

## Research Article

# miRNA as Potential Tool for Thyroid Cancer Diagnostics and Follow up: Practical Considerations

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**Abstract**

MicroRNAs are powerful regulators of gene expression having crucial impact on cell differentiation, proliferation and survival. Their dysregulation is implicated in carcinogenesis including development and progression of thyroid cancer. Since the discovery of pivotal role of miRNAs in thyroid carcinogenesis, a number of studies have explored the miRNAs assessment as a promising tool of the thyroid cancer screening, diagnostic and management. These studies were carried out using different analytic approaches and different sources of miRNAs that complicates comparison, analysis and practical application of the results. The purpose of this review is to systematically evaluate the utility of miRNAs assessment for thyroid cancer management and to summarize advantages and disadvantages of methods can be applied. In this Review, we discuss the practical aspects of miRNA-based diagnostic and prognostic approaches that may be of interest for both physicians and researchers dealing with thyroid cancer.

**INTRODUCTION****Thyroid cancer clinical issues**

**Origin:** Thyroid cancer (TC) is a group of neoplastic disease arising from thyroid gland. Follicular cells of the gland give a rise for most of the TC (95%) while small portion of cancers (5%) is developing from para-follicular C cells and referenced as medullary TC. Carcinomas originated from follicular component are subdivided in according to differentiation rate as well-, purely- and undifferentiated cancers. Most common group of well-differentiated TC includes papillary and follicular types which account for about 75-80% and 10-15% of total TC occurrence, correspondently. Undifferentiated (anaplastic) tumors present rare (0.2-2%) and most aggressive group of TC. So far, classification of TC is based on histological grading and growth pattern, however, controversial issues still exist in classification of borderline cases. Moreover, cancer with well-defined histological characteristics may exhibit heterogeneity of clinical behavior that indicates a need for mere extended classification [1].

**Epidemiology:** TC accounts for 95 % of all endocrine malignancies and its incidence is constantly increasing [2]. In

countries of North America [3,4], West Europe [5] and Australia [6] the incidence of TC has increased approximately threefold over the last two decades. In territory of Russian Federation, TC incidence rate has increased from 47,3 to 86,5 cases per 100 000 population over ten years period 2001-2011 [7]. According to different national statistics, the five-year relative survival rate for people with papillary, follicular, and medullary TC that is confined to the thyroid gland and doesn't invade its capsule is close to 100%. Spreading of TC to the regional lymph nodes reduces survival rate down to 90-70%. Appearance of distant metastases further reduces survival rate of TC patients however in strong accordance with histological type of cancer. About half of the patients diagnosed with well-differentiated (papillary and follicular) TC at stage IV will reach 5-years survival rate, while this statistics drops down to 25-40% for medullary thyroid cancer. Anaplastic (undifferentiated) cancer has poor prognosis in almost all cases with median survival close to 6-12 months.

In according with recent analytic reviews [8], the global increase of TC incidence isn't associated with increase of mortality and is rather caused by diagnostics improved over last decades. The spread of ultra-sonographic and cytological procedures led to diagnostic advance: to the discovery of occult micro-carcinomas and to a better preoperative selection

of patients [9]. Although current standard strategies for the management of thyroid cancer offer good outcomes for patients with favorable histological types at early stage, challenges arising from diagnosis and therapy still exist.

**Diagnostics:** TC is typically manifesting as thyroid nodule, which are very common and their prevalence varies from 3% to 76%, depending on the detection methods and the population evaluated [10,11]. However, only 5 % of thyroid nodule cases are malignant and most nodules are benign [12]. Currently, fine needle aspiration cytology (FNAC) of thyroid nodules is the “gold standard” diagnostic method providing with acceptable sensitivity and specificity (65-98% and 72-100%, respectively) [13-15]. Because of obvious technical reasons and inherent methodological limitations, substantial portion of FNAC provides with diagnosis as “atypia of undetermined significance” or “follicular lesion of undetermined significance” [16,17]. Thus, from 3-6 up to 10-25 % of the FNAC investigations are interpreted as indeterminate without a definitive diagnosis [18,19]. Misdiagnosis of the thyroid nodules results either in delay of necessary surgery or in unnecessary thyroid resections, which harbor risks like hypo-parathyroidism, recurrent laryngeal nerve palsy, and the need for lifelong thyroid hormone replacement [20,21]. Thus, achieved improvements of diagnostic of small and predominantly indolent thyroid nodules require of development of reliable biomarkers that can efficiently select the minority of patients with malignant tumors.

**Management:** The management of thyroid cancer may appear challenging issue because the tumors comprise a wide range of biologic behaviors, from small papillary microcarcinomas that pose little or no risk to survival for the patient, to anaplastic thyroid cancers that are arguably the most lethal tumor [22]. As it was mentioned, tumors within well-defined histological group may considerably vary in terms of clinical behavior. Therapeutic standards for particular clinical situations are hard to be established largely because randomized controlled trials are lacking as a result of the low incidence and generally favorable prognosis of the disease. The current guidelines of most national and international associations (American Thyroid Association [23], European Thyroid Association [24], and Russian Association of Endocrinologists [25]) are based predominantly on clinical studies and suppose a limited application of molecular genetics and/or epi-genetics. For instance, The American Thyroid Association (ATA) advised to consider the use of commercially available genetic tests (like status of TSH receptor mRNA, BRAF, RAS, RET/PTC, and PAX8/PPAR $\gamma$  genes) with appropriate caution due to absence of evidence-based recommendations and until an expert consensus review of existing data can be completed [26]. Guidelines regarding testing of RET mutations in frame of the medullary thyroid cancer management are still discussed between American Thyroid Association (ATA) and European Panel of Experts (EPE) while C-cell-derived carcinomas presents as hereditary form in 25-30% of cases [27].

Considering a number of genetic and epi-genetic alterations have been described in thyroid cancer over the last decades, the need for a shift of algorithms in the management of thyroid cancer patients is obvious. In order to personalize therapy of TC, novel molecular markers need to be identified and introduced to

clinical practice. Among promising candidates, small regulatory RNAs (microRNAs) attract a great interest.

## MiRNA in thyroid cancer (TC)

**MiRNA biology and clinical utility:** MicroRNAs (miRNAs) are small non-coding RNAs (19–25 nucleotides) that regulate gene expression at the transcriptional or post-transcriptional level. Perfect or imperfect complementary miRNAs bind to the 3'-untranslated region (3'-UTR) of an mRNA transcript and block its translation, thus leading to the control of various cellular processes including cell differentiation, cell cycle progression, and apoptosis. Over 2000 miRNA regulating the expression of the human genome have been identified so far, and they are believed to regulate around 60% of the protein-coding genes.

Half of the human miRNA genes are located in cancer-associated genomic regions or in fragile sites [28,29]. Over the recent years, an increasing number of studies have indicated a causal contribution of miRNAs in tumorigenesis, local progression and metastatic spread of cancer [30,31]. Moreover, several features of miRNAs make them attractive biomarkers for cancer. First, they are upstream regulators of mRNA, and each miRNA is able to target multiple protein-coding genes, resulting in substantial functional effects [32]. Thus, detection of certain miRNAs may have a robust relevance to certain phenotypic characteristics compared to mRNA. Next, in contrast to mRNAs, miRNAs do not need to be translated to proteins in order to exert their effects. The expressional status of miRNA may correlate closely with the functional status of the regulated gene(s), and its biological effect can be experimentally tested by generic sequence-based methods [32]. Finally, miRNAs are remarkably stable and maintain their expression profiles in various biological materials such as plasma, serum, residual cells obtained by FNA biopsy, archival formalin-fixed paraffin-embedded and frozen samples [32-34].

**General suppression of miRNA biogenesis associated with TC:** In their seminal paper published in 2005, He et al. were the first to profile miRNA in TC [35]. One of their significant findings was that in papillary TC the global suppression of miRNA patterns is associated with overexpression of several key cancer-driving miRNAs. This finding has since been confirmed in a recent study revealing decreased Dicer gene expression in malignant thyroid tissue and its association with aggressive features: extrathyroidal extension, angiolymphatic invasion, multifocality, distant metastasis, and fast recurrence [36]. These data suggest that disruption of normal Dicer miRNA processing may play a role in thyroid cancer progression. Overall dysregulation of the miRNA-based gene regulatory machinery in thyroid cancer is comprehensively analysed by Marina N. Nikiforova et al., and James C. Lee et al., in recent reviews [37,38].

**Specific miRNA implicated in thyroid cancer:** Between 2005 and 2014, a considerable number of studies reported the involvement of specific miRNAs in thyroid cancer. However, these reports are quite heterogeneous in terms of the patient cohorts included, tissue samples, and methodology applied. Most studies were focused on types of well-differentiated TC, since they are most common [39,40]. Some investigations relied on specific histological types of TC like medullary [41], anaplastic

[42], or follicular variants of papillary thyroid carcinoma [43]. The control or comparison groups varied from normal thyroid tissue [44] to multi-nodular goitre (MNG) [45,46] or benign follicular lesions [46]. Despite the great inconsistencies, some general trends of miRNA deregulation can be observed. The most consistently overexpressed miRNAs in well-differentiated TC types appear to be miR-221 [35,36,43-45,47-50], miR-222 [35,36,43-45,48-54], and miR-146b [35,36,43,44,46-49,52-56]. Other commonly reported overexpressed miRNAs were miR-155 [44,47,50,57] and miR-181 [43,48,52,58,59]. There was less consistency of downregulated miRNAs and miRNA implicated in other histological types of TC. Current knowledge in the field is comprehensively reviewed by Stefano Forte et al., [60].

### Diagnostic/prognostic potency of tumour-derived miRNA

**Diagnostics:** Considering the broad applicability of fine needle aspiration cytology (FNAC) for TC diagnostics, the inherent 'bottle-neck' of this method provides space to develop an miRNA-based diagnostic approach. Indeed, correlation between miRNA profiles obtained by analysis of surgical and FNA samples was first indicated by M. Nikiforova et al., [44]. Later, Shen et al., developed a set of four miRNAs (miR-146b, -221, -187, -30d) that could differentiate a malignant thyroid lesion from a benign tumour with a validated diagnostic accuracy of 85.3%, sensitivity of 88.9% and specificity of 78.3% [61]. Meanwhile, Keutgen et al., reported overall good diagnostic characteristics: 86.0% sensitivity and 85% specificity for another set of four miRNAs (miR-328, miR-222, miR-21, and miR-197) [52]. Mazeh et al., have achieved a specificity of 100%, sensitivity of 88%, and accuracy of 90% in differentiation of benign from malignant thyroid nodules by profiling of miR-21, -31, -146b, -187, -221, and -222 in FNA material [62]. These and other results are obviously promising, but are hard to directly apply to clinical use due to inherent statistical limitations. An alternative approach is a meta-analytic compilation of the data from various studies [63-65]. Most profiling studies in thyroid cancers were performed using oligo DNA microarrays [66]. Thus, Stokowy et al., have developed two-miRNA-classifiers based on the microarray data set, resulting in a specificity of 49% and a sensitivity of 82% in discriminating follicular adenomas from the follicular TC [67]. In contrast to others investigations, these classifiers consider both over- and under-regulated miRNA. However, excellent results are still required to be validated in independent sets of samples. When FNAC is a common approach to evaluate definitive diagnosis of a benign or malignant nodule in the majority of cases, the multi-gene next-generation sequencing (NGS) assay can offer significant improvement in diagnosis in AUS/FLUS (atypia of undetermined significance / follicular lesion of undetermined significance) nodules [68].

**Prognostic:** In addition to diagnostic purposes, altered miRNA expression in TC tissue was shown to be associated with certain aspects of clinical behaviour. For instance, overexpression of miR-146b is a prognostic factor associated with BRAF mutation and an aggressive tumour pattern [55,56]. Aggressive tumour behaviour can be predicted by the assessment of miR-146b, -222, -34b and -130b expression [54]. MiRNA-199b-5p was overexpressed in papillary TC patients with extra-thyroidal

invasion and cervical lymph node metastasis [46]. The tendency of papillary TC to metastasize to cervical lymph nodes was shown to be associated with overexpression of miR-2861 and miR-451 [69]. Significant associations were identified between miR-21 overexpression and lymph node metastasis [49]. These and other results indicate that miRNA profiling of thyroid cancer tissue may be useful to improve prognosis and to personalize the management of TC patients. Since the current therapeutic strategy is mostly defined by histological grading and the growth pattern of a tumour, further studies will have to adopt miRNA assessment approaches to histological parameters and to actual clinical algorithms.

### Circulating miRNA: perspectives of clinical application

Based on the suggestion that miRNAs are released from the primary tumour to the interstitial space and the circulation, the profile of blood-derived miRNA may also provide clinically relevant information. For instance, blood markers indicating the risk of malignant transformation would be helpful for clinical follow-up of benign nodules, while markers of recurrence are in demand during post-surgery management of TC patients. Currently, serum thyroglobulin (Tg) is used to evaluate the effectiveness of treatment and to monitor for recurrence. However, Tg assessment cannot be applied in many clinical situations such as low-differentiated non-producing Tg tumours, presence of Tg antibodies, performance of less than total thyroidectomy or metastatic stage of disease. Thus, circulating miRNA could present alternatives to conventional Tg test. While several reports focused on extra-cellular miRNA associated with TC have been published, further effort should be made to prove the clinical value of circulating miRNA.

Yu et al., measured miRNA expression in the serum of large cohorts of patients with either malignant or benign thyroid nodules and healthy donors using Solexa sequencing followed by RT-PCR validation. They found that serum levels of let-7e, miR-151 and miR-222 were significantly overexpressed in TC patients [70]. Moreover, expression of miR-151 and -222 in plasma was found to be reduced after surgery and associated with increased expression in tumour tissue [70]. In another study comparing plasma miRNA expression before and after total thyroidectomy, Lee et al., reported a significant reduction in miR-146b, -221 and -222 levels (5.1, 10.8 and 2.7 -fold correspondently) [53]. However, a similar pattern was observed in a group of patients with multi-nodular goitre that reduces the applicability of this analysis in a clinical setting. Lee and co-authors evaluated levels of miR-146b, -221, -222, and -155 expressions in the blood of patients with benign lesions, and papillary TC with and without lymph node metastases before surgery [71]. The authors reported that miR -146b and -155 can be used to discriminate between benign and malignant tumours with sensitivity/specificity indexes of 61.4/57.9% and 74.3/63.2%, respectively. Correlation between miR-146b and -155 blood level and presence of lymph node metastases and tumour size were also reported. However, all conclusions were made by comparison groups of patients with benign and malignant tumours, which diminishes the diagnostic applicability of these results. Differential expression of miRNA-579, -95, -29b and 190 in the serum of papillary TC patients



versus healthy individuals or patients with nodular goitres was reported by Cantara et al. [72]. Although all cited studies were focused on the same type of TC (papillary) and miRNA expression was measured/verified by the same method (RT-qPCR), their results are still far from consensus. An absolute quantification of circulating miRNA by droplet digital PCR (ddPCR) technology in groups of patients with different cancer types (including thyroid cancer) was recently reported [73]. Using this advanced method, authors revealed over-representation of miRNA-181a-5b in the plasma of thyroid cancer patients compared to healthy donors and patients with other cancers. Although these results did not clarify an insight of TC-associated miRNA in peripheral circulation, one very important observation came from this report. Similar levels and characters of data distribution were revealed for each of the nine miRNAs tested across all groups of patients, while these parameters varied considerably between different miRNAs. For instance, the plasma level of miRNAs -378a-3p and -766-3p varied in a range up to 2-4 copies per uL, while miRNAs -125a-5p and -21-5p were counted within the limit of 100 and 200 copies per uL, respectively. The presence of some miRNAs (for instance miRNA-21a-5b) differed in an order of magnitude between plasma and serum samples, while the amount of other miRNAs revealed little or no dependency on thrombotic event. Taken together, these results indicate that the pattern of circulating miRNA is not random, and each miRNA seems to have a certain range of physiological (and pathological) fluctuations. This conclusion justifies further input to evaluate cancer-associated signatures of circulating miRNA.

Thus, miRNA may be isolated from both thyroid gland tissue and circulating plasma, and miRNA analysis may provide with clinically relevant results at various situations including small asymptomatic thyroid nodes management, diagnostic of thyroid cancer, prognosis and therapy individualization and post-surgery follow up (Figure 1).

### Challenges of miRNA quantification: pre-analytical issues

Despite the promising data mentioned above, many concerns still exist regarding the methods of miRNA analysis including isolation, quantification and evaluation. Most of the reports published to date utilize TC tissue frozen after surgery. This is a most reliable approach providing sufficient amounts of cancerous and normal thyroid tissue, and this analysis is easily accompanied by routine histological assessment. miRNA can be also isolated from formalin-fixed/paraffin-embedded tissues after long storage, which enables large retrospective studies [45]. miRNA can be assessed in the material of preoperative FNA [62,74]; however, parallel performance of routine cytological tests and miRNA analysis requires increased amounts of aspirated tissue. In a clinical setting, miRNA analysis of material obtained after non-informative cytological tests would be in the greatest demand, allowing additional diagnostic iteration for cases undetermined by a standard approach. Thus, methods of miRNA isolation from routine air-dried and stained slides are under active development [75,76].

The impact of source choice and material processing has special importance in the case of circulating miRNA analysis





Clinical status	Source of miRNA	Approach
 Small asymptomatic nodes	Plasma miRNA	Screening
 Growing thyroid nodes	Tumor tissue (material of FNAC)	Diagnostic
 Thyroid cancer	Tumor tissue (resected gland)	Prognosis / therapy personalization
 Local recurrence or distant metastasis	Plasma miRNA	Post-surgery follow up / recurrence control

Figure 1 miRNA analysis.

[77,78]. Extracellular miRNA in blood circulation does not present a homogeneous population, and there still is no common opinion regarding its predominant cellular origin, mode of release into the bloodstream, packaging forms and dynamic of circulation (half-life). Circulating in blood of healthy individuals, miRNA are likely derived from endothelial cells, blood cells, and platelets. Injury of any tissue can lead to the release of tissue-specific miRNAs, as was reported for cordial [79], renal [80] or hepatic [81] pathology. Perturbations in blood cell count and haemolysis can alter plasma miRNA levels up to 50-fold [82] while certain miRNAs may be more or less sensitive to such impacts [73]. Even being associated with cancerous process, miRNA may be released by immune cells or other cells/tissues implicated in tumour invasion and inflammatory responses [83]. Thus, the cancer specificity of circulating miRNA markers must be evaluated with great caution and with respect to other tissue- and blood cell-based phenomena [84-86].

Besides concerns of cellular origin, the form of miRNA release may considerably influence purification efficacy and sensitivity of the analysis. Circulation miRNAs can be packaged into apoptotic bodies, shedding micro-vesicles, exosomes, or bound with high-density lipoprotein particles or complexed with AGO proteins. Experimental data reporting the prevalence of extra- [87,88] or intra- [89] vesicular forms of the extracellular miRNA packaging are still controversial. In the recent years of active research, exosomes were found as a specific form of cell-to-cell communication, and their miRNA cargo is supposed to have an essential biological and diagnostic significance. Exosomes are 30-100 nm vesicles consisting of a lipid bilayer membrane surrounding a small cytosol, they are derived from the microvesicle body (MVB) sorting pathway and contain various molecular components that are derived from their cell of origin. Cancer cells actively release exosomes that are able to suppress host immunity and induce tumour progression [90-92]. Many functional effects of exosomes are mediated by their miRNAs transferred from tumour cells to recipient host cells [93]. For instance, exosomal miRNA contribute, or even define, the metastatic potency [94] and drug resistance [95] of tumours. Importantly, circulating exosomes can be extracted from blood and their miRNA content can be assayed separately from the rest of the circulating miRNA population. Exosome isolation is supposed to improve the sensitivity of following miRNA analysis, and this promising approach is being explored now in order to develop novel markers for various types of cancer [96]. In the

field of TC, one report revealing the relative abundance of miRNA -222 and -146b in exosomes derived from papillary CT cells (TPC-1) in vitro has been published [97]. New research into circulating exosomal miRNA in TC patients is anticipated in the near future.

Total RNA or short RNA-enriched fractions are usually isolated from tissues or bodily fluids by the classic phenol-chloroform method or by conventional spin column-based isolation technologies explored in many commercially available kits. Despite the small fraction and short length of miRNA, degradation of total RNA crucially impacts miRNA profiling [98]. As was recommended, RNA integrity number (equal or above seven) should be used to control the quality of starting material before miRNA fraction analysis.

### Challenges of miRNA quantification: analytic approaches

Regardless of the source and method of RNA isolation, further analysis can be performed by number of approaches: hybridization-based (microarray), sequencing-based (massive parallel/next generation sequencing), amplification-based (real-time reverse transcription-PCR) and some advanced combinatorial techniques. However, the specific nature of miRNA faces methodological challenges affecting the sensitivity and specificity of its assessment:

- i. The short length of mature miRNA makes it difficult to design of specific primers and probes
- ii. The variability of GC content leads to different melting temperature across miRNA population
- iii. Lack of a common sequence feature prevents miRNA-selective processing
- iv. Close homology of miRNAs within the same family may lead to differences of a single nucleotide that is hard to distinguish
- v. Co-existence of mi-RNAs in different stages of maturation (pri-miRNA, pre-miRNA, mature miRNA) that share a common sequence are hard to distinguish

These features affect methods of miRNA analysis differently and should be resolved by the selection of an optimal analytic approach.

### miRNA microarrays

As with gene expression profiling, microarrays are still the best choice for a standardized multiplex assay that is amenable to high-throughput applications. Over 20 studies of TC miRNA have successfully utilized commercial miRNA microarrays. However, in addition to the known merits and drawbacks of microarray-based expression profiling, there are specific challenges relevant to miRNA nature. The short length of mature miRNA restricts the design of probes and difficult achievement of equal melting temperature across the chips. Members of miRNA families with limited sequence difference however differential expression pattern and biological functions are rather not distinguishable. These issues require specific efforts to be overcome and to reach an adequate specificity of arrays-based analysis. Systematic comparisons of the main commercially available miRNA

microarray platforms in terms of their reproducibility, specificity and reliability have been reported [99,100]. Comparative analysis of advanced techniques aimed at improving the performance of array-based miRNA assessment-Locked Nucleic Acid (LNA) microarray, beads array, and TaqMan quantitative real-time PCR Low Density Array (TLDA) was performed in another study [101]. Taken together, these data overall demonstrated good intra-platform reproducibility; however, the results obtained by different platforms appeared to be less reproducible. Thus, the optimization of a normalization method and combination with non array-based assays are both required to increase the fidelity of array-based miRNA profiling irrespective of the platform used.

### Sequencing

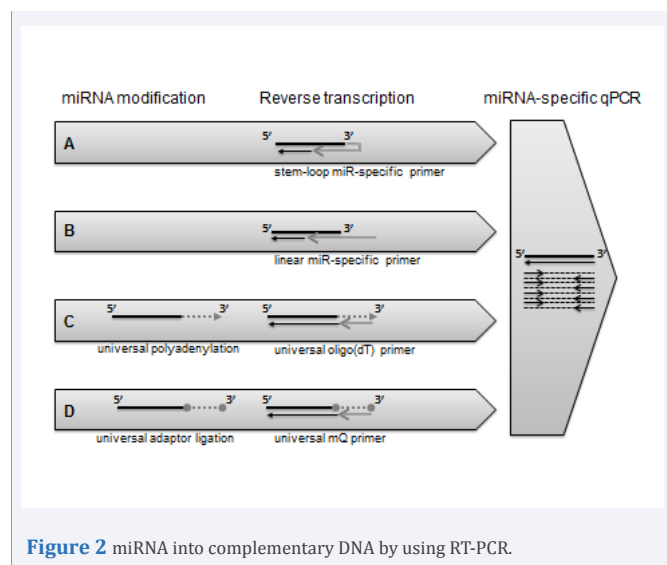
Despite being relatively expensive, the sequencing-based analysis of miRNA involved in TC seems to be utilized more frequently than arrays-based assays. The major theoretical advantage of sequencing over microarrays is that it is not biased and it helps to explore all miRNAs that exist in a sample. Important practical advantages of next generation sequencing techniques compared with arrays- and RT-qPCR-based analysis were recently evaluated using a set of samples from benign follicular adenoma and follicular thyroid carcinoma [102]. The authors of this study proposed three different approaches of sequencing data analysis: direct mapping that allows expression profiling of known miRNA (pipeline A), isoform analysis that allows discovery of new miRNAs with close similarity to that known (pipeline B), and analysis focused on seed region that allows exploration of the functional link between discovered miRNAs and their regulatory targets (pipeline C). Thus, sequencing analysis has broader applicability compared with arrays. Moreover, the saturation of miRNA array signal intensity was indicated by the authors of this report. Consequently, profiling overexpressed miRNA by microarrays may not be accurate enough, while deep sequencing provides a much broader range of signal intensity and may present an optimal analytic approach.

Differences between analytical performances of various sequencing platforms is further described in [103]; most of these sequencing platforms (Illumina [102,104], SOLiD [105] and Ion Torrent PGM [106,107]) are applied successfully for TC research.

### Reverse transcription-quantitative PCR

Most of the data describing the role of the miRNA in TC are obtained or validated using reverse transcription followed by real-time quantitative PCR (RT-qPCR). This method is traditionally established as a 'gold standard' of gene expression analysis including miRNA expression. Considering the relative low cost, high fidelity and easy interpretation of RT-qPCR data, this method will likely provide a basis for first miRNA-based clinical assays.

The first important step of RT-PCR is the complete and correct conversion of miRNA into complementary DNA, which can be performed in one of two ways (Figure 2). In the first approach, miRNA of interest is transcribed using specific reverse transcription primers. The 5'-end of one primer is complementary to the 3'-end of the specific miRNA, while the other primer is a universal PCR primer. The universal primer-binding sequence



may be designed to form a loop (Figure 2A) or as a linear primer (Figure 2B). The stem-loop structure of an RT primer reduces its annealing to pre- and pri-miRNAs and increases its specificity to mature miRNA molecules. A second approach to reversely transcribe miRNA uses its modification that allows the annealing of a universal RT primer. Two methods of miRNA modifications are described: polyadenylation by *E. coli* (A) Polymerase (PAP) (Figure 2C) [108] or ligation with a short linker by T4RNA ligase (Figure 2D) [109]. In both cases, the equal modification of all RNA molecules creates a binding site for the RT primer that allows the performance of universal (non-miRNA-specific) reverse transcription.

The second step of analysis is quantitative PCR. Since the miRNA pool is very heterogeneous in terms of GC content, the design of primers for PCR may be a challenging issue in this case, especially if many miRNAs are assayed in parallel. The temperature of primer annealing can be adjusted either by shortening primer length (decreased T) or by introducing LNAs (Locked Nucleic Acids) into the primer sequence (increased T) [110]. Among the existing fluorescent technologies for tracking PCR efficacy, SYBR Green I, and TaqMan probes are the most common for miRNA analysis.

The final challenge of RT-qPCR-based assessment of miRNA is correct normalization of results. The most reliable approach was proposed by Mestdagh et al., and uses the mean expression value of all miRNA in a given sample as a normalization factor [111]. However, this approach is applicable only when a large enough number of miRNAs is assayed in parallel. Diagnostic application measures few miRNA markers, and should include some reference invariant miRNA/miRNAs. Unlike mRNA expression analysis, no reference miRNA have so far been identified. Small nucleolar RNAs (snoRNAs)-RNU44, RNU48, RNU43, RNU6B-are frequently used as references; however, alteration of their expression may be associated with the cancer process [112]. Since each tissue is supposed to have a typical pattern of miRNA, references may be selected from a list of stably expressed tissue-specific miRNA. For instance, miR-16, miR-223, miR-103a, miR-124 were proposed as a reference for the analysis of plasma/serum-circulating

miRNA [113,114]. In order to define a reliable miRNA reference for thyroid gland tissue, deep sequencing analysis of tissue miRNAome should be performed using a sufficient number of samples. Such a study was recently performed [105]; however, the results of this investigation indicated great variability in the co-existing isoform of known miRNAs that presents difficulties in the identification of a 'bona fide' reference. Thus, the selection of a method for normalization of miRNA expression profiling is still an issue that needs to be resolved. Development of new high-throughput analytical approaches for the simultaneous assessment of a large number of miRNAs may provide an option to apply the mean expression value through the sample as a normalization factor.

### Advanced high-throughput technologies of miRNA analysis

Since the problematic issue of data normalization has still not been resolved, the results of the miRNA analysis methods described above remain hardly comparable and frequently contradictory. This issue may theoretically be overcome by the absolute quantification of specific miRNA molecules in the tested sample. Several new approaches allowing the capture and detection of individual miRNA molecules were developed in recent years. Thus, droplet digital PCR (ddPCR) is based on the amplification of each individual molecule inside a small droplet created by a massive sample partitioning in the form water-oil emulsion. Amplification inside the droplet can be detected by conventional TaqMan probe or EvaGreen dye-based assays [115]. Using this method, miR-181a-5p was quantified in plasma samples of thyroid cancer patients (n.27) and healthy donors (n.20) [106]. The results were presented as miRNA copies per plasma microliter. Despite the relatively low number of samples assayed, expression difference was estimated as statistically significant ( $p < 0.0005$ ). NanoString technology uses colour-coded molecular barcodes that can hybridize directly to different types of target molecules. Capture and detection of miRNA is mediated by two target-specific probes: a 3' capture probe containing biotin to allow absorbance to the solid phase via streptavidin, and a second 5' probe with an individually barcoded sequence. This method does not require amplification or labelling, and is considered one of the most reliable approaches for miRNA assessment [116]. Using this technique, a pattern of 18 miRNAs was reported to be sufficient in order to distinguish papillary TC from non-malignant thyroid tissue. Moreover, the involvement of miR-339-5p in the regulation of NIS-mediated radio iodide uptake by thyroid cancer cells, and hence the sensitivity of TC patients to radioiodine therapy was evaluated [117]. Another promising method exploring surface plasmon resonance sensors is still under active development. This approach provides high specificity, and multiplex sensing capacity coupled with a wide dynamic sensitivity range that should allow the reliable assessment of biological fluids containing a mixture of miRNAs at a wide range of concentrations [118]. First attempts to apply a plasmonic biosensor for cancer-related miRNA detection were recently reported [119,120].

### CONCLUSION

Thyroid cancer is heterogeneous disease in terms of clinical behaviour and prognosis. Currently management of



this disease heavily relies upon the histological classification and measurement of the protein Thyroglobulin (Tg) in blood. However, personalised management of patients with TC requires more detailed evaluation of tumour characteristics. Assessment of tumour-derived and/or circulating miRNA presents a promising approach to estimate presence of TC, to define its malignant and metastatic potency and to develop personalized therapeutic strategy. It is hoped that with further investigation of TC-associated miRNAs and development of new analytic methods, miRNA-based diagnostic and predictive tests will be soon introduced in to clinical practice.

## COMPLIANCE WITH ETHICAL STANDARDS

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## ETHICAL APPROVAL

This article does not contain any studies with human participants or animals performed by any of the authors.

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## Cite this article

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