Review Article

miRNA biomarkers for NPC diagnosis and prognosis

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Abstract: MicroRNAs (miRNAs) are short non-coding RNAs that have been termed "master regulators of the genome" given their significance in post-transcriptional gene regulation, and roles in a multitude of normal and disease processes. In cancer, dysregulation of miRNAs can facilitate disease progression and therapeutic resistance, affecting tumour-suppressors and oncogenes. Nasopharyngeal carcinoma (NPC) is a unique head and neck cancer that is frequently associated with the Epstein-Barr virus (EBV). Advances in miRNA profiling techniques have highlighted the significance of both EBV- and human genome-encoded miRNAs in the pathogenesis of NPC. These miRNAs have been implicated in critical NPC processes such as epithelial-to-mesenchymal transition (EMT) and metastasis, while further promoting chemoresistance and radioresistance. MiRNA signatures derived from profiling data and bioinformatics/statistical analyses may be particularly useful for the diagnosis of NPC, as well as the stratification of patients into clinically relevant groups to guide treatment selection. The identification and characterization of biologically relevant biomarkers will be crucial to improving patient outcomes and achieving greater understanding of the molecular underpinnings of NPC. This review integrates the literature on EBV-miRNAs, cellular miRNAs, and miRNA signatures to guide future research, while acknowledging challenges in the selection and clinical implementation of relevant miRNA biomarkers and signatures.

Keywords: Nasopharyngeal cancer; miRNA; biomarkers; Epstein-Barr virus (EBV)

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Introduction

MicroRNAs (miRNAs) are short non-coding RNAs, typically 21–23 nucleotides long, that function in post-transcriptional gene regulation typically through translation inhibition and/or mRNA degradation (1). Since their discovery in *Caenorhabditis elegans*, miRNAs have been extensively investigated as master regulators of gene expression in a variety of animal, plant, and human models. MiRNA expression is frequently altered during cancer development, associated with dysregulated expression of a plethora of different miRNAs, their biogenesis or processing proteins, such as DROSHA and DICER1 (1). These miRNA alterations are potential diagnostic and prognostic biomarkers when assessing patient disease progression or guiding clinical management. Since their development in the early 2000s, several groups in both academia and industry have published papers and produced miRNA panels as potential products, which are available for clinical use, although these are still in clinical testing and not yet FDA-approved (2). Similarly, miRNA-based therapeutics are also in development for the treatment

of a variety of human diseases. These clinical evaluations demonstrate the importance and potential of further miRNA research for clinical use, especially in the diagnosis and treatment of cancer.

Head and neck cancers (HNCs) are the seventh most common malignancy worldwide (3). One type of HNC is nasopharyngeal carcinoma (NPC), which arises from the nasopharyngeal mucosal lining often at the pharyngeal recess (4). The geographic distribution of NPC is notably unique wherein more than 70% of cases arise in east/southeast Asia, likely attributable to both genetic and environmental factors. NPCs are categorized into three subtypes (keratinising squamous, non-keratinising and basaloid squamous) (5). Of note, the non-keratinising subtype contributes to more than 95% of NPCs developing in endemic regions and is almost always associated with the Epstein-Barr virus (EBV), the most prevalent etiologic agent of NPC (6). The development of EBV-associated NPC is characterized by critical genomic alterations and aberrant signalling activities that ultimately ushers a normal, healthy epithelium towards malignancy (5). NPC miRNA expression profiling and signalling pathway elucidation suggest a dysregulated state caused by both cellular and viral-encoded miRNAs. While certain individual miRNAs may be informative of changes with respect to cellular growth, proliferation, metastases, and apoptosis for use in diagnostic or prognostic assessments, other specific miRNAs might guide the course of treatment by reflecting NPC resistance to radiotherapy (7-9) or chemotherapy (6,9-11). Advances in miRNA profiling, bioinformatics, and statistical analyses have permitted the identification of miRNA signatures with the potential to accurately diagnose or stratify patients into clinically relevant groups. Taken together, the identification of novel miRNA biomarkers and signatures may be crucial to clarify the underlying mechanisms of NPC pathogenesis and improve clinical outcomes.

miRNA biogenesis

Human miRNA biogenesis begins in the nucleus with the transcription of primary miRNA (pri-miRNA) transcripts, predominantly by RNA polymerase II, although some RNA polymerase III-mediated transcription has also been reported (12,13) (*Figure 1*). Transcription most commonly occurs at designated miRNA promoters but has also been observed in the introns of protein-coding regions. These pri-miRNA transcripts can vary substantially in length and commonly adopt stem-loop secondary structures (14). The Microprocessor complex, comprised of the RNase III DROSHA and RNA-binding protein DiGeorge syndrome critical region 8 (DGCR8), subsequently cleaves the pri-miRNA at the base of the stem-loop structure to generate a ~60-70 nucleotide hairpin-shaped precursor miRNA (pre-miRNA) (6). Pre-miRNAs are then exported from the nucleus by exportin-5 and GTP-bound Ras-related Nuclear Protein (RAN-GTP) for further modification by the RNase III DICER1 and its associated cofactor transactivation-responsive RNA-binding protein (TRBP) (13). DICER1 processing produces a ~21-23 nucleotide miRNA duplex and one of the strands, termed the "guide strand", is selectively integrated into the miRNA-inducible silencing complex (miRISC). Among other constituents, the major miRISC component is the Argonaute (AGO) protein (in humans, one of AGO 1–4) (12). The miRISC complex uses the incorporated guide strand to target mRNA transcripts and in turn, post-transcriptionally regulate its expression (13). A given miRNA guide strand may be complementary to numerous different transcripts and could possess several binding sites within a given mRNA (14). When partial complementarity exists between the guide strand and the target mRNA strand, AGO 1–4 can inhibit protein translation or direct the mRNA for non-specific degradation *via* cytoplasmic processing-bodies (P-Bodies) (14). Alternatively, when extensive or perfect complementarity exists between the guide strand and the target strand, the AGO2 protein may facilitate degradation of the mRNA through its "slicer" activity (*Figure 1*). In either case, these miRISC-mediated events primarily reduce the gene expression of the targeted mRNA transcript(s).

The functional roles of miRNAs in cancer

Given the broad repertoire of miRNAs identified in humans and their even more diverse array of target mRNAs, miRNAs are fittingly referred to as "master regulators" of gene expression. There are an estimated 2,000 miRNAs encoded in the human genome, which are further estimated to regulate at least one third of human genes (15,16). While global miRNA expression is typically downregulated in human cancer when compared to healthy tissues, aberrant expression of tumour-suppressor and oncogenic miRNAs can be critical in driving cancer initiation and subsequent progression (13). For example, two major oncogenic miRNAs are miR-21 and the miR-17-92 cluster, both of which are known to target tumour-suppressor genes

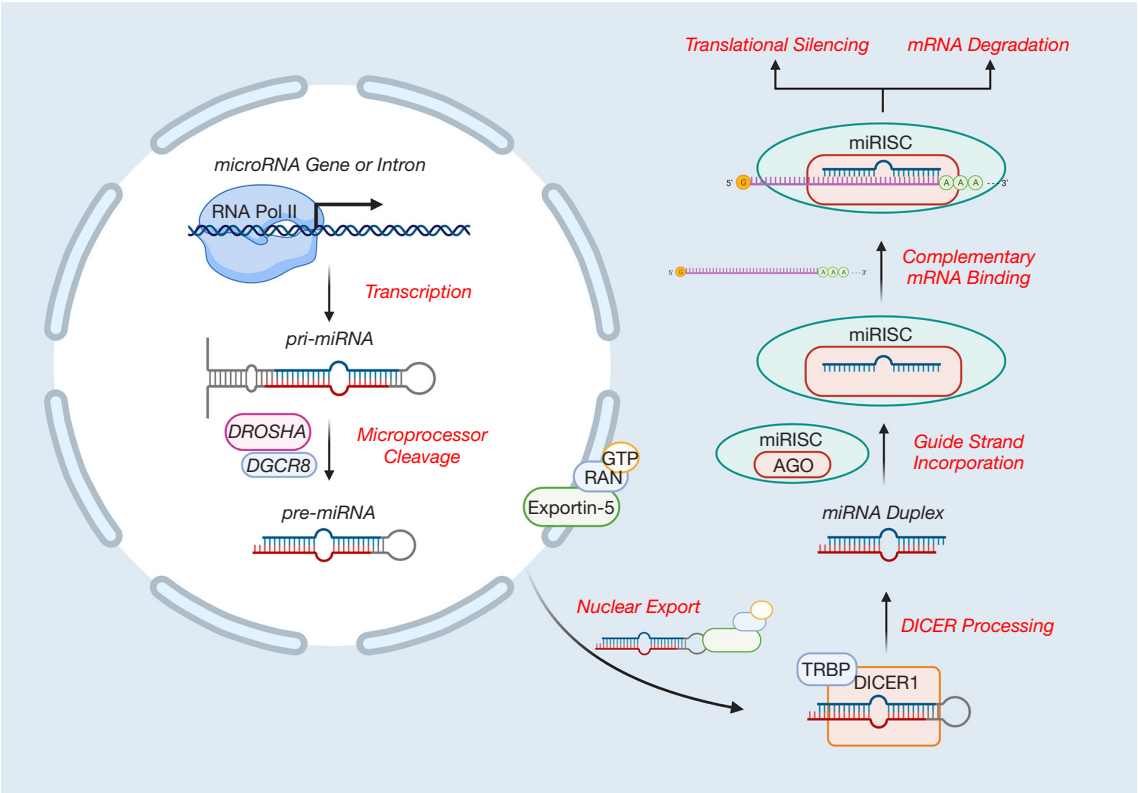

Figure 1 MicroRNA biogenesis. MiRNAs are transcribed/processed in the nucleus, exported into the cytosol for further processing, and incorporated into the miRISC complex. The miRISC post-transcriptionally regulates gene expression through translational silencing and/or mRNA degradation. AGO, Argonaute; DGCR8, DiGeorge syndrome critical region 8; GTP, guanosine tri-phosphate; miRISC, miRNA-inducible silencing complex; pre-miRNA, precursor microRNA; pri-miRNA, primary microRNA; RAN, Ras-related nuclear protein; RNA Pol II, RNA Polymerase II; TRBP, Transactivation-Responsive RNA-Binding Protein. Created with BioRender.com.

involved in cell cycle regulation and apoptosis (17). Conversely, miRNAs of the let-7 family are regarded as tumour-suppressors, as they are known to target oncogenes, such as Myc and Ras family members, and are often downregulated in cancer cells (18). Similarly, downregulated expression of miRNA biosynthesis proteins (such as DROSHA and DICER1) has been associated with poorer clinical outcome in cancer patients (13). This may be in part due to an impaired DNA damage response, which decreases radiosensitivity (14). In EBV-associated NPC, both viral- and cellular-encoded miRNAs are pivotal in defining the cancer cell phenotype and predicting its development and response to treatment.

The role of EBV-encoded miRNAs in NPC

The EBV genome possesses two well-characterized regions, the BART and the *BHRF1* genes, which encode 44 and 4 mature miRNAs (19), respectively. Notably, BHRF1 miRNA expression has not been observed in clinical NPC samples (20), while an overexpression of BART miRNAs has been well documented (21). BART miRNAs have also been detected in serum exosomes, vesicles, and soluble ribonucleoprotein complexes (22). Functionally, BART miRNAs are known to target an assortment of viral and cellular mRNAs to alter cellular proliferation, survival, and host immune response evasion (6,21,23). Consequently, BART-encoded miRNAs may be relevant as non-invasive biomarkers in the diagnosis and prognosis of NPC (*Table 1*).

A study investigating serum and plasma miRNA expression in NPC observed that miR-BART7-3p, 9-3p, and 13-3p were elevated in plasma, likely associated with extracellular vesicles or soluble ribonucleoprotein complexes (52,53). While the overexpression of these three miRNAs successfully distinguished NPC from healthy individuals and/or patients with non-NPC EBV-associated disease,

Table 1 An overview of EBV BART-microRNAs with identified cellular targets and their biological significance

EBV-encoded miRNAs	Altered cellular target(s), if known	Biological Significance in NPC	Reference(s)
miR-BART1	PTEN, PTEN-dependent pathways	Promotes cell migration, invasiveness, and metastasis. Role in regulation of cancer cell metabolism.	(21,24,25)
miR-BART2	RND3 Rho Family GTPase, ROCK signalling	Promotes cell migration, invasiveness, and metastasis. Inhibits cell apoptosis.	(26)
miR-BART3	Deleted in Cancer 1 (DICE1)	Promotes cell growth and proliferation.	(21,23,27)
miR-BART5	p53 upregulated modulator of apoptosis (PUMA), p53	Inhibits cell apoptosis.	(28,29)
miR-BART6	Long non-coding RNA LOC553103, DICER1	Inhibits cell invasion, proliferation, and metastasis. May modulate radiosensitivity by targeting DICER1.	(30-32)
miR-BART7	PTEN, Smad7, c-myc, c-Jun, E-cadherin, GFPT1	Promotes cell growth, proliferation, migration, and invasion. May contribute to cisplatin resistance, regulation of radiosensitivity and EMT induction.	(21,33-37)
miR-BART8	RING Finger 38 (RNF38), NF-κB and Erk1/2 Signalling	Promotes cell migration, invasion, metastasis and facilitates EMT.	(38)
miR-BART9	E-Cadherin, Bcl-2-interacting mediator of cell death (BIM), ATM	Promotes cell invasion, migration, and metastasis. Inhibits apoptosis. Modulates DNA double-strand break response and maintains viral latency.	(39,40)
miR-BART10	βTrCP	Promotes cell migration, invasion and facilitates EMT.	(41)
miR-BART13	NFκB inhibitor interacting Ras-like 2 (NKIRAS2), ABI2	Promotes cell growth, proliferation, metastasis and facilitates EMT via upregulation of c-Jun/SLUG signalling.	(42,43)
miR-BART15	NLRP3 Inflammasome[†]	Promotes growth of EBV-associated tumours.	(21,44)
miR-BART16	Translocase of Outer Mitochondrial Membrane 22 homolog (TOM22)	Inhibits apoptosis.	(45)
miR-BART17	Adenomatous Polyposis Coli (APC), Wnt Signalling, BRCA1	Promotes cell growth and proliferation. Associated with metastasis. Suppression of DNA repair that may increase cisplatin sensitivity.	(22,46,47)
miR-BART19	APC, Wnt Inhibitory Factor 1 (WIF1), BRCA1	Promotes cell proliferation. Suppression of DNA repair that may increase cisplatin sensitivity.	(47-49)
miR-BART22	May target MAP3K5	Promotes immune evasion, inhibits apoptosis, and contributes to cisplatin resistance.	(21,50,51)

[†], NLRP3 targeting was observed in monocytes rather than NPC cells.

circulating miR-BART13-3p was found to possess impressive specificity for NPC detection (97%) (52). MiR-BART13 has been previously implicated as a promoter of cell growth and metastases in NPC through an NFκB-dependent mechanism (42). Other investigations of plasma miR-BARTs similarly described an upregulation of miR-BART7 and miR-BART13, compared to healthy individuals (33,54). One study reported that plasma miR-BART7-3p, in conjunction with plasma EBV DNA, was associated with distant metastasis-free survival (DMFS), as patients with detectable abundances of both miR-BART7-3p and EBV DNA experienced poorer DMFS compared to patients with either factor independently or neither (54). In addition, miR-BART7 was identified as an *in vitro* promoter of proliferation (34), migration (33,35), and invasion (33), and could contribute to cisplatin resistance (36). Notably, miR-BART7-3p targets the tumour suppressor PTEN, and may augment PI3K/Akt signalling to facilitate nuclear accumulation of Snail and β-catenin to promote epithelial-to-mesenchymal transitions (EMT) (35,36) and metastases (35). MiR-BART7-3p targeting of Smad7 may

promote transforming growth factor beta (TGF-β) signalling to mediate EMT and confer stemness, thereby enhancing the metastatic phenotype (36). One study reported an alternative mechanism whereby miR-BART-7 expression downregulates TGF-β1 through glutamine-fructose-6-phosphate transaminase 1 (GFPT1) targeting to increase radiosensitivity (37), although further investigation will be necessary to clarify these conflicting observations.

Elevated miR-BART2-5p levels have been detected in patient sera samples, and its abundance has been associated with poorer clinical outcomes, particularly as a result of elevated migration, invasion, and distant metastasis (26). The underlying mechanism may relate to miR-BART2-5p binding of RND3, a negative regulator of Rho signalling, which could indirectly promote ROCK signalling and increase the aggressiveness of cancer cells (26). MiR-BART2-5p upregulation has also been observed in other EBV-associated conditions (52). Nonetheless, miR-BART2-5p has demonstrated potential as a biomarker for early NPC diagnosis, and more studies are necessary to confirm its significance (55).

Overexpression of miR-BART10-3p is associated with downregulation of beta-transducin repeat containing E3 ubiquitin protein ligase (βTrCP), which may dysregulate downstream β-catenin and Snail, thereby promoting EMT in NPC (41). Indeed, miR-BART10-3p expression has been described as a negative prognostic marker, associated with lower disease-free survival (DFS) and overall survival (OS) rates (41). The negative correlation between miR-BART10-3p and βTrCP expression in NPC cells suggests that both molecules warrant further investigations as possible biomarkers.

Taken together, these findings demonstrate the biological significance of EBV-encoded BART miRNAs and their potential as informative biomarkers for NPC diagnosis and predictors for patient outcome. Among the plethora of BART miRNAs whose expression is altered in NPC, further investigation is necessary to clearly elucidate the functional underpinnings of these miRNAs and their role in NPC development (*Table 1*). In addition to identifying cellular factors and pathways that may be dysregulated as a consequence of BART miRNAs, developing miRNA signatures composed of several viral miRNAs could potentially unify the observations described in the literature, thereby enhancing the diagnostic efficacy of miRNA assays in NPC.

Although not the focus of this review, there are also EBV-encoded small RNAs (EBERs) transcribed in abundance from the EBV genome in NPC, which possess a variety of functional roles including regulation of cell growth and survival, host innate immunity and oncogenesis (56-58). EBERs, in addition to miRNAs and other interacting viral and host factors, underscore the complexity of molecular interactions underlying NPC.

Techniques employed in human genome-wide miRNA profiling and signature derivation

MiRNA profiling is typically conducted through a general technical workflow that spans from sample preparation and RNA extraction to subsequent profiling and analysis [reviewed in (59)]. Each step in this sequence necessitates care and quality control to ensure that the results generated by the assay are truly representative of the original sample and not obscured by 'noise'. While both serum and plasma samples are commonly extracted from patients and directly profiled, tissue samples are often either freshly-extracted, frozen, or formalin-fixed paraffin-embedded (FFPE), thus introducing variability among samples that may affect profiling results (1,59). Numerous techniques exist for miRNA profiling, most prominently quantitative reverse transcription PCR (qRT-PCR), microarrays, and high-throughput RNA sequencing (RNA-seq), as well as other unique human miRNA assays such as Nanostring and Taqman Low-Density Arrays (TLDAs); however, each technique differs in its methodology (60-62). While qRT-PCR employs reverse transcription of miRNAs to cDNAs and subsequent qPCR to quantitatively monitor specific products generated, hybridization-based methods such as microarrays rely on fluorescent labelling and probe hybridization to quantify miRNAs (59). qRT-PCR, for example, is capable of measuring smaller miRNA panels with high sensitivity and specificity (62). Conversely, microarrays may measure greater numbers of miRNAs and more readily enable comparison of abundance between different samples (for example, a "healthy" *vs.* "diseased" sample), albeit with less sensitivity and specificity (62). A detailed discussion of miRNA profiling techniques is reviewed in Pritchard *et al.* (59) and Dave *et al.* (62).

Once profiling is completed, each dataset is subjected to statistical analyses and modelling, for example univariate/multivariate Cox analyses (63-65) or Risk Score calculations (63-65), dependent on the phenotypic variable being investigated to identify miRNAs that may be significant. Of note, certain bioinformatic investigations of miRNA signatures in NPC were conducted using datasets available

from the Gene Expression Omnibus (GEO) database. The data produced and analyzed by Liu *et al.* (GSE32960) for example, was re-analyzed by Wang *et al.* and Zhang *et al.* (discussed below), and interestingly, yielded partially-overlapping miRNA signatures, which were subsequently validated using independent validation sets (63,64,66). Overall, while previous investigations of miRNAs in EBV-positive NPC sought to identify consistent biomarkers and miRNA signatures, they do not necessarily examine the same type of specimens or employ the same profiling techniques or statistical analyses throughout their workflow. The differences between their findings, as well as their methodologies, warrant consideration when selecting the most meaningful miRNA biomarkers for clinical and/or scientific use.

miRNA signatures in NPC

In NPC, miRNA expression profiles are known to be significantly different between healthy tissues and tumours, and furthermore, between tumour subtypes (6); hence, the diversity of tumours that may develop in NPC poses a significant challenge in accurately assessing patient outcomes (1). Rather than replacing conventional TNM staging, these miRNA signatures may be used in a complementary fashion to better assess patient prognosis and guide treatment selection (60,64,65). *Table 2* summarizes all the published NPC miRNA signatures to date. Several of these studies employed pathway enrichment analyses to identify plausible targets of the miRNAs included in their signatures. These targets tended to be molecules or signalling pathways that might enhance cancer progression through alterations in cell cycle regulation, motility, survival, proliferation, or the extracellular matrix. While these signatures comprise a statistically significant selection of miRNAs with altered expression (either up- or downregulated) in NPC, it is also worth noting that individual miRNAs within these signatures have been implicated in the pathogenesis of NPC (6).

Liu *et al.* investigated miRNA expression profiles in 312 paraffin-embedded NPC samples (evenly randomized into a 156 sample training set and internal validation set), and 18 non-NPC samples using an 873 probe microarray and qRT-PCR, and subsequently, an independent set of 153 samples (64) (*Table 2*). A 5-miRNA signature was thus identified (miR-93, miR-142-3p, miR-29c, miR-26a, miR-30e) in which each miRNA was significantly associated with DFS, and as secondary outcomes, DMFS and OS (64). The prognostic value of this signature was greater when used in combination with TNM staging, while TNM staging performed less well alone, reiterating the enhanced prognostic value conferred by the addition of the miRNA signature (64). The dataset produced and analyzed by Liu *et al.* was uploaded to the GEO database (GSE32960) and was re-analyzed by Zhang *et al.* and Wang *et al.* to identify a partially-overlapping 4-miRNA signature and 3-miRNA signature, respectively (63,64,66). Zhang *et al.* analyzed the GSE32960 microarray dataset to identify 46 statistically significant differentially-expressed miRNAs (63). A weighted co-expression network was used in combination with univariate Cox regression analysis to identify 4 miRNAs (miR-142-3p, miR-150, miR-29b, miR-29c) that were most significantly associated with clinical traits such as DMFS, DFS, and OS, and also possessed the ability to classify patients into low-risk or high-risk groups (63). The 4-miRNA signature and its associated Risk Score generated by Zhang *et al.* was better able to predict the survival of NPC patients than TNM staging alone, similar to the signature identified by Liu *et al.* (63,64). While Zhang *et al.* identified potential mRNA targets of the miRNAs comprising their signature and consequently, protein pathways which may be altered, this study did not include a validation set to test the efficacy of their model with an independent set of samples (63).

Conversely, Wang *et al.* analyzed the GSE32960 dataset to produce a 3-miRNA signature (miR-142-3p, miR-29c, and miR-30e) which was subsequently validated using the independent GSE70970 dataset produced by Bruce *et al.* (60,66) (*Table 2*). This validation set demonstrated robustness of the signature in distinguishing survival between low-risk and high-risk groups (66). Of note, the 3-miRNA signature identified by Wang *et al.* overlapped with the 5-miRNA signature originally identified by Liu *et al.*, while the 4-miRNA signature identified by Zhang *et al.* only partially overlapped. The independent validation that was conducted by Wang *et al.* and Liu *et al.* reiterates the significance of their overlapping miRNAs, particularly miR-142-3p, miR-29c, and miR-30e, and should therefore prompt further investigation into the significance of these miRNAs in NPC pathogenesis or potential for use as biomarkers.

Another study conducted by Liu *et al.* retroactively analyzed 512 serum specimens from newly-diagnosed, non-metastatic NPC patients pre-treatment (65) (*Table 2*). These samples were randomly allocated into a training set and validation set and four identified differentially-expressed miRNAs (miR-22, miR-572, miR-638, and miR-1234) were constructed into a statistically significant signature,

Table 2 A summary of published miRNA signatures identified for diagnostics or prognostics in NPC.

Author	Publication year	Origin of samples	miRNA signature	Clinical significance	Equation	Pathway analysis miRNA targets
Zeng *et al.* (61)	2012	Serum	miR-17, miR-20a, miR-29c, miR-223	Ability to discriminate between healthy subjects and NPC patients for diagnostic purposes.	$A = (Ct_{miR\text{-}29c} + Ct_{miR\text{-}223}) - (Ct_{miR\text{-}17} + Ct_{miR\text{-}20a}) = -3.30$	Pathway analysis was not performed in this study
Liu *et al.* (64)	2012	Formalin-Fixed Paraffin-Embedded NPC Tissue Biopsy	miR-93, mir-142-3p, miR-29c, miR-26a, miR-30e	Predicts disease-free survival and secondarily, distant-metastasis free-survival and overall survival.	Risk score = $(miR\text{-}93_{Expression} \times 0.212) - (miR\text{-}142\text{-}3p_{Expression} \times 0.154) - (miR\text{-}29c_{Expression} \times 0.183) - (miR\text{-}26a_{Expression} \times 0.116) - (miR\text{-}30e_{Expression} \times 0.141)$	Pathway analysis was not performed in this study.
Liu *et al.* (65)	2014	Serum	miR-22, miR-572, miR-638, miR-1234	Predicts overall survival and distant metastasis-free survival.	Risk score = $(miR\text{-}22_{Expression} \times 0.146) + (miR\text{-}572_{Expression} \times 0.288) + (miR\text{-}638_{Expression} \times 0.182) - (miR\text{-}1234_{Expression} \times 0.272)$	Focal Adhesions, MAPK Signalling, ErbB Signalling. Genes associated with invasion, metastasis, and proliferation.
Bruce *et al.* (60)	2015	Formalin-Fixed Paraffin-Embedded NPC Tissue Biopsy	miR-154-5p, miR-449b-5p, miR-140-5p, miR-34c-5p	Predicts the likelihood of distant metastasis, and disease-specific survival.	Risk score = $(miR\text{-}154\text{-}5p_{Expression} \times 0.417) + (miR\text{-}449b\text{-}5p_{Expression} \times 0.280) - (miR\text{-}140\text{-}5p_{Expression} \times 0.653) - (miR\text{-}34c\text{-}5p_{Expression} \times 0.311)$	Cyclins (CCND1/D2), Cyclin-Dependent Kinases (CDK4/6), S-phase Promoting Transcription Factors (E2F1/3). Genes involved in cell cycle regulation.
Zhang *et al.* (63)†	2019	Formalin-Fixed Paraffin-Embedded NPC Tissue Biopsy	mir-142-3p, miR-150, miR-29b, miR-29c	Predicts overall survival, disease-free survival, and distant metastasis-free survival.	Risk score = $-(miR\text{-}142\text{-}3p_{Expression} \times 0.6) - (miR\text{-}150_{Expression} \times 0.37) - (miR\text{-}29b_{Expression} \times 0.42) - (miR\text{-}29c_{Expression} \times 0.66)$	Focal Adhesions, PI3K/Akt Signalling, p53, mTOR Signalling. Genes involved in ECM production and organization, blood vessel development.
Wen *et al.* (67)‡	2019	Whole Blood	miR-4665-3p, miR-4433-5p, miR-3935, miR-188-5p, miR-513b, miR-3196, miR-1908, miR-4284	Ability to discriminate between healthy subjects and NPC patients for diagnostic purposes.	$f(x) = \sum_{i=1}^{M} \alpha_m G_m(x)$, Linear Combination of M Base Regressors to develop a boosted model. Scores ≥0.708 would diagnose a patient with NPC for the 8-miRNA signature, while scores ≥0.5 would diagnose a patient with NPC for the 16-miRNA signature.	Pathway analysis was not performed in this study.
Wen *et al.* (67)‡	2019	Whole Blood	miR-296-5p, miR-361-3p, miR-4665-3p, miR-4439, miR-155-5p, miR-5091, miR-4706, miR-4436b-5p, miR-4284, miR-1224-3p, miR-4740-3p, miR-425-5p, miR-1973, miR-513b, miR-1908, miR-1280	Ability to discriminate between healthy subjects, other HNTs, and NPC patients for diagnostic purposes.		Pathway analysis was not performed in this study.
Zhang *et al.* (68)	2020	Plasma	let-7b-5p, miR-140-3p, miR-17-5p, miR-20a-5p, miR-20b-5p, miR-205-5p	Ability to discriminate between healthy subjects and NPC patients for diagnostic purposes. No significant associations with patient prognosis.	$Logit(P) = -0.981 + (let\text{-}7b\text{-}5p_{Expression} \times 0.207) - (miR\text{-}140\text{-}3p_{Expression} \times 0.066) - (miR\text{-}144\text{-}3p_{Expression} \times 0.005) + (miR\text{-}17\text{-}5p_{Expression} \times 0.277) - (miR\text{-}20a\text{-}5p_{Expression} \times 0.013) - (miR\text{-}20b\text{-}5p_{Expression} \times 0.004) + (miR\text{-}205\text{-}5p_{Expression} \times 0.003)$	P53 Signalling, FoxO Signalling, Viral Carcinogenesis. Genes involved in ion binding, cell cycle, cell death, and immune system processes.
Wang *et al.* (66)†	2021	Formalin-Fixed Paraffin-Embedded NPC Tissue Biopsy	miR-142-3p, miR-29c, miR-30e	Predicts overall survival, distant metastasis free survival, recurrence-free survival, and disease-free survival.	Risk score = $-(miR\text{-}142\text{-}3p_{Expression} \times 0.124) - (miR\text{-}29c_{Expression} \times 0.219) - (miR\text{-}30e_{Expression} \times 0.336)$	Pathway analysis was not performed in this study. However, NF-κB signalling and PI3K/Akt signalling are proposed.

†, these studies were performed using the GSE32960 dataset [originally deposited in the GEO Database by Liu *et al.* (in 2012)]. ‡, this study identified two novel microRNA signatures with seven and fourteen microRNAs, respectively.

which predicted poorer survival independent of clinical stage and elevated risk of distant metastasis (65). Notably, this 4-miRNA signature did not overlap with the previous 5-miRNA signature identified by Liu *et al.* and could thus, be an alternative means of assessing patient prognosis and guiding treatment selection, particularly among patients from the high-risk group who may benefit from more aggressive therapies (64,65).

Bruce *et al.* profiled the expression of 734 miRNAs from 135 diagnostic biopsy samples in a training set and 131 diagnostic biopsy samples in an independent validation set using the Nanostring Human miRNA Assay (60) (GSE70970 Dataset) (*Table 2*). The Cox Proportion Hazard Regression Model was used to develop a 4-miRNA signature that was associated with the risk of developing distant metastases, which was statistically significant in both the training (P<0.001) and validation (P=0.01) sets (60). Intriguingly, this signature could also discern 'lower risk' patients, who may be better treated with radiotherapy alone as opposed to chemoradiotherapy, thereby alleviating potentially unnecessary toxicity and the burden of treatment (60). When compared to the 5-miRNA signature identified by Liu *et al.*, the signature identified by Bruce *et al.* seemed to perform better in terms of hazard ratio and statistical significance, with Bruce *et al.*'s model possessing a greater area-under-curve (AUC); however, due to extensive differences in the sample populations and methodologies employed between the studies, neither model can be described as superior over the other (60,64).

Zeng *et al.* analyzed serum miRNA expression using a TLDA and qRT-PCR to identify differentially-expressed miRNAs, and developed a 4-miRNA diagnostic signature (miR-17, miR-20a, miR-29c, miR-223) using the "Ct difference method" (61) (*Table 2*). When this diagnostic model was validated using an independent cohort of 74 NPC serum samples and 57 healthy serum samples, the sensitivity was 97.3% and specificity was 96.5% (61). The miRNAs identified in this study, particularly miR-17, miR-20a, and miR-29c, have been implicated in other signatures both derived from serum and other tissue sources (*Table 2*), suggesting that they may be useful as general biomarkers for NPC diagnosis across a variety of sample methods, although further investigations would definitely be necessary.

Zhang *et al.* identified a 7-miRNA plasma diagnostic signature by analyzing 200 NPC plasma samples and 189 healthy donors, which was found to be statistically significant following multi-phase validation (68) (*Table 2*). While six of these miRNAs were found to be significantly altered in tissue specimens, none of the seven miRNAs were significantly altered in plasma exosomes (68). A logistic regression statistical model was developed using the signature [Logit(P) equation] to distinguish NPC patients from healthy individuals, which possessed notable diagnostic ability based on receiver operating characteristic (ROC) curve analyses; however, the seven miRNAs comprising this signature were not significantly correlated with NPC prognosis (68). Nonetheless, further research into these seven identified miRNAs may shed light on molecular alterations in NPC tissues, and the differential miRNA expression/transport mechanisms that may distinguish miRNA profiles between tissues, exosomes, and plasma.

Wen *et al.* identified two miRNA signatures using microarray and qRT-PCR profiling of whole blood samples acquired from patients diagnosed with NPC, head-neck tumours (HNT), and healthy subjects (HSs) (67) (*Table 2*). A diagnostic model was constructed using an 8-miRNA signature in a training set of 84 NPC and 21 HSs samples, then validated using 36 NPC and 9 HSs independent samples (67). When applied to the validation set, this diagnostic model possessed notable accuracy, sensitivity, and specificity, when distinguishing NPC patients from HSs, and the AUC determined by ROC analyses was significant (P<0.01) (67). In an attempt to develop a signature that could distinguish NPC from other HNT, Wen *et al.* randomized a second training (84 NPC, 20 HNT, 22 HSs samples) and validation set (36 NPC, 10 HNT, 8 HSs samples), and identified 16 differentially-expressed miRNAs with diagnostic value for NPC, distinguishing from other HNT patients or HSs (67). When these miRNAs were constructed into a diagnostic model and validated, the 16-miRNA signature similarly possessed good sensitivity and specificity, with a statistically significant AUC (P<0.01) again, suggesting that this signature was able to adequately discern NPC cases from other HNT patients and HSs (67). Four miRNAs overlap between the 8-miRNA signature and the 16-miRNA signature (miR-4665-3p, miR-513b, miR-1908, miR-4284). Of these miRNAs, the variable importance plots for both signatures highlight miR-4665-3p as one of the most significant miRNAs in both models, while the non-overlapping miR-296-5p and miR-361-3p were most significant in the 16-miRNA signature (67). Further investigation into these miRNAs may provide important insights in distinguishing the molecular differences between healthy, HNT, and NPC tissues.

miRNA-associated cellular dysregulation in NPC

Numerous cellular miRNAs have been implicated for their role in altering NPC cellular phenotypes (69). Specific cellular targets of these miRNAs have been identified, in addition to roles in a variety of other malignancies. For example, miRNAs such as miR-34c (10), miR-200c (11), miR-205-5p (70), miR-223 (71), miR-296-5p (72), miR-379-5p (73) and miR-449b (74) may induce or suppress EMT. Others may be involved in the development or suppression of radioresistance, such as miR-17 (7), miR-20a-5p (8,75), miR-29c (9), miR-150 (76), and miR-205 (77), or chemoresistance, such as miR-29c (9,78), miR-34c (10), miR-200c (11) miR-449b (74), and miR-1278 (79). Notably, several miRNAs from the aforementioned signatures have been reported to adopt pathogenic roles, which have been subsequently validated *in vitro*. Given the plethora of dysregulated miRNAs in NPC, we will focus on the cellular role of miRNAs described in the various signatures.

Metastasis remains one of the major motivations in the development of miRNA signatures, given that many of the identified miRNAs regulate proliferation, migration, and invasion through a variety of signalling mechanisms. MiR-93 is one such example, having been reported to promote cell growth (80), proliferation (81,82), and invasion (80). Studies conducted *in vitro* have identified several targets of miR-93 including cyclin-dependent kinase inhibitor 1A (CDKN1A) (81), transforming growth factor beta receptor 2 (TGFβR2) (83), programmed cell death protein 4 (PDCD4) (82), and disabled homolog-2 (DAB2) (80). Collectively, miR-93 expression was upregulated in NPC cells and inversely correlated with the expression of these targets, which may favour proliferation as a consequence of aberrant cell cycle regulation and invasion through augmented TGF-β/Smad signalling, and in turn, PI3K/Akt signalling (83). These findings are consistent with the role of miR-93 in the signature proposed by Liu *et al.* (in 2012), wherein elevated expression of miR-93 contributed to higher risk scores, and in turn, increased the risk of distant-metastases (64).

As previously described, miR-29c, miR-30e, and miR-142-3p overlapped between several signatures, which were associated with distant metastasis, and treatment resistance. MiR-29c, in addition to its role as a regulator of chemosensitivity (9,78) and radiosensitivity (9), may suppress metastasis in a T-cell lymphoma invasion and metastasis-inducing protein 1 (TIAM1)-dependent manner (84). MiR-29c is significantly downregulated in NPC, which may thereby increase TIAM1 levels, and in turn, facilitate metastasis (84). This finding is consistent with the role of miR-29c in several signatures, as previously described, whereby lower levels of miR-29c were associated with poorer DMFS. Likewise, miR-30e-5p was also reported to inhibit migration and metastasis by targeting the metastasis-associated 1 (MTA1) protein (85), and ubiquitin-specific peptidase 22 (USP22) (86). Downregulated miR-30e-5p was associated with poorer prognosis, as would have been predicted by higher signature risk scores. While several signatures noted that elevated miR-142-3p would correspond to reduced risk scores, the literature is limited and controversial. One study proposes that downregulated or epigenetically silenced miR-142-3p, mediated by enhancer of zeste homolog 2 (EZH2)-recruited DNA methyltransferase 1 (DNMT1) in NPC cells may promote EMT and metastasis by upregulating zinc finger E-box-binding homeobox 2 (ZEB2) (87). Conversely, another study identified suppressor of cytokine signaling 6 (SOCS6) as a direct target of miR-142-3p, and postulated that miR-142-3p overexpression in NPC would promote tumorigenesis (88). This finding is conflicting given that several studies, including those that constructed signatures, noted miR-142-3p downregulation in NPC tissues was associated with poorer prognosis. Further research investigating expression profile alterations of individual miRNAs and their cellular consequences would be necessary to clarify this discrepancy. Regardless, the biological significance in metastasis of the miRNAs implicated in the proposed signatures is generally corroborated by independent studies noted in the literature.

TGF-β signalling has been implicated as a critical player in the development of various cancers, owing to its pleiotropic roles in cell proliferation, differentiation, migration, and survival (89). In EBV-positive cancers such as NPC, TGF-β signal transduction is further dysregulated by the interaction of both viral and host factors, including miRNAs (90) (see Section "The role of EBV-encoded miRNAs in NPC"). Notably, TGF-β functions in a biphasic manner during cancer development, acting as a tumour suppressor in the early stages of disease, but promoting tumor progression and aggressiveness in later stages, particularly through EMT (89,90). MiRNA-mediated regulation of TGF-β signalling and its downstream effectors may thus be a key contributor to NPC development and the successful prediction of clinical outcomes.

Our group conducted the global miRNA profiling in Bruce *et al.* (*Table 2*) and subsequently elucidated the biological

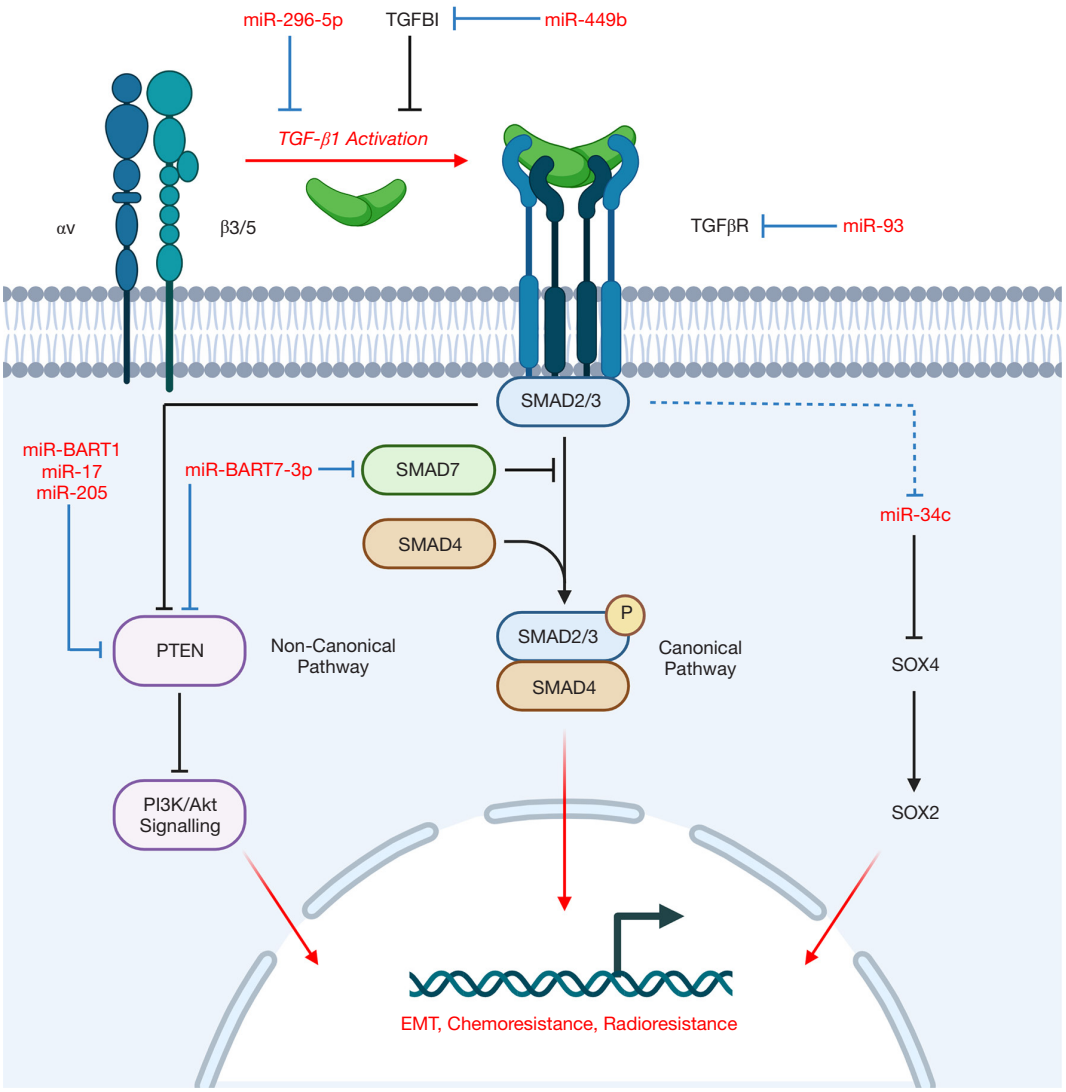

Figure 2 TGF-β canonical and non-canonical signalling-dependent induction of EMT, chemoresistance, and radioresistance in NPC. TGFBI competes with TGF-β1 for binding of αvβ3/5 integrin. MiR-449b indirectly promotes canonical and non-canonical TGF-β signalling by directly targeting TGFBI. Similarly, miR-BART7-3p targets PTEN and regulatory SMAD7 to promote both canonical and non-canonical TGF-β signalling. Through an unknown mechanism (dashed line), TGF-β signalling downregulates miR-34c expression leading to the expression of SOX4/2, key regulators of EMT. Also denoted are other EBV- and cellular-encoded miRNAs, which are known to target molecules in these pathways. Collectively, these miRNAs may augment TGF-β and downstream signalling to promote the development of EMT, chemoresistance, and radioresistance. αv, integrin alpha V subunit; β3/5, integrin beta 3 or 5 subunit; EMT, epithelial-to-mesenchymal transition; TGF-β, transforming growth factor beta; TGFBI, transforming growth factor beta induced; TGFβR, transforming growth factor beta receptor. Created with BioRender.com.

significance of both miR-449b (74) and miR-34c (10) in NPC, two miRNAs further implicated in TGF-β signal transduction (*Figure 2*). Higher levels of miR-449b were reported in NPC cells, and patients presenting with elevated miR-449b expression experienced poorer 5-year OS (74). MiR-449b promoted chemoresistance *in vitro*, particularly cisplatin-resistance, by directly targeting transforming growth factor beta induced (TGFBI) protein, which resulted in downstream PTEN inactivation and Akt activation. Inhibition of either miR-449b or Akt was found to restore cisplatin sensitivity, as

was observed when TGFBI was overexpressed.

We also described a role of TGFBI-mediated inhibition of TGF-β1 signalling activity, in which TGFBI competes with extracellular latent TGF-β1 for binding of αvβ3/5 integrin (74). Downregulated TGFBI, as a consequence of miR-449b, would increase TGF-β1-integrin binding and subsequent TGF-β1 activation. Thus, canonical (Smad2/3) and non-canonical TGF-β signalling would be promoted and facilitate EMT and Akt activation, further enhancing chemoresistance. This outcome is consistent with the miRNA signature, as elevated miR-449b expression similarly corresponded to higher patient risk scores. These findings not only underscore the importance of both miR-449b and TGFBI as regulators of chemoresistance in NPC, but their potential value as biomarkers to inform treatment selection.

Our group identified that another miRNA from the Bruce *et al.* signature (60), miR-34c, targeted SOX4, a critical regulator in EMT, which was itself regulated by TGF-β1 further upstream (10). MiR-34c was found to be downregulated in NPC cells, relative to a normal nasopharyngeal cell line, which was simultaneously accompanied by elevated SOX4 and downstream SOX2 expression. EMT may be facilitated by SOX2/4 as suggested by increased expression of EMT markers in these cells (10). Furthermore, miR-34c inhibition conferred cisplatin-resistance to cells while its overexpression increased chemosensitivity. Thus, cells with low levels of miR-34c would likely possess a greater tendency to undergo EMT and develop chemoresistance, a finding consistent with the Bruce *et al.* signature (60). A study conducted by another group demonstrated that downregulated miR-34c-3p in NPC was associated with upregulated NOTCH1 and promoted cell growth, invasiveness, and EMT, further corroborating the relevance of miR-34c in NPC pathogenesis (91). While the precise mechanism of miR-34c downregulation could not be confirmed in our study, we proposed that TGF-β1 was a negative regulator of miR-34c that could alter SOX2/4 expression further downstream. Treatment with a TGF-β Receptor 1 inhibitor reduced SOX4 expression, and in turn, led to increased chemosensitivity.

When the two studies conducted by our group are considered in tandem (10,74), it is mechanistically plausible that miR-449b overexpression in NPC, and its corresponding downregulation of TGFBI and accumulation of TGF-β1, could downregulate miR-34c expression and upregulate SOX2/4 downstream to promote EMT and chemoresistance. Interestingly, the proposed miR-449b/miR-34c mechanism parallels the mechanism of miR-BART7-3p action described by Cai *et al.* (see Section "The role of EBV-encoded miRNAs in NPC") wherein miR-BART7-3p similarly promotes canonical TGF-β signalling albeit by targeting regulatory Smad7 and promoting PI3K/Akt signalling through PTEN, ultimately conferring chemoresistance, stemness (as suggested by elevated stemness markers, including SOX2) and potentially EMT (36) (*Figure 2*). The convergence of these miRNAs, both cellular and viral-encoded, on TGF-β signalling, the PTEN/Akt axis and SOX2/4 expression, with their resulting consequences on chemoresistance and EMT, collectively underscores the biological significance of these pathways, and warrants further investigation into these molecules not only as biomarkers but potential therapeutic targets.

Challenges and future directions

Despite the great strides that have been achieved in understanding miRNA biology, there remains many challenges in elucidating the roles of miRNAs in NPC. The vast repertoire of miRNAs, both cellular and viral-encoded, is definitely daunting when attempting to identify the most significant players in the initiation or progression of this disease (6). Indeed, a plethora of individual miRNAs has been proposed as potential biomarkers in the literature. To this end, miRNA profiling techniques (59,62) and subsequent analyses aim to shed light on the most meaningful molecules or signatures that could be useful in the diagnosis, prognosis, or treatment selection of NPC patients. However, many studies remain hindered by differences in tissue type, tumour heterogeneity, and diverse profiling and analytical techniques employed (1). Consequently, the varying findings between studies may appear disparate and difficult to compare due to the differences that exist in methodologies. While some overlapping miRNAs exist between the signatures that have been highlighted in this review, the signatures themselves remain particularly diverse with their own unique miRNAs. Even so, the utility of these signatures is underscored by their significance with independent validations (60,61,64-68). Further investigation of the individual miRNAs comprising these signatures would be necessary to confirm their biological significance in NPC, especially *in vivo* studies to complement the multitude of miRNA investigations that have been conducted *in vitro*. At the time of this writing, no miRNA signatures have yet been identified with respect to EBV miR-BARTs,

although future studies may provide additional insights given the high frequency of EBV-associated NPCs. Moving forward, unravelling the molecular events influenced by the intersection of miRNAs in NPC may help identify novel biomarkers, and additionally, therapeutic targets to improve clinical outcomes.

Conclusions

The biological significance of miRNAs in NPC is highlighted through their dual origin, both encoded by host cells or EBV, and the numerous cellular processes they influence as "master regulators" of the genome. Further investigations will be crucial to identify the most significant miRNA biomarkers and signatures in addition to their underlying relevance in NPC biology, potentially towards improved treatment, disease management, and potential therapeutic targets.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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