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Human Papillomavirus and non-coding RNAs: from basics to diagnostic

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ABSTRACT

Human Papillomavirus (HPV) is the etiological agent of cervical cancer. Like other oncogenic viruses HPV encode oncoproteins and the role of HPV oncogenes in cellular immortalization and transformation has been extensively investigated. HPV E6 and E7 proteins disable tumor suppressors p53 and Rb and up-regulate telomerase, fundamental changes for cell immortalization. Another important step in the induction of cancer by oncogenic viruses seems to be the specific interaction of some viral proteins with mitochondria, an organelle that has been implicated for decades in carcinogenesis. The identification of non-coding RNAs (ncRNAs) has opened new research areas and there has been an explosive increase of reports showing that the expression of these RNAs is deregulated in many different human diseases, including cancer. The ncRNAs can be classified into two groups based on their length: small transcripts (20–200 nucleotides), such as microRNAs (miRs), piwi-interacting RNAs and long transcripts (higher than 200 nucleotides). While the function of small ncRNAs has been well documented, the role of long ncRNAs is still not completely understood. MicroRNAs are small 21–22 nt non-protein-coding RNAs that regulate mRNA translation and decay. It has become evident that miRs plays a pivotal role in the development of human cancer. Some miRs have been characterized as tumor suppressors and others as oncogenic (onco-miRs). MiR patterns are tissue specific, and the expression profile could allow to distinguish carcinomas from normal cells. Moreover, miR expression profiles of cervix, head and neck cancers have been carried out in different studies associated to HPV infection. In this scenario, the HPV E5, E6 and E7 proteins modulate the expression of several cellular miRs. Deregulation of microRNAs expression might be used to identify cancer progression and also as potential target for therapy against HPV infection and cervical cancer development. The differential expression of a family of long non-coding mitochondrial RNAs (ncmtRNAs) in response to HPV infection has been recently reported. The expression profile of these transcripts allows distinguishing between normal, pre-tumoral and cancer cells. One of these transcripts, SncmtRNA-1, has been characterized as a regulator of cell cycle progression while two others, ASncmtRNA-1 and 2, has been suggested to act as

tumor suppressors. HPV E2, E6 and E7 modulate the expression of this family of mitochondrial long ncRNAs. Further evidence suggests that other viruses such a Hepatitis B virus, Human T-cell lymphotropic Virus type 1 and Epstein Barr virus can also modulate the expression of these long ncRNAs in human cells. In this chapter we will discuss how the differential expression of both, microRNAs and long ncRNAs, in response to HPV infection, might serve as early biomarkers for progression of cervical dysplasia in PAP smears and biopsies allowing the detection of precursor lesion of cervical cancer.

Non-coding RNAs and cancer

The central dogma of molecular biology indicates that the functional importance of genetic information lies in proteins encoded within it [1]. According to this concept, cellular functions would be defined by the presence or absence of certain proteins and RNAs would be mere intermediates in the process of gene expression [2]. Nevertheless, after the publication of the human genome in 2001 and a profound analysis of open reading frames (ORFs) of different organisms, it has been concluded that more than 90% of the genome are non-protein-coding sequences [3, 4]. Within the non-coding sequences, elements non-translated into proteins, we can find several RNAs with structural or regulatory functions [5-7]. Within the group of structural RNAs we can mentione for example transfer RNAs (tRNAs), ribosomal RNAs (rRNAs) and small nuclear RNAs (snuRNAs) [5-7]. On the other hand, regulatory RNAs, or non-coding RNAs (ncRNAs) mainly modulate gene expression and can be classified according to their sizes, as small non-coding RNAs (sncRNAs) [8, 9] and long non-coding RNAs (lncRNAs) [6, 10, 11]. Small ncRNAs can be subdivided in two groups: microRNAs (miRs) with an average size of 22-23 nt and piwi-interacting RNAs (piRNA) with an estimated size of 26-31 nt [12-14]. Conversely, long ncRNAs correspond to molecules with a size over 200 nt long [15, 16]. There is a group of lncRNAs that correspond to antisense RNAs, which are complementary to coding transcripts, and thereby induce their degradation or negatively regulate their translation [17-19]. Regarding the synthesis of ncRNAs, the participation of the enzymes RNA pol II and RNA pol III have been described [6, 20-22]. Moreover, there are reports indicating that these classes of transcripts are processed including splicing, polyadenilation and addition of 5'cap [7, 23]. Giving the increasing importance that ncRNAs and its relationship to cancer during the past years (Figure 1), this chapter will be focused on the state of the art on this topic, with emphasis on the role of miRs and lncRNAs in cancer development associated to the HPV.
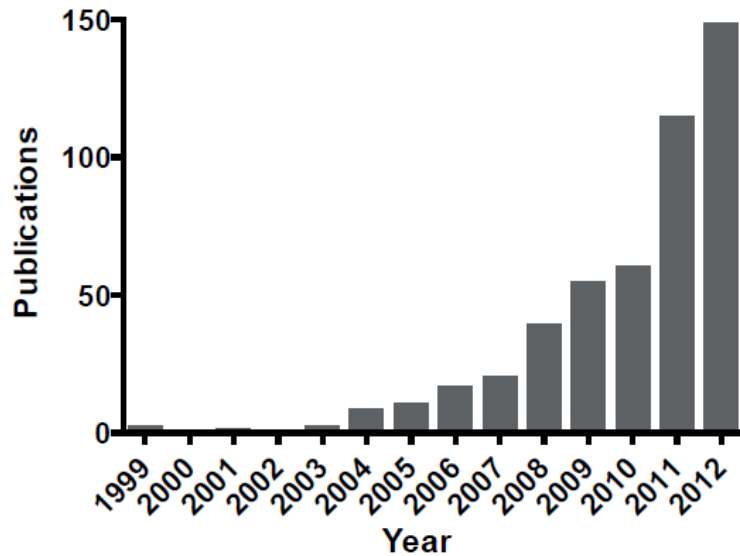
Figure 1.- Number of publications related to non-coding RNAs associated to cancer. A search on the Pubmed database from the National Center for Biotechnology of Information (NCBI) was performed using the terms "ncRNA" or "non-coding RNA" or "noncoding RNA" or "non-protein-coding RNA" AND cancer, with limit date December 31, 2012. Data was corroborated performing the same search on the Gopubmed database. The data was plotted as number of publications per year, starting from 1999.

MicroRNAs and their role in cancer

MiRs are small non-coding RNAs, which regulate gene expression at a post-transcriptional level [12]. The first findings that indicate the abundance of miRs genes came from sequencing small RNAs from mammals, flies, and worms [13, 14]. Hundreds of mammalian miRs have now been identified by Sanger sequencing of cloned small RNA-derived cDNAs [12-15, 17, 24, 25]. Some miR, however, are expressed only in a limited number of cells or at a limited time during development, arising a problem for their detection. MiRs are involved in a series of cellular processes such as differentiation, proliferation and apoptosis [13, 14]. Interestingly, the expression profile of these molecules is deregulated in a large number of pathologies, such as psoriasis [26], cardiovascular disease [27, 28] and also in different types of cancer [29-32].

The genesis of these molecules involves RNA pol II that transcribes a primary miR (pri-miR), which contains one or more segments that fold into an imperfect hairpin (Figure 2). For canonical metazoan miRs, the RNase III enzyme Drosha together with its partner, the RNA-binding protein DGCR8, recognize the hairpin and subsequently Drosha cleaves both strands 11 bp from the base of the stem, leaving a 5' phosphate and 2-nt 3' overhang [33, 34]. The released precursor miR (pre-miR) hairpin is then exported to the cytoplasm by Exportin-5 [35, 36], where the RNase III enzyme Dicer cleaves off the loop of the pre-miR, ~22 nt from the Drosha cut again leaving a 5' monophosphate and 2-nt 3' overhang [37, 38]. The resulting miR:miR* duplex, comprising ~22-bp is associated with the Argonaute (Ago) proteins in such a manner that the miR strand is usually the one that is stably incorporated, while the miR* strand dissociates and is subsequently degraded. In addition to canonical miRs, some miRs mature through pathways that bypass Drosha/DGCR8 recognition and cleavage [39]. Finally, partial complementarity of the miR and its mRNA target, usually at the 3' UTR, induce translational repression and in some cases, degradation of the transcript [29, 30].
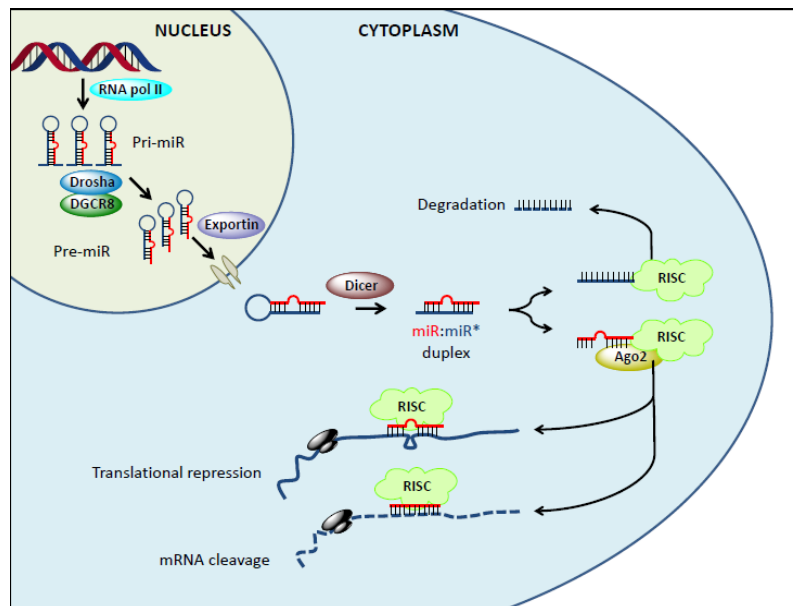

Figure 2.- Biogenesis and functions of cellular microRNAs. In the nucleus, RNA pol II transcribes a pri-miR, which folds as an imperfect hairpin. Drosha, together with its partner DGCR8, recognizes the hairpin and subsequently cleaves both strands from the base of the stem leaving a 5' phosphate and 2-nt 3' overhang in a structure named pre-miR, which is exported to the cytoplasm by

Exportin-5. There, Dicer cleaves off the loop of the pre-miR, 22 nt from the Drosha cut, again leaving a 5' monophosphate and 2-nt 3' overhang. The resulting miR:miR* duplex, comprised about 22-nt taken from each arm of the original hairpin, associates with the Ago protein in such a manner that the miR strand is usually the one that is stably incorporated, while the miR* strand dissociates and is subsequently degraded. The mature miR will then associate with other proteins of the RNA Induced Silencing Complex (RISC), which directs it to a target mRNA. Finally the perfect base complementarity of the miR and its target, usually the 3' UTR of an mRNA, induce the degradation of the transcript, while partial complementarity induces translational repression.

Since the first reports describing the existence of miRs in mammals [39, 40], there has also been an increasing number of publications showing a deregulation of a great number of miRs associated to human cancer [29, 30]. Some of the different types of cancer associated to deregulation of miRs are non-melanoma skin cancer [41], thyroid [42], breast [43], ovarian [44], glioblastome [45], lung [47], gastric [48, 49], melanoma [50], kidney [51] and cervical cancer [52, 53]. Some of the best-characterized miRs involved in cancer are summarized in table 1. In all these types of cancers there appears to be a common feature: deregulation of miRs that participate in tumorigenesis [54, 55], apoptosis [56, 57] and in metastasis capacity [58, 59]. Interestingly, up or down-regulation of miR expression associated to malignant processes now is being used to improve diagnostic and prognosis of different types of cancer. For examples: miR-200, has being used in ovarian cancer diagnostics [44], miR-34c, miR-375, miR-17-5p in lung cancer [47, 60, 61], miR-148b in breast cancer [62], miR-146, miR-371a-3p and miR-372 in thyroid cancer [42, 63]. Additionaly, miR-182 and miR-96 has being studied for their role in ovarian cancer development [64, 65], miR-148a, miR-210, miR-21, miR-10b, miR-8, and let-7 in breast cancer [66, 67], miR-10b, miR-34a, miR-376a, miR-21 and miR-211 in glyoblastoma [68-72]. A number of miRs related to cervical cancer have been analyzed and the great majority of them participates in cancer development or has metastatic potential. Furthermore, miR-21, miR-143, miR-200a, miR-345, miR-9, miR-372, miR-218, miR-133b, miR-19a, miR-19b and miR-203 have been implicated in different aspects of cervical cancer development and most of them are deregulated by one of the HPV oncoproteins and are being used for diagnostic and prognosis [52, 53, 73-75].

Name	Use and/or Function	Associated cancer	References
miR182	Cancer progression	Ovarian	64, 65
miR96	Cancer progression	Ovarian	64, 65
miR371a-3p	Diagnostic/Cancer progression	Thyroid	42, 63
miR19a	Diagnostic/Cancer progression	Adenomas/Cervical	52, 53
miR200a	Cancer progression	Cervical	52, 53
miR146b	Diagnostic/Cancer progression	Thyroid	42, 63
miR210	Diagnostic/Cancer progression	Lung	47
miR183	Diagnostic/Cancer progression	Lung	47
miR200	Diagnostic/Tumor suppressor	Lung/Ovarian	44, 47
miR486-5p	Diagnostic/Cancer progression	Lung	61
miR148b	Diagnostic/Cancer progression	Breast	62
miR17-5p	Diagnostic/Cancer progression	Lung	47
miR34	Diagnostic/Tumor suppressor	Gastric/Lung	48, 60
miR133b	Cancer progression	Cervical	52, 53
let7	Tumor suppressor/ Cell differentiation	Breast	66, 67
miR125b	Cancer progression	Cervical	79, 86
miR21	Diagnostic/Cancer progression	Glioblastoma/Cervical	45, 208
miR143	Diagnostic/Cancer progression	Cervical	52, 53
miR23b	Cancer progression	Cervical	204
miR29	Tumor suppressor	Cervical	80
miR27a	Cancer progression	Gastric	49

Table 1.- Representative microRNAs associated with human cancer

MicroRNAs, which are involved in cell proliferation, such as miR-15a, miR-16, miR-17 and miR-148a are up-regulated in cervical cancer cell lines [53, 76-78]. Oppositely, miR-29, miR-218, miR-125b, miR-145, miR-328, miR-199, miR-574 and miR-455, which suppress cell proliferation, are down-regulated. [76, 79-81] Concerning the ability of papillomaviruses to deregulate the expression of miRs, the oncoproteins E5, E6, E7 and the capsid protein L2 are involved in down or up-regulate of a subset of miRs [82-87]. The functional importance of these processes during viral replication and malignization of cervical cells is discussed in detail in the following sections of this chapter. Currently, worldwide researchers are investigating the ability of oncogenic or not oncogenic viruses to modulate the expression of cellular miRs and the ability to generate their own repertoire of miRs in order to regulate the transcriptional machinery of the infected cell. The consequence is the ability to escape immune vigilance, to maintain long latency periods or to regulate their own transcriptional machinery [88-90].

Long non-coding RNAs and their role in cancer

LncRNAs are considered molecules with a size over 200 nt [16, 91]. Currently more than 3,000 lncRNAs have been described in humans [17-19, 24, 25]. The majority of lncRNAs are transcribed by RNA pol II and then processed as an mRNA precursor, including splicing, polyadenylation and 5′-capping [6, 7, 21, 22, 26]. In addition, has been demonstrated that some types of lncRNAs are transcribed by RNA pol III indicating an over activation of lncRNAs in cancer cells [20, 92, 93]. For example, BC200, a cytoplasmic lncRNA found in neurons of primate nervous systems and human cancers, but not in non-neuronal organs [92, 93]. Unlike the majority of lncRNAs, BC200 is transcribed by RNA pol III and shares unique homology with human Alu elements [92, 93]. Similarly, the lncRNA HULC also shares homology with mobile DNA, containing a long terminal repeat (LTR) retroelement [94, 95]. The BC200 lncRNA has been characterized as a negative regulator of eIF4A-dependent translation initiation [96]. Due to the fact that many whole transcriptome sequencing methods were developed to enrich poly(A) purified transcripts, RNA pol III transcripts may have been excluded from previous analysis. This suggests that other, as yet unidentified RNA pol III depending lncRNAs, which are over-expressed in cancer might be participating in tumorigenesis [97].

Similar to miRs, many of the lncRNAs are expressed in different stages of development or are tissue-specific [98-102]. The majority of them is involved in cellular differentiation and also has been described in human pathologies such as cancer, participating in pathogenesis and maintenance of the tumorigenic potential of transformed cells [103, 104]. In the past few years a large number of lncRNAs have been identified, and the specific function of only some of them are known [6]. The expression profiles of well characterized lncRNAs such as HOTAIR [22, 105], ANRIL [97, 106-109], MALAT-1 [97, 109-114], AK023948 [115], PANDA [116], SRA-1[117], PCAT [118], MEG3 [97, 109], GAS5 [97, 109, 119, 120], H19 [121, 122], UCA-1 [123], P15AS [124], HULC [94] and CDRU [125] have been associated to different types of cancer and are summarized in table 2.

Name	Function	Associated cancer	References
PTENP1	Tumor suppressor	Prostatic/Lung	102, 105
HULC	Metastasis	Liver/Colorectal	94
HOTAIR	Metastasis	Breast/Colorectal/Pancreatic/Liver	22, 105
ANRIL	Cancer progression	Prostatic/Glioma/Leukemia/Breast	97, 106-109
MALAT1	Metastasis/Cancer progression	Lung/Colon/Prostatic/Cervix/Liver	97, 109-114
AKO23948	Tumor suppressor	Thyroid	115
PANDA	Resistence to chemotherapy	Breast	116
SRA1	Metastasis	Breast	117
PCAT1	Cell proliferation	Prostatic	118
GAS5	Tumor suppressor	Prostatic/Melanoma/Lymphoma/Breast	97, 109, 119, 120
H19	Cell proliferation	Breast/Colon	121, 122
UCA1	Cell proliferation/Cancer progression	Bladder	123
P15AS	Cell proliferation	Leukemia	124
CUDR	Resistence to chemotherapy/Cancer progression	Squamous carcinome	125
MEG3	Tumor suppressor	Brain/Bladder/Liver	97, 109
SncmtRNA	Cell proliferation	Leukemia/Cervical/Breast/Prostatic/liver/Ovarian	126, 213
ASncmtRNA	Tumor suppressor	Leukemia/Cervical/Breast/Prostatic/liver/Ovarian	126, 213

Table 2.- Some long non-coding RNAs associated with human cancer

An increasing number of lncRNAs correspond to antisense ncRNAs with perfect complementarity with their respective coding or sense counterparts. The formation of a double-stranded RNA can negatively regulate gene expression by inducing degradation of the mRNA [100, 101]. This antisense lncRNAs can act in cis, when transcribed from the opposite strand of the same genomic loci, or in trans, when transcribed from a distant one [100-102]. Many tumor suppressor genes express antisense ncRNAs, such as p15INK4B/ ANRIL and AIR/HOTAIR [98, 99]. Another interesting example is the tumor suppressor PTEN (Phosphatase and Tensin) and PTENP1 (PTEN pseudogene 1). PTENP1 is a lncRNA transcribed from a pseudogene with a high complementarity to PTEN. This tumor suppressor is involved in cell cycle regulation, preventing cells cell division and is one of the targets of the oncomiR miR-21 [102]. PTENP1 acts like a "sponge" that trao or absorve miR-21 diminishing the effect of oncomiRs on PTEN [102]. PTENP1 is down-regulated in prostatic and lung cancer [102, 105]. A new family of sense and antisense non-coding transcripts derived from mitochondria has been described. Within this family, Sense non-coding mitochondrial RNA (SncmtRNA) is associated to cellular proliferation and is found over-expressed in leukemia, breast, prostatic, colon, hepatocarcinoma, ovarian, brain, kidney cancer, lymphoma and cervical cancer [126]. Antisense non-coding mitochondrial RNA (ASncmtRNA) has a tumor suppressor function and has been found repressed in leukemia, breast, prostatic, colon, hepatocarcinoma, ovary, brain, kidney cancer, lymphoma and cervical

cancer [126], representing the most universally suppressed molecule across a high number of different types of cancer. The role of these molecules in cervical cancer is discussed in detail on this chapter.

Concomitantly with the basic biology and the understanding of the functions of many of the molecules described here, researchers have focused their efforts in evaluating the diagnostic potential of the expression profiles of non-coding transcripts. Thus, expression patterns of MALAT-1, HOTAIR y HULC have been used to evaluate the prognosis of patients with lung cancer [102, 105], and hepatocarcinoma [94, 102, 105]. The expression patterns of the SncmtRNA and ASncmtRNAs are also being evaluated, mainly in progression of cervical cancer [126], as well as in other pathologies of the urinary tract [127]. Probably, in a near future the use of expression profiles of ncRNAs will be as important or complementary to current detection systems based on the presence or absence of proteins or in morphological changes analyzed by H&E [128].

Non-coding RNAs encoded by oncoviruses

The first report on agents capable of transmitting cancer was described in 1909 by Rous [129]. Up to date, it is recognized that about 20-25% of human cancers are associated with viral infections [130]. Human T-cell Leukemia Virus type 1 (HTLV-1) was the first described retrovirus associated to adult T-cell leukemia [131, 132]. Later, another retrovirus, HTLV-2, was also associated with human diseases [133]. Another group of viral agents involved in human pathologies are the DNA viruses, responsible of a great number of mammalians cancers. Thus the Herpes Simplex Virus type 2 (HSV2) was associated to cancer development, including cervical cancer [134, 135]. Years later, HPV, another DNA virus, was found to be the etiological agent of cervical cancer [136]. This major breakthrough in cervical cancer carried out by zur Hausen was recognized by the Nobel academy in 2008.

Depending on the type of genetic materials, human oncogenic viruses can be divided into DNA or RNA oncogenic viruses. Examples of the first group are the Epstein-Barr virus (EBV), the etiological agent of Burkitt lymphoma, nasopharyngeal carcinoma (NPC) and a type of Hodgkin disease [137-144], the Kaposi Sarcoma associated Herpesvirus (KSHV), involved in the development of Kaposi´s sarcoma and a type of B-cell lymphoma [145-147], the Hepatitis B virus (HBV), the etiological agents of liver cancer [148-150] and HPV, the etiological agent of cervical cancer [136, 151, 152]. The RNA oncogenic viruses include HTLV-1, associated to Adult T-cell leukemia/lymphoma and Tropical Spastic Paraparesis [131, 153] and Hepatitis C virus (HCV), whose persistent infection causes cirrhosis and liver cancer [154-156].

MicroRNAs and Oncovirus.

EBV was the first reported oncogenic virus that encodes miR [157, 158]. Currently, there are more than twenty five described miRs encoded by EBV [158], sixteen miRs encoded by HSV-1, thirteen miR encoded by HSV-2 and twelve miR encoded by KSHV [158]. In contrast to other DNA viruses, HPV is a paradigm due to the fact that it is the most characterized etiological agent associated with cervical cancer development and yet, there are no reports revealing miRs encoded by this virus [158]. A summary of the main miRs encoded by human DNA and RNA oncoviruses is shown in Table 3 and we will briefly describe some of their main functions.

miRNA	Virus	Function	References
BART-5	EBV	Antiapoptotic	163, 164, 165
BART-6	EBV	Latency	163, 164, 165
BART-2	EBV	Immune evasion	163, 164, 165
BHRF-1	EBV	Latency	163, 164, 165
miR-k12	KSHV	Viral replication	159, 160, 161
miR-k2	KSHV	Viral replication	159, 160, 161
miR-k4	KSHV	Latency	159, 160, 161
miR-k8	KSHV	Viral replication	159, 160, 161
miR-k7	KSHV	Latency	159, 160, 161
miR-k5	KSHV	Antiapoptotic	159, 160, 161
miR-k9	KSHV	Antiapoptotic	159, 160, 161
miR-k3	KSHV	Antiapoptotic	159, 160, 161
miR-k1	KSHV	Antiapoptotic	159, 160, 161
miR-k10	KSHV	Antiapoptotic	159, 160, 161
miR-k10	HSV	Viral replication	167, 168
miR-H6	HSV	Viral replication	167, 168
miR-k12	HSV	Viral replication	167, 168
miR-H3	HSV	Neurovirulence	167, 168
miR-H4	HSV	Neurovirulence	167, 168
miR-93	HTLV-1	Antiapoptotic	173, 174
miR-130b	HTLV-1	Antiapoptotic	173, 174
miR-21	HTLV-1	Antiapoptotic	173, 174
miR-24	HTLV-1	Antiapoptotic	173, 174
miR-146A	HTLV-1	Antiapoptotic	173, 174
miR-155	HTLV-1	Antiapoptotic	173, 174
miR-29	HTLV-1	Immune evasion	173, 174
miR-142-5p	HTLV-1	Latency	173, 174

Table 3.- Principal microRNAs encoded by human oncogenic viruses

During the latency period, KSHV differentially express twelve miRs besides the latency proteins [159]. Thus, miR-K4 and miR-K7 regulate the expression of viral mRNAs and the later is involved in immune evasion [160, 161]. On the other hand, miR-K5, miR-K9 and miR-K10 inhibit the apoptotic ability of the infected cells [161]. MiR-K10 and miR-K12 are involved in the latency-lytic replication transition [159]. Furthermore, small antisense RNAs that target miR-K2, miR-K4 and miR-K8 are expressed from the viral genome and are associated with the restriction of lytic replication [159]. MiR-K1, miR-K3 and miR-K10 are involved in apoptosis inhibition [159] and attenuation of cell cycle arrest [159], besides inhibiting the p21 protein located down-stream in the activation pathway of P53 [162].

EBV encodes three miR-BHRF1s and twenty-two miR-BARTs, which are differentially expressed during the viral cycle [163-165]. MiR-BHRF1 is expressed during late latency and miR-BART, in previous phases [163-165]. MiR-BART2 has a role in the immune evasion of infected cells [163-165]. EBV also induces over-expression of miR-155, a cellular transcript involved in viral replication [166].

HSV express six miRs, involved in different stages of the viral cycle. MiR-H6 maintains a latent infection and miR-H10 and miR-H12 are associated to the activation of the lytic phase of viral replication [167]. HSV-1 and 2 encode miR-H3 and miR-H4, which are associated to the neurovirulence [168] and HSV-2 also expresses miR-H6, which participates in viral replication [168].

Although there are no reports on HCV-encoded miRs, the virus induces over-expression of miR-122, a cellular transcript necessary for viral replication [169, 170]. The cellular miRs, miR-196b, miR-199a-3p and miR-29 are also involved in antiviral defense, inhibiting replication and viral pathogenicity [171].

To date, there are no reports on the existence of HBV-encoded miRs. Nevertheless, this virus down-regulates the expression of cellular miR-199a-3p, miR-210, miR-575, miR-4294 and miR-125a-5p, which inhibit HBV replication [172]. HBV also induces over-expression of the cellular transcripts miR-122, miR-22 and miR-99 [172].

HTLV-1 encodes miR-93 and miR-130b that target the tumor suppressor TP53 [173] and induces over-expression of the cellular miR-93, miR-21, miR-24, miR-146a, miR-155 and miR-130b involved in the restriction of tumor suppressor activity [174]. Interestingly, cellular miR-15a and miR-16-1 are down regulated in leukemia cells [173].

Long non-coding RNA and oncovirus.

In addition to microRNAs, EBV encode two lncRNAs, named EBER1 and EBER2, which are highly expressed in latently infected cells [175]. They form complexes with many cell proteins, such as RNA-activated protein kinase R (PKR) [176, 177], ribosomal protein L22 [178-180], La [181] and the retinoic acid-inducible gene I (RIG-I) [182]. In this manner, EBERs act upon direct binding with their targets [175]. It has been demonstrated that EBERs confer resistance to interferon (IFN-gamma)-induced apoptosis by binding PKR and inhibiting its phosphorylation [177]. Although EBERs are not essential for cell transformation [183], it has been reported that a recombinant EBV, lacking EBERs, have a diminished capacity of transformation compared to wild-type EBV [184]. Although there is a debate concerning the real tumorigenic potential of EBERs, it has been demonstrated that EBER2 plays a critical role in efficient B-cell growth transformation, whereas EBER1 is dispensable [184].

To date, there are no reports on lncRNAs encoded by HBV. However, this virus is capable to modulate the expression of the lncRNA known as HULC (highly up-regulated in liver cancer), which is strongly associated to the development of liver cancer and is over-expressed in HBV-infected cells [185]. A new lncRNA related to liver cancer and HBV pathologies and referred to as lncRNA-HEIH (long non-coding RNA highly expressed in hepatocarcinoma) is polyadenilated and it might be transcribed by the polymerase II [186]. LncRNA-HEIH is found in the nucleus and the cytoplasm, where promotes cell proliferation and control of cell cycle arrest [186].

KSHV encodes a lncRNA known as PAN RNA (poly-adenylated nuclear RNA) [187], which is polyadenilated and transcribed by RNA polymerase II. In the nucleus, PAN forms a speckled pattern typical of U snuRNA [187], and the KSHV protein ORF57 induces the nuclear accumulation of PAN [187]. ORF57, also known as MTA, is the earliest KSHV regulatory genes to be induced [188, 189] and required for lytic virus replication, suggesting that PAN RNA is involved in this process [190, 191]. Whether the study of ncRNAs encoded by viruses will be in the future used as tools for virus related cancer diagnostic is still a matter of discussion.

Deregulation of microRNAs by Human Papillomavirus

HPV is the etiological agent of cervical cancer development. Neoplastic progression occurs through the concerted action of the viral oncoproteins E6 and E7, which bind and stimulate the degradation of the tumor suppressor p53 and retinoblastoma (Rb), respectively [192-195]. These two events lead the cell to genomic instability due to an impaired DNA response to damage and a dysfunctional G1/S checkpoint. However, increasing amounts of evidence suggest that HPV infection by itself is not enough to provoke malignant changes and thereby additional host genetic variations induced by the viral oncoproteins would be important in the development of cervical cancer. In this section we will discuss the basic biology related to deregulation of miR expression in response to HPV infection. The potential use of these differential profiles as a tool for diagnosis, prognosis and management of cervical dysplasia and cancer is discussed in the final section of this chapter. Some of the best-characterized microRNAs modulated by HPV proteins are summarized in figure 3.

The E6 protein and microRNAs

Only recently, studies on the role of cellular miRs in the regulation of the HPV replicative cycle and carcinogenesis have been reported. The first publication showing a relationship between HPV infection and deregulation of miRs expression was miR-218, which is strongly down-regulated by the HPV-16 [83]. Down-regulation of miR-218 was induced only by the expression of the E6 protein from HPV-16. In striking contrast, the E6 protein from low-risk (lr) HPV-6 did not alter the expression levels of miR-218. Interestingly, the target of miR-218 is the mRNA of laminin 5 β3 (LAMB3), a protein that when over-expressed can potentiate viral infection of surrounding tissues and tumorigenesis [83]. A recent clinical study evaluates the expression levels of miR-218 in 78 samples from patients with different degrees of cervical intraepithelial neoplasia (CIN). The results show that miR-218 was down-regulated in samples from patients infected with high-risk (hr)

HPV compared to those infected with low or intermediate-risk HPV, or non-infected samples [196]. A later study also shows down-regulation of miR-218 in samples from patients with HPV-associated cervical cancer tissues. In addition, miR-433 was also down-regulated, while miR-16, miR-21, miR-106b, miR-135b, miR-141, miR-223, miR-301b and miR-449a were over-expressed in cancer and cervical dysplasia compared to normal cervical tissue [197]. Zheng *et al* described up-regulation of miR-15b, miR-16, miR-146a, miR-155 and down-regulation of miR-126, miR-143, miR-145, miR-424 and miR-218 in samples obtained from cervical cancer biopsies. Interestingly, no differences in expression patterns could be observed when comparing cells with integrated or episomal HPV genome [87]. From the list, miR-143 and miR-145 were analyzed in detail and they were found to be suppressors of cell growth, while miR-146a is involved in cell proliferation, suggesting that the differential expression of these molecules is a fine-tuning in carcinogenesis. Furthermore, miR-143 and miR-145 were down-regulated in HPV-induced pre-neoplastic lesions, suggesting that down-regulation of these molecules is an early step in cancer development and thereby can be potentially used as early markers [87].

A recent study established the miRs expression profiles of biopsies obtained from patients with oral (OSCC) and pharyngeal cell carcinoma (PSCC), compared to normal epithelium. Surprisingly, many of the miRs that presented deregulation associated to HPV infection were similar to those found in cervical cancer samples. Thus, miR-145, miR-125a, miR-126, miR-127-3p and miR-379 were down-regulated in cancer tissues [198]. Up-regulation of miR-363 was also found in PSCC and confirmed by the group of Khan *et al* [199]. This group determined that miR-363, miR-33 and miR-497 were up-regulated, whereas miR-155, miR-181a, miR-181b, miR-29a, miR-218, miR-222, miR-221 and miR-142-5p were down-regulated in HPV-positive cells compared to both HPV-negative SCC of head and neck (SCCHN) and normal oral keratinocytes. HPV E6 silencing in SCC2 cells or expression of E6 in HFK cells allowed the authors to suggest that E6 protein is involved in deregulation of the above-mentioned miRs [199]. More recently, down-regulation of miR-145 in HPV-positive cancer cells was proposed as a novel target

for cervical cancer therapy due to its role in the suppression of the p53 protein [200].

In 2009, Zheng *et al* described that E6 protein from HPV-16/18 inhibits the expression of the tumor suppressor miR-34a. MiR-34a is a transcriptional target of the transcription factor p53, and thereby the destabilization of p53 by E6 results in promotion of cell proliferation [201]. Reinforcing this data, the expression level of pri-miR-34a was found reduced in cervical tissues of patients with cervical cancer and pre-neoplastic lesions compared with normal cervical epithelium. Down-regulation of miR-34a also correlates with the malignancy of the lesion, from CIN 1 to CIN 3 [202]. Thus, expression levels of miR-34a can be used as precancerous lesion detection, even before observation of morphological changes.

Finally, a recent report indicates that HPV-16 E6 decreases the expression levels of miR-23b, which has been previously associated to cervical cancer [203]. The target of miR-23b is the mRNA of urokinase-type plasminogen activator (uPA), and consequently down-regulation of miR-23b induces up-regulation of uPA, increasing migration of human cervical carcinoma cells. Interestingly, p53 acts as transcriptional factor for expression of miR-23b. Thus, degradation of p53 mediated by HPV E6 indirectly affects miR-23b expression. In this manner, the p53/miR-23b/uPA triad is involved in cervical cancer development [204].

The E7 protein and microRNAs

In 2010, a report indicated that the E7 protein from HPV-16 and 31 was responsible for down-regulation of miR-203, with E6 having a minimal effect. One target of miR-203 is the transcription factor p63, which regulates the balance between epithelial proliferation and differentiation. Thus, in HPV-infected epithelia, the expression of E7 blocks expression of miR-203, which leads to increased levels of p63, causing cells to remain active in cell cycle and HPV genome amplification [84]. An interesting finding is that miR-34b, miR-124a and miR-203 gene are hypermethylated in samples from cancer precursor lesions and cervical tumors versus normal control samples, demonstrating that epigenetic alterations of

miRs are also involved in cervical carcinogenesis associated to hr-HPV genotypes [205]. Moreover, miR-15b was up-regulated in biopsies from patients with anal carcinomas associated to hr-HPV infections, which leads to a down-regulation of cyclin E1, which plays a crucial role in G1/S transition. Knockdown of E7 induce down-regulation of miR-15b with a simultaneous down-regulation of cyclin E1, as observed in CasKi cells, suggesting a role for this molecule as an inhibitor of cell cycle progression [85].

Other HPV proteins involved in microRNAs deregulation

E6 and E7 proteins are key players during cellular transformation upon HPV infection, and thereby a direct modulation of miRs expression by these proteins would be expected. Thus, it was described that the expression of miR-125b was diminished in CIN 1 lesions, specifically in koilocytes, where the productive infection of HPV takes place. Remarkably, down-regulation of miR-125b was associated to the structural protein L2. The expression of HPV-16 L2 protein in NIH3T3 cells was enough to induce down-regulation of miR-125b. This process occurs in cells involved in productive infection of HPV virions and thereby a role of miR-125b in regulation of the viral DNA and proteins synthesis was suggested [86].

In 2011 the group of Auvinen *et al* described for the first time the involvement of the E5 protein in deregulation of miRs. A direct correlation between down-regulation of miR-203 and miR-324-5p and up-regulation of miR-146a in HaCaT cells, depending on E5 expression was found [82]. It is interesting to note that down-regulation of miR-203 is also a property of the E7 protein [84], suggesting that the oncogenic properties of both proteins are at least partially, mediated by miR-203. Interestingly, up-regulation of miR-146a was also observed in breast, pancreas and prostate cancers [206], suggesting that cellular factors none related to HPV E5 are able to induce similar molecular changes on cell transformation.

Another interesting finding is the discovery that in beta HPV-8 infected cells, miR-17-5p, miR-21 and miR-106a were up-regulated while, miR-155 and miR-206

were down-regulated in HPV8-CER (E6/E7/E1/E2/E4=*CER)* mice after UV irradiation being the first report on how a cutaneous HPV modulate the expression of miRs [207]. Other authors reported up-regulation of miR-21 in cervical cancer lesions associated to HPV, a molecule that regulates proliferation, apoptosis and migration of HPV-16 cervical squamous cells and probably by targeting Chemokine (C-C motif) ligand 20 (CCL20) [197, 208]. Thus, miR-21 could also represent a universal miR target for potential clinical diagnosis of different types of HPV.

Two independent investigations made by the group of Xie *et al* demonstrated deregulation of miR-100 and miR-29 [80, 209]. Mir-100 was reduced in cervical cancer in a gradual tendency from low-grade CIN to high-grade CIN and carcinoma in situ associated to HPV infection. Interestingly, changes in miR-100 expression were not attributable to E6 or to E7 oncoproteins [209]. On the other hand, miR-29 was down-regulated in samples from patients with different degrees of lesion suggesting that this molecule could be associated to cell cycle progression and promotion of malignant transformation induced by HPV [80].

Conclusively, miR expression profiles will be in the near future a complementary diagnostic or prognostic tool in cancer associated to HPV. In addition, rigorous and strict detection techniques and controls must be used in order to corroborate up-or down-regulation of a specific miR, since expression levels of these molecules are quite variable between tissues. The use of miR arrays has also been proposed, due to the inherent problem of the internal controls when using qRT-PCR [210]. Much work has to be done in this area, but we anticipate that increasing amount of clinical investigations will improve the diagnosis and prognosis of HPV-associated cancer based on miR expression. Principal cellular microRNAs deregulated by HPV are summarized in table 4.
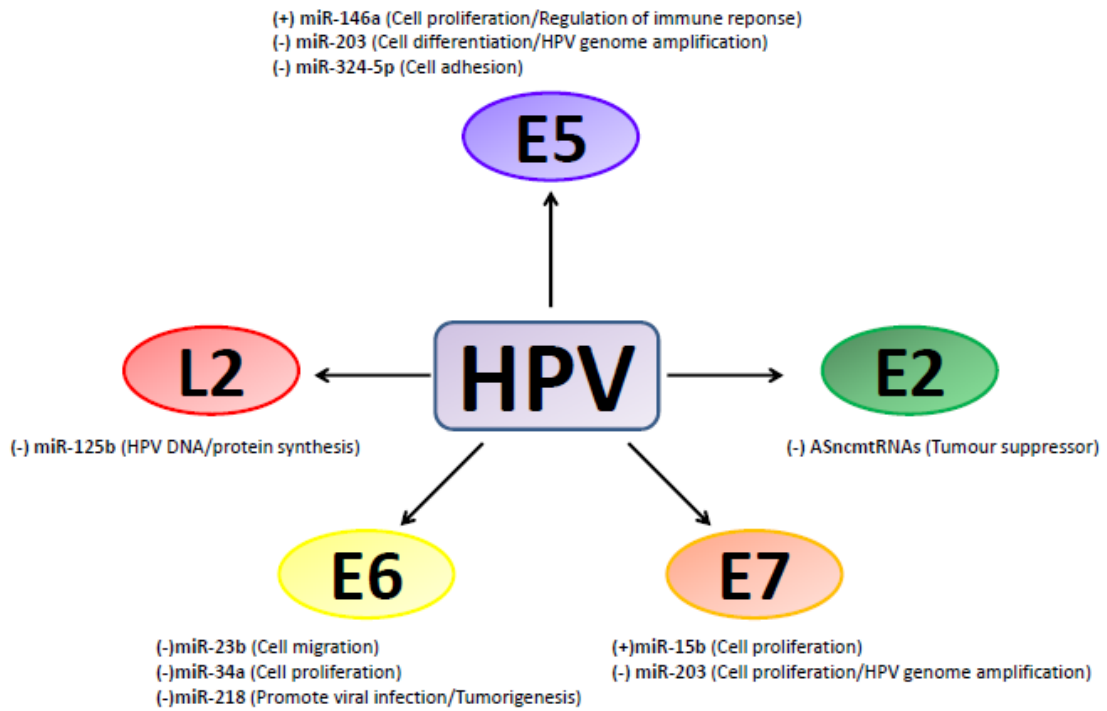
Figure 3.- Principal microRNAs modulated by HPV proteins. Only HPV proteins that directly influence the expression of cellular miRs are indicated. In brackets, the putative function of these miRs are shown

Methodology	Up-regulated	Down-regulated	References
miRNA array	miR-193b, miR-200c, miR-205	miR-143, miR-145,miR-218,miR-368,miR-497	83
qRT-PCR			
Northern Blot			
miRNA array	miR-15b, miR-16, miR-146a, miR-155	miR-126, miR-143, miR145, miR-218, miR-424	87
Northern Blot			
Northern Blot		miR-34a	201
miRNA ligation assay			
qRT-PCR		miR-203	84
Northern blot			
In situ RT-PCR		miR-125b	86
qRT-PCR	miR-363, miR-33, and miR-497	miR-155, miR-181a, miR-181b, miR-29a,	199
miRNA array		miR-218, miR-222, miR-221, miR-142-5p	
miRNA array		miR-23b	204
qRT-PCR			
qRT-PCR		miR-100, miR-29	80, 209
miRNA array	miR-16, miR-21, miR-106b, miR-135b,	miR-218, miR-433	197
qRT-PCR	miR-141, miR-223, miR301b, miR-449a		
miRNA array	miR-146a	miR-203, miR-324-5p	82
qRT-PCR			
qRT-PCR	miR-17-5p, miR-21, miR-106a	miR-155, miR-206	207
In situ hibridization			
qRT-PCR	miR-15b		85
miRNA array			
miRNA array	miR-363	miR-145, miR-125a, miR-126, miR-127-3p, miR-379	198
qRT-PCR			
miRNA array	miR-181a, miR-125a-5p, miR-502-3p,	miR-558, miR-576-3p, miR-606, miR-886-3p,	210
	miR-923, miR-92a-1, miR-500	miR-888, miR-1255a, miR-1274b	
qRT-PCR	miR-21		208
Northern Blot			

Table 4.- Cellular microRNAs deregulated by HPV infection.

Human Papillomavirus and long non-coding RNAs

Non-coding mitochondrial RNAs

More than ten years ago, a research group described for the first time a particular mitochondrial non-coding RNA in mouse cells [211]. This transcript contains a region corresponding to the 16S mitochondrial ribosomal RNA (16S mtrRNA) and an inverted repeat (IR) linked to the 5' end of the 16S mtrRNA and generated by transcription of the L strand of the mtDNA [211]. A related transcript associated to cell prpliferation was described in human [212]. Later, it was reported that human cells express a family of ncmtRNAs, which are differentially expressed in normal, and tumor cells [126]. This family of long ncmtRNAs comprises sense and antisense transcripts. The first described transcript, the Sense ncmtRNA-1 (SncmtRNA-1) contains an IR of 815 nt linked to the 5' end of the 16S mtrRNA [213]. The IR is fully complementary to an internal region of the 16S mtrRNA, forming a long stem-loop structure resistant to RNase A digestion. The SncmtRNA-1 is expressed in normal proliferating cells and tumor cells but not in resting cells. Furthermore, normal proliferating cells express two antisense transcripts named ASncmtRNA-1 and -2 that contain IRs of 316 and 545 nt, respectively [126, 212]. In striking contrast, in tumor cell lines as well as in tumor cells present in human biopsies of different types of cancer the ASncmtRNAs are down-regulated [126]. These observations suggest that down-regulation of the ASncmtRNAs is an essential step during neoplastic transformation and progression and led the authors to suggest the possibility that these transcripts are tumor suppressors derived from the mitochondrial genome [126, 213]. Down-regulation of the ASncmtRNAs is very similar to the fate of protein tumor suppressors [214] or miRs [215] during neoplastic transformation. This is due to the fact that tumor suppressors lose their function as a result of mutations or deletions of the coding locus. Furthermore, the synthesis of ncmtRNAs require mitochondrial transcription since HeLa cells treated with ethidium bromide, a chemical agent capable of blocking mitochondrial transcription [216-218], leads to down-regulation of the

SncmtRNAs, without changes in the expression of nuclear transcripts, such as 18S rRNA [212]. Additionally, these mitochondrial transcripts exit the organelle and are found in the cytoplasm and in the nucleus associated to chromatin and nucleoli [219].

HPV E6 and E7 induce expression of a new Sense ncmtRNA

The ability of the hr-HPV oncogenes E6 and E7 to block the function of the tumor suppressor p53 and pRb together with up-regulation of telomerase are critical steps for human keratinocytes immortalization [136, 152, 220-222]. Keratinocytes immortalized with HPV-16/18 express high levels of the SncmtRNA-1 and down-regulate the expression of ASncmtRNAs, like their tumor counterparts SiHa and HeLa cells (derived from patients with cervical carcinoma infected with HPV-16 and HPV-18, respectively)[213]. The exclusive expression of both, hr-HPV E6 and E7, is not enough to down-regulate the ASncmtRNAs in human keratinocytes [213]. Therefore, down-regulation of ASncmtRNAs seems to be one key early event during immortalization and neoplastic transformation. In addition, these results reinforce the hypothesis that the antisense transcripts would be acting like tumor suppressors, because the virus suppresses its expression in a similar manner to the functional deletion of the classic tumor suppressors p53 and pRb. Interestingly, keratinocytes expressing hr-HPV E6/E7 or hr-HPV-immortalized keratinocytes also express a second sense transcript, named Sense ncmtRNA type 2 (SncmtRNA-2) [213]. This transcript is over-expressed in HPV-16/18 immortalized cells, but not in normal or tumor cells, such as HFK, SiHa and Hela cells. Since the SncmtRNA-2 exhibits 99,7% identity with the sequence of the human 16S mtrRNA gene and 100% identity with SncmtRNA-1, one would expect that this transcript will be localized in mitochondria, as is the case of SncmtRNA-1 and the ASncmtRNAs. Interestingly however, SncmtRNA-2 is found only in the cytosol fraction. The IR of SncmtRNA-2 is 63 nt shorter than the IR of the SncmtRNA-1 (752 and 815 nt, respectively), suggesting that the SncmtRNA-2 originates from SncmtRNA-1 [213]. Therefore, we postulated it seems reasonable

to postulate that SncmtRNA-2 could originate from SncmtRNA-1 by an unknown reaction similar to the cleavage-and-ligation reactions (editosome) necessary for the edition of kinetoplastid transcripts in *Trypanosoma* and *Leishmania* [223-225]. Furtheremore, the results suggest that this type of processing occurs outside mitochondria [213].

Previous reports have described miRs encoded within lncRNAs or functional regulation of cellular and viral miRs by the lnc transcripts [226]. *In silico* analysis of this 63 nt sequence revealed that this fragment is highly complementary to miR-620. MiR-620 is involved in the silencing of more than 100 target mRNAs, including the mRNA of promyelocytic leukemia (PML) protein, which is the core component of PML nuclear bodies found in tumor cells [227, 228]. PML nuclear bodies are important structures involved in HPV replication and several reports indicate that the E6 and E7 oncoproteins are localized in these nuclear structures [229-231]. E6 and E7 are needed for the expression of SncmtRNA-2 and therefore it is tempting to suggest that the 63-nt fragment released from SncmtRNA-1 is required to work as "sponge" [232-234] to trap miR-620 and consequently relieve the negative effect of this miR on the expression of PML protein (Figure 4). Interestingly, a ncRNA encoded by Herpesvirus saimiri binds and induces degradation of miR-27 to facilitate infection and viral transformation [235].

Villota *et al* showed that over-expression of RAS induces cell transformation of HPV-immortalized cells together with down-regulation of SncmtRNA-2 (Villota, unpublished results). The expression of HPV oncoproteins in addition to the activation of oncogenes, such as telomerase and RAS, are crucial for cervical transformation [236]. Therefore, it is possible that telomerase and RAS might block the expression of SncmtRNA-2 in HPV-infected tumor cells [213]. Why both oncoproteins are needed for the expression of SncmtRNA-2 is unclear but it is of great interest that efficient keratinocytes immortalization as well as full cell transformation also requires both E6 and E7 expression. The fact that SncmtRNA-2 is expressed in immortalized but not in tumorigenic cells might contribute to the screening of early cervical intraepithelial premalignant lesions, as discussed later [213].
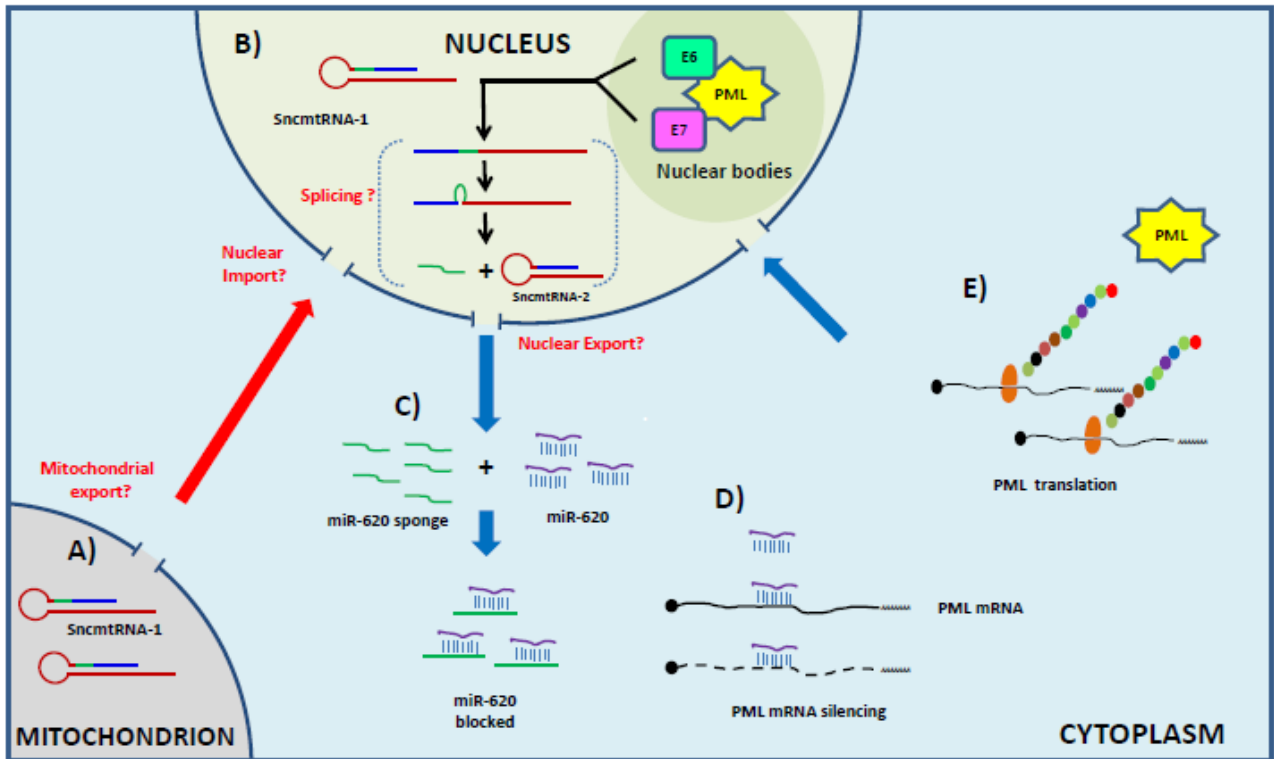
Figure 4.- Hypothetical mechanism on the modulation of SncmtRNAs by HPV. A) The SncmtRNA-1 is synthesized in the mitochondria and then the transcript exits the organelle to the cytoplasm and to the nucleus. B) HPV E6 and E7 proteins induce processing of SncmtRNA-1 to synthesize SncmtRNA-2 together with a 63 nt RNA fragment. C) The 63 nt RNA fragment would act like a miR "sponge" specific to trap miR-620. D) In a normal condition, miR-620 induces inhibition of translation and degradation of the mRNA of PML, hindering production of PML proteins. E) The 63 nt RNA fragment traps miR-620 allowing the synthesis of PML proteins which are required for HPC replication.

HPV E2 is involved in down-regulation of the ASncmtRNAs

Villota *et al* described for the first time that HPV oncoproteins are involved in modulation of the ncmtRNAs expression during viral infections [213]. These results indicate that E2 is involved in the down-regulation of the ASncmtRNAs in keratinocytes immortalized with HPV-16 and HPV-18. E2 is essential for viral genome replication and regulation of E6 and E7 expression in early stages after HPV infection [237]. In addition, regulation of transcription factors, cell proliferation, apoptosis, cell differentiation and chromosome instability seem to be the most significant functions of E2 [237-239]. The structure of the amino (N) and carboxyl (C) domains of E2 are relatively conserved among human and animal papillomaviruses, whereas the Hinge (H) domain is highly variable in sequence and length. Little is known about the function of the H domain, and it is generally considered a flexible linker between the two functional domains [240-242]. Interestingly, E2 seems to have an oncogenic potential by itself. Expression of the HPV-8 E2 gene in transgenic mice results in increased skin cancer development, which is enhanced by UV irradiation [243]. On the other hand, the low risk HPV virus HPV-11 E2, does not induce cell transformation [244]. Whether the induction of skin cancer is related to the ability of HPV-8 E2 to induce knockdown of the ASncmtRNAs warrants future research. Interestingly however, the ASncmtRNAs are also down-regulated in 293T cells (transformed with large T antigene of SV40) and the lymphoma cell line Devernelle (transformed with EBV) (Villota *et al.*, unpublished results). These results suggest that oncogenic proteins of these viruses could be involved in down-regulation of the ASncmtRNAs. Previous works have shown nucleo-cytoplasmic localization of HPV-16/18 E2 [245]. E2 does not interact with mitochondria and therefore a pertinent question is how E2 alone or in combination with other cellular factors induces down-regulation of ASncmtRNAs. In addition, cellular localization of hr-HPV E2 protein is different to that of lr-HPV E2. The E2 protein of HPV-6 and HPV-11 viruses is exclusively nuclear, whereas E2 proteins from high-risk viruses are located in both, the nucleus and in the

cytoplasm. HPV-18 E2 protein actively shuttles between the nucleus and the cytoplasm of infected cells and seems that partial cytoplasmic localization of hr-HPV E2 proteins correlates with the induction of apoptosis involving caspase 8 activation. In contrast, lr-HPV E2 proteins do not induce apoptosis due to their exclusive nuclear localization [245], determined by the H domain of HPV-11 E2 [246]. Electron microscopy ISH showed that the SncmtRNAs and the ASncmtRNAs in normal human kidney exit the organelle and are found localized in the cytoplasm and nucleus associated to chromatin and nucleoli [219]. In renal cell carcinoma, the SncmtRNA shows similar localization, while few copies of the ASncmtRNAs are mainly found in the cytoplasm [219]. Therefore, an intriguing question is how these mitochondrial transcripts containing long double-stranded regions can escape from the processing activities of Dicer and Drosha [224]. Hypothetically, the double-stranded region of these mitochondrial transcripts binds to the double stranded binding domain (DRBD) of Dicer and/or Drosha [223, 224] resulting in inhibition of their activities. Adenovirus ncRNAs containing double-stranded structures bind and inhibit Dicer or Drosha [223, 224]. Another interesting example is rncs-1 ncRNA of *Caenorhabditis elegans*, which contains a long double-stranded structure and binds and inhibits Dicer [247]. Perhaps then, the ASncmtRNAs in keratinocytes are able to form complexes with Dicer inhibiting its dicing activity. Hypothetically then, the expression of E2 in HPV-immortalized keratinocytes would relieve the inhibition on Dicer resulting on the degradation or processing of the double-stranded structure of the ASncmtRNAs. Nevertheless, down-regulation of ASncmtRNAs seems to be a potent diagnostic tool for early cervical cancer detection.

Non-coding RNAs in HPV-associated cancer diagnostics, prognosis and management

Cancer is the result of alteration of many cellular processes, which have been classified as cancer hallmarks by Hanahan and Weinberg [248]. Genes that induce cell proliferation or oncogenes are usually up-regulated whereas tumor suppressors genes are down-regulated [248]. As described before, ncRNAs represent a large family of molecules that behave as tumor suppressors or oncogenes roles. The expression profiles of ncRNAs revealed correlation with cancer progression and the potential use of these molecules as biomarkers has been extensively described over the past five years [248]. In fact, many clinical studies quantified miRs levels in different types of cancer as biomarkers of progression of the disease or predictors of conventional treatments. In the last section of this chapter, we will focus on recent published studies on the expression profiles of ncRNAs and their potential use as biomarkers of cancer prognosis, diagnostic or even management.

MicroRNAs and their clinical potential

Analysis of the expression profiles of one hundred sixty six miRs in six tumor cell lines (SiHa, C33A, SW756, CasKi, C41 and ME-180) and five normal cervical samples, revealed that let-7b, let-7c, miR-23b, miR-196b and miR-143 were down regulated in tumor cells [203]. In contrast, miR-21 was up-regulate in cervical tumor cell lines compared to normal tissues [203]. These results were confirmed later [52, 197, 198]. Remarkably, and in addition to HPV-associated cancer, miR-21 is also found up-regulated in glioblastoma, breast cancer and other cancer cell lines, while miR-143 is down-regulated in colorectal tumors, sarcomas, breast and prostatic cancer [67, 249]. The expression profiles of miR-21 and miR-143 were recently evaluated by Deftereos *et al* (2011) in HeLa, SiHa and Caski cell lines, normal samples infected and not infected by HPV and one hundred thirty three clinical samples including CIN 1 to 3 and cervical cancer. Interestingly, miR-21 was up-

regulated in severe lesions such as CIN 2 and higher compared to normal tissues and moderate dysplasia. Conclusively, despite the fact that miR-21 and miR-143 do not seem to be a specific biomarker for cervical cancer, up-regulation of miR-21 and down-regulation of miR-143 fulfill the requisites to be considered as markers to assess the development of the disease.

Analysis of miR expression in ten invasive squamous cervical carcinoma (ISCC) samples and ten normal cervical tissues showed that sixty eight miRs were up-regulated in ISCC compared to normal tissues, including miR-9, miR-127, miR-133a, miR-145, miR-199 and miR-214. Only miR-149 and miR-203 were down regulated in ISCC samples. The authors concluded that miR-127 could be a useful marker for lymph node metastasis of ISCC tissues. Up-regulation of miR-9 and down-regulation of miR-203 was later confirmed [52]. However, other groups reported down-regulation of miR-145 in cervical cancer [83, 87, 198]. The obvious concern on the analysis of this kind of transcripts is the technique used to identify their expression the use of appropriate experimental controls. Quantitative real-time RT-PCR (qRT-PCR) is the primary technical choice used to determine the expression profile of microRNA, but there are inherent problems with internal controls of this technique [210, 252]. In our opinion, intense research in qRT-PCR applied to clinical specimens has to be carried out to establish miRs expression profiles and probably more than one technique will be required.

Alteration of miRs expression in cervical cancer cell lines (CasKi, SiHa, HeLa, C33A and W12) with episomal or integrated HPV genome, as well as three CIN 3 samples and five cervical carcinomas, revealed that miR-126, miR-143, miR-145 and miR-195 were down-regulated in integrated HPV-16 cervical cell lines, whereas miR-182, miR-183 and miR-210 were up-regulated. Surprisingly, miR-218 was down-regulated in CINs and cervical cancer samples compared to the normal cervix [83]. From this list, miR-218 appears to be an excellent candidate for cancer diagnostics since down-regulation of this transcript in cervical cancer associated to HPV has been well documented [52, 83, 87, 197, 199].

In a small cohort study of five HPV-negative cervical squamous carcinoma and five normal samples it was reported that miR-494 and miR-61 were down-

regulated in tumor tissues, while miR-189, miR-202, let-7c and let-7a were up-regulated in tumor cells [254]. A year later, Hu *et al* (2010) described a study with one hundred and two cervical samples, using 60 samples as training for their predicting model and finally 42 for testing [52]. By using qRT-PCR they identified miR-9, miR-21, miR-200a, miR-218 and miR-203 associated to cancer survival [251]. The authors concluded that the expression of miR-200a and miR-9 are predictive of patient survival since down-regulation of these miRs is associated to metastasic potential [52].

In oral and pharyngeal carcinoma, changes in miR expression profile compared to controls patients were repoted [198]. One hundred and fourteen miRs were differentially expressed between OSCC and normal epithelium, and the most significant result was down-regulation of miR-375 and up-regulation of miR-31 in OSCC compared to normal tissues. As discussed before, HPV infection induces up-regulation of twenty one miRs and down-regulation of miR-127-3p, miR-125, miR-126, miR-145 and miR-363. Interestingly, miR-187, miR-181b, miR-21 and miR-345 were up-regulated in all OSCC samples. This is an important finding since suggests that miR expression profiles are in somehow similar between cervical and non cervical cancer associated to [198]. Pereira *et al* analyzed the miR expression profiles in four cervical squamous cell carcinomas, five high-grade intraepithelial lesions, nine low-grade intraepithelial lesions and nineteen normal cervical tissues [53]. The results indicate that miR-26, miR-99a and miR-513 were down-regulated in pre-neoplastic and cancer compared to normal cervical tissues. Down-regulation of miR-513 suggest that this molecule has tumor suppressor activity because targets the oncogene Kras, was also down-regulated. In addition, miR-106a, miR-205, miR-197, miR-16, miR-27a and miR-142-5p were down-regulated in normal and dysplasia samples, but up-regulated in cervical cancer.

A recent analysis of one hundred and fifty oropharyngeal SCC samples, including 101 cases for training and forty nine cases to validate a new miR-based prognostic system, revealed that six miRs retained their prognostic significance [255]. The expression of miR-142-3p, miR-31, miR-146a, miR-26b, miR-24 and miR-193b were up-regulated in surviving patients, whereas miR-31, miR-24 and

miR-193b were over-expressed in patients with low survival rate. Interestingly, the expression of miR-9, miR-31, miR-223, miR-155 and miR-18a correlates with HPV transcriptional activity, suggesting that miR-31 has a prognostic value in OSCC while miR-9 represent a good prognostic target in cervical cancer induced by HPV [255]. Taken together, the results described in this section suggest that the expression profile of miRs may be useful to differentiate cervical cancer and dysplasia from normal cervical tissue.

Long non-coding RNAs and their clinical potential

Recently, the relationship between cervical cancer progression and expression of lncRNAs was reported. Gibb *et al* analyzed lncRNA expression profiles in four normal samples, three CIN1 and 2 and six CIN3 samples by Long Serial Analysis of Gene Expression (L-SAGE) [108]. Analysis six hundred and sixty eight lncRNAs expression profiles revealed that one hundred twenty three lncRNA were differentially expressed between CIN1-3 compared to normal tissue, while thirteen aberrantly expressed lncRNAs were common to all CIN grades. While Xist (X-inactive specific transcript) is expressed "constituvely" in all samples, MALAT1 is down-regulated in all CIN2 and higher lesions and GAS5 was down-regulated from CIN1 to cervical cancer. The de-regulation of MALAT-1 and GAS5 in CIN may be contributing to the dynamics of precursor cervical cancer lesions. Taken together, these results suggest that the lncRNA expression profiles can be used to differentiate normal tissue from dysplasia and high-grade lesions [108].

Mitochondrial long ncRNAs and their clinical potential.

Recently, Villota *et al* (2012) described for the first time that HPV oncoproteins modulate the expression of mitochondrial ncRNAs in keratinocytes immortalized with the virus. Immortalization with the whole genome of HPV 16 or 18 induces down-regulation of the ASncmtRNAs and the E2 oncogene is involved in this process (Villota *et al.*, 2012). Taken together, these results indicate that the

differential expression of the SncmtRNA and the ASncmtRNAS offers a potent opportunity to detect tumor cells or cells at early stage of neoplasic transformation. Indeed, we have used the differential expression of these transcrits to detect tumor cells in voided urine of patients with bladder cancer (BC). A pilot study using FISH shows that in the urine of 24 patients with BC there are cells that express the SncmtRNA but not the ASncmtRNAs and therefore, they correspond to tumor phenotype [127]. In contrast, hybridization of the few exfoliated cells recovered from healthy donors revealed no expression of these mitochondrial transcripts [127]. Preliminary studies show that the differential expression of the mitochondrial transcripts can also be used to detect tumor cells present in cervical cells (liquid cytology). The LncmtRNAs expression profile has also been analyzed in normal, CIN1, 2, 3 and invasor squamous carcinoma (Villota *et al*, unpublished results). The results showed that SLncmtRNA-1 expression is maintained in all tissues (normal to ISCC) whereas the AsncmtRNAs are downregulated from CIN1 to ISCC.

On the other hand, the oncoproteins E6 and E7 are both involved in induction of SncmtRNA-2 in HPV-immortalized cells. Interestingly, SncmtRNA-2 is expressed in CIN 1 and 2. Normal tissues, as well as CIN 3 and ISCC do not express this transcript. These results support the idea that expression of SncmtRNA-2 might contribute to the screening of early cervical intraepithelial lesions. Taken together, the data indicates that miRNA and lncRNA expression profiles could potentially help to differentiate between normal, dysplasia and high-grade lesions of cervical cancer.

Figure legends

Table 1.- Representative microRNAs associated with human cancer

Table 2.- Some long non-coding RNAs associated with human cancer

Table 3.- Principal microRNAs encoded by human oncogenic viruses

Table 4.- Cellular microRNAs deregulated by HPV infection.

Figure 1.- Number of publications related to non-coding RNAs associated to cancer. A search on the Pubmed database from the National Center for Biotechnology of Information (NCBI) was performed using the terms "ncRNA" or "non-coding RNA" or "noncoding RNA" or "non-protein-coding RNA" AND cancer, with limit date December 31, 2012. Data was corroborated performing the same search on the Gopubmed database. The data was plotted as number of publications per year, starting from 1999.

Figure 2.- Biogenesis and functions of cellular microRNAs. In the nucleus, RNA pol II transcribes a pri-miR, which folds as an imperfect hairpin. Drosha, together with its partner DGCR8, recognizes the hairpin and subsequently cleaves both strands from the base of the stem leaving a 5' phosphate and 2-nt 3' overhang in a structure named pre-miR, which is exported to the cytoplasm by Exportin-5. There, Dicer cleaves off the loop of the pre-miR, 22 nt from the Drosha cut, again leaving a 5' monophosphate and 2-nt 3' overhang. The resulting miR:miR* duplex, comprised about 22-nt taken from each arm of the original hairpin, associates with the Ago protein in such a manner that the miR strand is usually the one that is stably incorporated, while the miR* strand dissociates and is subsequently degraded. The mature miR will then associate with other proteins of the RNA Induced Silencing Complex (RISC), which directs it to a target mRNA. Finally the perfect base complementarity of the miR and its target, usually the 3' UTR of an

mRNA, induce the degradation of the transcript, while partial complementarity induces translational repression.

Figure 3.- Principal microRNAs modulated by HPV proteins. Only HPV proteins that directly influence the expression of cellular miRs are indicated. In brackets, the putative function of these miRs are shown

Figure 4.- Hypothetical mechanism on the modulation of SncmtRNAs by HPV. A) The SncmtRNA-1 is synthesized in the mitochondria and then the transcript exits the organelle to the cytoplasm and to the nucleus. B) HPV E6 and E7 proteins induce processing of SncmtRNA-1 to synthesize SncmtRNA-2 together with a 63 nt RNA fragment. C) The 63 nt RNA fragment would act like a miR "sponge" specific to trap miR-620. D) In a normal condition, miR-620 induces inhibition of translation and degradation of the mRNA of PML, hindering production of PML proteins. E) The 63 nt RNA fragment traps miR-620 allowing the synthesis of PML proteins which are required for HPC replication.

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