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REVIEW ARTICLE

Circulating biomarkers for nonfunctional gastroenteropancreatic neuroendocrine neoplasm: Where do we stand?

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Abstract

Gastroenteropancreatic neuroendocrine neoplasms (GEP-NENs) encompass a heterogeneous group of tumors associated with variable presentations, growth rates, and prognoses. The majority of GEP-NENs are nonfunctional, and their diagnosis remains challenging given the often subtle and variable clinical manifestations of these tumors. As a consequence, GEP-NENs are often recognized at an advanced stage; indeed, most patients with nonfunctional GEP-NENs exhibit metastatic disease at diagnosis. Lack of treatment options as well as limitations in currently available imaging modalities and biomarkers make it challenging to manage NENs. Thus, novel biomarkers are needed to provide high sensitivity and specificity for minimum disease detection and to predict treatment efficacy and prognosis. Although tissue-based biomarker data can provide such information, circulating biomarkers such as NETests, circulating tumor cells, and microRNAs, are superior owing to their easy accessibility and the ability for repeated real-time sampling.

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Neuroendocrine neoplasms (NENs) constitute a heterogeneous group of tumors, and the tumor phenotype can range from indolent to almost completely unregulated growth, resulting in aggressive invasion and metastasis [1]. GEP-NENs are the most common sites, accounting for 55%–70% of all NENs [2]. The incidence of all GEP-NENs has increased markedly from 2.48 per 100,000 persons in 1994 to 5.86 per 100,000 in 2009 [3]. GEP-NENs can be defined as functioning or nonfunctioning depending on the presence of a syndrome related to inappropriate hormone secretion. Most GEP-NENs are nonfunctional and lack specific manifestations; thus, they are often diagnosed at a late stage at which metastatic progression is observed. Two critical unmet needs are the inability to establish an early and accurate diagnosis and the evaluation of NEN therapeutic responses. The latter is mainly based on Response Evaluation Criteria in Solid Tumors (RECIST) criteria, which is difficult to assess in indolent lesions. Overall, the current criteria for the assessment of NEN progression and therapeutic responses is suboptimal. Given the limited accuracy of currently available biomarkers, NEN-specific biomarkers are necessary to ensure scientific and clinical value.

Biomarkers are classified into three categories by the National Institutes of Health. Type 0 biomarkers suggest the natural history of the disease, type I biomarkers reflect interventional effects, and type II biomarkers are surrogate clinical endpoints [4]. Ideal biomarkers should be multidimensional, providing information on the specific diagnosis, proliferative and metastatic capacity, presence of residual lesions, and therapeutic responses [5]. Recent molecular studies have investigated the genomic landscapes of these tumors. These studies have resulted in the identification of mutations and expression anomalies in genes and pathways, such as the ATRX-DAXX, multiple endocrine neoplasia type 1 (MEN1), and phosphoinositol 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) pathways, as well as epigenetic alterations, such as DNA methylation, histone modification, chromatin remodeling, and extension of alternative telomerase activation mechanism [6–9]. These discoveries in turn may lead to new and better prognostic biomarkers and

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additional candidates for targeted therapies. However, it is still unclear whether such molecular biomarkers may be correlated with biological behaviors and clinical features. Compared with tissue biopsies, sampling the blood is minimally invasive and allows for dynamic monitoring of molecular changes in the tumor rather than relying on a static time point. In this review, we summarize the approach for rational validation of potential circulating candidates that may be involved in nonfunctional GEP-NENs management. We also focus on recent advances in our understanding of the roles of these biomarkers as diagnostic/prognostic factors and the optimal therapeutic approaches for management of GEP-NENs.

Current biomarkers

Current neuroendocrine tumor biomarkers include chromogranin A (CgA), neuron-specific enolase (NSE), pancreatic polypeptide, gastrin, and 5-hydroxyindoleacetic acid (5-HIAA). However, these biomarkers are not sufficient for accurate identification of the primary tumor site and prediction of prognosis. Immunohistochemistry for CgA is not sufficiently specific for the diagnosis of NENs and may be negative in poorly differentiated neuroendocrine carcinomas (NECs). Moreover, chromogranin B is more prevalent in colorectal and appendix NENs [10]. Ki67 antigen, a proliferation-related protein, is associated with biological behavior, treatment responses, and prognosis. However, morphologically well-differentiated NENs can have high Ki67-labeling indices (20%–50%). In fact, about 40% of grade 3 GEP-NENs are well differentiated and could be better designated as "NET G3". Notably, studies have shown that NET G3 lesions have a less aggressive phenotype and exhibit sensitivity to platinum-based chemotherapy compared with poorly differentiated high-grade NECs [11]. Alternative evaluation methods, such as circulating biomarkers, have been investigated. The best known circulating biomarker is plasma CgA. Increased CgA is generally considered to be sensitive (60%–90%) and accurate once an NEN has been identified [12]. Moreover, an early decrease in CgA after treatment is positively correlated with survival rate [13]. However, measurements are usually nonspecific (10%–35% specificity) since CgA is elevated in other conditions, including neoplasia, renal failure, cardiac and inflammatory diseases, and proton pump inhibitor (PPI) administration [14]. A standard cutoff value does not exist, and variations occur in measurements across different laboratories. Thus, novel biomarkers associated with accurate diagnosis and assessment of treatment and prognosis are needed. Current mono-analyte blood-based biomarkers for diagnosis and follow-up of NENs do not achieve satisfactory metrics of sensitivity and specificity.

Multi-analyte assays with algorithmic analytics and NETest

Specific mono-analytes, which define the secretory status of a tumor, have been proven to be useful in diagnosis but are disappointing in the assessment of disease progression. Current scientific analyses of biomarker identification have focused on the development of multi-analyte assays with algorithmic analytics (MAAAs). This strategy facilitates the coupling of integral aspects of disease represented by individual markers into a mathematical algorithm that provide multidimensional clinical and pathobiological information inaccessible in a mono-analyte approach [15–16]. MAAAs have been used to identify circulating NET transcripts and have shown that blood measurements are correlated with tumor tissue transcript analysis. The latter is segregated into six gene clusters, which differentiate SD from PD [17]. The MAAA-derived values allow for a sensitive, noninvasive approach for detection of NENs. Such an approach may be rendered even more informative in combination with assessments provided by integration with objective data obtained from imaging and nuclear medicine scanning.

Blood-based multi-analyte algorithm analysis polymerase chain reaction (PCR)-based tests (NETests) show potential for GEP-NEN management. NETest assays are not affected by factors unrelated to NEN disease and exhibit a high specificity of 97% and sensitivity of 98%. For example, the NETests are not elevated in patients receiving PPIs, making them superior to plasma CgA [18]. Under such conditions, NETests may be more reflective and specific in comparison with mono-analyte tests. Moreover, NETests provide additional information that can be used for the detection of disease recurrence and prediction of the therapeutic response of stabilized analogs of somatostatin (SSA) and peptide receptor radionuclide therapy (PRRT) [19]. Considering the relatively indolent and slow progressive behaviors of GEP-NENs, the RECIST criteria and mono-analyte markers are not sufficient to accurately assess residual lesions and recurrence. In addition, NETests are considered as useful in assessment of the adequacy of operative resection and radiofrequency ablation [20]. Therefore, NETests have the potential for precise determination of residual disease, minimal disease detection, and recurrence for patients after R0 resection. However, due to the limited data and short follow-up period, additional studies are needed to establish the most accurate timing of blood collection and other metrics in the prediction of residual/recurrent disease.

MicroRNA (miRNA)

miRNAs are short, noncoding RNAs of approximately 21–23 nucleotides that can interfere with protein expres-

sion either by inducing cleavage of their specific target mRNAs or inhibiting their translation. miRNAs have been shown to regulate a rapidly increasing list of complex biological processes, including cell proliferation, the cell cycle, and apoptosis. miRNAs play an important regulatory role in tumor development and progression, suggesting a wide spectrum of novel diagnostic and therapeutic opportunities. Because of the rarity of the tumor and lack of cell lines, few studies have reported the miRNA signatures of GEP-NENs, and most studies have concentrated on neuroendocrine tumors located in the pancreas and small intestine. Unique miRNAs expression profiles have been shown to be associated with different types and subsets of GEP-NENs. Roldo [21] investigated the global miRNA expression patterns in the normal pancreas and pNENs; the results demonstrated that the upregulation of miR-103/107, associated with a lack of miR-155 expression, was greater in pNENs than in the normal pancreas. The data showed that miRNA expression could distinguish pNENs from normal pancreas tissues. Moreover, overexpression of miR-21 was found to be associated with high rates of pNEN tumor proliferation and liver metastasis. miR-133a was shown to be downregulated during progression from primary to metastatic SI-NENs, suggesting that this miRNA may have an important role in tumor development and progression with utility for prognosis [22]. Li [23] characterized nine miRNAs in well-differentiated SI-NENs, five (miR-96, miR-183, miR-196a, and miR-200a) were upregulated during tumor progression, whereas four (miR-31, miR-129-5p, miR-133a, and miR-215) were downregulated.

miRNAs have potential applications as novel diagnostic and predictive biomarkers. Additional studies are needed to clarify the roles and mechanisms of miRNA expression in biological behavior and may improve early detection rates and accurate assessment of prognoses. In contrast to DNA or mRNAs, miRNAs have long half-lives in vivo and are stable *in vitro*, enabling miRNA profiling techniques to be extremely sensitive, objective, and standardized, even in formalin-fixed tissues. Indeed, miRNAs can be extracted from various specimen types, including fresh or formalin-fixed paraffin-embedded (FFPE) samples, and body fluids, such as plasma, serum, urine, and sputum [24].

Recently, miRNAs have been shown to be differentially expressed and have roles in the regulation of oncogenes or tumor-suppressor genes. The modulation of miRNAs may affect tumor proliferation, and this approach could be transferred to the clinic setting [25]. The therapeutic application of miRNAs involves two strategies. The first is directed against gain-of-function and aims to inhibit oncogenic miRNAs using miRNA antagonists. The second strategy, miRNA replacement, involves the reintroduction of a tumor-suppressive miRNA to restore a loss of function [26]. However, the association between miRNA concentrations in sera and tissues is weak. Both up- and downregulation of miRNA expression have been noted in NENs, suggesting that the use of this marker could be more complex than expected. Moreover, the roles of miRNAs in various genetic networks and regulatory pathways need to be analyzed in larger cohort neoplastic and normal tissues. From a therapeutic standpoint, adequate assessment of the functional effects after miRNA inhibition and antagonism *in vivo* are critical for the clinical application of anti-miR-based therapies.

Circulating tumor cells (CTCs)

CTCs are known to shed into the peripheral blood from solid tumors and therefore provide a less invasive and easily accessible source of tumor material that can be collected in a serial fashion. The presence and persistence of CTCs have been associated with decreased progression-free and overall survival in patients with metastatic breast, colorectal, and prostate cancer [27]. Currently, CTCs have been reported in blood samples from a number of patients with metastatic GEP-NENs (43% in the midgut and 21% in pNENs). In addition, CTCs are associated with progressive NENs and could be used as prognostic markers [28].

The CellSearch platform detects CTCs with high sensitivity and specificity and is the only system approved by the US Food and Drug Administration. The CellSearch platform requires the cellular expression of epithelial cell adhesion molecule (EpCAM), and the majority of NENs exhibit strong expression of EpCAM. Khan [29] analyzed 176 patients with metastatic NENs and showed that 49% of patients had at least one CTC in 7.5 mL blood. CTCs remain significant when other prognostic markers, including grade, tumor burden, and CgA levels, are considered. A liver metastatic burden of over 25% has been shown to be correlated with increased CTCs.

Studies have shown that CTCs in NENs may be heterogeneous. The heterogeneity may have important implications as mutations may arise when cells are shed from the primary tumor or could occur in the circulation; the latter may represent an escape mechanism from therapy. The CTCs of NENs correlate with prognosis and even have a role in adjuvant therapy through reflecting the response to chemotherapy. For example, in patients with GEP-NEN treated with SSA therapy, expression of somatostatin receptor 2 (SSTR2) and SSTR5 can predict treatment response [30]. Thus, the molecular characterization of CTCs could potentially assist in understanding NET metastasis and resistance to therapy in addition to their utility as biomarkers. However, additional studies are needed to determine how to cluster the entire spectrum of CTCs accurately and how to analyze specific subtypes. Moreover, the relationships among pathological and prognostic information need to be verified. CTCs

are not sensitive to detect of different types of NENs and are not specific for subgroups of NENs; thus, these cells should be evaluated in future studies.

Other circulating biomarkers

Recent studies have discovered more potential circulating biomarkers and solidified the potential utility of these approaches to more precisely define tumor dynamic behaviors. Circulating tumor DNA (ctDNA) is shed into the bloodstream by cells undergoing apoptosis or necrosis, and the load of ctDNA correlates with tumor staging and prognosis [31]. Moreover, recent advances in the sensitivity and accuracy of DNA analysis have allowed for genotyping of ctDNA for somatic genomic alterations. However, lower-stage tumors and even advanced cases involving low-level micrometastatic disease have reduced numbers of ctDNA fragments. In 2016, the European Neuroendocrine Tumor Society (ENETs) [32] reported three novel potential biomarkers in serum. High levels of DcR3 and TFF3 were found to be correlated with poor survival in SI-NENs, and DcR3 was shown to be a marker of liver metastasis. TFF3 and Mindin are sensitive, specific, novel diagnostic biomarkers of SI-NENs found circulating in the serum. However, the molecular mechanisms of those circulating biomarkers remain unclear, and additional studies are needed to validate these markers for clinical applications.

Conclusions

GEP-NENs are heterogeneous tumors that exhibit different characteristics based on disease subtype and have heterogeneous features within individual patients. Considering the flaws of current biomarkers, identification of efficient molecular profiling and liquid biopsy techniques is critical for providing valuable information for diagnosis, classification, monitoring of treatment responses, and determining prognoses in patients with GEP-NENs. Genomic studies and molecular profiling have revealed a number of genomic alterations. Such analyses can identify prognostic and predictive genetic alterations, though these approaches are not currently used to inform the initial treatment decisions. The relationships between tumor behaviors and specific genes, however, remain unclear. Given that repeated biopsies are not always feasible clinically, the development of blood-based strategies to measure changes in circulating molecular signatures is relevant for disease management and analysis of treatment response and outcomes. Such approaches, including analyses of CTCs, circulating RNA, NETests, and miRNAs, may be clinically relevant. Compared with mono-analyte biomarkers, NETests can define multiple variables that represent tumor growth and are applicable in the assessment of multidimensional information for monitoring tumor response to therapy and defining ambiguous clinical scenarios, such as stable disease or mixed responses. Furthermore, NETests may predict treatment response early in the course of therapy, which allows for real-time modification of treatment regimens. miRNAs have primarily been studied in the context of pNENs and SI-NENs, which have the potential for early diagnosis and therapeutic applications. CTCs are associated with progressive behaviors and have roles in adjuvant therapy. All these potential benefits of novel circulating biomarkers will have to be evaluated in appropriately designed clinical trials.

Conflicts of interest

The authors indicated no potential conflicts of interest.

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