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ORIGINAL ARTICLE

Predicting Human miRNA-like Sequences within Human Papillomavirus Genomes

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Background. This study presents a prediction of putative miRNA within several Human Papillomavirus (HPV) types by using bioinformatics tools and a strategy based on sequence and structure alignment. Currently, little is known about HPV miRNAs.

Methods. Computational methods have been widely applied in the identification of novel miRNAs when analyzing genome sequences. Here, ten whole-genome sequences from HPV-6, -11, -16, -18, -31, -33, -35, -45, -52, and -58 were analyzed. Software based on local contiguous structure-sequence features and support vector machine (SVM), as well as additional bioinformatics tools, were utilized for identification and classification of real and pseudo microRNA precursors.

Results. An initial analysis predicted 200 putative pre-miRNAs for all the ten HPV genome variants. To derive a smaller set of pre-miRNAs candidates, stringent validation criteria was conducted by applying <-10 ΔG value (Gibbs Free Energy). Thus, only pre-miRNAs with total scores above the cut-off points of 90% were considered as putative pre-miRNAs. As a result of this strategy, 19 pre-miRNAs were selected (hpv-pre-miRNAs). These novel pre-miRNAs were located in different clusters within HPV genomes and some of them were positioned at splice regions. Additionally, the 19 identified pre-miRNAs sequences varied between HPV genotypes. Interestingly, the newly identified miRNAs, 297, 27b, 500, 501-5, and 509-3-5p, were closely implicated in carcinogenesis participating in cellular longevity, cell cycle, metastasis, apoptosis evasion, tissue invasion and cellular growth pathways.

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Conclusions. The novel putative miRNAs candidates could be promising biomarkers of HPV infection and furthermore, could be targeted for potential therapeutic interventions in HPV-induced malignancies. © 2018 IMSS. Published by Elsevier Inc.

Key Words: Apoptosis, Bioinformatics, Carcinogenesis, HPV, *In silico*, Longevity pathway, miRNA, Self-splicing introns, Pre-miRNA.

Introduction

MicroRNA (miRNA) integral elements are a family of evolutionarily conserved and abundant type of short non-coding single-stranded RNA molecules, comprised of approximately 22 nucleotides (nts) long that are present in all metazoan and plant species so far tested (1−5). MiRNAs repress the expression of protein-coding genes acting at the post-transcriptional level either by inhibiting translation or by degrading mRNA as a result of the partial or complete complementarity to specific mRNA targets, respectively (2,6). The primary miRNAs (pri-miRNAs) are transcribed and processed into the nucleus and exported as individual double-stranded precursor miRNAs (pre-miRNAs), to the cytoplasm (7). Each pre-miRNA, comprised approximately of 41−180 nts in humans, forms a typical stem-loop secondary structure that is subsequently transported to the cytoplasm and processed by Dicer into an 18−22 nts long mature miRNA (7−10).

The miRNAs have been identified as one of the most abundant gene expression regulators in different species; including, animals, plants and more recently in viruses, affecting among others, cell metabolism, development, differentiation, and apoptosis (9,11,12). Considering the importance of these short non-coding RNA molecules in regulating vital cellular processes, their association with several diseases including cancer is evident (8,11). Nowadays, 2588 mature miRNAs have been identified in *Homo sapiens,* listed in miRBase *(mirbase.org)*, and this number will continue to grow as more sensitive approaches appear for the discovery of new miRNAs. On the other hand, 502 mature miRNAs have been discovered in 29 different viruses, most of them carrying double-stranded DNA genomes *(mirbase.org)*. Recent evidence from members of the herpes virus family has revealed that their miRNAs suppress both viral and cellular genes, regulating establishment and maintenance of viral latency and control of host immune responses (12−14).

Due to the problematic and limits of systematically identifying miRNAs from a genome by current experimental methods, emerging computational techniques play an important role in the identification of new putative miRNAs. As a characteristic secondary structure, the hairpin of the pre-miRNA is an important feature used in the computational identification of miRNAs (7). Moreover, miRNAs seed regions, essential conserved heptameric sequences (6−8 nts) within the miRNA, are also of central importance in the recognition of miRNA target genes due to their exceptional complementarity to their mRNA targets (2,15,16). Likewise, thermodynamic stability of the interaction between miRNA-mRNA targets also contributes to the recognition of potential miRNA target candidates (17,18). In this context, there are several algorithms available online to detect miRNAs and their target genes, for instance: TargetScan, miRanda, miRbase target database, sequence and annotation, and mFold software, among others (19−22). All those existing methods utilize comparative genomic information besides structural features to predict new miRNAs.

To date, more than 150 genotypes of Human Papillomaviruses (HPV) have been identified (23), being HPV-16 and -18 the most persistent worldwide, found in 70% of all cervical cancers (24). At present, little is known about miRNAs encoded by HPV. In 2011, Gu W, et al., predicted miRNAs from 8 HPV-affecting skin and mucosal genotypes using comparative genomics. This group was able to identify one of the predicted miRNAs in three different cervical cancer cell lines by northern blot hybridization (25). In addition, in 2013 Qian K, et al., sequenced RNA libraries from different HPV-related cervical tissue samples, and also from well-established cell lines, predicted several putative HPV miRNAs: four of those miRNAs were confirmed by qPCR, two of them corresponding to HPV-16, one to HPV-38, and another one to HPV-68 (26). Nevertheless, despite the efforts of these two groups, to date, there is not a single well-established miRNA from HPV reported in miRBase, due in part to the absence of an effective viral-replication model.

In the present study, we applied an *in silico* comparative genome-based homology search strategy to identify potential human miRNA-like sequences within HPV-6, -11, -16, -18, -31, -33, -35, -45, -52, and -58 genomes linked to anogenital lesions. The new putative miRNAs identified in this study, and its future potential validation, can provide new insights on understanding the development of cervical cancer and thus, contribute t(7)o the potential discovery of novel therapeutic targets to alleviate HPV-induced diseases. Moreover, these novel HPV miRNAs could be possibly implicated in regulatory pathways used by HPV virus to establish its pathogenic effects.

Materials and Methods

Strategic Approach to Identify New miRNA Sequences in Full-length Viral Genomes

A flowchart diagram is depicted in Figure 1, providing an effective guided example of the strategy employed to identify

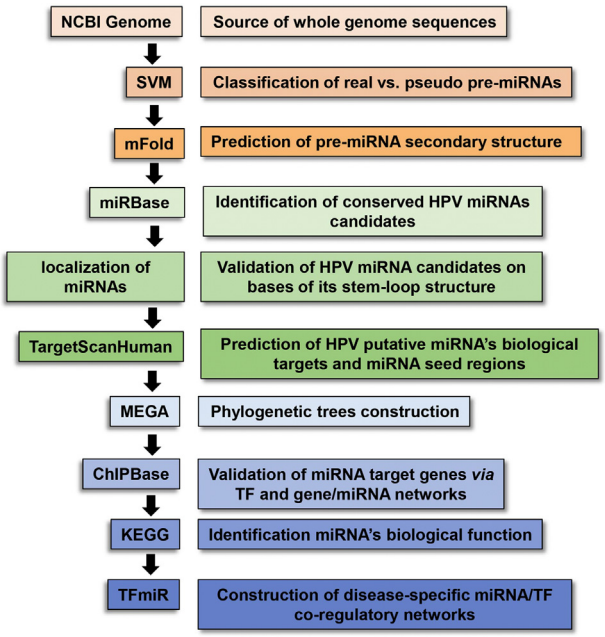

Figure 1. A guided flowchart of the methodology utilized in this study. Depicted is a delineated genome-wide strategy to identify miRNAs and miRNAs seeds, and their potential target genes, as well as their possible implications on co-regulatory pathophysiological networks. In the left column of the flowchart diagram are annotated the interconnected databases or the software used in this study, whereas in the right column are displayed a brief description of each bioinformatics tool function.

novel HPV miRNAs (hpv-miRNAs) sequences by using a selected collection of online bioinformatics tools; software combinations and interconnected databases.

In silico *Analyses Searching for Pre-miRNAs within HPV Genomes*

Ten whole HPV genome sequences, as well as three HPV-16 genome variants, were acquired from National Center for Biotechnology Information (NCBI) and were divided into two groups according to their association with cancer, low and high-risk types. Their accession numbers are as follows: the low-risk HPV-6bR X00203, HPV-11R M14119; and the high risk HPV-16R AY686579, HPV-18R X05015, HPV-31 J04353, HPV-33 M12732, HPV-35 X74477, HPV-45 X74479, HPV-52 X74481, HPV-58 D90400, HPV-16 Asian-American variant AF402678, HPV-16 European variant AY686580 and HPV-16 African variant AF472508. Once downloaded, all genome sequences were subjected to bioinformatics analysis *via* computational *ab initio* and support vector machine (SVM) software, to identify and classify the real and pseudo pre-miRNAs (7). The approach involved was to use consensus genome alignments generated from the ten HPV sequences to identify miRNA homologs into the HPV selected sequences, based on local contiguous structure-sequence features. These *in silico* analyses were conducted in both forward and backward directions and then analyzed by using a miRBase database and mFold software (22).

Prediction of Pre-miRNA Secondary Structures

The single-stranded RNA sequences of the newly discovered pre-miRNA were subjected to analysis for secondary structure prediction by using the mFold software tool (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi), searching for the typical stem-loop structure. This program predicts the most stable secondary structure, with minimum free energy and base pair probabilities from single RNA sequences in a dot-bracket notation.

Identification of Cellular miRNA in HPV-pre-miRNA Candidates

MiRBase is a searchable database of published miRNA sequences, with three main functions; registration of novel miRNA genes, sequence data and annotation, and target prediction (19,20). Pre-miRNA candidate sequences were evaluated for the purpose of finding already described miRNA sequences by using the miRBase sequence search tool (http://www.mirbase.org/search.shtml). The hpv-pre-miRNA sequences that exhibited 50% or higher homology to previously described human miRNAs were identified as ''HPV-miRNA candidates'', which were subsequently manually projected on the stem-loop structures within their pre-miRNA sequences.

Finding Potential miRNA Targets and Their Function

Potential biological target genes were identified for the hpv-miRNA candidates using the TargetScanHuman software (http://www.targetscan.org). This useful tool predicts accurately the miRNA's biological targets by examining for the presence of conserved 8 mer, 7 mer, and 6 mer sites that match complementarily the seed region of each miRNA query (27,28). By using this software, large lists of potential gene targets were obtained.

Identifying miRNA Seed Regions and Their Phylogenetic Trees

The miRNA seed regions within the hpv-pre-miRNA candidate sequences were searched by operating the TargetScanHuman software (http://www.targetscan.org) (28). This international database contains the sequences of miRNA seeds already published. Then, by using the identified miRNAs seeds a phylogenetic tree was constructed to distinguish if specific seeds were grouped according to oncogenicity viral types. Subsequently, the MEGA, molecular evolutionary genetics analysis (http://www.megasoftware.net) program, was also employed (29). This MEGA software is an integrated tool for conducting sequence alignment, inferring phylogenetic trees, estimating divergence times, mining online databases, estimating rates of molecular evolution, inferring ancestral sequences, and testing evolutionary hypotheses (v. MEGA 5.2).

Identifying HPV-miRNA Co-regulatory Networks

ChIPBase (http://deepbase.sysu.edu.cn/chipbase/) database, a platform that deciphers transcription factor (TF) binding sites, expression profiles, and transcriptional regulation of miRNAs, was used to generate miRNA → TF and or miRNA → mRNA co-regulatory networks of some of the predicted miRNA seeds (30). To identify the miRNA candidates biological function, KEGG (Kyoto Encyclopedia of Genes and Genomes; http://www.genome.jp/kegg/) database was employed (31). This database uses systematic analysis for understanding high-level gene functions and their utilities within a biological system context. Additionally, to better comprehend the potential implications of the newly identified miRNA seeds at the subcellular level, the TFmiR target network database was utilized. TFmiR is a server for the examination of regulatory interactions between miRNAs, transcription factors and target genes linked to specific diseases (http://service.bioinformatik.uni-saarland.de/tfmir/); (32).

Results

Prediction of Pre-miRNAs within HPV Genomes Based on Sequence and Structure Alignment

In order to predict putative HPV pre-miRNAs from the low-risk HPV-6 and -11, or high-risk HPV-16, -18, -31, -33, -35, -45, -52 and -58 genomes, *in silico* analyses were performed. These analyses were accomplished by using an SVM-based software tool which has been successfully employed for human genome analysis and for predicting new viral miRNAs sequences (7,33).

The initial approach was performed to identify the typical stem-loop structures within the HPV genomes, which are distinctive structures of the well-known pre-miRNAs (8). Approximately 200 potential stem-loop sequences were detected. Moreover, according to MiRscan program analyses, there were not any similarities between these stem-loop sequences and the pre-miRNAs already described, suggesting the presence of novel potential HPV-specific sequences. Applying a more stringent criterion to redefine the stem-loop sequences obtained, 19 of them were selected as potential pre-miRNA candidates (Table 1; structure-sequence features are shown in Figure 2).

The number of pre-miRNA candidates found within each of the HPV genomes was variable. For instance, the HPV-6 and some high-risk (members of clade A9) HPV-31, -33, -35, and -58 presented one or two candidates, whereas other high-risk (members of clade A7) HPV-18, -45, and HPV-16 (from clade A9) showed three or more pre-miRNAs candidates per genome. Interestingly, HPV-11 and -52 did not present any putative candidate (Table 1). In general, the pre-miRNA candidates were mapped within all HPV viral genes, however, an enrichment of candidates in E1 gene was observed. Furthermore, the hpv-pre-miRNA candidates that were localized inside E1, E6 and L genes, were also found in different positions within the gene sequences, suggesting that none of them shared pre-miRNAs sequences (data not shown).

Table 1. Profiling of pre-miRNAs from low to high-risk Human Papillomaviruses

HPV accession number	Start nt	End nt	Length nts	HPV type	CLADE	ΔG value	miRNA nomenclature candidate
X00203	5056	5122	67	6	A10	−29.5	hpv6-pre-miRNAs5056
	6823	6882	60	6	A10	−18.6	hpv6-pre-miRNAs6823
M14119	-	-	-	11	A10	-	-
AY686579	552[a]	599	48	16	A9	−12.6	hpv16-pre-miRNAs552
X05015	1574	1633	60	18	A7	−13.3	hpv18-pre-miRNAs1574
	6261	6353	93	18	A7	−28.2	hpv18-pre-miRNAs6261
	7318	7373	56	18	A7	−21.9	hpv18-pre-miRNAs7318
J04353	5750	5835	86	31	A9	−24.4	hpv31-pre-miRNAs5750
M12732	1136	1200	65	33	A9	−23	hpv33-pre-miRNAs1136
X74477	1528	1590	63	35	A9	−22.7	hpv35-pre-miRNAs1528
X74479	401	473	73	45	A7	−19.87	hpv45-pre-miRNAs401
	1979	2034	56	45	A7	−18.2	hpv45-pre-miRNAs1979
	4880	4923	44	45	A7	−29.9	hpv45-pre-miRNAs4880
	6266	6334	69	45	A7	−21.1	hpv45-pre-miRNAs6266
X74481	-	-	-	52	A9	-	-
D90400	3526	3580	55	58	A9	−17.7	hpv58-pre-miRNAs3526
AF402678	552[a]	599	48	16	A9	−12.6	hpv16-pre-miRNAs552
AY686580	2644[b]	2708	65	16	A9	−19.8	hpv16-pre-miRNAs2644
	3682	3733	52	16	A9	−16.8	hpv16-pre-miRNAs3682
AF472508	552[a]	599	48	16	A9	−12.6	hpv16-pre-miRNAs552
	2644[b]	2708	65	16	A9	−19.8	hpv16-pre-miRNAs2644

[a]Pre-miRNA of HPV16.

[b]Pre-miRNA of HPV16 European and African variants; nt = nucleotide, whereas nts = nucleotides; ΔG value = free Gibbs energy physicochemical value. HPV-6 and 11 are low-risk HPV types, whereas the remaining HPV-16, 18, 31, 33, 45, 52 and 58 are high-risk HPV types.

>sl_f_5056_5122_X00203
CCUGUUCCUGGUACUGCACCUCGGCCUCGUGUGGGCCUAUAUAGUCGUGCAUUGCACCAGGUGCAGG
(((((.((((((.((((((..((((((.....)))))).....)..)))))..).)))))).)))))

>sl_f_6823_6882_X00203
UACAUCUUCCACAUACACCAAUUCUGAUUAUAAAGAGUACAUGCGUCAUGUGGAAGAGUA
(((.(((((((((((. (..(((((........))))).....).)).)))))))))))))

>sl_f_552_599_AY686579
AGCUGUAAUCAUGCAUGGAGAUACACCUACAUUGCAUGAAUAUAUGUU
(((((((.(((((((((. ((......)).)).)))))))).)))).)))

>sl_f_1574_1633_X05015
ACAUAUGGGCUAUCAUUUACAGAUUUAGUUAGAAAUUUUAAAAGUGAUAAAACCACGUGU
(((..(((.(((((((((..((((((.....))))))...)))))))...)))..)))

>sl_f_6261_6353_X05015
AAAUGUGAGGUACCAUUGGAUAUUUGUCAGUCUAUUUGUAAAUAUCCUGAUUAUUUACAAAUGUCUGCAGAUCCUUAU
GGGGAUUCCAUGUUU
(((((((.. ((.((((.((((...((.(((.(.((((((((((((.....)))))))))))).)))))).))))..))))..)).)))))))

>sl_f_7318_7373_X05015
GCCCUAGUGAGUAACAACUGUAUUUGUGUUUGUGGUAUGGGUGUUGCUUGUUGGGC
((((.((..(((((((.(((((((.........)))))))..)))))))..))))))

>sl_f_5750_5835_J04353
UCAGGUUACAAUAUAGGGUAUUUAGGGUUCGUUUACCAGAUCCAAACAAAUUUGGAUUUCCUGAUACAUCUUUUUAUA
AUCCUGA
(((((((((.((...(((((((((.((((...........(((((((....)))))))))))))))))).)))..)).)))))))))

>sl_f_1136_1200_M12732
ACGAAAGUUUGCCGCAUGUUCACAAAGUGCUGCGGAGGACGUUGUUGAUCGUGCUGCAAACCCGU
(((...((((((.(((((.((((((.((.((....)).)).))).))).)))))).)))))).)))

>sl_f_1528_1590_X74477
GAUUGGUGUGUGGCCGCAUUUGGAAUAGCCCAAGUGUGGCGGAAAGUUUAAAAACAUUAAUU
(((((((((.(. ((((((((((.......))))))))))).)..........)))))))))

>sl_f_401_473_X74479
UUUGUUAAUAAGGUGCCUGCGGUGCCAGAAACCAUUGAACCCAGCAGAAAAACGUAGACACCUUAAGGACAAA
((((((..(((((((.(((((.(((.................))).....)))))).)))))))..))))))

>sl_f_1979_2034_X74479
UGGGCAUUUGAUAAUGACCUUACAGAUGAAAGUGAUAUGGCAUUUCAAUAUGCCCA
((((((((((.((((.((((((........)))).))).)))))))))).)))))))

>sl_f_4880_4923_X74479
CCCUACUGUGCGGCGGGUACGGGGUCCCCGCCUGUAUAGUAGGG
(((((((((((((((((.((...)).)))))).)))))))))))))

>sl_f_6266_6334_X74479
AGGUUCCAUUAGACAUUUGUCAAUCCAUCUGUAAAUAUCCAGAUUAUUUGCAAAUGUCUGCUGAUCCCU
(((...((.((((((((((.(((...((((((........)))))).)))))))))))))))).)))...)))

>sl_f_3526_3580_D90400
AGGACUACACAGUACAACUAACUGUACAUACAAAGGGCGGAACGUGUGUAGUUCU
((((((((((((.(..((...(((....))))...)).).)).)))))))))))

>sl_f_552_599_AF402678
AGCUGUAAUCAUGCAUGGAGAUACACCUACAUUGCAUGAAUAUAUGUU
(((((((.(((((((((.((......)).)).)))))))).)))).)))

>sl_f_2644_2708_AY686580
UUUGACGAAAACGGAAAUCCAGUGUAUGAGCUUAAUGAUAAGAACUGGAAAUCCUUUUUCUCAAG
(((((.((((((.(((..((((((.(((.(......).)))...)))))))..)))).)))))))))))

>sl_f_3682_3733_AY686580
UACAUUGUAUACUGCAGUGUCGUCUACAUGGCAUUGGACAGGACAUAAUGUA
(((((((((...((((((((((((....))))))))))..)))...)))))))))

>sl_f_552_599_AF472508
AGCUGUAAUCAUGCAUGGAGAUACACCUACAUUGCAUGAAUAUAUGUU
(((((((.(((((((((.((......)).)).)))))))).)))).)))

>sl_f_2644_2708_AF472580
UUUGACGAAAACGGAAAUCCAGUGUAUGAGCUUAAUGAUAAGAACUGGAAAUCCUUUUUCUCAAG
(((((.((((((.(((..((((((.(((.(......).)))...)))))))..)))).)))))))))))

Figure 2. Representation of local structure-sequence features by using the dot-bracket notation of the predicted pre-miRNAs from HPV genomes. The triplet unit "…" denotes the stacking of paired bases and the unit "…" means the interior or bugle loops. The triplet element is comprised of the 3 adjacent sub-structures and the nucleotide type at the middle. Thus, these triplet elements replicate information of the local contiguous fine-structures and the nucleotide sequence composition. Also, as an example, the nomenclature description of the putative pre-miRNA f_5056_5122_X00203 is as follows: f, indicate forward direction; 5056, is the initial nucleotide, whereas, 5122 is the end nucleotide, and X00203 is the accession number of the HPV type in NCBI. Notice that all the included pre-miRNA sequences in this figure are in the forward direction.

Looking for Cellular miRNAs into HPV-16-pre-miRNA Candidates

As mentioned before, HPV-16 is the most studied HPV type, belonging to a big family of viruses (23,24). By means of the microRNA database, miRBase.org annotation tool, a detailed analysis was applied to identify cellular miRNAs (previously identified miRNAs in different species) into hpv-16-pre-miRNA candidate sequences. The following predicted hpv-16-pre-miRNAs were included in this analysis: pre-miRNA 522, 2644 and 3682; as well as the following HPV-16 sequence variants; Asian-American 1 (As-Am), African 1, African 2, and European (E).

More than 100 cellular miRNA candidates were found; however, only 14 corresponded to *Homo sapiens* miRNA sequences (hsa-miRNAs), exhibiting from 50–75% of homology (Figure 3A), being the hsa-mir-487a the only one candidate with a different precursor. We noticed that some of these potential hpv-miRNAs were located at the same position within the pre-miRNA stem-loop structure, suggesting possible clusters (Figure 3B). To identify if those hpv-miRNA candidates are evolutionarily conserved between species, we sought for orthologous sequences. The hsa-mir 15a, hsa-mir 107 and hsa-mir 146 a were highly conserved, presenting 20, 19 and 10 orthologue species, respectively (Supplementary Table 1). Interestingly, hsa-mir 548 g did not present any orthologous sequence (Supplementary Table 1). Moreover, according to the stem-loop secondary structure of the pre-miRNAs, the majority of these candidates were located in the stem structure, while mir-620 and mir-590-5p were found in the loop motif (Figure 3B). Taken together these results indicate that hpv-16-pre-miRNAs could harbor previously known cellular miRNAs.

Identification of Potential Gene Targets for HPV-16-miRNA Candidates

To identify potential HPV miRNA target genes that could be involved in cervical cancer, the hpv-16-miRNA candidates were subjected to analysis by using the miRBase target database and TargetScanHuman software. Around 900 target genes were obtained for each miRNA candidate, from which interestingly, 28 were normally expressed in cervical cells (Supplementary Table 2). In contrast, most of the targets were found to participate in cell proliferation, translation, cellular homeostasis, cell cycle, immune response, among others (Supplementary Figure 1).

Searching for miRNA Seed Sequences

Here we aimed to identify miRNA seed regions inside the sequences of the predicted pre-miRNAs, as well as in the HPV-6, -11, -16, -18, -31, -33, -35, -45, -52, and -58 whole genome sequences by using the miRBase database. As expected, we found that all the HPV genomes harbor miRNA seed sequences. For each HPV genome, approximately 82 ± 6 seeds were found (data not shown). Altogether, 952 seeds were identified, from these, 680 (71.4%) were orthologous sequences, while the 272 (28.5%) remaining sequences corresponded to *H. sapiens*. Among them, 160 were present only in high-risk HPVs, while 100 were shared between high and low-risk HPVs, and only 12 were found in low-risk HPVs (Figure 4). Interestingly, none of the seed sequences identified were present in all the HPV genomes studied. On the other hand, out of the 19 different hpv-pre-miRNA sequences analyzed (Table 1), only hpv-16-pre-miRNAs contained miRNA seed sequences (Supplementary Figure 4), whereas the rest of the HPV pre-miRNAs did not harbor any seeds, so that they could be considered as virus-specific sequences. Furthermore, the number of seeds inside the hpv-16-pre-miRNAs was variable with a single or multiple (as cluster) seeds within the stem or loop structure (Supplementary Figure 2).

In addition, we examined if the seed sequences found in the different HPV genomes could be shared between low and high-risk HPVs. For this analysis, the different HPV genotypes were grouped according to phylogenetic clade assignments (Supplementary Table 3). We observed that a specific group of seeds was related to a specific HPV clade. For example, the seed sequences of hsa-mir-568, hsa-mir-485-5p and hsa-mir-574-5p were members of the clade 9, which includes HPV-16, -33, -52, -58 types. Those miRNA seeds that were shared between clades were analyzed by using the TFmiR target network database to look for target genes being affected by them. TFmiR combines seven different methods for finding crucial genes interconnected with miRNAs and TF during pathological co-regulatory networks and can serve as disease-specific drug targets for potential therapeutic interventions. Interestingly, clear differences on these were seen among clades, groups of at least two seeds were shared among each clade. Remarkably, the clade A7 displayed a large number of target genes, many of these including transcription factors (blue boxes Supplementary Figure 3), suggesting an extremely complex mechanism of regulation. In contrast, clade A10 showed only a few targets, being also most of the transcription factors (Supplementary Figure 3). These findings suggest that each HPV clade has their own gene regulation pathway, as well as different molecular mechanisms of action.

HPV-miRNA Candidates Target Genes and Their potential Mechanisms of Carcinogenesis

Due to that the hpv16-miRNA candidates; 297, -27b, -500, -501-5b and -509-3-5p, are located between the oncogenes E6 and E7, and intercalated among the early and late splicing regions of the HPV-16 virus (Figure 5), they were subjected to analysis *via* chipBase tool to identify their

A

Pre-miRNA	# of cellular miRNAs	*Homo sapiens* miRNAs	% of homology	Total # of target genes	Location of miRNAs	Orthology
E2, A2	35	hsa-mir-570	55	775	stem	2
		hsa-mir-146b-5p	60	941	stem	2
		hsa-mir-146[a]	65	992	stem	10
		hsa-mir-487[a]	60	991	stem	5
		hsa-mir-590-5p	60	1148	hairpin	3
		hsa-mir-548f	65	0	stem	1
		hsa-mir-548g	70	0	stem	0
		hsa-mir-145*	60	831	stem	2
E1	70	hsa-mir-107	55	1004	stem	19
		hsa-mir-103	55	1040	stem	11
		hsa-mir-575	50	1023	stem	1
		hsa-mir-15[a]	55	1112	stem	20
VE, AA, A1	10	hsa-mir-48[a]	75	991	stem	5
		hsa-mir-620	65	896	hairpin	0

B

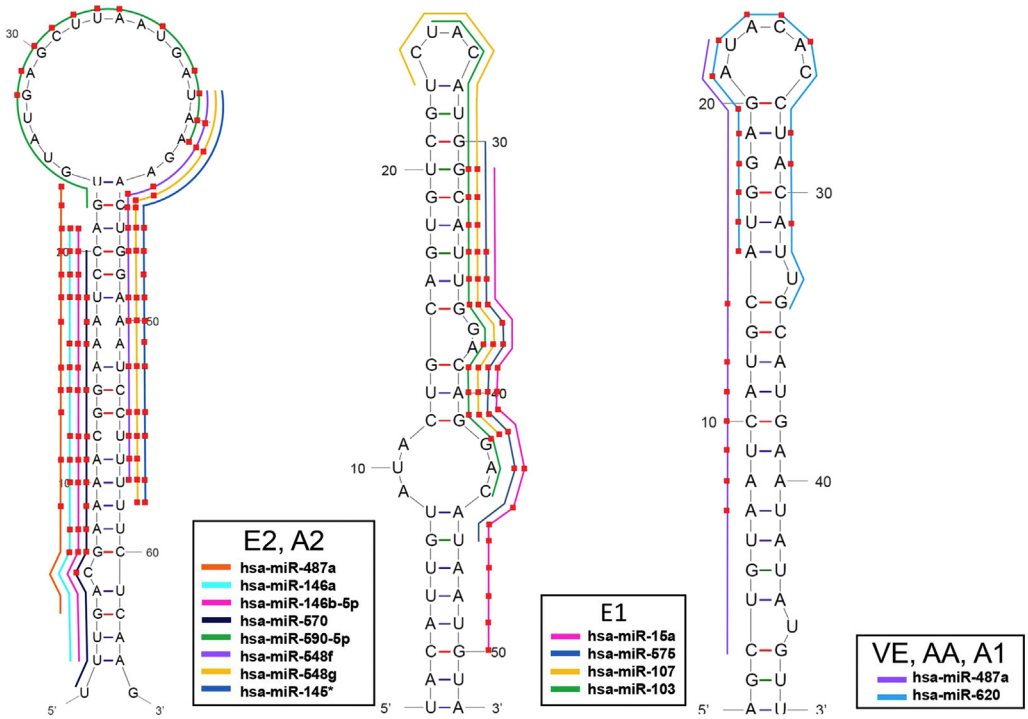

Figure 3. Description and structural feature illustrations of HPV-16 variants and their miRNA candidates. (A) Description of the HPV-16 miRNA candidates. Fourteen well-known *Homo sapiens* (hsa) miRNAs that presented homology to pre-miRNA sequences from HPV-16 are annotated and categorized as HPV-16 miRNA candidates. Homology percentage, number of targeted genes, location within the pre-miRNA structure and orthologues are included. (B) Putative miRNAs positions and homology within the pre-miRNA stem-loop structures are depicted with solid color lines and red squares, respectively.

potential target genes (Supplementary Table 4). Subsequently, the KEGG software tool was employed to identify the biological function of the predicted hpv-miRNA targets. Remarkably, some of these genes were found to be involved in the longevity-regulating pathway (data not shown). And interestingly, stimulation of the longevity pathway could avoid cell death, favoring cell cycle activation, resulting in cell survival, which could contribute to cancer development. Overall, the target genes of the aforementioned miRNA candidates were predicted to be implicated in cell survival, proliferation, metastasis, apoptosis evasion, tissue invasion, and cell-cycle transition.

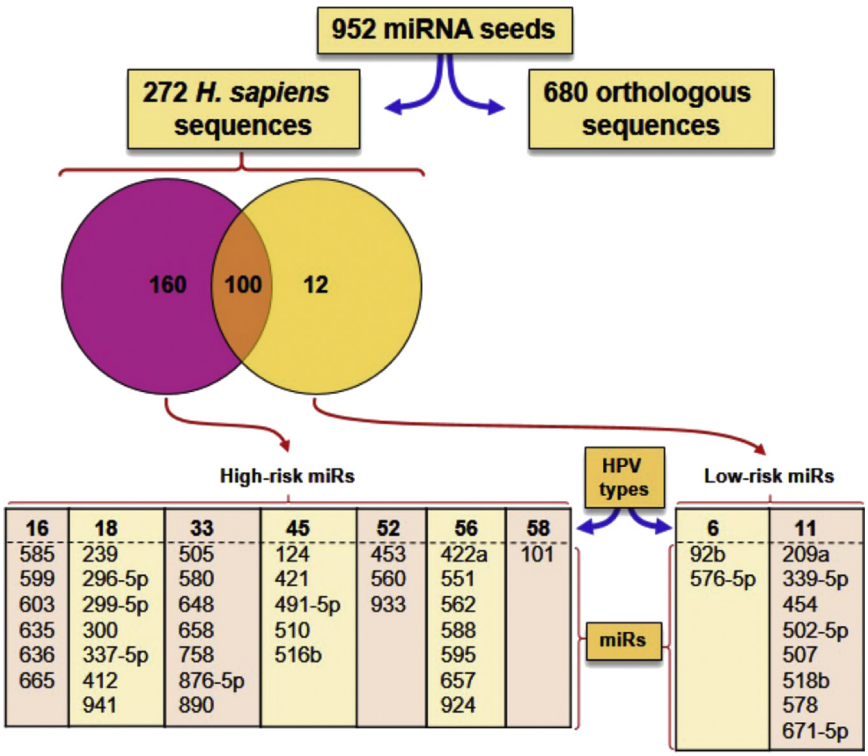

Figure 4. Predicting the existence of miRNAs seeds into HPV-6, -11, -16, -18, -33, -45, -52, -56 and -58 genomes and their homology with well-defined *Homo sapiens* miRNA sequences. The Venn diagram is displaying the total number (272) of seed sequences within the predicted pre-miRNAs, which has homology with *Homo sapiens* miRNA seed sequences. The magenta circle corresponds to pre-miRNAs inside of high-risk HPV, the overlapping orange portion of the diagram embrace pre-miRNAs sequences shared between high and low-risk HPVs, whereas the yellow circle represents the seeds sequences present inside of low-risk HPV. The boxes on the bottom are representative of miRNAs seeds inside of high and low-risk HPV types.

Discussion

Although numerous HPV genomes have been sequenced, information of their miRNA profile content is elusive. In this study, ten HPV genome sequences posted in the National Center for Biotechnology Information (NCBI) were used as templates to discover novel miRNAs based on sequence alignment strategy and bioinformatics tools.

HPV is one of the main contagious causative agents of sexually transmitted disease provoking the development of cervical lesions, which consequently progress to cervical cancer, among others health disorders (34–38); being the leading origin of death among females worldwide (24,38). Additionally, HPV afflicts indistinctly both men and women (39). Epidemiological evidence concluded that nearly 70% of the cases of cervical cancers could be attributed to both HPV-16 (~60%) and HPV-18 (~10%) viruses (24,35). Some others HPV types, like HPV-31, -33, -35, -45, -52, and -58 are also capable to lead cell transformation, while HPV-6 and -11 cause benign epithelial proliferation (37,38).

In the past, several miRNAs have been detected in a variety of viruses such as Herpesvirus, Polyomavirus, HIV, and Adenovirus, indicating that both DNA and RNA viruses contain miRNA sequences (12–14,40). Therefore, HPV viruses might not be the exception for this occurrence. In the present study, we show *in silico* evidence that suggests that HPV harbor several miRNAs that according to their predicted target genes, could favor the development of cervical cancer, however, experimental data is necessary for their validation.

Nowadays, computational and bioinformatics free-available analysis programs provide powerful tools to predict pre-miRNAs in the genomes, which importantly have contributed to the discovery of novel miRNAs (6,10,25,26). The support vector machine (SVM) software applies a set of features based on secondary structures to classify real and pseudo pre-miRNAs, attaining about 90% of accuracy on human data (7).

Here we identified 19 putative pre-miRNA candidates within HPV genomes (Table 1). Furthermore, by means of comparative genomics, we found several human miRNAs that presented high homology to the hpv-pre-miRNA predicted sequences from different HPV types and variants, which we named ‘‘hpv-miRNA candidates’’. A large number of target genes were also identified for each potential candidate, which were found to be involved in important biological functions, such as those that are normally expressed in cervical tissue (Supplementary Table 2).

In the past, *in-silico* evidence has suggested that viral miRNAs could act as potential oncogenes that could lead

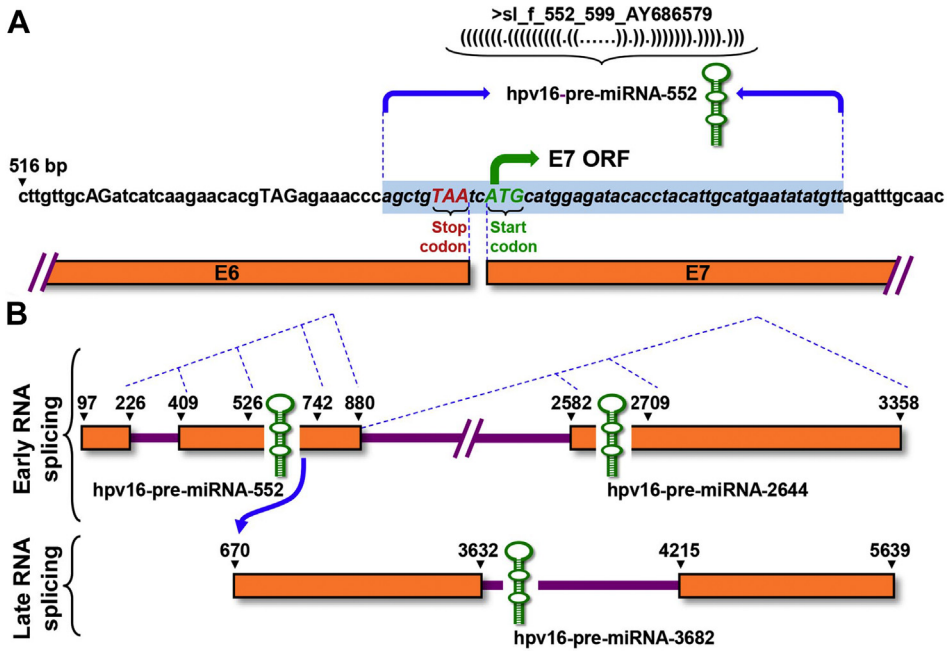

Figure 5. Potential mechanism of splicing associated with the E6* and E7* oncoprotein formation, implicating a predicted pre-miRNA (sl_f_552-599-AY686579) from HPV-16 genome. (A) The sequence and location of the hpv16-pre-miRNA-552 are depicted between the adjacent E6 and E7 oncogenes; the predicted hpv16-pre-miRNA-552 sequence is displayed with italic font and blue background; the E6 stop codon (red font) and the E7 start codon (green font) are indicated with capital letters. (B) Illustrations of the early and late splicing mechanism. The rectangular orange boxes represent exons, whereas the purple lines between them indicate the viral intronic region; the dotted blue lines specify the splice sites and the nucleotide numbers indicate their positions on the genomic map. The positions of hpv16-pre-miRNA-552, hpv16-pre-miRNA-2644, and hpv16-pre-miRNA-3682 are intercalated into the genomics map of both early and late virus regions (in green).

to the development of cancer (41−44). For example; target genes that were predicted in the Epstein-Barr virus for miR-BHRF1-1 and miR-BART-1 include the tumor suppressor p53 and Bcl-2, respectively (41). The first is mutated in 50% of human tumors (45), and the last is well known to be a cell death regulator, either as a pro or anti-apoptotic inducer (46). Despite the *in-silico* evidence found in these analyses, experimental data is needed to corroborate the contribution of viral miRNAs in cancer development.

The seed region of a miRNA is a highly conserved sequence, a short stretch of six to eight nucleotides, crucial for binding to its mRNA target. Moreover, even if the whole sequence of a miRNA does not match perfectly to its target, the base-pairing interactions of the seed region to its mRNA have to be exactly the Watson and Crick (G:C and A:U) complementary (2,15,16,47). In this study we identified a total of 952 miRNA-like seeds sequences in all the HPV genomes studied (Figure 4), those sequences were orthologous in different species including human. The fact that miRNA seed regions are highly conserved phylogenetically, suggests that they have evolved jumping from one species to another, establishing alternative gene regulation patterns, which could also be the case for HPV. Moreover, these miRNA seed regions, together with their miRNAs, could be sharing similar roles and mechanisms of action in cellular processes across different species.

Based on the identified seed sequences we generated HPV-miRNA regulatory-network maps, focusing on seeds affecting both transcriptional factors (TF) and gene expression (Supplementary Figure 3). The TF and target genes altered by HPV miRNA seeds varied for each particular clade. For instance, hsa-miRNA-640, hsa-miR-574-5p, hsa-miR-938 and hsa-miR-625 appeared to regulate mainly TF (blue boxes; Supplementary Figure 3); whereas hsa-miR-367 and hsa-miR-568 mainly affected mRNA gene expression (yellow boxes; Supplementary Figure 3). However, hsa-miR-24-1 and hsa-miR-568 increase a balanced number of TF and mRNA targets. Additionally, the total number of targets modified by each individual miRNA seed was variable: hsa-miR-367 and hsa-miR-485-5p perturbed the lowest and biggest number of targets, respectively (Supplementary Figure 3). These results propose that the newly identified miRNA seeds could act as regulatory molecules, modulating TF and/or mRNA targets in intricated cellular biochemical networks.

Other interesting miRNA-related seed sequences found in this study are HPV-16 hsa-miR-27b, hsa-miR-hsa-500, hsa-miR-501-5p and hsa-miR-509-3-5p, which were found to be situated within the E6 and E7 oncogenes, into the splice sites. This data correlates with Weng SL, et al. (2017), where a pre-miRNA was found inside the E6 open reading frame (ORFs) within the HPV-16 genome (48).

Interestingly, hsa-miR-27b, hsa-miR-500, have been described in cervical cancer cells (49,50).

Our group recently reported that several already known miRNAs seeds from Epstein Barr, Cytomegalovirus, Kaposi's sarcoma viruses were also contained within the HPV genomes, suggesting that these viruses could share similar post-transcriptional regulation strategies (51). For example, the viral oncogenic proteins from Polyomavirus (Large T antigens) and the viral proteins E6/E7 from HPV can form protein complexes with p53 or pRb (52,53). In this theoretical context, the possibility that the presence of human miRNAs seeds within HPV genomes is a co-evolutionary event among several viruses should be considered. In addition, some of the HPV-16 variants that were analyzed shared a consensus miRNA sequence (AY686579 prototype, AA variant AF402678, and Af variant AF472508), these not only indicate that these sequences are highly conserved, but also that those variants with consensus miRNA sequences could share molecular mechanisms of genetic regulation. It is clear that HPV has extremely complex regulation machinery, even among their variants and evidently among types and clades. Thus, the risk factors for HPV infection are difficult to control, due to a combination of serious and unexpected challenges to mitigate its pathogenic effects.

Currently, it is well known that the viral oncoproteins E6 and E7 inactivate p53 and pRb cellular regulatory proteins, respectively, promoting carcinogenesis (53). However, the role of the potential HPV miRNAs might be another important gene regulation mechanism. Based on the miRNAs found for each HPV genome, and the important biological role of the predicted target genes we postulated that the HPV oncogenicity could also be related to those miRNAs. Moreover, we did not detect potential miRNA sequences shared between the different types of HPV genomes. This heterogeneity suggests that each HPV type could have their own ''oncogenicity pathway'' distinct from the classic E6 and E7 roles. This suggestion can be supported by the differences found in clinical lesions, where HPV-18 is associated with a more aggressive cervical cancer than any other HPV viral type (35).

Additionally, studies based on chromosomal imbalances, microarrays and Serial Analysis of Gene Expression have revealed that HPV lesions in cervical tissue exhibited great heterogeneity, without sharing an altered gene as a molecular marker (49,54,55). Altogether, these differences could represent several pathways linked to cervical cancer development. However, we should not discard the possibility that different viral loads or multiple HPV infections, on tested samples, possess important additional implication (56). Thus, all these molecular events will point out a more complex phenomenon of HPV carcinogenesis. Several reports have indicated that the E6 oncogene transcriptional process generates by splicing more than five E6* isoforms, which modulate the E6-mediated degradation of p53 during viral replication (57–60), which predispose HPV-infected cells to genetic changes that can lead to malignant conversion. It has also been reported that the E6 oncoprotein alone is sufficient to provoke carcinomas in transgenic animals (61).

In this study, we present *in silico* evidence of the intronic presence of hpv-miRNAs within of E6 spliced region, and we hypothesize that the HPV-16 intronic putative pre-miRNAs can be ''eliminated by splicing'' to form the E6* isoforms. In this context, the proposed hpv16-pre-miRNA-552 intronic sequence could release miRNA-297 during the E6 and E7 splicing process. Interestingly, it has been documented that miRNA-297 down-regulates the multidrug resistance protein 2 (MRP-2) expression, sensitizing colorectal carcinoma cells to anti-cancer drugs (62), and also, probably acting as a tumor suppressor decreasing the survival of glioblastoma cells (63). Moreover, miRNA-297 decreases the androgen receptor expression reducing androgen-induced proliferation in prostate cancer suggesting the use of miRNA-297 as a drug in prostate cancer therapy (64). Thus, further studies to validate the presence of miRNA-297 in HPV-16 could benefit our knowledge in understanding the mechanisms implicated in carcinogenesis and most importantly could provide a potential target for therapies against HPV-16-infected individuals. However, experimental studies still await to corroborate this proposed hypothesis.

In summary, we provide evidence *via* computational prediction of the existence of new viral miRNAs within HPV genomes, as a specific type of clusters. Also, a large number of target genes with relevant biological functions were identified for each miRNA candidate. Some of the predicted miRNAs were found to be originated from their target genes *via* self-splicing intron sequences. Furthermore, we propose that the putative miR-297, miR-27b, miR-500, miR-501-5, and miR-509-3-5p from HPV-16 could be implicated in a new HPV-associated mechanism of carcinogenesis. However, experimental validation of these miRNAs candidates still needs to be accomplished. Moreover, the new miRNAs molecules could be used as potential therapeutic targets against HPV-induced malignancies. Additionally, the novel HPV miRNAs could be also used for diagnostic purposes, as risk factors markers, as well as predictors of the clinical evolution of the diseases inflicted by the HPV family.

Competing Interests

The authors have declared that no competing interests exist.

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Supplementary Data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.arcmed.2018.10.008.

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