by performing a wound healing assay. As we can observe in Figure 5(b), BON1-shA1 cells migrated slowly if compared with BON1-Scr cells, at the same time (Figure 5(b)). This trend was even more pronounced at 48 hours (Figure 5(b)). Recently, our group has found that EZH2, one of the key regulators of cellular migration and invasion, is transcriptionally activated by HMGA1 in human lymphomas [22,25]. As reported in Figure 5(c,d), EZH2 expression was strongly downregulated in both BON1 and QGP cells silenced for HMGA1, thus suggesting that HMGA1 is able to regulate GEP-NET migration property by controlling the expression of its transcriptional target EZH2.

Therefore, these functional results indicate that HMGA1 is not only important for proliferation of GEP-NET cells but its expression is also required for the acquisition of a malignant phenotype, as observed through the evaluation of migration ability.

3.3. Evaluation of HMGA1 in Merkel cell carcinomas

Finally, to uncover whether the overexpression of HMGA1 is restricted only to neuroendocrine tumors of the gastroenteropancreatic districts or, on the contrary, this occurrence is more general and concerns also neuroendocrine tumors of other anatomical district (being related to the neuroendocrine cell of origin), we evaluated a panel of Merkel cell carcinomas of the skin (n = 57 total cases, n = 49 evaluable, Table 2).

After performing immunohistochemical staining on whole sections, we found that HMGA1 was overexpressed (at least 50% of the positive cells, see above) in n = 46 out of

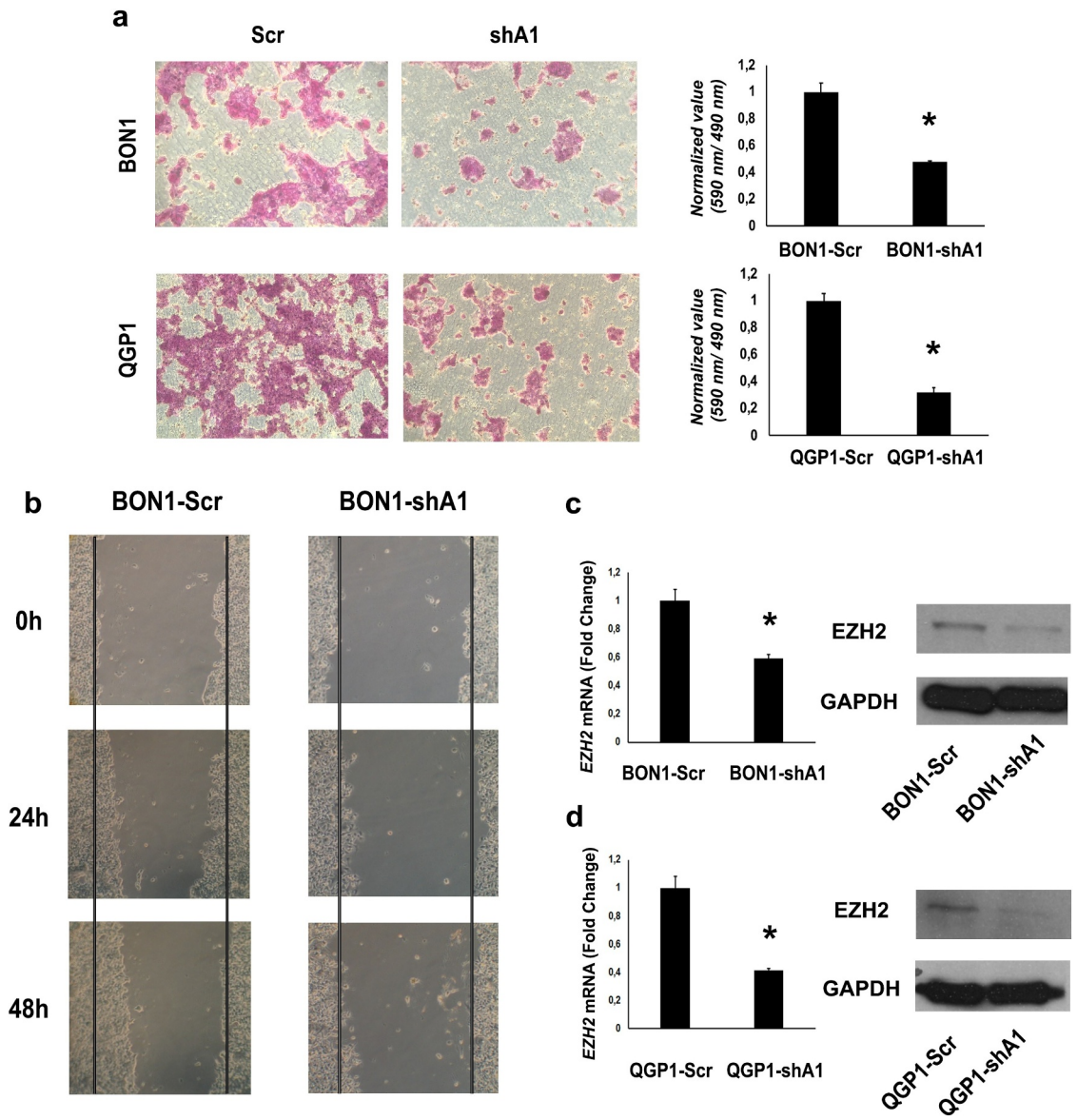

Figure 5. Evaluation of migratory abilities after the silencing of HMGA1 protein expression. (a) Migration assays performed in BON1-shA1 and QGP1 shA1 cell clones in comparison with their controls BON1-Scr and QGP1-Scr, respectively. Cells were plated in the upper chamber of a transwell containing medium deprived of fetal bovine serum and allowed to migrate toward the lower chamber containing complete medium. Evaluation of migration was performed 24 hours later. Migrated cells were fixed with crystal violet solution. After elution from transwell, it was quantified by reading its absorbance at 590 nm. (b) Wound healing assay evaluating the propensity to migrate of BON1-shA1 versus BON1-Scr cells. Wound was inflicted in each full confluent plate with a P200 tip and images were taken 24 and 48 hours later. (c) mRNA (upper panel) and protein (lower panel) levels of EZH2 in BON1 cell clones transfected with shRNA targeting HMGA1 (BON1-shA1) versus the corresponding cell clones transfected with the control vector (BON1-Scr). Cell lysates are the same of Figure 4(a). (d) mRNA (upper panel) and protein (lower panel) levels of EZH2 in QGP1 cell clones transfected with shRNA targeting HMGA1 (QGP1-shA1) versus the corresponding cell clones transfected with the control vector (QGP1-Scr). Cell lysates are the same of Figure 4(b).* $p < 0.05$.

n = 49 evaluable samples of Merkel cell carcinomas analyzed (Table 2).

Therefore, we can conclude that HMGA1 is also overexpressed in this kind of tumors, confirming its relation with the neuroendocrine cell of origin rather than the tumor histotype.

4. Discussion

Neuroendocrine tumors still represent quite mysterious entities for which useful diagnostic markers and effective therapies are still lacking. It is also worth to note that they very often arise

asymptomatically, and, thereby, are even more difficult to handle.

Here, we report that HMGA1 is overexpressed in GEP-NET samples, either at mRNA and protein levels, and these results certainly propose the HMGA proteins as diagnostic markers of GEP-NETs. Moreover, since HMGA1 is overexpressed in the great majority of the tumors analyzed, we can hypothesize that HMGA1 overexpression represents an early-onset marker, which in the future can be inserted into the diagnostic panels to be used in clinical practice. Interestingly, HMGA1 protein appears to be overexpressed early in another type of endocrine tumors, such as Merkel cell skin carcinomas, and this result further reinforces the importance of the evaluation of HMGA1 in tumors originating from neuroendocrine cells.

The causes of HMGA1 protein overexpression can be various and, recently, it has also been observed that several members belonging to the class of non-coding RNAs, including microRNA (miRNA) and long non-coding RNA, contribute to its deregulated expression. However, based upon our observation in GEP-NETs, we can exclude the involvement of HMGA1 pseudogenes. In fact, no overexpression of the HMGA1-pseudogenes HMGA1P6 and HMGA1P7 has been detected in GEP-NETs (data not shown): it is likely that this occurrence is more frequently associated to highly aggressive phenotype, as already described in anaplastic thyroid carcinomas [26], larynx carcinomas [27], endometrioid endometrial carcinomas [28] and ovarian carcinomas [29]. This means that they do not contribute to HMGA1 overexpression, which, on the other hand, could be supported by the downregulation of miRNAs targeting HMGA1 protein [30,31]. However, studies are in progress to evaluate their expression in NETs of additional histotypes other than gastroenteropancreatic one.

Moreover, our results also indicate a critical role of HMGA1 overexpression in the acquisition of a malignant phenotype other than a simple diagnostic marker in GEP-NET. Indeed, we reported that the suppression of HMGA1 expression in NET cells deriving from pancreas, negatively modulated proliferation and migration abilities (phenotypic characteristics of malignant tumors).

5. Conclusions

In conclusion, the data reported in this manuscript bring new insights in the management of neuroendocrine carcinomas. In particular, the evaluation of HMGA1 protein expression might help the diagnosis of NET in a well-refined fashion. Additionally, due to the involvement of HMGA1 protein in the mechanisms leading to NET carcinogenesis, the elucidation of this intricate relationship could certainly improve the actual prognostic evaluation protocols. Finally, the possibility of knock-down of the expression of HMGA1 gene also opens new perspectives of a more accurate and personalized therapy of neuroendocrine carcinomas.

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Disclosure statement

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Author contributions

Conceptualization, P.P., A.F. and L.M.T.; methodology, S. P. and M.De M.; software, F.E. and V.C.; validation, N.T., C.Q. and F.E.; formal analysis, F.E. and C.Q.; investigation, S. P.; M.De M., F.E. and C.Q.; resources, V.C. and S.E.C.; data curation, P.P. and N.T.; writing—original draft preparation, P.P., A.R., P.C. and A.F.; writing—review and editing, P.P., P. C., A.F. and L.M.T.; visualization, P.P., S.P. and M.De M.; supervision, A.F. and L.M.T.; project administration, N.T.; funding acquisition, L.M.T.; All authors have read and agreed to the published version of the manuscript.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Institutional review board statement

"The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethical Committee of North-West and Central Switzerland (EKBB nr 361/12)."

Informed consent statement

"Informed consent was obtained from all subjects involved in the study."

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