Chapter 7

The Role of miRNAs in Diagnosis, Prognosis and Treatment Prediction in Cervical Cancer

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Additional information is available at the end of the chapter

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Abstract

Cervical cancer represents one of the major problems of health women worldwide, especially in the developing countries. If discovered in its earliest stages, cervical cancer is successfully treatable; however, due to lack of proper implementation of screening programs, the majority of cervical cancer patients are diagnosed in advanced stages, which dramatically influence their outcome. Almost a half of these patients will suffer recurrence or metastasis in the following 2 years after therapy. If there are no immediate prospects in terms of developing new or more effective therapies, identifying new tools for early diagnosis, prognosis and treatment prediction remains a big challenge for cervical cancer. miRNAs have been validated to be key players in cell physiology, alterations in miRNA expression being associated with cancer progression and response to therapy. Cervical cancer studies have showed that alterations of miRNA expression can be identified in tumor tissues, exfoliated cervical cells and patients serum and that their transcription pattern is regulated by the present HPV genotype. Furthermore, miRNAs have been associated with patients response to therapy, therefore suggesting their potential to be used as biomarkers for cervical cancer diagnosis, prognosis and treatment response.

Keywords: cervical cancer, miRNA, HPV, Diagnosis, Prognosis, treatment response

1. Introduction

With an incidence of over a half million new cases every year, cervical cancer still represents a major health problem for women worldwide. The last collected data from 2012 show that the incidence is not proportionally distributed, 84% of new cases (444,500 out of 527,600)

being recorded in developing countries and only 16% new cases in developed ones [1]. Consequently, the mortality rate is pretty similar, with 86.63% deaths (230,200 of 265,700) recorded in developing areas, compared with 13.47% deaths registered in developed ones.

This burden is related to the dis-proportional implementation of screening programs (Pap smear and HPV tests), which allow the early detection of cervical precancerous and cancerous lesions that can be successfully treated surgically. Previous data revealed that cervical cancer control programs based on Pap test reduce the risk of cervical cancer with 25–36% [2]. To maximize the screening effect, World Human Organization (WHO) recommended Pap test for women 30 years or older, considered at high risk for developing cervical cancer. Moreover, testing and following the patients with persistent high-risk HPV infections could identify the early stages of cervical cancer.

Unfortunately, due to lack of proper implementation of screening programs in developing areas, the majority of cervical cancer patients are diagnosed in locally advanced stages (IIB–IIIB). Depending on the stage of the disease, the treatment includes radiotherapy concomitant with adjuvant or neoadjuvant chemotherapy, associated or not with surgery [3]. Nevertheless, the advanced stages will dramatically influence their response to the therapy, and almost a half of these patients will suffer recurrence or metastasis in the following 2 years after treatment.

In cervical cancer, as in the other cancers, the identification of new competitive drugs and new accurate biomarkers for diagnosis and prognosis is challenging. If there are no immediate prospects regarding developing new or more effective drug-based therapies, identifying new tools for early diagnosis, prognosis or treatment predictions, has become a necessity.

The genomics revolution has uncovered new molecular data that advanced the characterization and the understanding of the complex regulatory signaling networks that drive cancer growth and development. Molecular findings related to both coding and non-coding transcriptome were investigated in order to identify new cancer biomarkers, including in cervical cancer. Of these, non-coding RNAs related to cell functionality, such as micro-RNA (miRNA), have became important pieces of puzzle to characterize cervical cancer phenotype or to investigate their involvement in prognosis and treatment response.

The subsequent chapter will present the existent data regarding the involvement of miRNAs in cervical carcinogenesis, including their synthesis, stability and specificity, as well as the role of HPVs infection in modulating host's miRNAs expression. Furthermore, we will discuss the latest updates on the miRNAs clinical applications as additional valuable markers for diagnosis, prognosis and treatment prediction in cervical cancer.

2. Micro-RNAs

MiRNAs represent a class of non-coding RNAs, evolutionary conserved between species, which negatively regulate gene expression at both transcriptional and post-transcriptional levels, during normal and pathological conditions [4, 5]. Functional studies indicate that up to 60% of protein-coding genes (PCGs) are regulated by miRNAs, which "gained" them the

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name of "master modulators" of the human genome [6, 7]. An interesting aspect of miRNAs is that each miRNA can target up to 200 mRNAs on average or different miRNAs can target the same mRNA [8]. In physiological conditions, miRNAs play important roles in the modulation of many cellular processes and mechanisms such as angiogenesis, cell cycle regulation, differentiation, apoptosis, DNA repair or stress response. Subsequently, when alterations occur in their expression, miRNAs become key regulators of many diseases, including cancer. Several genetic and epigenetic events induce changes in miRNAs expression, including DNA amplifications or deletions that lead to gain or loss of miRNAs loci regions. Moreover, DNA mutations or SNPs (single nucleotide polymorphisms) inside of miRNAs coding genes could modify the miRNAs specificity by changing their structure [9]. Epigenetic modifications are related to hypermethylation of CpG islands of the transcriptional factors and promoters of miRNA-coding genes resulting in silencing of these genes, leading to miRNAs expression alteration [10]. Croce's group demonstrated for the first time an association of miRNAs with cancer, identifying deletion and down-regulation of two miRNAs (miR-15 and miR-16) in 13q14 LOH (Loss of Heterozygosity) of chronic lymphocytic leukemia [11]. The same group demonstrated that the majority of miRNAs genes are located in cancer-associated genomic regions, considered genomic fragile sites [12]. Taking advantage of high-throughput microarray technology, Calin et al. [13] demonstrated that genome-wide miRNA profiling represents a reliable tool to characterize and investigate tumor phenotypes, deepening the understanding of the molecular mechanisms of cancer. Since 1993, when the first miRNAs were discovered, extensive research and tremendous efforts were put into identifying and characterizing new miRNAs. Due to the identification of growing numbers of miRNAs, a miRNA database was assembled, the miRBase Release 2.0 including 506 miRNA sequences from six organisms became firstly available in 2004 via web interface [14]. Currently, 26,654 entries from 223 species, including 2588 mature human miRNAs, have been deposited in the newest data from miRBase Release 21 (http://www.mirbase.org/).

2.1. Biogenesis of miRNA

It is estimated that only a small fraction (less than 2%) of the human genome is represented by coding DNA (exons from PCGs), while the rest of 98% include sequences of non-coding DNA such as introns, non-coding RNAs, regulatory DNA sequences, and other DNA sequences with unknown functions [15]. Non-coding DNAs play important roles in controlling all steps of gene expression, when PCGs are transcribed into mRNAs and translated into proteins. The investigation of functions of non-coding DNAs during the ENCODE (Encyclopedia of DNA Elements) project revealed new data related to how genetic information is converted into living cells and also the fact that almost 80% of human genome is activate in cell physiology [16]. Part of non-coding RNAs, miRNAs represent the most studied components being widely accepted that they are major players in cell cycle dynamics, regardless of the physiological or pathological status.

Generally, almost all regions of the genome may encode miRNA. As much as 40% of miRNAs are located in intragenic regions, within the introns or even in the exons of the PCGs. The majority of the miRNAs (50%) are encoded by both intronic (40%) and exonic (10%) regions of non-protein coding genes also known as non-protein coding RNAs (npcRNAs) (**Figure 1**) [17].

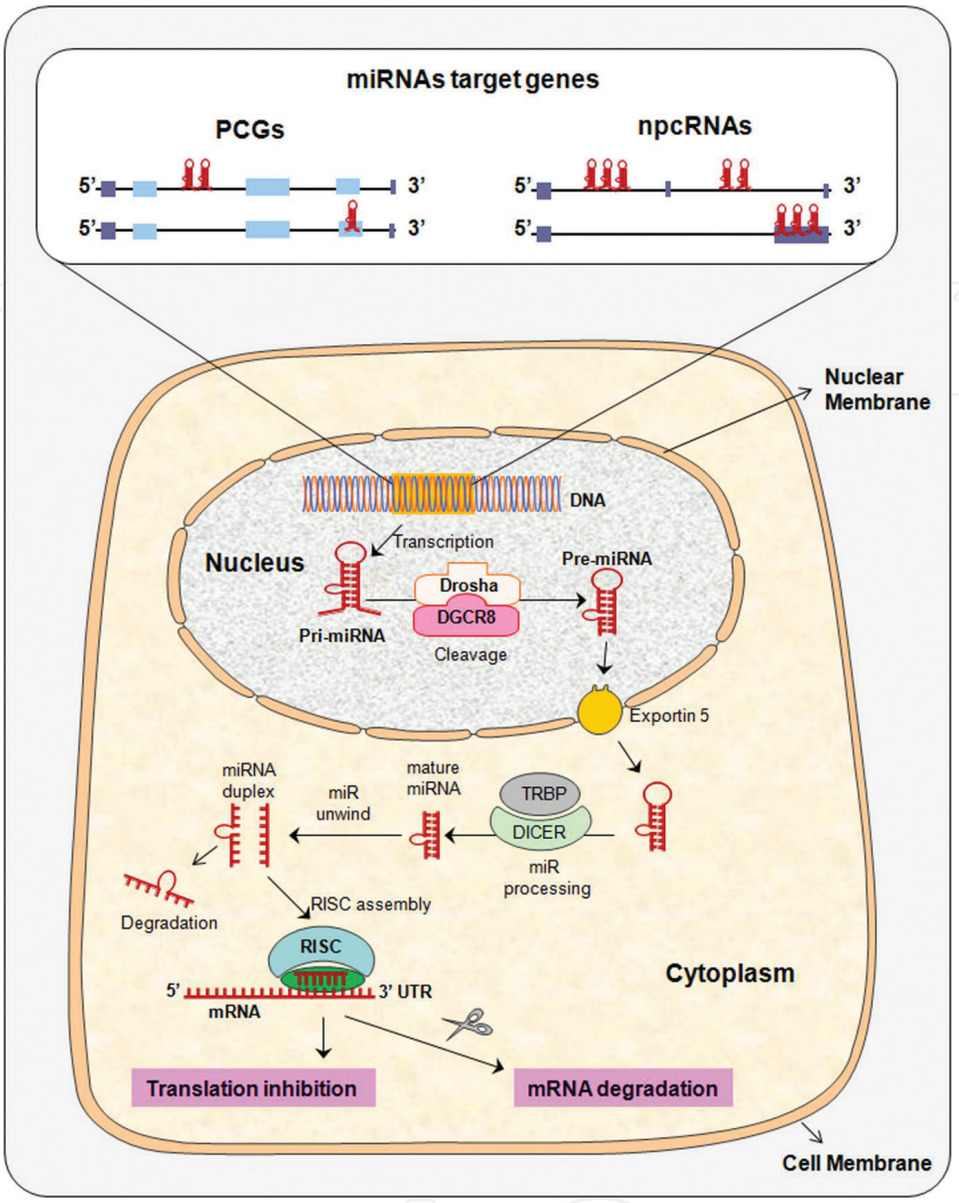

Figure 1. The biogenesis of miRNAs. MiRNAs are transcribed from intra- and intergenic regions of DNA, from both PCGs and npcRNAs. The long hairpins structures (pri-miRNA) transcribed from DNA are cleaved in pre-miRNAs smaller transcripts (~70 nucleotides long) by a microprocessor complex that includes Drosha and DGCR8 enzymes. After nuclear export through exportin 5, pre-miRNAs are processed to mature miRNAs. One miRNA strand is loaded in RNA-induced silencing complex (RISC) complex, while the other is degraded. RISC complex aligns by sequence complementarity to the target mRNA and negatively regulate their expression by degradation and/or translational repression.

The miRNAs transcription starts in the nucleus being mediated by RNA polymerase II [18], but there is also some evidence that miRNAs could be transcribed by RNA polymerase III when are associated with Alu family repetitive elements [19]. The first miRNA transcripts (pri-miRNAs) transcribed from DNA are long hairpin structures that include hundreds or thousands nucleotides. Upon recognition of a microprocessor complex, including RNA polymerase III Drosha and DGCR8 (DiGeorge syndrome critical region 8), pri-miRNAs are processed into one or more smaller hairpins of about 70 nucleotides long called precursor

miRNAs (pre-miRNAs) [20]. Forward, pre-miRNAs are exported from nucleus to the cytoplasm by the nuclear export receptor exportin 5 [21]. After their export in cytoplasm, pre-miRNAs are processed by cytoplasmic RNA polymerase III Dicer to generate 21–23 nucleotides mature miRNA duplexes [22]. The miRNA strand with less stable paired 5′ end will be preferentially loaded into Argonaute 2 (AGO2) proteins that possess cleavage activity, while the other miRNA strand will be degraded [23]. The miRNA-loaded AGO2 protein will be incorporated into RNA-induced silencing complex (RISC) that will target by sequence complementarity to the 3′UTR of specific mRNA target, leading to mRNA degradation or translational repression.

2.2. MiRNAs in cancer

Non-coding RNAs, especially miRNAs, have changed the sense of the "central dogma of molecular biology." In accordance with central dogma of molecular biology, RNAs represent intermediary information in the genetic flow, by copying genetic information (transcription) from DNA and transform it (translation) to cellular effectors, the proteins:

$$\mathbf{DNA} \rightarrow \text{transcription} \rightarrow \mathbf{mRNA} \rightarrow \text{translation} \rightarrow \mathbf{Proteins} \quad \text{(the central dogma of molecular biology)} \tag{1}$$

Following discovery and characterization of small non-coding RNA, such as miRNAs, siRNAs and piwi RNAs as well as long non-coding RNAs and ultraconserved elements (UCEs), the central dogma of molecular biology has became more complex [24]. Actually, the genetic information from DNA is transcribed in both coding RNAs (mRNAs) and non-coding RNAs (miRNAs, siRNAs, piwiRNAs, lncRNAs and ECEs). Furthermore, ncRNAs actively participate both in the regulation of protein synthesis by regulation of the DNA transcription and mRNA translation as well as by regulating their expression by complex cross-regulation [16] (**Figure 2**).

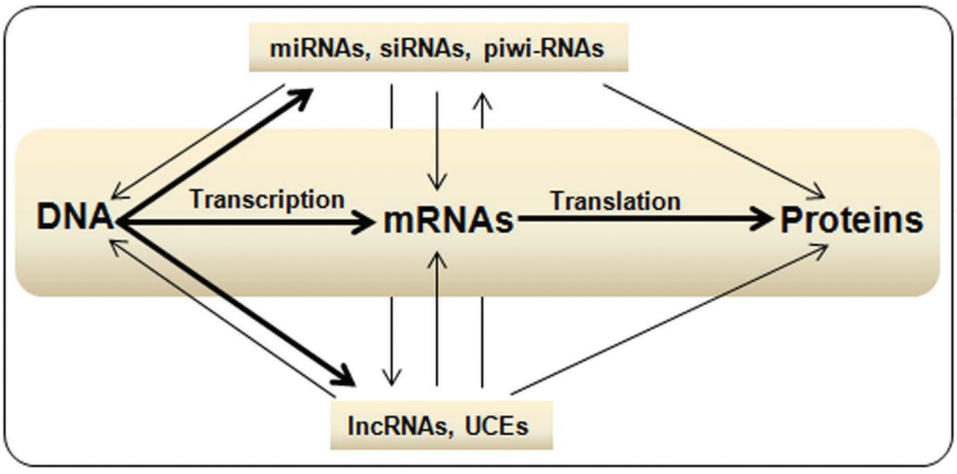

Figure 2. The new concept of the central dogma of molecular biology. The genetic information from DNA is transcribed (bold arrows) in both coding RNAs (mRNAs) and non-coding RNAs (miRNAs, siRNAs, piwiRNAs, lncRNAs and ECEs), while the mRNA translation in their specific proteins includes complex regulations (regular arrows) performed by all type of ncRNAs.

After their discovery, miRNAs have become a hot subject for many researchers worldwide, in the attempt to establish their role in cell functionality, especially in cancer. Croce's lab was the first that established that miRNAs alteration can be associated with cancer [11], showing that miR-15 and miR-16 were deleted or down-regulated in the majority of patients with chronic lymphocytic leukemia. Furthermore, they demonstrated that these miRNAs act as tumor suppressor genes, inducing apoptosis of leukemic tumor cells by negatively regulation of the expression of BCL2 oncogene (mRNA), which encodes a protein that blocks the apoptotic death [25]. The expression of both miR-15 and miR-16 was inversely correlated with Bcl2 expression, strengthening the hypothesis that miRNAs are post-transcriptional negative regulators their mRNA targets and can be successfully used to characterize malignant hematological tumors [13]. Immediately afterwards, miRNA profiling has quickly become an important tool to characterize tumor phenotype and identify specific miRNA biomarkers. MiRNAs were clearly related to cancer development by three landmark studies published in Nature. In one of these studies, Lu et al. [26] demonstrated that poorly differentiated tumors are successfully classified when miRNA profiling is used; moreover, miRNA profiling represents better classifiers than mRNA profiling. In the second landmark study, He et al. [27] demonstrated that miR-17-92 cluster has an oncogenic potential, and its expression can modulate carcinogenesis in B-cell lymphoma in synergy with c-myc transcriptor factor. Although the mechanism of the oncogenic effect has not been fully elucidated, the authors suggested a decrease of apoptosis in these cells. The mechanisms that underlie the synergy between c-myc and miR-17-19b overexpression in B-cell lymphomas were demonstrated 10 years later by Mihailovich et al. [28] based on a comprehensive analysis integrating proteomics, transcriptomics and miRNAs prediction analysis. The third landmark study performed by O'Donnell et al. [29] demonstrated that the activity of E2F1 transcriptional factor can be controlled by two clusters of miRNAs (mir-17 and mir-106a) whose expression is activated by c-myc oncogene. After miRNAs were definitively linked to cancer development, a worldwide research effort has been made to characterize and establish the miRNAs roles in cancer.

Collectively, all previous data showed that miRNAs represent key ubiquitous players involved in cancer development. MiRNAs regulate molecular pathways involved in all cancer hallmarks such as self-sufficiency in growth signals, indolence to antigrowth signals, evasion from apoptosis, limitless potential replicative, angiogenesis, invasion and metastasis, reprogramming energy metabolism and evading immune destruction [30].

The growing miRNA profiling data, target prediction and data validation led to the development of specialized online miRNA databases that can be easily accessed in a user-friendly manner. Until now, there are at least 14 online open-access databases containing extensive information about miRNAs [31]. Some of these databases (*SomammiR DB2.0*) are useful to search for miRNAs somatic mutations and to predict their functional analysis based on these mutations, to identify miRNAs alterations involved in protein regulation (CancerNet) or to review the annotation changes for each miRNAs entry (miRbase Tracker). Additional databases focus on miRNAs roles in particular types of cancer such as head and neck and oral carcinomas (HNOCDB), endometrial (miREC), renal (Renal Cancer cell database), pancreatic (Pancreatic Cancer Database), sarcoma (Sarcoma microRNA Expression Database) or breast cancers (OncomiRdbB). Certain miRNA databases such as canEvolve include recent information from next-generation sequencing (NGS) technology in different type of cancers,

healthy people and other pathologies, while miRCancer stores data about miRNAs in cancers collected by data mining. Other miRNAs databases are focused on the role of miRNAs in diagnosis and overall survival (PROGmiR) and their presence in extracellular vesicles (miRandola) as well as their role in autophagy activation (AutomiRDB).

2.3. MiRNAs can function as oncogenes or tumor suppressor genes

It is well known that cancer is a disease characterized by abnormal cell grown caused by uncontrolled division of cells. Two main classes of genes such as proto-oncogenes (ex KRAS, MYC, Her-2/neu, EGFR) and tumor-suppressor (ex RB, TP53, PTEN) are involved in the regulation of cell cycle in normal conditions.

When cancer occurs, multiple genetic and epigenetic alterations disrupt the normal function of these two classes of genes leading to their abnormal expression and therefore, uncontrolled cell division [32]. Cancer cells appear when proto-oncogenes are converted in oncogenes by "gain of function," and tumor-suppressor genes are inactivated or deleted resulting in "loss of function." From a functional perspective, the oncogenes and tumor suppresser genes expression can be modulated by miRNAs toward mRNA degradation or translational repression. Many previous studies have shown that miRNAs play both oncogene (oncomiR) and tumor-suppressor (tumor-suppressor miRNAs, TS-miRNAs) roles. By their up-regulation, onco-miRNAs negatively modulate the expression of tumor suppressor genes, while the down-regulation of TS-miRNAs will result in the absence of regulation or increased expression of oncogenes. A selection of oncomiRs and tumor-suppressor miRNAs, their mRNA targets as well as the pathologies where they are involved are presented in **Table 1**.

OncomiR or/tumor suppressor miR (TS-miR)	MiRNAs/ expression	Validated mRNA targets/regulation	miRNAs Function	Pathologies associated with miRNAs alteration	Ref.
oncomiR	miR-21↑	BCL2↓ PTEN↓ PDCD4↓ BTG2↓ TPM1↓	Promote cell survival and proliferation by antiapoptotic effects	Overexpressed in a variety of human tumors including breast, melanoma, ovarian, head and neck, colon, prostate, lung, pancreas and cervix	[33–37]
oncomiR	miR-155↑	PTEN↓ BCL2↓ SOCS1↓ SOCS6↓ BLC6↓	Induces cell proliferation, differentiation and migration, as well as inhibits apoptosis	Up-regulated in different cancers such as, breast, liver, hematological malignancies and cervix	[37–40]
oncomiR	miR-221/222↑	PTEN↓ TIMP3↓ $p27^{Kip1}$↓ CDKN1C/p57↓ BBC3/PUMA↓	Promotes cell proliferation, regulate cell cycle phase distribution, and inhibits apoptosis	Overexpressed in glioblastomas, thyroid papillary carcinomas, breast cancer, glioma, hepatocellular carcinoma, and lung cancer	[41]

OncomiR or/tumor suppressor miR (TS-miR)	MiRNAs/ expression	Validated mRNA targets/regulation	miRNAs Function	Pathologies associated with miRNAs alteration	Ref.
oncomiR	Cluster miR-17-92↑	PTEN↓ TGFBR2↓ SMAD2↓ SMAD4↓ P21↓	Promote tumorignesis, cell cycle progression, cell proliferation and survival	Up-regulation in glioblastoma, lymphomas as well as thyroid, pancreas, colorectal, breast, lung, and stomach cancers	[44, 45]
TS-miR	miR-221/222↓	c-Kit↓	Inhibits of erythropoiesis	Loss in erythroblastic leukemia	[42]
TS-miR	Cluster miR-17-92↓	AIB1↑	Suppress proliferation	Down-regulation in breast cancer	[46]
TS-miR	miR-15a↓ miR16-1↓	BCL2↑	Induces apoptosis, inhibits carcinogenesis	Deleted (loss) in chronic lymphocytic leukemia, prostate, breast and cervical cancers	[11, 47, 48]
TS-miR	miR-34↓	CCDC↑ CDK4↑ CDK6↑ HMGB1 Wnt/β↑	Induces apoptosis and inhibits cell proliferation, invasion and metastasis	Down-regulated in laryngeal, liver, pancreatic, colorectal, breast and cervical cancers	[50–52]
TS-miR	miR-143↓	KRAS↑ MMP-13↑ CD44v3↑ COX2↑ ERK5↑	Suppress proliferation, tumor development and proliferation, invasion and metastasis and induces apoptosis	Down-regulated in prostate, bladder, colon, breast and cervical cancers	[53, 54]
TS-miR	miR-145↓	ERG↑ FLI-1↑ KRAS↑ MUC1↑ IRS-1↑ VEGF-A↑	Suppress cell growth tumor invasion, proliferation angiogenesis and promotes apoptosis	Down-regulated in prostate, colon, breast, ovarian and cervical cancer	[55–57]

Table 1. Selection of oncomiRs and tumor suppressor miRNAs (TS-miR), their mRNA targets, and the pathologies were they are involved.

MiR-21, one of the most oncogenic miRNAs, is frequently overexpressed in many tumor types [33] including cervical cancer cells [34]. MiR-21 can down-regulate multiple tumor suppressor genes such as phosphatase and tensin homolog (PTEN), B-cell lymphoma protein 2 (BCL2), programmed cell death 4 (PDCD4), BTG2 or tropomyosin 2 (TPM2), leading to cell survival, increased proliferation and decreased apoptosis [35, 36].

MiR-155 is another oncomiR highly expressed in tumors, which negatively regulated multiple tumor suppressor genes such as PTEN, suppressor of cytokine signaling 1(SOCS1), suppressor

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of cytokine signaling (SOCS6), and B-cell CLL/lymphoma 6 (BCL6) [37–39]. In cervical cancer, high expression of miR-155 is associated with poor prognosis [40].

Two highly homologous microRNAs, miR-221 and miR-222, are key miRNAs deregulated in many types of cancers, having a double role as oncogenes or tumor suppressors depending on the cellular context and tumor type. In the majority of tumors where mir221/222 were identified, these act as oncomiRs by promoting cell proliferation, regulating cell cycle phase distribution, and inhibiting apoptosis. Their validated targets include several tumor suppressor genes such as PTEN, BCL2 binding component 3 (BBC3/PUMA) and TIMP metallopeptidase inhibitor 3 (TIMP3) tumor suppressor genes [41]. Contrary, in erythroleukemic cells, mir-221/222 has been showed to act as tumor-suppressors, by down-regulating proto-oncogene receptor tyrosine kinase (c-Kit), leading to inhibition of erythropoiesis [42].

Similar with miR221/222, miR-17-92 cluster was validated for both oncogenic and tumor suppressive roles [43]. The oncogenic activity of miR-17-92 cluster is related to the negative modulation of PTEN tumor suppressor gene [44], and multiple components of TGFβ signaling pathway, including transcriptional modulators such as SMAD2/SMAD4 and CDKNIA (p21) which are involved in negative regulation of cell cycle progression [45]. The suppressor role of miR-17-92 cluster was proved by its miR-17-5p member that negatively regulates the nuclear receptor coactivator 3 (NCOA3/AIB1), leading to decreased proliferation of breast cancer cells [46].

While some miRNAs promote carcinogenesis by their up-regulation, several studies have shown that the majority of tumors present deletions of miRNAs tumor suppressor genes, due to the fact that they are located in or near fragile sites of cancer-associated genomic regions, genomics rearrangements (chromosomal and/or genes) [12]. For example, the first cluster of tumor suppressor miRNAs (mir-15a/miR-16-1) was related to deletion of 13q14.3 region in chronic lymphocytic leukemia. Calin et al. [11] demonstrated that the expression of BCL2 oncoprotein (anti-apoptotic regulator), target of mir-15a/miR-16-1, is inversely correlated with these miRNAs. The role of miR-15 and mir-16 tumor suppressor genes was also demonstrated in prostate and breast cancer cells [47, 48] as well as in cervical cancers [49].

A component of TP53 tumor suppressor network, mir-34a represents another tumor suppressor gene that regulates cell proliferation by targeting cyclin D1 (CCDC1), cyclin-dependent kinase 4 (CDK4) and cyclin-dependent kinase 6 (CDK6) proteins or Wnt/β-catenin signaling pathway in laryngeal [50], breast [51] colon and cervical cancers [52].

Down-regulation or loss of function of miRNA-143 was identified in multiple cancers including cervical cancer, being associated with carcinogenesis and tumor progression [53]. Some important targets of miR-143 include KRAS proto-oncogene conducting cancer development, matrix metallopeptidase 13 (MMP-13) with role in metastasis, cluster of differentiation 44v3 (CD44v3) involved in migration and invasion, cyclooxygenase 2 (COX2) supporting tumor metastasis by tumor proliferation, migration and epithelial-mesenchymal transition (EMT) [54].

Tumor suppressor miR-145 has important roles in the regulation of cell proliferation, and its loss or down-regulation has been associated with development and progression as well as invasion and metastasis of different type of malignancies such as breast, ovarian, colon, prostate and cervical cancer [55, 56]. The mir-145 targets include erythroblast transformation-specific (ETS) family of transcriptions factors (ERG),

KRAS, fms-related tyrosine kinase 1 (FLT1), mucin 1 (MUC1), vascular endothelial growth factor A (VEGFA) and insulin receptor substrate 1, involved in different pathways of tumor progression and metastasis [57].

3. High-risk HPV infection modulates miRNA expression in cervical cancer

Human Papillomaviruses (HPVs) represent the most important risk factor for developing cervical cancer. Papillomaviruses are small (50–60 nm) DNA viruses, counting over 200 members that include more than 150 HPVs [58]. Depending on their type of infection, HPVs are divided into two subgroups: cutaneous, which infects cutaneous skin producing benign papillomas (warts), and mucosal, infecting mucosal epithelial cells, which provoke epithelial dysplasia and invasive carcinoma. Currently, 12 HPVs that include 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 subtypes are confirmed oncogenic and referred to as high-risk HPVs (HR-HPVs) [59]. The infection and minimum 2 years persistence with HR-HPVs is a prior condition of cervical cancers. As a result of the discovery that HR-HPVs are causative agents of cervical cancer, Harald zur Hausen was awarded the Nobel Prize in 2008. Data from epidemiological studies revealed that HPV 16 and 18 subtypes are associated with the majority of cervical cancers [60]. Moreover, previous data showed that HPV detection increases during transition from precancerous lesions to cervical cancers. If in CIN1 (cervical intraepithelial neoplasia) characterized by low-grade dysplasia, HPV is present in proportion of 50–70%, in middle dysplasia (CIN2), it increases to 85%, while in severe dysplasia (CIN3) and cervical cancer, the HPV presence raises to almost 100% [61]. This observation was further exploited to identify HPV-dependent-specific biomarkers for cervical cancer detection.

Because the HPV genomes do not possess their own enzymes necessary for viral replication, these viruses will use the enzymatic machinery of the host infected cells (basal layer of squamous epithelia) to sustain DNA viral replication. HPVs possess small genomes, of about 8 kb in size, including a well-conserved core set of genes involved in replication (E1 and E2) and packaging (L1 and L2). The remaining HPV genome includes genes (E4, E5, E6, and E7) that modify cellular environment, cell cycle regulation, immune evasion, and virus release. The detailed roles of the proteins encoded by these genes, involving host cells infection, evading the immune system and the modulation of carcinogenic pathways were recently presented [59]. Shortly, following infection, HPVs have the ability to insert their genetic material into host's cell genome, leading to expression and replication of viral DNA by the host cell. Expression of viral genes E1 and E2 leads to the production of oncoproteins E5, E6, E7. These oncoproteins act in a cooperative way to promote malignant transformation of the host cell by inhibiting p53 and pRB tumor suppressor proteins, altering the apoptosis and the cell-cycle control, activating telomerase and promoting the proliferation of infected cells.

Cellular changes due to HR-HPVs are also visible in microRNA regulation. Several studies reported aberrant miRNA expression profiles in HPV positive cervical cancer when compared to HPV-negative cervical cancers. In particular, E6 and E7, but also E5 HR-HPV oncoproteins, are capable of modulating the expression of different miRNAs in host infected cervical cancer cells (**Figure 3**).

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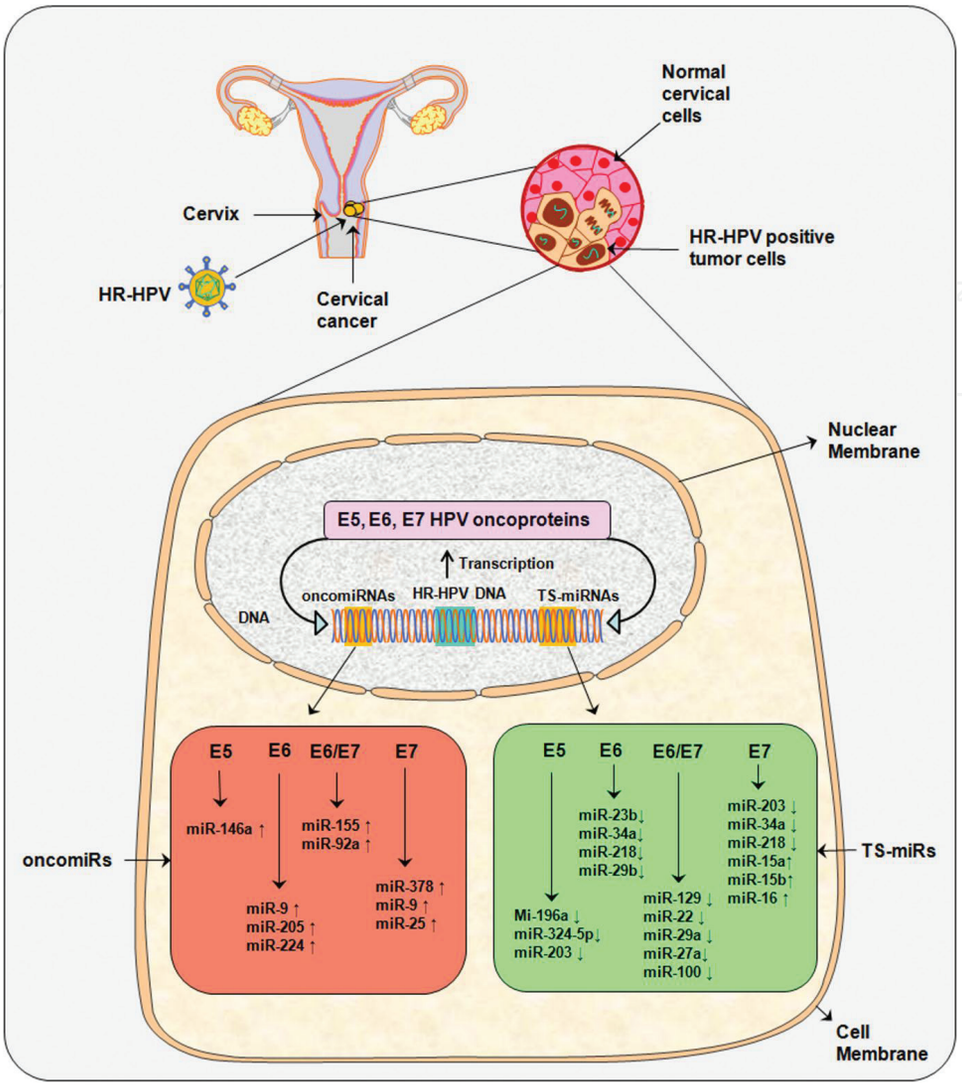

Figure 3. miRNAs modulated by HR-HPV E5, E6 and E7 oncoproteins in cervical cancer. After infecting the basal layer of squamous epithelia, HPV virus modifies the cellular environment, cell cycle regulation, cell proliferation and migration, immune evasion and altering the apoptosis by regulation of specific miRNAs. HPVs insert their genetic material into host's cell genome, leading to expression of specific sets of oncomiRs and blocking or modulating certain miRNAs, in a cooperative way that promote malignant transformation of the host cell.

In a study comprising 101 cervical cancer cases, Liu et al. [62] identified the E6 and E7 HPV transcripts in all the HPV-positive cases (86% of all cervical cancer samples). Four miRNAs, including miR-9, miR-205, miR-224 (up-regulated) and miR-29b (down-regulated), were correlated with HP-positive expression using HPV-negative cancers as controls. MiR-9 was selected for further investigations and validation as it was the most aberrantly expressed miRNA. Their results showed that miR-9 is mostly expressed by HPV16 infected cells, and it is mainly activated by E6, (up-regulated 45-fold as a result of E6 expression), but also due to E7 (5-fold up-regulation). Based on target prediction algorithms, RNA-seq data and RT-PCR experiments, they identified FASTL1 (involved in cellular proliferation) and ALCAM (involved in cell migration) as miR-9 targets in cervical cancer.

Au Yeung et al. [63] demonstrated in vitro that HPV16 E6 protein leads to the down-regulation of tumor suppressor miR-23b, through p53 degradation. The decreasing expression of

miR-23b results in increasing expression of urokinase-type plasminogen activator (uPA) that leads to increased migration of cervical tumor cells. Moreover, the reduce expression of miR-23b was related to invasion and metastasis, being considered a prognostic factor of cervical carcinoma [64].

In a similar study, Raver-Shapira's group [65] demonstrated that HPV E6 oncoprotein can decrease the expression of miR-34a through p53 destabilization during viral infection. MiR-34a represents an important tumor suppressor miRNA, whose alteration leads to cell proliferation, by activating the genes involved in cell cycle regulation such as cyclins (E2 and D1), cyclin-dependent kinases (CDK4, CDK6), transcription factors (E2F1, E2F3, E2F5), and decreased apoptosis by up-regulation of antiapoptotic Bcl-2 [66, 67].

Another target of HPV16 E6 oncoprotein is miR-218. In vitro and in vivo studies demonstrated that miR-218 is down-regulated by E6 oncoprotein in both precancerous lesions and cervical cancers. In an in vivo study, Li's group [68] proposed to investigate whether there is a correlation between HPV16 infection and miR-218 down-regulation and to evaluate the relationship between miR-218 expression and CIN staging. Seventy-eight CIN cases were included in this study. Of all the cases, 66% presented infection with a single HPV type, 24% presented infection with multiple HPV subtypes, and 9% were HPV free. Quantitative RT-PCR measurements showed a down-regulation of miR-218 in CIN cases presenting HR-HPV infections compared to HPV-free CIN cases. MiR-218 expression levels dropped further down with CIN stage evolution, CIN3 cases presenting a significantly lower level of miR-218 than CIN1. One of the targets of miR-218 is LAMB3 with roles in cell migration and carcinogenesis of cervical cancer. Another study revealed that the expression of miR-218 can lead to EMT inhibition, migration and invasion by targeting pro-tumorigenic genes such as SFMBT1 and DCUN1D1 [69]. Recently, the expression of miR-218 in cervical cancer was associated with radiosensitivity via promoting radiation induced apoptosis, down-regulation of miR-218 significantly reduced the radiation-induced apoptosis [70].

If HR-HPV E6 oncoprotein mediates the miRNAs targets through p53 destabilization, the HR-HPV E7 oncoprotein modulates the miRNAs targets by increasing the transcription of E2F family of transcription factors through degradation of pRB from pRB-E2F complex, leading to releases and activation of transcription factor E2F [71]. In this regard, several miRNA targets of HR-HPV E7 oncoprotein such as miR-15a, miR-15b and miR-16 were identified as targets of E2F transcription factors [72, 73]. These miRNAs, considered tumor suppressor miRNAs (TS-miRNAs), were identified as overexpressed in cervical cancers. Interestingly, miR-15b was also suggested to play an important role in cervical carcinogenesis, regulating mechanisms such as angiogenesis, invasion and metastasis by targeting and down-regulating the RECK gene, which can negatively affect the transcription and activity of MMPs [74]. Another target of E7 oncoprotein is represented by miR-203, identified by Yi et al. [75]. Mir-203, by its p63 target, has an important role in regulating the proliferation of undifferentiated basal cells and repress "stemness" of epithelial cells.

To identify new targets of HR-HPV E6 and E7 oncoproteins, Wang et al. [76] established an in vitro study based on miR-array and miR-seq exploratory analysis, followed by data validation on human samples. They identified in cell culture a set of 13 statistically significant miRNAs (miR-16, miR-25, miR-92a, miR-83, miRr-106b, miR-210, miR-224, miR-378, miR-22,

miR-24, miR-27a, mR-29a and miR-100) specifically regulated by HR-HPV E6 and E7 oncoproteins. Based on their preliminary data, eight miRNAs including miR-16, miR-22, miR-25, miR-27a, mR-29a, miR-92a, miR-100 and miR-378) were selected for further investigation, to determine which of the two E6 and E7 oncoproteins are their modulators.

HR-HPV E7 oncoprotein had a stronger effect than HR-HPV E6 oncoprotein on the positive regulation of miR-25, miR-378 and miR-16, while both viral oncoproteins regulate a moderate overexpression of miR-92a. The role of mir-25 in cervical cancer is assigned to invasion and metastasis by negatively regulation of RECK gene [77], while miR-16 by its CCNE1 target is involved in modulation of cell cycle progression [78].

Although miR-378 was associated with cervical cancer, its precise role has not yet been specified. Mir-92a promotes cell proliferation and cell migration as well as apoptosis blocking by down-regulation of PTEN [79]. The last four miRNAs investigated (miR-22, miR-27a, miR-29a and miR-100) were down-regulated by both E6 and E7 viral oncoproteins. While miR-22 inhibits tumor growth through a pro-apoptotic effect by targeting ATP citrate lyase [80], down-regulation of miR-27a led to overexpression of B4GALT3 that mediates malignancies of cervical cancer by β1-integrin pathway [81].

Cervical cancer progression is also mediated by the down-regulation of miR-29a resulting in HSP47 overexpression, which contributes to migration and invasion of cervical cancer [82], while down-regulation of miR-100 activates PLK1, increasing cell proliferation [83]. A common target of both E6 and E7 viral oncoproteins is miR-129, with tumor suppressor activity. In cervical cancer, miR-129 leads to up-regulation of CDK6 and therefore to increasing cell proliferation by G1-S cell cycle progression [84]. An important oncomir modulated by either E6 or E7 is miR-155, found to have high expression, and also associated with poor prognosis in cervical cancers [40]. MiR-155 is involved in proliferation of cervical tumor cells by down-regulation of LKB1 tumor suppressor gene [85]. Recent evidence demonstrates that not only HR-HPV E6 and E7 modulate the miRNAs expression in cervical cancer, but also HR-HPV E5 oncoprotein is involved in miRNAs targeting in cervical tumors. In a recent study, Liu et al. [86] demonstrated that E5 viral oncoprotein can target and down-regulate miR-196a, leading to increasing proliferation and cell growth by expression of HOXB8 and reduce apoptosis by modulating the caspase 3/7. In another study, Greco et al. [87] showed that in HaCaT human keratinocytes transfected with HPV 16 E5 (HaCaT-E5), the E5 oncoprotein can down-regulate miR-324-5p and miR203 and can up-regulate miR-146a. While miR-146 is involved in negative regulation of immune response in cervical cancer by targeting IRAK1 and TRAF6, miR-324-5p represents a negative modulator of the oncogenic Hedgehog pathway, contributing by its down-regulation leading to carcinogenesis and progression of cervical cancer. As we presented above, miR-203 is also targeted by E7 viral oncoprotein, being involved in epithelial cell differentiation and tumor progression [75].

4. MiRNAs expression in pre-neoplasic lesions and cervical cancer

Similar to other cancers, miRNA profiling in cervical cancer tissues was a topic intensively studied. Different studies involving different designs, from comparing normal and cancer

tissues, primary and metastatic lesions, samples collected before and after surgery or therapy reported distinct miRNA profiles in cervical cancer.

There were studies, which used cervical cancer tissue and normal tissue samples from adjacent spots. The normal tissues were harvested from spots located 2–5 cm beyond the boundary of the tumor of the same individual or from healthy individuals. Therefore, the cervical cancer samples and the normal tissue samples were very likely to have similar histological structures. Patients that were enrolled in these studies had not received chemo- or radiotherapy prior to sample collection, which in most cases, occurred during surgery. In this regard, Rao et al. [88] determined miRNA profiling of 26 matched cervical cancer and normal tissue samples collected from 13 patients. MiRNA gene expression of cervical cancer tissues and normal adjacent tissues was assessed using CaptialBio mammalian miRNA array V3.0 (CapitalBio, Beijing, China). Microarray data analysis revealed that 18 miRNAs were significantly up-regulated and 19 miRNAs were significantly down-regulated in cervical cancer tissue when compared to normal tissue (**Table 2**). Significant analysis of microarray (SAM) using false discovery rate (FDR) showed that the expression levels of the microRNAs identified in this study were not dependent on lymph node metastasis (FDR = 0.529), vascular invasion (FDR = 0.371), or pathological differentiation (FDR = 0.163).

Author	Up-regulated miRNAs	Down-regulated miRNAs	Samples/cell lines	Method	Ref.
Rao et al.	miR-7, miR-429, miR-141, miR-142-5p, miR-31, miR-200a, miR-224, miR-20b, miR-18a, miR-200b, miR-93, miR-146b, miR-200c, rno-miR-93, miR-210, miR-20a; PREDICTED_MIR189, rno-miR-31, rno-miR-93	miR-127, rno-miR-140, miR-376a, miR-214, miR-218, miR-1, miR-368, miR-145, miR-100, miR-99a, miR-195, miR-320, miR-152, miR-497, miR-143, miR-99b, miR-10b, rno-miR-10b, mmu-miR-140	13 CC tissues/13 pair normal tissues	Microarray	[88]
Shen et al.	miR-224	–	126 CC/126 pair normal tissues	qRT-PCR	[89]
Lui et al.	miR-21	let-7b, let-7c, miR-23b, miR-196b, miR-143	Six cervical cell lines and 5 normal cervical tissues	Sequencing, Northern Blot	[90]
Han et al.	miR-21	–	30 CC tumor/30 normal tissue	qRT-PCR	[91]
Ding et al.	miR-657, miR-490-5p, miR-323-3p	miR-126, miR-96, miR-144	4 CC, PLN+/6 CC, PLN-	Microarray qRT-PCR	[92]
Cheung et al.	miR-518a, miR-34b, miR-34c, miR-20b, miR-338, miR-9, miR-512-5p, miR-424, miR-345, miR-10a	miR-193b, miR-203	24 CIN/9 healthy controls; validation 24 CIN; cross validation 51 CC	qRT-PCR	[93]

Author	Up-regulated miRNAs	Down-regulated miRNAs	Samples/cell lines	Method	Ref.
Ruiz et al.	miR-196a, miR-18b, miR-183, miR-500, miR-18a, miR-25, miR-182, miR-20b, miR-106a, miR-20a	miR-125b, miR-10b, miR-143, miR-337-5p, miR-199a-5p, miR-199b-3p, miR-127-3p, miR-214, miR-379, miR-145	4 CC/4 healthy controls and 12 tumoral cervical cell lines	Microarray	[94]
Pereira et al.	miR-148a, miR-302b, miR-10a, miR-196a, miR-132	miR-26a, miR-29a, miR-99a, miR-143, miR-145, miR-199a miR-203, miR-513	19 normal tissue, 4 squamous CC, 5 HSIL, 9 LSIL	Microarray, qRT-PCR	[95]
Li et al.	miR-155, miR-92a	mir-29a, miR-375, miR-195, mir-99a	51 CC, 51 CIN2/3, 21HR-HPV infected normal cervix, 49 normal specimens	Microarray, qRT-PCR	[96]

Abreviations: CC, cervical cancer; CIN, cervical intraepithelial neoplasia; HSIL, high-grade squamous intraepithelial lesion; LSIL, low-grade squamous intraepithelial lesion.

Table 2. MiRNAs expression in cervical cancer/CIN compared with normal tissues.

Another comparative study on cervical cancer and normal tissue was performed by Shen's group [89]. The qRT-PCR results showed a higher expression of miR-224. Moreover, miR-224 was significantly higher in advanced cervical cancers compared with early cervical cancers ($p = 0.02$) as well as in lymph node of metastatic-positive patients compared with lymph node metastatic-negative patients positive ($p = 0.008$), suggesting its role in cervical cancer progression. Furthermore, miR-224 was associated with less differentiated tumors ($p = 0.03$) and vascular invasion ($p = 0.01$), being proposed as an independent prognostic marker of cervical cancer.

Lui et al. [90] adopted a sequencing approach to identify new miRNAs in six human cervical cancer cell lines when compared with five normal cervical tissues. Their results revealed six miRNAs statistically significantly expressed in cervical tumor cell lines compared to normal cervix (**Table 2**). Five out from these miRNAs, such as let-7b, let-7c, miR-23b, miR-196b and miR-143, were down-regulated, while miR-21 was up-regulated in cervical tumor cells. MiR-21 and miR-143 were cross-validated in a new set of 29 pairs of cervical cancers and their matched normal tissues. Both these miRNAs have confirmed higher, respectively, lower expressions when compared to normal cervical tissue, qualifying them as markers for cervical cancer.

Another approach was to assess single miRNAs in cervical cancer tissue samples as biomarkers for diagnosis, and prognostic. In line with this view, Han et al. [91] compared the expression levels of miR-21 in 30 pairs of cervical cancer and normal tissue samples. Cervical tumor samples and normal tissue were collected from the same patients. The normal tissues were harvested beyond a 5 cm borderline from the tumor, in order to ensure the structure similarity between the tissue samples. MiR-21 quantification by qRT-PCR showed a higher expression level in cervical cancer tissues than in the normal ones with an average fold change of 4.02 and

p value <0.05. Moreover, miR-21 up-regulation was correlated with HPV16 infection by a relative expression level of 2.37 in HPV16 positive cases and only 1.94 in HPV16 negative cases. MiR-21 activity was also correlated with clinicopathological parameters including depth of invasion $p = 0.031$ and lymph node metastasis $p = 0.015$.

Ding et al. [92] reported a miR-microarray analysis where they identified a specific miRNA profile for metastatic cancers (PLN-positive) compared with non-metastatic cases (PLN-negative). Thirty-nine miRNAs with FC > 4 were included in this molecular signature; 22 miRNAs were significantly up-regulated, and 17 miRNAs were significantly down-regulated in the PLN-positive group when compared with PLN-negative group (**Table 2**). Six of these miRNAs including miR-126, miR-96, miR-144, miR-657, miR-490-5p and miR-323-3p for which were identified tumor associated putative target genes, involved in cell proliferation, apoptosis, tumor invasion and metastasis, were validated by qRT-PCR. However, they suggested that their data have to be confirmed on larger studies before making a reliable hypothesis related to these miRNAs.

Another important approach was to identify miRNAs for early diagnosis of cervical cancer. Cheung et al. [93] tried to identify a specific miRNA signature for CIN and to reveal what miRNAs could be involved in cervical carcinogenesis. Twenty-four high-grade cervical intraepithelial carcinoma (CIN 2/3) and nine normal cervical epithelium samples were used in a testing study for miRNA profiling, using qRT-PCR method for screening the expression of 202 target miRNAs. The obtained results, a set of 12 miRNAs (**Table 2**) statistically significantly expressed ($FC \geq 2$, $p < 0.05$) between CIN and normal tissue, were further validated in a validation set of 24 high-grade CIN samples. Because permutation analysis returned 0.0% FDR for this set of miRNAs, a new analysis on an independent cohort of 51 squamous cell carcinomas was proposed, to reveal the miRNAs associated with cervical carcinogenesis. The fold change values of up-regulated miRNAs between CIN patients and normal subjects were ranging between 2.07 and 3.53 and between 2.67 and 2.81 for down-regulated ones. In the case of squamous cell carcinoma (SCC), seven miRNAs of 12 validated for CIN samples were identified as important for cancer progression. From these, miR-9, miR-20b, miR-345, miR-338, miR-518a and miR-512-5p were up-regulated, and miR-203 was down-regulated in cervical cancers versus normal epithelium control. Consequently, a specific miRNA signature that can distinguish CIN and cervical tumors from normal cervical epithelium was proposed. In the same line, Ruiz's group [94] used a microarray approach to identify differentially expressed miRNAs in cervical cancer cell lines as well as in cervical cancer and normal tissues. They studied miRNA expression on 12 cervical cancer cell lines, four cervical cancer tumor tissues and four normal tissue samples. They identified a set of miRNAs with significantly abnormal expression, of which, miR-196a had the highest expression level for cervical cancer tissues ($p = 4.75E{-}04$) and cancer cell lines ($p = 1.32E{-}07$). Up-regulation of miR-196a was confirmed by qRT-PCR analysis in cervical cancer tissues, low-grade squamous intraepithelial lesion (LSIL) and high-grade squamous intraepithelial lesion (HSIL) and cervical cancer cell lines. A highest up-regulation of miR-196a was observed in cervical cancer cell lines and a slight up-regulation in cervical cancer tissues. Other 19 miRNAs were found statistically significantly expressed in microarray analysis; nine of them were up-regulated, while the rest of 10 were down-regulated (**Table 2**).

Pereira et al. [95] profiled miRNA expression in a heterogenous set of 25 cervical tissues including 19 normal cervical tissue, four squamous cervical carcinoma, five high-grade squamous

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intraepithelial lesions (HSILs) and nine low-grade squamous intraepithelial lesions (LSILs). MiRNA profiling revealed a high variability of their expression among normal samples and a lack of clear separation of normal, pre-neoplasic and cervical cancer samples. One of the reasons claimed by authors was related to the fact that a part of normal cervical epitheliums harvested from area adjacent to the tumors were HPV infected. However, they identified two sets of statistically up-regulated and down-regulated miRNAs between precancerous and cancerous samples when compared with normal cervical tissues. Eight down-regulated miRNAs including miR-26a, miR-29a, miR-99a, miR-143, miR-145, miR-199a miR-203, miR-513 and five overexpressed miRNAs including miR-148a, miR-302b, miR-10a, miR-196a and miR-132 were associated with transition from normal cervix to both precancerous stages (atypical dysplasia) and cancer.

Ly et al. [96] profiled miRNA-microarray expression in six HPV16-positive cervical cancers, six HPV16-positive intraepithelial neopasia (CIN2/3) and six normal cervix tissues. They identified a set of 31 statistically significant miRNAs that distinguish cervical tumors from precancerous and normal samples. Six miRNAs (miR-155, miR-92a mir-29a, miR-375, miR-195 and miR-99a) were further successfully validated in a panel of 91 samples including 24 HPV16-positive cervical cancers, 24 HPV 16-positive CIN2/3 and 43 normal cervical tissues. Considering that cervical cells can be infected by different HR-HPV not only by HPV16, they also investigated and validated the expression of these six miRNAs in a larger group of 45 HR-HPV + cervical cancer, 45 HR-HPV + CIN2/3 and 43 normal cervical tissues.

5. MiRNAs expression in serum of patients with pre-neoplasic lesions and cervical cancer

Due to their high stability in easy accessible body fluids and their proved differential expression between cervical cancer and normal tissue, miRNAs have arisen as potential biomarkers for diagnosis and prediction of this disease.

In this regards, Jia et al. [97] performed a sequencing experiment to identify specific miRNAs in the serum of cervical cancer patients and normal subjects. They identified a set of 12 miRNAs differentially expressed between two groups. Cross-validation on a second set of 103 patients and 74 controls confirmed the significant up-regulation of five microRNAs (miR-21, mIR-29a, miR-200a, miR-25 and miR-486-5p) in cervical cancer when compared to the negative controls. MiR-21, one of the highest up-regulated microRNAs, was previously reported as overexpressed in cervical tumors, being an oncomir associated with poor prognosis [90, 91]. The diagnostic value of selected miRNAs was assessed by ROC analysis, showing that with each subsequent miR addition, the biomarker panel gained a higher sensitivity and specificity in discriminating cervical cancer from controls. With an AUC value of 0.908 (95% CI: 0.868–0.948), these results suggest the potential of the five microRNAs as noninvasive markers in cervical cancer.

Serum miRNAs could become valuable prognostic biomarkers (**Table 3**), aiding clinicians in treatment decision, to distinguish between cases that could be treated by using the classic therapy and the ones that might need a different approach because of a higher risk of developing lymph node metastasis.

Author	Serum microRNAs	Cases	Technology	Validation/cross validation	Test accuracy	Ref.
Jia et al.	↑miR-21 ↑miR-29a ↑miR-200a ↑miR-25 ↑miR-486-5p	213 CC patients 158 controls	Solexa sequencing qRT-PCR	103 patients 74 controls	CC vs. healthy AUC 0.908; 95% CI: 0.868-0.948; sensitivity: 81.0%, specificity 88.6%	[97]
Chen et al.	↑miR-205	60 CC patients60 controls	qRT-PCR	Same subjects 60 CC60 controls	LN+ vs LN− 0.694; sensitivity: 71.1%, specificity: 72.7% moderately differentiated vs poorly differetiated 0.717; sensitivity: 76.5%, a specificity: 73.1%	[98]
Zhao et al.	↑miR-1246 ↑miR-20a ↑miR-2392 ↑miR-3147 ↑miR-3162-5p ↑miR-4484	40 CC LN+ 40 CC LN− 20 controls	Microarray qRT-PCR	–	LN+ vs LN− AUC 0.932; 95% CI: 0.884-0.980, sensitivity 0.856, specificity 0.850	[99]
Ma et al.	↑miR-20a ↑miR-203	40 CC LN+ 40 CC LN− 20 controls	qRT-PCR	–	miR-20a LN+ vs LN− AUC 0.734; 95% CI: 1.137–2.118, sensitivity: 75%, specificity: 72.5% miR-203 LN+ vsLN− AUC 0.658; sensitivity: 65%, specificity: 62.5%	[100]
Yu et al.	↓miR-218	90 CC 50 controls	qRT-PCR	–	cc vs normal expression levels 1.000 vs 0.392 LN+ vs LN− expression levels 0.235 vs 0.468	[101]
Zhang et al.	↑miR-16-2 ↑miR-497 ↑miR-2861 ↓miR-195	184 CC patients 186 CIN 193 controls	qRT-PCR	85 CC 91 CIN 93 controls	CC vs healthy AUC 0.849; 95% CI: 0.813–0.886; sensitivity: 73.1%, specificity: 88.4% CC vs CIN AUC 0.829; 95% CI: 0.794–0.865; sensitivity: 71.4%, specificity: 67.2% cin vs healthy AUC 0.734; 95% CI, 0.683-0.784; sensitivity: 62.6%, specificity: 88.9%	[102]

Author	Serum microRNAs	Cases	Technology	Validation/cross validation	Test accuracy	Ref.
Nagamitsu et al.	↑miR-1920	70 CC patients 55 CIN 31 controls	Microarray qRT-PCR	45 CC patients 55 CIN 31 controls	CC vs healthy0.7957; 95% CI: 0.6937-0.8977; sensitivity: 90.3%, specificity: 62.2%,	[103]
Liu et al.	↑miR-196a	105 CC patients 86 CIN 50 controls	qRT-PCR	–	–	[104]

Abbreviations: CC, cervical cancer; CIN, cervical intraepithelial neoplasia; LN+, lymph node positive; LN−, lymph node negative; AUC, area under curve.

Table 3. MiRNAs expression in serum serum of patients with pre-neoplasic lesions and cervical cancer.

Using microarrays screening tools, Chen et al. [98] identified 89 miRNAs that had different expressions both in serum and in tissue samples of positive lymph node metastasis cervical cancer patients when compared to healthy controls. For further analysis, they restricted the number of microRNAs of interest, by choosing the microRNAs with the highest expression levels that are presented in both serum and tissue samples. They identified a six-miRNA panel (miR-1246, miR-20a, miR-2392, miR-3147, miR-3162-5p and miR-4484) with a diagnostic value for lymph node metastasis in cervical cancer patients, having a sensitivity of 0.856 and a specificity of 0.850.

Consistent cervical cancer serum levels of miR-20a were reported by Zhao et al. [99]. miR-20a being significantly up-regulated in serum samples collected from females with cervical cancer stages I–IIA, when compared to healthy donors. Moreover, higher differences were reported in serum samples from patients with positive lymph node metastasis (LN+) than in the ones without lymph node metastasis (LN−). They showed that miR-20a expression level could be used as a lymph node metastasis diagnosis tool, distinguishing LN+ from LN− patients with a moderate accuracy (AUC = 0.734) the test having a sensitivity of 75%, and a specificity of 72.5%. They also measured the expression level of miR-203 in serum samples of cervical cancer patients and healthy donors. Although it was observed a significant up-regulation of serum miR-203 in cervical cancer patients, miR-203 showed a low accuracy for distinguishing LN+ from LN− patients.

Ma's group [100] measured miR-205 level in blood and tissue samples of cervical cancer patients and paired healthy controls. They found a fivefold up-regulation of miR-205 in serum from cervical cancer patients, comparing to normal, and threefold up-regulation in tissue samples. Moreover, a higher expression level of miR-205 was correlated with an advanced cancer stage, a worse overall survival rate, and metastasis. As a prognosis biomarker, miR-205 expression level could differentiate between metastatic and non-metastatic cases with a sensitivity of 71.1% and specificity of 72.7%, and distinguish poorly differentiated tumors from that moderately differentiated ones with a sensitivity of 76.5% and a specificity of 73.1%.

Yu's group [101] investigated if serum miR-218 can be associated with cervical cancer. Accordingly, they identified reduced level of miR-218 in serum samples of 90 cervical cancer patients (expression level 0.392 ± 0.021) when compared to 50 healthy controls (expression level 1.000 ± 0.062). Quantitative RT-PCR showed even more decreasing levels of miR-218 in cervical cancer patients with advanced stages (0.128 ± 0.016) compared to earlier ones (0.425 ± 0.033). Moreover, they observed the same tendency for those patients presenting lymph node metastasis (0.235 ± 0.020) compared to non-metastatic cases (0.468 ± 0.018), and it suggested that miR-218 could represent a marker for prognosis. An interesting observation was that miR-218 expression level was lower for adenocarcinoma (0.216 ± 0.016) than for squamous cell cervical cancer (0.399 ± 0.019).

Timely biomarker detection would be of a great importance, considering the increased chances of survival and lower recurrence rates at early stages. Also, a blood-based biomarker that has a comparable or higher sensitivity and specificity than Pap test would be of a great benefit especially in the areas where high-quality medical care and screening are not available.

In line with this view, Zhang et al. [102] performed a qRT-PCR-based screening study evaluating the performance of circulating miRNAs as diagnostic biomarkers in the serum of patients with cervical cancer and patients with CIN. The study followed a multi-step approach: screening, testing and validation. For validation, they used randomly selected cervical cancer patients, CIN subjects and healthy individuals as negative controls. MirRNAs identified in the first cohort of patients in the screening step were tested in a second cohort and finally cross-validated on a third group of patients. A panel of four miRNAs (miR-16-2, miR-497, miR-2861, miR-195) with significantly aberrant expression was selected to discriminate cervical cancer from healthy subjects (AUC: 0.849; 95% CI: 0.813–0.886; sensitivity: 73.1%, specificity: 88.4%) and CIN (AUC: 0.829; 95% CI: 0.794–0.865; sensitivity: 71.4%, specificity: 67.2%). This 4-miRNA signature also distinguishes CIN from healthy subjects (AUC: 0.734; 95% CI: 0.683–0.784; sensitivity: 62.6%, specificity: 88.9%).

Following a similar workflow, Nagamitsu et al. [103] reported a recent microarray study that identified a panel of four up-regulated miRNAs (miR-485-5p, miR-1246, miR-1275, miR-1290) in the serum of cervical cancer patients. Mir-1290 was particularly up-regulated, so the group further investigated its expression level in the sera of CIN individuals and cervical cancer patients at different stages. The results showed that miR-1290 levels could differentiate cervical cancer patients from healthy subjects with an AUC value of 0.7957 (95% CI: 0.6937–0.8977; sensitivity: 90.3%; specificity: 62.2%). Also, miR-1290 up-regulation was correlated with cancer progression, with lowest expression in the control group and gradually increased expression from CIN2 to locally advanced cervical cancers, showing biomarker potential for cervical cancer diagnosis in early stages.

In another study, Liu's group [104] evaluated the expression of serum miR-196a in 105 cervical cancer patients, 85 CIN individuals and 50 healthy subjects. Their data revealed a significantly higher relative expression level of miR-196a in cervical cancer patients than in both healthy and CIN individuals, and higher expression in CIN than in healthy subjects. The study aimed to identify the clinical significance of serum miR-196a in cervical cancer and CIN patients. Their results showed an association between miR-196a and clinical parameters of

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cervical cancer patients, such as tumor size, lymph node (LN) metastasis, FIGO stage and cancer grade, but no association with HPV infection, age, or cell type. Also, they observed different levels of miR-196a expression depending on CIN grade, with a lower expression in CINI and CINII and a significantly higher expression level in CINIII. Moreover, overall survival of cervical cancer patients was negatively correlated with higher miR-196a expression. These results suggest that serum miR-196a could represent a reliable biomarker for early diagnosis and prognosis of cervical cancer.

6. MiRNAs expression in exfoliated cells of cervix

In addition to cervical cancer screening tests by using serum-based diagnosis biomarkers, in a recent study, Tian et al. [105] have approached a new screening method for early detection of cervical cancer. This group collected residual exfoliated cell samples from HPV-positive subjects that underwent Pap test. Samples were triaged by Pap test and colposcopy in six groups, depending on the lesions grade, ranging from normal to ASCUS, LSIL, ASC-H, HSIL, and finally CC. Sampled cells from 1021 HPV-positive women were used for measuring the expression levels of several microRNAs, which were previously detected as abnormally regulated in cervical cancer (miR-424/miR-375/miR-34a/miR-218/miR-92a/miR-93). Detailed cytological examination classified the subjects by CIN grade. Quantitative RT-PCR analysis showed that relative expression levels of miR-218, miR-34a, miR-424 and miR-375 were significantly lower in more advanced CIN grades (CIN2+ and CIN3+) than in the incipient ones (CIN-1 and CIN-2), suggesting that those microRNAs could be used as candidate biomarkers in cervical cancer screening. For determining the diagnosis value, the group performed ROC analysis for miR-424, miR-375, miR-34a and miR-218 and compared them to the ROC curve for Pap test. Both miR-424 and miR-375 detection in cervical exfoliated cells had a greater AUC (0.828 for miR-424 and 0.760 for miR-375) and a higher sensitivity (82.3% for miR-424 and 80.9% for miR-375, respectively) and specificity (70% for miR-424 and 71.2% for miR-375, respectively) in identifying high-grade CIN (CIN3+) than Pap test (AUC: 0.699; sensitivity 69.8%, specificity 70%). A lower performance was registered for miR-34 and miR-218. In conclusion, this study opens future challenges in non-invasive diagnosis procedures. MicroRNA detection in cervical exfoliated cells could be an effective option for triage of HPV-positive women and incipient cancer detection, especially for those areas where well-trained cytologists are lacking.

7. MiRNAs modulate treatment response in cervical cancer

The molecular mechanisms of resistance to radiation in cervical cancer are not well understood. The discovery of miRNAs opened the opportunity to research for new molecules that mediate treatment response, with potential to be developed into new targeting strategies and/or prediction algorithms in cervical cancer treatment. However, the current knowledge is still limited, and there are just a few reports describing the role of miRNAs as modulators of treatment response in cervical cancer.

In a recent study, Song et al. [106] investigated the role of miR-375 in radiotherapy resistance in HR-HPV-positive cervical cancers. They evaluated tissue and serum expression of miR-375 in both cervical cancer patients ($n = 22$) and healthy control subjects ($n = 20$) highlighting significantly down-regulated expression also in tissue ($p < 0.001$) and serum ($p < 0.001$) of cancer patients when compared to normal individuals. The authors also assessed the miR-375 expression level in serum and tissue samples at 6 months after completion of the radiotherapy treatment and correlated its expression with clinical and histological data. Their data showed low expression of miR-375 in tissue ($p < 0.001$) and serum ($p < 0.001$) of radioresistant patients when compared with radiosensitive patients. Further investigations have proved that miR-375 may induce radioresistance in cervical cancer cells by targeting UBE3A and BIRC5 (surviving) and regulate apoptosis through the 53 pathway.

Ye et al. [107] reported decreased miR-145 expression in tumors and cervical cell lines when compared with cervical non-tumor tissue and normal cell lines. Five potential targets of miR-145 including HLTF, CUT2, BCR, BUFIP2 and ZCH11A were identified by in silico analysis, but only HLTFs were further investigated due to its mRNA highest expression (1.96-fold). The expression of HLTF was higher in cervical tumors than in normal tissues and significantly negatively correlated with miR-145 in cervical cancers. Moreover, performing luciferase reporting assay, this group has proved that HTLF is a specific target of miR-145. HTLF influences the outcome of radiotherapy in cervical tumors playing an important role in chromatin remodeling and enhancing DNA damage repair capacity of cervical tumor cells, therefore miR-145 down-regulation could lead to radioresistance in cervical cancers.

Ke et al. [108] investigated the role of miR-181a in radiotherapy resistance of cervical cancers. MiRNA microarray profiling in seven radio-resistant cervical samples and 11 radio-sensitive cervical tumors led to the identification of eight miRNAs (miR-181a, miR-21, miR-30, miR-23a, miR-16-2, miR-378, miR-18a and miR-221) significantly expressed between groups. Further investigation showed that miR-181a has no effect on cell proliferation but lead to inhibition of apoptosis by targeting the PRKCD gene and decreasing the caspase 3/7 activity. The authors suggested that miR-181a protects cervical tumor cells from radiation-inducing death by inhibiting of apoptosis.

8. Conclusion

Data presented above provide evidences that miRNAs modulated by HR-HPV E5, E6 and E7 oncoproteins could be investigated as potential biomarkers for early diagnosis of pre-neoplasic and neoplasic lesions of the cervix. Moreover, miRNAs identified in serum and exfoliated cervical cells could be taken into account as valuable minimal invasive markers for monitoring cervical cancer progression and its treatment response.

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