Circulating tumor cells and miRNAs as prognostic markers in neuroendocrine neoplasms

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Abstract

The prognosis of neuroendocrine neoplasms (NENs) is widely variable and has been shown to associate with several tissue- and blood-based biomarkers in different settings. The identification of prognostic factors predicting NEN outcome is of paramount importance to select the best clinical management for these patients. Prognostic markers have been intensively investigated, also taking advantage of the most modern techniques, in the perspective of personalized medicine and appropriate resource utilization. This review summarizes the available data on the possible role of circulating tumor cells and microRNAs as prognostic markers in NENs.

Key Words

- ▶ neuroendocrine tumors
- ▶ circulating biomarkers
- ▶ prognosis
- ▶ miRNA
- ▶ CTC

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Introduction

Neuroendocrine neoplasms (NENs) are heterogeneous tumors with widely different behavior and prognosis, that to date had been related mainly to the grade of neuroendocrine differentiation as well as the proliferative attitude of these tumors, measured by means of the Ki67 labeling index. However, as underlined by the ENETS guidelines, NEN prognosis may also depend on patient age, tumor site, metastatic spread and hormonal production. Several methods have been proposed to predict overall survival (OS) and progression-free survival (PFS) of NEN patients, including clinical nomograms taking into

account the number of liver metastases, tumor size and Ki67 index (Ruzzenente *et al.* 2016) or blood neutrophil-to-lymphocyte ratio, Ki67 index and lymph node ratio (Cao *et al.* 2016), positron emission tomography results by means of new tracers, such as (18)F-fluorothymidine (Johnbeck *et al.* 2016), or molecular markers, such as *HOPX* promoter methylation/gene expression (Ushiku *et al.* 2016) or phosphorylated histone H3 assessment (Villani *et al.* 2016). Besides tissue-based characteristics, blood-based biomarkers have been considered, also due to easy accessibility of the compartment and possibility

of multiple sampling. Among circulating biomarkers, chromogranin A has been measured in several NEN settings, but its value as a prognostic biomarker in NEN patients is limited (Capdevila *et al.* 2014), similar to other monoanalyte biomarkers (Oberg *et al.* 2015, Verbeek *et al.* 2016). The identification of prognostic factors capable of predicting the outcome of NENs is crucial in order to plan an adequate clinical management. Therefore, other attempts have been made, also on the basis of the results of studies performed in cancer fields different from NENs.

Aim

The aim of this review is to summarize the available data on two new putative prognostic biomarkers for NENs, such as circulating tumors cells (CTCs) and microRNAs (miRNAs).

Methods

Among the six authors, four (MCZ, EMG, EG and CS) independently searched MEDLINE (PubMed database) to detect articles published in the English language reporting on the prognostic value of CTCs and miRNAs in NENs, excluding Editorials and Letters. The search was last updated February 23, 2017. Additional studies were identified by reviewing the references of all selected articles. Three authors (MCZ, EG and CS) managed articles dealing with CTCs and four authors (MCZ, EMG, EG and CS) managed articles dealing with miRNA.

Part 1. Circulating tumor cells

Biogenesis and detection of circulating tumor cells

Circulating tumor cells (CTCs) are released into the circulatory system from primary or metastatic tumors, depending on the disease stage. The biogenesis of CTCs can be summarized in three steps: intravasation, migration and extravasation (Chen & Bai 2015). During the first step, tumor cells may transform to mesenchymal-like cells and intrude the blood vessel wall, entering the blood stream (Thiery 2002). Then, tumor cells migrate as single elements or in cellular clusters (microemboli). Finally, after reaching appropriate distant sites of the body, cells exit blood vessels and seed new tumor lesions (Chen & Bai 2015). The population of CTCs includes both epithelial and mesenchymal-like cells, non-stem and stem-like cells. Most of CTCs departing the primary tumor will die, whereas about 0.01% of CTCs are likely to form metastases,

as observed in preclinical models (Fidler 1970, Langley & Fidler 2011).

CTC detection consists of two main steps: enrichment and identification procedures. Enrichment techniques are based on cellular physical (size/deformability, density and electric charge) or biological properties (expression of specific antigens on the surface of neoplastic cells) (Alix-Panabieres & Pantel 2013). Most of the studies concerning the prognostic role of CTCs in cancer have been performed by using the Cell Search system (Veridex), which is the only currently FDA-approved system for the detection of CTCs in patients with metastatic breast, prostate and colorectal cancers (Cristofanilli *et al.* 2004, Danila *et al.* 2007, Cohen *et al.* 2008, de Bono *et al.* 2008, Olmos *et al.* 2009). Cell Search is a semi-automated system of CTCs detection from a small volume of blood sample (7.5 mL), based on immunomagnetic assays that target the antigens through magnetically bound antibodies and allows cell capture after exposure to a magnetic field. A positive selection of cells with the ferrofluid-bound antibody against epithelial cell adhesion molecule-1 (EpCAM) is carried out. After enrichment, a substantial number of leucocytes is still to be depleted. Subsequently, a strategy for CTC identification is performed: enriched cells are permeabilized, fixed and labeled with the fluorescent nuclear dye DAPI and fluorescent antibodies directed against the leukocyte antigen CD45 and the epithelial markers cytokeratins (CK) 8, 18 and 19. A semi-automated fluorescence microscope scans the sample and produces images that are then selected by trained operators. CTCs are DAPI+ (contain a nucleus), CK+ (express epithelial markers) and CD45– (negative for leukocyte common antigen). Analysis of neuroendocrine CTCs has been performed by using fluorescent antibodies against synaptophysin and CD56 (Khan *et al.* 2011), but they are not routinely used. Cell Search equipment can be used by applying protocols for molecular profiling, flow cytometry and FISH techniques (Sieuwerts *et al.* 2009). Enrichment methods based on physical properties include density gradient centrifugation, filtration, label-free biochips or dielectrophoresis (Parkinson *et al.* 2012); those based on biological properties are antibody-based magnetic or antibody-based microfluidic platforms (Alix-Panabieres & Pantel 2013). The functional EPISPOT assay can be added to the enrichment step, in order to identify only vital cells by measuring secreted, shed and released proteins in 24–48 h of short-term culture (Alix-Panabieres 2012). Cell Search system received regulatory approval for routine clinical use due to the high reproducibility and

purity yields (Wang *et al.* 2016). For all other systems, limited data are available concerning their clinical applications; therefore, results still must be validated in large clinical studies. At the same time, Cell Search system has an inherent limitation that hampers the detection of EpCAM-negative CTCs. This drawback is overcome by a negative enrichment method (CD45[−]) combined with immunostaining-fluorescence ISH, which is able to yield CTCs independently of EpCAM expression and cell size (Gao *et al.* 2016). A possible limitation, in case of early-stage cancer, is the difficulty to count CTCs as they are expected to be few, corresponding to a low sensitivity of the method. A possible efficient strategy to overcome this problem is to directly target the cells *in vivo*. GILUPI GmbH approach is based on the application of an antibody-coated wire into a peripheral arm vein for a duration of 30 min (Saucedo-Zeni *et al.* 2012). After removing the wire, isolated CTCs can be removed for immunocytochemical and molecular analysis. This innovative method needs to be validated in order to establish its clinical relevance.

Other detection methods are Epic sciences and Maintrac. Epic sciences (Bethel *et al.* 2014) separates nucleated cells from red blood cells and the former are exposed to four fluorescent markers. Each cell is then analyzed for 90 parameters including the fluorescence intensity of the 4 markers and other 86 different morphologic parameters; an image analysis system identifies each cell with specific coordinates and a hematopathology-trained algorithm proposes candidate CTCs that are confirmed by a trained reader. Maintrac (Pachmann *et al.* 2008) applies an approach based on microscopic identification of CTCs: it does not purify or enrich them, but it identifies them among other blood compounds by the use of a fluorescent marker (EPCAM). Only vital cells are counted by this system, and the nuclear staining with propidium iodide is used to distinguish vital from dead cells. With this method, the prognostic marker is represented by the cell count dynamics and not by the single cell count. Other techniques used to detect CTCs are available (Fabisiewicz & Grzybowska 2017), but they lack the robust validation and reproducibility of Cell Search, and their description is beyond the scope of the present review.

Circulating tumor cells as prognostic markers in human cancers The role of CTCs in cancer has been extensively investigated, especially in breast cancer. The American Society of Clinical Oncology guidelines (Harris *et al.* 2007) did not recommend the use of CTC

measurement for diagnosis nor for treatment modifications in breast cancer. However, there is solid evidence showing its value as stable prognosticator both in early stage and metastatic disease. Indeed, a meta-analysis (Zhang *et al.* 2012) conducted on 49 relevant publications pointed out that >1 CTC/7.5 mL represents a significant risk factor for both PFS in case of metastatic disease, disease-free survival (DFS) in case of early-stage disease and OS. A 5/7.5 mL cut-off has been cleared by the FDA only for metastatic breast disease. In order to predict tumor response to drugs, an assay based on both measurement and evaluation of protein expression (Hormone receptor, Her2) in CTCs has been developed (Mathew *et al.* 2015). On the basis of a well-recognized prognostic role for CTCs in breast cancer, many 'interventional' trials have been designed in metastatic disease settings (Bidard *et al.* 2013, Smerage *et al.* 2014). Similarly, CTCs have been approved by the FDA as a valid prognostic factor in progressive prostate cancer (Danila *et al.* 2007), where morbidity is mainly related to disease progression despite hormonal treatment (castration-resistant prostate cancer (CRPC)). CRPC patients can be categorized prospectively as having either unfavorable (baseline CTC level count >5/7.5 mL) or favorable outcome (baseline CTC count <5 CTC/7.5 mL). It has also been demonstrated that post-treatment CTC count can be predictive of survival (de Bono *et al.* 2008). Also in the settings of metastatic colorectal cancer (mCRC), CTCs have been recognized by the FDA (Cohen *et al.* 2008) as having an independent prognostic value. Therefore, CTCs may represent a prognostic marker in several cancer patients and may be useful to guide treatment approaches and evaluate patients response (Wang *et al.* 2017).

Circulating tumor cells as prognostic markers in neuroendocrine tumors On the basis of the evidence that CTCs have a prognostic role in several human cancers, this aspect has been investigated also in the field of NENs. The first study addressing this issue was published in 2011 (Khan *et al.* 2011), investigating the feasibility of CTC detection in patients with metastatic NENs and its potential prognostic role. The authors were capable of demonstrating strong EpCAM protein expression in formalin-fixed, paraffin-embedded (FFPE) gastroenteropancreatic (GEP) NEN tissues by immunohistochemistry and therefore set out to assess CTCs in 79 patients with metastatic NENs by using the Cell Search system. They found a variable number of CTCs in nearly 1 out of 2 patients with midgut NENs and 1 out of 5 patients with pancreatic

NENs (pNENs). Stable disease strongly associated with the absence of CTCs, whose levels correlated with urinary 5-hydroxyindole acetic acid levels and liver metastases extension, providing the first evidence that CTCs may be considered as a prognostic marker also in NENs. However, the patients were highly heterogeneous and therefore the authors performed a second study on 175 patients with metastatic NENs (Khan et al. 2013), that confirmed the feasibility of CTC count in these settings. In addition, the study showed that patients with more than one CTC displayed worse PFS and OS as compared to patients with <1 CTC, independently of other prognostic markers. To clarify whether CTCs may also predict the response to treatment, the same authors (Khan et al. 2016) evaluated CTC count before and after treatment in 138 metastatic NEN patients (31 pNEN including 9 G1, 7 G2 and 15 G3; 81 midgut NENs including 48 G1, 28 G2 and 5 G3; 12 lung NENs including 4 G1, 6 G2 and 3 G3) and found that first post-treatment CTC count was significantly associated with progressive disease. Indeed, a better outcome was recorded in patients that did not have CTCs before and after therapy and in patients displaying a >50% reduction in CTC count after treatment. The prognostic value of CTC count was confirmed by the evidence that CTCs strongly correlated with OS also in multivariate analysis, indicating that CTC count, independently of tumor grade, may be a useful tool to help guiding therapeutic decisions. However, this study groups different types of NENs, that have very different clinical behavior and follow-up schedules that may hamper the clinical applicability of this method. This limitation is being dealt with in a phase IV, multicenter, open-label, single-group exploratory study that is now ongoing in order to evaluate whether monitoring CTCs count can predict clinical symptomatic response, as well as quality of life and PFS, in patients with functioning midgut NENs treated with a somatostatin analogue (circulating tumor cells in Somatuline Autogel-treated neuroendocrine tumors patients (CALM-NET); ClinicalTrials.gov Identifier: NCT02075606). In addition, the employed dynamic approach overcomes the limit of applying a single CTC threshold level, as in previous studies performed in the settings of epithelial tumors (Cristofanilli et al. 2004, de Bono et al. 2008, Cohen et al. 2009) as it analyzes the percent CTC changes following treatment.

Concerning specific NEN histotypes, the utility of CTC count has been explored in patients with Merkel cell carcinoma (MCC) in two different studies employing the Cell Search system (Blom et al. 2014,

Gaiser et al. 2015). Their findings support the prognostic role for CTCs also in MCC, since Blom and coworkers (Blom et al. 2014) showed that patients with a negative CTC count display a better prognosis. However, the difference in the time of blood withdrawal with respect to disease stage and treatment limits the value of these findings. Gaiser and coworkers (Gaiser et al. 2015) evaluated CTCs in 30 patients with MCC confirming that these patients display a high CTC count. However, the study has important technical limitations as the heterogeneity of the patient sample prevented the identification of a correlation between tumor stage and CTC and the employed method detected CTCs also in control subjects, indicating a low specificity. On the other hand, they found significantly higher CTC counts in patients with active disease as compared to controls, supporting the need for further studies to improve the prognostic significance of CTCs in the follow-up of MCC patients. CTCs have also been investigated as prognostic markers in small-cell lung cancer (SCLC) by Hiltermann and coworkers (Hiltermann et al. 2012) who found that CTC count, assessed by the Cell Search system, correlated with tumor bulk and confirmed that the absence of CTCs at diagnosis associates with prolonged survival. Although in these settings CTC count did not predict response to chemotherapy, it represented the strongest predictor of OS after the first cycle of chemotherapy. Along this line, a recent research highlighted that copy number aberrations (CNAs) in CTCs of SCLC patients could correctly identify patients as chemorefractory or chemosensitive, indicating that CTC CNA may represent a further predictive and prognostic marker in these settings (Carter et al. 2017). Similarly, in EGFR-mutant patients with non-small-cell lung cancer (NSCLC), prospective trials employing the Cell Search system showed that CTCs are valuable and non-invasive tools to evaluate acquired resistance to chemotherapy treatment (Yanagita et al. 2016). In addition, a cut-off value of >5 CTC/7.5 mL of blood using Cell Search has been shown to be highly significant in predicting worse prognosis in NSCLC patients (Krebs et al. 2011). The most relevant findings are summarized in Table 1.

There are still more questions than answers concerning optimal technologies for CTC detection, analysis, biological significance and clinical utility. However, they may represent an important tile in the future development of 'liquid biopsies' as they mark the presence of a tumor and/or its metastases, as recently underlined (Dive & Brady 2017).

Table 1 Prognostic role of CTCs in NENs.

Series	Findings	Reference
79 metastatic NENs: – 19 pancreatic – 42 midgut – 13 bronchopulmonary – 5 unknown primary	<ul style="list-style-type: none"> Moderate correlation ($r=0.5$, $P=0.007$) between CTCs count and urinary 5-HIAA in midgut and unknown primary NENs Significant association between CTCs count and tumor burden of liver metastase ($B=8.91$, 95% CI=4.3–13.5, $P<0.001$) No correlation between CTCs count and Ki67 ($r=0.08$, $P=0.59$) and low correlation between CTCs count and serum CgA ($r=0.246$, $P=0.03$) 0 CTCs associated with stable disease ($P<0.001$) 	Khan et al. (2011)
175 metastatic NENs: – 42 pancreatic – 101 midgut – 17 bronchopulmonary – 12 unknown primary – 3 hindgut	<ul style="list-style-type: none"> Significant association between CTCs count (≥ 1) and grade ($P=0.036$), tumor burden $>25\%$ ($P<0.001$) and serum chromogranin A >120 pmol/L ($P<0.001$), respectively CTCs count ≥ 1 related with worse PFS (HRs: 6.6, $P<0.001$) and OS (HRs: 8.0, $P<0.001$) Within grade 1, two CTCs-based subgroups: HRs=5.0 for PFS ($P=0.017$) and HRs=7.2 for OS ($P=0.023$) Within grade 2, two CTCs-based subgroups: HRs=3.5 for PFS ($P=0.018$) and HRs=5.2 for OS ($P=0.036$) 	Khan et al. (2013)
138 metastatic NENs: – 31 pancreatic – 81 midgut – 12 bronchopulmonary – 11 unknown primary – 3 hindgut	<ul style="list-style-type: none"> Significant association ($P<0.001$) between the first post-treatment (after 3–5 weeks) CTCs count and progressive disease (PD): PD in 8% of patients with 'favorable count' (0 CTCs at baseline and after treatment, or $\geq 50\%$ CTCs reduction after treatment) vs 60% in unfavorable group ($<50\%$ CTCs reduction or increase) Changes in CTCs count at the first post-treatment time (after 3–5 weeks) strongly associated with OS ($P<0.001$): the best outcome in group with 0 CTCs count at baseline, followed by the group with $\geq 50\%$ CTCs reduction after treatment (HRs=3.31; 95% CI=1.50–7.32) and then the group with $<50\%$ CTCs reduction or increase (HRs=5.07; 95% CI=2.48–10.38) In multivariate analysis changes from baseline CTCs count ($P=0.0002$) and grade ($P=0.0046$) resulted independent prognostic factors 	Khan et al. (2016)
34 Merkel cell carcinomas	<ul style="list-style-type: none"> Correlation between CTCs positivity (≥ 1 CTCs) and extent of disease ($P=0.004$) Statistically significant difference in median OS between CTCs positive and CTCs negative samples ($P=0.0003$), also in case of regional node metastases ($P=0.015$) 	Blom et al. (2014)
30 Merkel cell carcinomas	<ul style="list-style-type: none"> Significantly higher CTCs count in patients with active disease Increasing CTCs count associated with development of new metastases 	Gaiser et al. (2015)
59 Small cell lung cancers	<ul style="list-style-type: none"> Association between CTCs count <2 and prolonged OS and PFS ($P\leq 0.001$) CTCs count decrease after the first cycle of therapy correlated with longer OS and PFS ($P\leq 0.001$) CTCs count decrease after four cycles of therapy correlated with longer OS ($P=0.05$) and PFS ($P=0.007$) CTCs count <2 after the first cycle of therapy is an independent prognostic factor for OS in multivariate analysis (HRs=3.5, $P=0.09$) 	Hiltermann et al. (2012)
31 Small cell lung cancers	<ul style="list-style-type: none"> Identification of chemosensitive and chemorefractory patients by CTCs copy-number aberrations profile and observation of significant difference ($P=0.0166$) in PFS between these two groups Difference in CTCs copy-number aberrations profile between initial and acquired chemoresistance 	Carter et al. (2017)

Part 2. microRNAs

Molecular biogenesis and detection of miRNAs

miRNAs are endogenous small non-coding RNAs of 19–25 nucleotides in length that control eukaryotic gene expression post-transcriptionally through degradation or translation inhibition of specific mRNAs (He & Hannon 2004). The first miRNA was discovered in *Caenorhabditis elegans*, over 20 years ago (Lee et al. 1993)

and the following studies identified more than 300 miRNAs in animals and plants (Lagos-Quintana et al. 2001, Lau et al. 2001, Lai et al. 2003). There are now over 2500 mature human miRNAs annotated in the miRNA registry (<http://mirbase.org>). miRNA biogenesis is a multistep process composed by four phases: nuclear transcription, nuclear cropping, export to cytoplasm and cytoplasmic dicing (Lee et al. 2002, Kim 2004, 2005,

Kim *et al.* 2009). miRNA genes can be divided into intergenic, with independent transcription unit, and intragenic, located inside another gene in the introns or, in a minority, in the exons. Intergenic miRNA genes can be single-, bi- or poly-cistronic (Di Leva *et al.* 2014). In the nucleus, miRNA genes are transcribed by RNA polymerase II in primary miRNAs (pri-miRNAs); they have similar structure to mRNA such as 7-methyl guanylate (m7G) caps at 5'UTR and poly(A) tails at the 3'UTR (Lee *et al.* 2004). Some miRNAs, associated to Alu repeats and linked to cell cycle and growth regulation, are transcribed by RNA polymerase III (Borchert *et al.* 2006). The pri-miRNAs are usually several kilobases long and are characterized by a large stem-loop structure, with single-stranded RNA extensions at both ends. The pri-miRNA is then processed ('cropping') by the nuclear microprocessor complex, that includes RNase III endonuclease (Drosha) and the double-stranded RNA-binding protein DiGeorge syndrome critical region gene 8 (*DGCR8*), to form 60–100 nucleotide precursor miRNA (pre-miRNA) (Han *et al.* 2004, Yeom *et al.* 2006). Pre-miRNAs are then exported into the cytoplasm by the nuclear transport receptors exportin-5 (*XPO-5*) and its partner RanGTPase, which prevents nuclear degradation and facilitates translocation into the cytoplasm (Bohnsack *et al.* 2004). Finally, in the last processing step ('dicing'), pre-miRNA is cleaved by the cytoplasmic RNase III (Dicer) into ~22 nucleotides miRNA duplexes called miRNA/miRNA* (Kim 2005). Human dicer is associated with two proteins: TRBP (TAR RNA-binding protein) (Chendrimada *et al.* 2005) and the protein activator PACT (also known as PRKRA) (Lee *et al.* 2006). Both these proteins are responsible for recognizing the same binding domain of Dicer but are not essential for Dicer-mediated cleavage. At this point, the mature miRNA strand is incorporated into the RNA-induced silencing complex (RISC), whereas the other strand is usually degraded. miRNA guides RISC to target messenger RNA (mRNA). Perfect complementarity between miRNA and mRNA leads to cleavage and degradation of the latter, whereas imperfect complementarity results into suppression of the translation of the target mRNA (Tang 2005). Otherwise, mature miRNAs can be secreted by cells in biological fluids including plasma, serum, saliva and several other body fluids (Larrea *et al.* 2016). miRNAs can be released by passive secretion from cells that lost their integrity after tissue injury, cell apoptosis or necrosis (Laterza *et al.* 2009). An alternative hypothesis is that miRNAs are actively secreted from cells in microvesicles such as exosomes or shedding vesicles (Valadi *et al.* 2007) or

conjugated to RNA-binding proteins (such as nucleophosmin 1 and argonaute 2) (Arroyo *et al.* 2011, Vickers *et al.* 2011). Circulating miRNAs might have great potential as minimally invasive biomarkers. In fact, miRNAs are more stable than mRNAs. Circulating miRNAs can be quantified by standard methods such as quantitative real-time PCR (qRT-PCR), hybridization technology (microarray) or next-generation sequencing (NGS) and *in situ* hybridization (ISH) (Uso *et al.* 2014). The starting material can be isolated from cell lines, blood and fresh or FFPE tissues (Xi *et al.* 2007). After extraction, the time between blood collection and processing should be short to prevent lysis and cellular contamination, which can be a major source of variability between samples (McDonald *et al.* 2011). Furthermore, the anti-coagulant used in plasma collection and heparin-coated tubes should be avoided because they influence downstream detection technologies (Al-Soud & Radstrom 2001). The choice of the method to quantify circulating miRNAs depends on the required experimental design. Only well-known miRNAs can be examined by qRT-PCR and microarray technology. qRT-PCR detects and quantifies miRNAs by fluorescence. miRNA microarrays is based on hybridization between target molecules (miRNAs) and complementary probes. qRT-PCR is more specific and sensitive than microarray platforms, but the latter allows large-scale studies. Moreover, new qRT-PCR arrays have been developed for large-scale miRNA profiling (Mohammadian *et al.* 2013). NGS is used to detect both novel and known miRNAs, with the limit that this technique is relatively expensive (Larrea *et al.* 2016). Finally, ISH technology may provide information regarding the exact localization of miRNAs within the tumor cell itself or within tumor stromal cells (Nuovo 2010), but has limited relevance as to circulating miRNAs are concerned. It should be borne in mind that the choice of the technique can deeply influence measurements; indeed, several studies show a lack of concordance between different methods when using the same sample source (Watson & Witwer 2012). Therefore, the same method should always been used when performing clinical studies.

miRNAs as prognostic markers in human cancers Several studies have outlined an important role of miRNAs in prognosis and prediction of therapeutic outcome in several cancers. Both decreased and increased levels of various circulating miRNAs may be associated with poor outcome. Another potentiality is to predict

Table 2 Role of circulating miRNAs as biomarkers of cancer.

miRNA	De-regulation	Site	Role as biomarker	References
miR-126a	Up-regulated	Colon	Prognostic and potentially predictive	Hansen et al. (2013)
miR-20a	Up-regulated	Colon	Diagnostic	Chen et al. (2015)
miR-155	Up-regulated	Breast	Prognostic and predictive	Roth et al. (2010) , Sun et al. (2012)
miR-195	Up-regulated	Breast	Diagnostic	Heneghan et al. (2010)
miR-29a, miR21	Up-regulated	Breast	Diagnostic	Wu et al. (2011)
miR-16	Up-regulated	Breast	Diagnostic	Hu et al. (2012)
miR-25				
miR-222				
miR-324-3-p				
let-7	Up-regulated	Breast	Diagnostic and prognostic	Joosse et al. (2014)
miR-202				
miR-18b	Up-regulated	Breast	Diagnostic and prognostic	Kleivi Sahlberg et al. (2015)
miR-103				
miR-107				
miR-652				
miR-210	Up-regulated	Breast	Predictive	Jung et al. (2012)
miR-21	Up-regulated	Lung	Diagnostic, prognostic and predictive	Wei et al. (2011)
miR-155, miR-197, miR-182	Up-regulated	Lung	Diagnostic	Zheng et al. (2011)
miR-125b	Up-regulated	Lung	Prognostic	Zhao et al. (2016)
let-7a	Down-regulated	Prostate	Predictive of high grade tumor	Mihelich et al. (2015)
miR-24				
miR-26b				
miR-30c				
miR 93				
miR-100				
miR-103				
miR-106a				
miR-107				
miR-130b				
miR-146a				
miR-223				
miR-451				
miR-874				
miR375	Up-regulated	Prostate	Prognostic	Huang et al. (2015)
miR1290				

tumor chemosensitivity. The most relevant studies are listed in [Table 2](#).

miRNAs as prognostic markers in neuroendocrine tumors

Several studies evaluated miRNA expression levels in NENs ([Vicentini et al. 2014](#)), but few examined their potential role as prognostic markers ([Table 3](#)). The prognostic role of specific miRNAs in lung NENs has been reported for the first time by Ranade and coworkers ([Ranade et al. 2010](#)), who examined the expression levels of 880 validated human mature miRNAs and 473 validated human pre-miRNAs in 31 small-cell lung cancer (SCLC) samples and found that miR-92a2* levels were inversely correlated with survival. They also showed that expression levels of miR-92a2*, miR-147 and miR-574-5p were significantly associated with chemoresistance. Following studies confirmed the association between miRNA expression levels and survival, supporting the

prognostic role for miRNA signature in SCLC. Bi and coworkers ([Bi et al. 2014](#)) surveyed 924 known miRNAs in specimens deriving from 82 patients with SCLC, who were treated with surgical resection and adjuvant chemotherapy with platinum-based regimens. They discovered that lower miR-150 and miR-886-3p expression levels were associated with a poor OS and PFS. These findings retained statistical significance as independent predictors of survival after adjusting for age, gender and smoking status and confirmed the results of a previous study on the prognostic role of miR-886-3p levels in SCLC ([Cao et al. 2013](#)). In that study, miR-886-3p expression was found to be regulated by DNA promoter methylation and to potentially repress cell proliferation, migration and invasion. A prognostic role has also been indicated for miR-7, whose expression levels in 44 SCLC samples inversely associated with chemoresistance and OS ([Liu et al. 2015](#)). The authors also demonstrated that *in vitro* miR-7

Table 3 Prognostic role of tissue miRNAs in NENs.

miRNA	De-regulation	NEN histology	Number of cases	Prognostic role	References
miR-92a2*	Up-regulated	SCLC	31	Correlation with chemoresistance and reduced OS	Ranade et al. (2010)
miR-150 and miR-886-3p	Down-regulated	SCLC	82	Correlation with reduced OS and PFS	Bi et al. (2014)
miR-886-3p	Down-regulated	SCLC	82	Correlation with reduced OS and PFS	Cao et al. (2013)
miR-7	Up-regulated	SCLC	44	Correlation with chemoresistance and reduced OS	Liu et al. (2015)
miR-192, miR-200c, miR-205	Down-regulated	SCLC	50	Correlation with increased OS	Mancuso et al. (2016)
miR-21	Up-regulated	Lung NENs	63 (19 TC, 6 AC, 19 LCNEC, 19 SCLC)	Correlation with presence of lymph node metastases in TC and AC	Lee et al. (2012)
let-7d, miR-19, miR576-5p, miR-340*, miR-1286	Up-regulated	Lung NENs	12 (3 TC, 3 AC, 3 SCLC, 3 LCNEC)	Correlation with OS	Mairinger et al. (2014)
miR-409-3p, miR-409-5p, miR431-5p	Down-regulated	Lung NENs	37 (22 TC, 15 AC)	Correlation with presence of lymph node metastases	Rapa et al. (2015)
miR-21	Up-regulated	Pancreatic NEN	40	Correlation with Ki-67 index and liver metastases	Roldo et al. (2006)
miR-642, miR-210	Up-regulated	Pancreatic NEN	37	Correlation with Ki-67 index (miR-642) and with metastatic spread (miR-210)	Thorns et al. (2014)
miR 196a	Up-regulated	Pancreatic NEN	37	Correlation with advanced stage, lymph node metastases, higher mitotic count, higher Ki67 index, reduced OS and DFS	Lee et al. (2015)
miR-183, miR-375	Up-regulated	MTC	45	Correlation with lymph node, residual disease after surgery, distant metastases and survival	Abraham et al. (2011)
miR-224	Up-regulated	MTC	40	Correlation with the absence of node metastases, lower stage at diagnosis and biochemical cure during follow up	Mian et al. (2012)
miR-21	Up-regulated	MTC	64	Correlation with basal calcitonin levels, lymph node metastases and advanced disease at the end of follow up	Pennelli et al. (2015)
miR-1225-3p	Up-regulated	PC	34	Correlation with recurrence in sporadic PC	Tombol et al. (2010)
miR483-5p	Up-regulated	PC	24	Correlation with metastatic spread and shorter DFS	Meyer-Rochow et al. (2010)

AC, atypical carcinoids; DFS, disease-free survival; LCNEC, large cell neuroendocrine carcinomas; MTC, medullary thyroid carcinoma; OS, overall survival; PC, pheochromocytoma; PFS, progression-free survival; SCLC, small cell lung cancer; TC, typical carcinoids.

modulates chemoresistance by repressing MRP1/ABCC1, a mechanism known to be involved in chemoresistance in SCLC ([Hsia et al. 2002](#), [Kuo et al. 2003](#)). An inverse

correlation was found between OS and the expression levels of 3 miRNAs (miR-192, miR-200c and miR-205) in cytological samples from 50 SCLC patients (25 samples

from primary tumors and 25 samples from lymph node metastases) (Mancuso *et al.* 2016). These miRNAs were selected because the respective target genes are frequently altered in SCLC. On the contrary, no association between miRNAs expression levels and survival or drug resistance was found by Lee and coworkers (Lee *et al.* 2011) who investigated the expression of a panel of seven miRNAs (miR-21, miR-29b, miR-34a/b/c, miR-155 and let-7a) in 31 SCLC tumor samples. The prognostic role of miRNAs in lung NENs has also been explored in another study (Lee *et al.* 2012), which evaluated the expression pattern of three miRNAs (miR-21, miR155 and miR let-7a), selected on the basis of their close correlation to tumor development and progression, in a series of 63 lung NENs, including 19 typical carcinoids (TC), 6 atypical carcinoids (AT), 19 large cell neuroendocrine carcinomas (LCNEC) and 19 SCLC. The results showed that miR-155 and miR-21 levels were significantly higher in high-grade neuroendocrine carcinomas (LCNECS and SCLC) as compared to TC and AC. In addition, miR-21 expression levels correlated with the presence of lymph node metastases in TC and AC, supporting the prognostic role of this miRNA in lung NENs. Mairinger and coworkers (Mairinger *et al.* 2014) screened 763 miRNAs known to be involved in pulmonary carcinogenesis in 12 lung NENs (3 TC, 3 AC, 3 SCLC and 3 LCNECS) and found that five miRNAs (let-7d, miR-19, miR576-5p, miR-340* and miR-1286) significantly associated with survival. Moreover, eight miRNAs (miR-22, miR-29a, miR-29b, miR-29c, miR-367*, miR-504, miR-513C and miR-1200) negatively correlated with tumor grade, whereas four miRNAs (miR-18a, miR-15b*, miR-335* and miR-1201) positively correlated with tumor grade. On the contrary, no association between miRNA expression and disease-free survival was found by Deng and coworkers (Deng *et al.* 2014) who evaluated a panel of 1145 miRNAs in a series of 47 lung NENs (44 TC and 3 AC). Rapa and coworkers evaluated 56 cases of lung NENs (22 TC, 15 AC, 15 LCNECS and 4 SCLC) for the expression of 11 miRNAs (miR-15a, miR-22, miR-141, miR-497, miR-503, miR-129-5p, miR-185, miR-409-3p, miR-409-5p, miR-431-5p and miR-129*), selected on the basis of the results obtained in a previous pilot series. In agreement with the previous study by Mairinger and coworkers and by Lee and coworkers showing distinctive microRNA expression profiles in carcinoids as compared to high-grade neuroendocrine carcinoma, all but two miRNAs (miR-503 and miR-185) were significantly able to segregate different tumor histotypes. All but one miRNA (miR-431-5p) were found to be upregulated in the carcinoid group as compared to high-grade

neuroendocrine carcinomas. Moreover, when evaluating the association between the 11 selected miRNAs and clinical and carcinoid pathological characteristics, four miRNAs (miR-129-5p, miR-129*, miR-22 and miR-141) were found downregulated in high-stage tumors, four miRNAs (miR-129-5p, miR-409-3p, miR-409-5p and miR-431-5p) were found downregulated in cases with vascular invasion and three miRNAs (miR-409-3p, miR-409-5p and miR-431-5p) were found downregulated in tumors with lymph node metastases (Rapa *et al.* 2015).

Similarly, miRNA expression pattern in GEP NENs has been extensively investigated (Ruebel *et al.* 2010, Matthaei *et al.* 2012, Li *et al.* 2013a,b, 2015, Dossing *et al.* 2014, Miller *et al.* 2016), but few studies evaluated a possible prognostic role of miRNAs in GEP NEN. Roldo and coworkers (Roldo *et al.* 2006) evaluated the global miRNA expression in a series of 40 pNENs (28 non-functioning and 12 insulinomas) and found that miR-21 levels strongly associated with Ki67 labeling index and liver metastases. In addition, they have shown that the expression levels of programmed cell death 4 (*PDCD4*) gene were significantly downregulated in metastatic and highly proliferative tumors, where they inversely correlated with miR-21, in keeping with the hypothesis that *PDCD4* is a target of miR-21. *PDCD4*, in turn, represents a tumor suppressor gene involved in invasion and metastasis (Jansen *et al.* 2004). Thorns and coworkers (Thorns *et al.* 2014) investigated the expression levels of 754 miRNAs in tissue samples of 37 patients with pNEN. They found that miR-642 and miR-210 correlated with Ki-67 labeling index and with metastatic spread, respectively, but could not provide information concerning survival. Lee and coworkers (Lee *et al.* 2015) evaluated the expression levels of eight miRNAs (miRNA-27b, 122, 142-5p, 196a, 223, 590-5p, 630 and 944) in 37 pNENs. High miR 196a levels were significantly associated with advanced stage, lymph node metastases, higher mitotic count, higher Ki67 index, reduced OS and DFS. The negative prognostic power of miR 196a in pNENs was conserved also when only the group of G2 NEN patients was considered. In addition, high miR-27b levels associated with decreased OS. Therefore, miRNA profiling may represent a useful prognostic tool in GEP NENs.

On the contrary, miRNA expression profiles in medullary thyroid carcinoma (MTC) have been evaluated in few studies (Nikiforova *et al.* 2008, Abraham *et al.* 2011, Mian *et al.* 2012, Hudson *et al.* 2013, Santarpia *et al.* 2013, Gundara *et al.* 2014, Pennelli *et al.* 2015, Lassalle *et al.* 2016), out of which only three evaluated the prognostic role of miRNAs in MTC. Abraham and coworkers

(Abraham *et al.* 2011) evaluated the expression of four miRNAs (miR-183, miR 375, miR 9 and miR 9*) selected on the basis of their reported significance in other cancers. They found that in 45 cases (26 sporadic and 19 hereditary MTC) miR-183 and miR-375 overexpression predicted lymph node metastases and was associated with residual disease after surgery, distant metastases and mortality. On the basis of these results, the authors proposed a possible use of these miRNAs in patients that do not show clinical nor radiological lymph node involvement before surgery in order to select those to be submitted to prophylactic lateral neck dissection or to guide a more stringent post-operative surveillance of the lateral neck compartment for evidence of recurrent disease. The authors corroborated their results with a functional study in the TT cell line, proving that miR-183 knockdown significantly decreased the number of viable cells, potentially via autophagy. Mian and coworkers (Mian *et al.* 2012) analyzed the expression of nine miRNAs (miR-21, miR-127, miR-154, miR-224, miR-323, miR-370, miR-9*, miR-183 and miR-375), selected on the basis of previous profiling studies, in 34 sporadic and in 6 hereditary MTC. They found that miR-224 upregulation correlated with the absence of node metastases, lower stage at diagnosis and biochemical cure during follow-up. At variance with the study by Abraham and coworkers (Abraham *et al.* 2011), Mian and coworkers (Mian *et al.* 2012) did not find a correlation between miR-183 or miR-375 levels and TNM stage or patients outcome. Subsequently, the same authors (Pennelli *et al.* 2015) studied the PDCD4/miR-21 pathway in 64 MTCs (56 sporadic and 8 hereditary). In line with the previous results obtained in GEP NEN (Roldo *et al.* 2006), they found that miR-21 levels inversely correlated with PDCD4 levels, confirming the latter as a target of miR-21. In addition, they found that miR-21 levels directly correlated with basal calcitonin levels, lymph node metastases and advanced disease at the end of follow-up. Along this line, PDCD4 levels correlated inversely with disease stage at diagnosis and directly with biochemically persistent disease at the end of follow-up. These studies pave the way for further validation studies, where the prognostic value of miRNA profiles should be investigated for clinical purposes.

Even fewer studies have reported microRNAs expression in pheochromocytomas (PC) and paragangliomas, so far (Patterson *et al.* 2012, de Cubas *et al.* 2013, Zong *et al.* 2015). These studies were mainly focused on the different miRNA expression profiles of hereditary tumor syndromes associated with PC and of benign vs. malignant PC.

This issue is extremely important as there are no reliable histologic or molecular markers that allow distinguishing benign from malignant PCs. Tombol and coworkers (Tombol *et al.* 2010) evaluated 34 PC cases including 9 sporadic benign, 5 sporadic recurrent tumors, 8 lesions in the settings of multiple endocrine neoplasia type 2, 6 associated with von Hippel Lindau disease, 5 with type 1 neurofibromatosis and 1 malignant PC. They observed that miR-1225-3p levels are higher in sporadic recurrent as compared to sporadic non-recurrent PC, providing a threshold value for miR-1225-3p also that identified a PC as recurrent with good sensitivity and specificity. Meyer-Rochow and coworkers (Meyer-Rochow *et al.* 2010) evaluated 12 benign and 12 malignant PCs and found a total of 18 miRNAs differentially expressed in benign as compared to malignant tumors. Three miRNAs (miR-483-5p, miR-15a and miR 16) were selected for validation in a separate cohort of benign and malignant samples and confirmed to be diagnostic markers for malignant PC. In particular, high miR483-5p expression levels were found to be a negative prognostic marker as they were capable of predicting metastatic spread and reduced DFS.

In contrast with the evidence that tissue miRNAs may help in predicting NEN patient prognosis, few data are available concerning circulating miRNAs. The feasibility of measuring circulating miRNAs has been confirmed in studies performed in patients with small intestine NENs (Li *et al.* 2015) or pNENS (Li *et al.* 2013a). However, these studies do not provide information concerning patient prognosis. In addition, it has been reported that, as compared to healthy volunteers, patients with pNEN display very few different circulating microRNAs that are not the mostly deregulated in the tissue (Thorns *et al.* 2014). Moreover, a recent consensus on biomarkers for NENs (Oberg *et al.* 2015) concluded that circulating miRNA levels weakly associated with tissue miRNA levels that, in turn, have been found to vary widely. At present, clinical use of circulating miRNA is not recommended as the assessment needs further refinement, especially regarding normalization. Therefore, circulating miRNAs cannot be regarded as possible prognostic markers in NENs on the basis of available evidence.

Conclusions

The available literature data clearly show that tissue miRNA profiling may potentially represent a prognostic biomarker in NENs. However, the role of circulating miRNAs in these settings is far to be consolidated.

Studies prospectively evaluating circulating miRNA in different NEN types (and stages) and their levels after the different available therapeutic approaches are still lacking. On the contrary, studies employing CTC count as a marker of NEN prognosis report very promising results that need to be validated in further clinical trials. Studies on selected validation cohorts with long-term clinical follow-up are necessary to further qualify CTC as biomarkers in NENs.

In conclusion, the field of research for reliable prognostic markers in NENs remains open, with the future perspective to identify specific characteristics or clinical algorithms that may help in planning the most appropriate clinical management for NEN patients.

Declaration of interest

M C Zatelli has received consultant fees from Novartis, Pfizer and Genzyme. The other authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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Author contribution statement

Maria Chiara Zatelli wrote the abstract, the Introduction, the part related to the role of circulating tumor cells as prognostic markers in NENs, the conclusions and revised the manuscript. Erika Maria Grossrubatscher wrote the part related to the role of miRNAs as prognostic markers in NENs. Elia Guadagno wrote the part related to the detection of CTCs as well as the part related to miRNAs and CTCs as prognostic markers in human cancers. Concetta Sciammarella wrote the part related to the biogenesis of miRNA and CTCs as well as the part related to the detection of miRNA. Antongiulio Faggiano proof-read the manuscript. Annamaria Colao supervised the project.

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