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Authors: Maja Cokarić Brdovčak, Andreja Zubković, Antun Ferenčić, Ivan Šoša, Valter Stemberga, Dražen Cuculić, Filip Rokić, Oliver Vugrek, Michael Hackenberg, Igor Jurak

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Herpes simplex virus 1 miRNA sequence variations in latently infected human trigeminal ganglia

Maja Cokarić Brdovčak[a], Andreja Zubković[a], Antun Ferenčić[b], Ivan Šoša[b], Valter Stemberga[b], Dražen Cuculić[b], Filip Rokić[c], Oliver Vugrek[c], Michael Hackenberg[d], Igor Jurak[a]

[a]Department of Biotechnology, University of Rijeka, Radmile Matejčić 2, HR-51000 Rijeka, Croatia

[b]Department of Forensic Medicine and Criminalistics, University of Rijeka, Braće Branchetta 20, HR-51000 Rijeka, Croatia

[c]Laboratory for advanced genomics, Ruđer Bošković Institute, Bijenička cesta 52, HR-10000 Zagreb, Croatia

[d]Department of genetics, University of Granada, Campus de Fuentenueva s/n, ESP-18071 Granada, Spain

Corresponding author:

Igor Jurak
Department of Biotechnology, University of Rijeka, Radmile Matejčić 2, HR-51000 Rijeka, Croatia
igor.jurak@biotech.uniri.hr

Highlights

- - we analyzed human trigeminal ganglia infected with HSV-1
- only a small subset of HSV-1 miRNAs is expressed in latently infected human TGs

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- we revised the sequences of HSV-1 miRNAs
- HSV-1 expresses a high proportion of isomiRs
- No evidence for Varicella Zoster Virus miRNAs

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Abstract

Human herpes simplex virus 1 (HSV-1) expresses numerous miRNAs, the function of which is not well understood. Several qualitative and quantitative analyses of HSV-1 miRNAs have been performed on infected cells in culture and animal models, however, there is very limited knowledge of their expression in human samples. We sequenced small-RNA libraries of RNA derived from human trigeminal ganglia latently infected with HSV-1 and Varicella zoster virus (VZV) and detected only a small subset of HSV-1 miRNA. The most abundantly expressed miRNAs are miR-H2, miRNA that regulates the expression of immediate early gene ICP0, and miR-H3 and –H4, both miRNAs expressed antisense to the transcript encoding the major neurovirulence factor ICP34.5. The sequence of many HSV-1 miRNAs detected in human samples was different from the sequences deposited in miRBase, which might significantly affect targeted functional analyses.

Keywords: Herpes simplex virus 1; miRNAs; latency; human trigeminal ganglia; sequence variation

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1. Introduction

Herpes simplex virus 1 (HSV-1) is an important human pathogen widely recognized as the causative agent of cold sores (Roizman et al., 2013). HSV-1, in addition to self-limiting and clinically minor infections, can cause severe and life-threatening diseases like keratoconjunctivitis and encephalitis (Whitley and Roizman, 2017). Interestingly, it has been observed that throughout the last decades that the seroprevalence of HSV-1 is significantly decreasing in many developed countries, which coincides with the increased frequency of genital HSV-1 infections (Haddow et al., 2006; Roberts et al., 2003; Wald, 2006; Whitley and Roizman, 2017).

HSV-1, like other herpesviruses, replicates in two distinct phases, productive and latent. During the productive (lytic) phase the virus expresses its genes, immensely alters metabolism and inhibits defense mechanisms of infected cells resulting in new virus progeny and spread. Subsequently, the virus gains access to nearby peripheral ganglia in which it will establish a life-long latent infection (latency), and from where it can reactivate and cause recurrent diseases (Lee et al., 2015; Roizman et al., 2013). The major reservoir of the latent HSV-1 genomes is trigeminal ganglia (TG), which also frequently harbor varicella zoster virus (VZV), another neurotropic alphaherpesvirus (Croen et al., 1987; Mahalingam et al., 1992; Richter et al., 2009).

The molecular mechanisms that regulate establishment, maintenance and reactivation of HSV-1 from latency are still poorly understood. Nonetheless, it is well established that, in contrast to productive infection, during latency the virus genome is largely associated with heterochromatin and the expression of virus genes is broadly suppressed (reviewed in: (Bloom et al., 2010; Knipe and Cliffe, 2008; Nicoll et al., 2012)). The only abundantly expressed transcripts detected in latently infected neurons are multiple long non-coding RNAs arising from the latency-associated transcripts (LATs) locus, including the primary 8.3-kb LAT transcript (reviewed in (Bloom, 2004; Kent et al., 2003)). This transcript, also called the minor LAT, is rapidly spliced giving rise to remarkably stable 2-kb and 1,5-kb introns (Brinkman et al., 2013; Farrell et al., 1991; Zabolotny et al., 1997). The exact roles of LATs are not yet determined, however, several biological functions have been assigned to them including, repression of the productive gene expression (Chen et al., 1997; Garber et al., 1997), heterochromatin modulation (Cliffe et al., 2009; Kwiatkowski et al., 2009; Wang et al., 2005) and inhibition of cell death (Perng et al., 2000; Thompson and

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Sawtell, 2001). Recently, a number of HSV-1 miRNAs, small regulatory non-coding RNAs, encoded by HSV-1 have been discovered in productively and/or latently infected cells. In fact, HSV-1 has been shown to encode at least 20 miRNAs (miR-H1 – H29), some of which are conserved in HSV-2 (Han et al., 2016; Jurak et al., 2010; Munson and Burch, 2012; Umbach et al., 2008; Umbach et al., 2009). The genomic loci of most of these miRNAs are located within the inverted repeat of the genome and within the LAT locus (Jurak et al., 2011). During latency the expression of these miRNAs strongly depends on the activity of the LAT promoter (LAP), indicating that the 8.3kb transcript is also the primary transcript for the biogenesis of several miRNAs (Kramer et al., 2011). This might also explain the high instability of the minor LAT. The roles of HSV-1 encoded miRNAs are largely unknown, however for some miRNAs the genomic location strongly indicates their function. For example, miR-H2 is expressed antisense to ICP0, an important productive phase viral protein, and thus complementary to its mRNA (Umbach et al., 2008). Similarly, miR-H3 and –H4 are expressed antisense to ICP34.5, the main HSV-1 neurovirulence factor (Jurak et al., 2010; Umbach et al., 2008). The potential of miR-H2, H3 and H4 to regulate ICP0 and ICP34.5, respectively have been experimentally confirmed (Flores et al., 2013; Pan et al., 2017; Tang et al., 2008; Tang et al., 2009; Umbach et al., 2008), however the exact biological importance of this regulation is still puzzling. In addition, miR-H6, a miRNA that is transcribed from a locus upstream of the LAP and frequently detected in latently infected mouse ganglia, has been proposed to target another important immediate early gene ICP4 (Umbach et al., 2008). On the other hand, miR-H8 has been found to target phosphatidylinositol glycan anchor biosynthesis class T (PIGT), a host protein and a member of the complex that covalently attaches proteins to glycosylphosphatydilnositol to counteract innate defense mechanisms (Enk et al., 2016). Important to note, miRNAs recognize their targets through sequence complementarity binding, generally to the 3' untranslated region (3'-UTR) of mRNA, resulting in translational repression and mRNA degradation leading thus to reduced levels of proteins (reviewed in (Bartel, 2009; Bartel, 2018)). The nucleotides 2-8, called seed sequence, are the major determinants for miRNA target specificity, and even a single nucleotide polymorphism (SNP) can significantly alter the function and the specificity of miRNAs (Gong et al., 2012; Haas et al., 2012). Indeed, it has been shown recently that Kaposi's Sarcoma-Associated Herpesvirus (KSHV) miRNA SNPs

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in clinical samples can affect miRNA processing, level of expression and its silencing activity (Han et al., 2013). Moreover, Sungawa et al. have analyzed Epstein-Barr Virus (EBV) miRNAs in different clinical samples of EBV-associated lymophomas and found variations in miRNA sequences (Sunagawa et al., 2017). Although there were several studies on HSV-1 miRNAs using common laboratory strains and different latency models, only one study comprehensively analyzed HSV-1 miRNAs in clinical samples (Umbach et al., 2009). Interestingly, in contrast to latency models in animals, Umbach et al. found only a few miRNAs expressed in human samples (miR-H2 – miR-H8).

We questioned whether HSV-1 miRNAs show sequence variations in clinical isolates, which might relate to differences between different strains or clinical isolates in virulence and pathogenesis. We performed a comprehensive pilot study on two latently infected human trigeminal ganglia using massive parallel sequencing detecting only a subset of HSV-1 miRNAs (miR-H2, - H3, -H4, -H6 and –H7). MiRNAs expressed by clinical HSV-1 isolates show low sequence variation when compared to common laboratory strains, however, we identified several sequence differences between our study and miRNAs sequences deposited in MiRBase and previous studies.

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2. Results and discussion

To analyze HSV-1 miRNAs expressed in relevant human samples we obtained trigeminal ganglia (TG) from two recently deceased humans. Briefly, the autopsy was performed within 24 hours of death during which the TGs were removed, snap frozen in liquid nitrogen, and stored at -80ºC. At the time of death, patients did not show any symptoms of HSV-1 infection (**Table 1**). Next, we extracted DNA and RNA from the samples and analyzed the presence of HSV-1, HSV-2 and VZV genomes using PCR assays specific for each virus (Hukkanen et al., 2000; Watzinger et al., 2004). We were able to confirm HSV-1 and VZV DNA in both samples; however, the samples were negative or below the sensitivity of the assay for the HSV-2 genome (**Fig. 1**). These results were expected since both, HSV-1 and VZV, usually reside within the TGs, and the prevalence of HSV-2 is relatively low in the Croatian population (Rode et al., 2008; Vilibic-Cavlek et al., 2011). In addition, we determined the number of HSV-1 genomes in the sample by RT-qPCR using HSV-1 strain KOS genome cloned as a bacterial artificial chromosome (BAC) as a standard (Jurak et al., 2012). Our results show a relatively low number of HSV-1 genomes in both samples (100 and 71 genomes per µg of total DNA; data not shown), which is comparable to previous reports (Cohrs et al., 2000; Pevenstein et al., 1999; Umbach et al., 2009). We were unable to quantify the exact copy number of VZV, probably due to a low number of VZV genomes and/or limited sensitivity of the assay. It is also possible that the exact number of viral genomes is somewhat underestimated.

Next, to analyze HSV-1 miRNAs expressed in human tissues, we generated small-RNA libraries from the extracted RNAs (TruSeq small RNA sample preparation kit, Illumina) and sequenced using MiniSeq System (Illumina) in the Laboratory for advanced genomics, Ruđer Bošković Institute. The sequencing data were processed using sRNAtoolbox, a collection of bioinformatics tools for small RNA research (Rueda et al., 2015) under standard stringency parameters (**Supp. Data**). Briefly, only the first 19 nt of the sequence reads were used to map the reads simultaneously against the HSV-1 strain 17 (NC_001806.2), HSV-2 strain HG52 (NC_001798.2), VZV strain Dumas (X04370.1) and human genome (reference genome GRCh38), allowing only 1 mismatch with the reference sequences. After the initial cut-off, we obtained 1,103,250 and 1,250,593 high quality reads from subject 1 and 2, respectively (detailed analysis of sequencing results in **Suppl. table 1**). As expected,

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the vast majority of recovered reads were human, and only a small fraction (362, and 217, sample 1 and 2 respectively) represented the HSV-1 specific reads (**Suppl. Table 1**). Within the virus-specific reads, we detected 114 reads representing known HSV-1 miRNA sequences published in miRBase for sample 1 (representing 31 % of all HSV-1 reads) and 84 reads for sample 2 (representing 39 % of all HSV-1 reads) (**Table 2 and Supp. Table 1**). Curiously, although a large number of HSV-1 miRNAs has been found expressed in cultured cells, and many of these were confirmed in animal models, (Du et al., 2011; Flores et al., 2013; Han et al., 2016; Jurak et al., 2014; Jurak et al., 2010; Kalamvoki et al., 2014; Kramer et al., 2011; Munson and Burch, 2012; Umbach et al., 2008), only a small subset of miRNAs has been detected in human tissue by Umbach et al (Held et al., 2011; Umbach et al., 2009). Likewise, we detected only five miRNAs, miR-H2, -H3, -H4, -H6 and miR-H7, characteristic to the latent HSV-1 program (Jurak et al., 2010; Umbach et al., 2008). The most abundant miRNAs were miR-H2-3p (25 and 22 reads for subject 1 and 2, respectively) and miR-H4-3p (55 and 44 reads for subject 1 and 2, respectively). These miRNAs are expressed antisense to ICP0 and ICP34.5, respectively and have been shown to regulate these genes ((Pan et al., 2017; Tang et al., 2008; Tang et al., 2009; Umbach et al., 2008), Jurak et al. unpublished data). Slightly less abundant were miR-H3, which is also expressed antisense to ICP34.5 (20 reads for subject 1 and 8 reads for subject 2) and miR-H7, expressed antisense to intron 1 of the ICP0 mRNA (8 reads for subject 1 and 3 reads for subject 2). The fact that, among the few miRNAs detected in human ganglia, multiple miRNAs target ICP0 and ICP34.5 strongly indicates the importance of these genes for the maintenance/reactivation process, which is yet to be experimentally confirmed. Notably, miR-H6, the miRNA which is abundantly expressed and readily detected during latency in experimental models (Du et al., 2011; Jurak et al., 2010; Umbach et al., 2008), was barely detectable in our analysis. The expression pattern of the selected HSV-1 miRNAs in TGs was also confirmed by stem-loop RT-qPCR assays (data not shown). Taken together, these results resemble the HSV-1 miRNA expression pattern in human ganglia obtained by Umbach et al, however, we did not detect miR-H5 and miR-H8, which were detected previously through a low number of reads. Furthermore, we observed strikingly different frequencies of 5p and 3p strands for some miRNAs compared to the previous report (e.g. in our analysis miR-H4-3p is the prevalent

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strand, whereas in Umbach et al. it is miR-H4-5p). We and others have observed that HSV-1, similar to other viruses, but in contrast to human miRNAs, shows reduced "strand bias", a phenomenon that only one strand (guide strand) of the miRNA duplex is retained and functional, whereas the other strand is rapidly degraded (passenger or star strand). This finding could be due to technical artifacts (different sequencing protocols) but also biological reasons are conceivable. For example, small differences in the pre-miRNA sequences between different strains can affect the miRNA biogenesis, as it has been shown for other herpesviruses and humans (Han et al., 2013; Sun et al., 2009; Sunagawa et al., 2017), or this finding might even reflect a resourcefulness of the virus to efficiently exploit its genome and to maximize the amount of encoded genetic information. There is a limited knowledge on the degree of mutations of miRNA sequences obtained from clinical isolates (Szpara et al., 2014), and to our knowledge, only one study has investigated the HSV-1 miRNAs expressed in human samples using sequencing (Umbach et al., 2009). To address this, we analyzed the sequences of 26 publicly available strains of HSV-1 and found that pre-miRNA (100 bp sequence spanning pre-miRNA) and mature miRNA sequences are remarkably conserved among all HSV-1 strains (i.e. 97-100% and 100% respectively; Zubkovic et al. not published). To further test this hypothesis we compared the sequences of the most abundant reads with the reference miRNA sequences from miRBase and the sequences recovered by Umbach et al. (**Table 3**). Of note, we typically observe a relatively high frequency of isomiRs (length and/or sequence variants of the canonical reference sequence) for some HSV-1 miRNAs compared to host miRNAs (**not shown and Supp. Table 2**). This phenomenon has been also observed for other viruses (Hooykaas et al., 2016; Walz et al., 2010). Especially 5' length variants will affect the seed sequence and therefore very likely impact the target recognition. Most miRNAs recovered in our analysis match the sequences in miRBase, however there were several exceptions. For example, 5' sequence of miR-H2-3p in our analysis matched the reference sequence but has one nucleotide shift compared to the sequence published by Umbach et al. On the other hand, the sequence of miR-H4-5p in our analysis is shifted by one nucleotide compared to both, miRBase and Umbach et al. Moreover, miR-H7 in one of the samples has one nucleotide difference to miRBase and Umbach et al, and also compared to the other sample in our analysis (**Table 3**). Of note, we have recovered

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the sequence reads identical to sequences of miRNAs in the miRBase or published by Umbach et al, but at much lower frequencies. We also observed fluctuations at the 3' end of HSV-1 miRNAs (**Table 3 and Supp. Table 2**.), namely differences in the terminal "U" which might also represent posttranscriptional modifications (Brennecke et al., 2005) affecting the stability of miRNAs, however it is less likely that it will affect the targeting of miRNAs. The molecular basis and biological significance of plausibly imprecise miRNA biogenesis is not clear.

In addition, we have observed relatively frequent A→G nucleotide transitions within the seed region of miR-H2-3p and several sporadic C→U transitions at the 3' end of other HSV-1 miRNAs (Supp. table 2). These sequence variations resemble well described adenosine-to-inosine editing of the miR-376 cluster in the human brain ((Kawahara et al., 2007) and Suppl. table 4), and might be, likewise, arising from the posttranscriptional RNA editing by ADAR (Adenosine deaminase acting on RNA) family members that mediate adenosine-to-inosine editing. There is a mounting body of evidence that posttranscriptional miRNA editing has an important role for their functions by broadening silencing targets and has been linked to several diseases (Choudhury et al., 2012; Paul et al., 2017; Pinto et al., 2018). Nonetheless, would the seed sequence alteration of miR-H2 change its selectivity for the ICP0 transcript is yet to be determined.

In contrast to most herpesviruses, VZV and its closely related simian varicella virus (SVV) have been analyzed but not found to encode miRNAs (Umbach et al., 2009; Umbach et al., 2010). Since we showed that both our samples contained latent VZV, we specifically looked for the VZV specific reads under the same stringency conditions that were applied for the HSV-1 analysis. We found only a small number of the virus-specific reads (i.e. 5 and 6 reads in sample 1 and 2, respectively; **Supp. Table 3**). Only one of these reads was 22 nt, the size-range of an average miRNA, but did not satisfy other criteria for a genuine miRNA (not shown). The other reads were 18 nt or excessively long (>35 nt). Furthermore, by applying the same stringency analysis but including a more broad size spectrum (i.e reads 15-36 nt) we found 436 and 327 reads in sample 1 and 2, respectively. Again, the vast majority of reads were only 15-17 nucleotides long indicating that these reads likely represent degradation products **(Supp. Figure 1)**. However, the precise alignment to the VZV genome was not possible due to shortness of the reads which aligned also to the

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human genome, and are thus likely of human origin (not shown). Recently, Marcus et al. have found multiple small RNA encoded by VZV in the VZV infected human fibroblasts in culture and human embryonic stem cell-derived (hESC) neurons, some of which are predicted to have stem-loop precursors potentially representing miRNAs (Markus et al., 2017). We have not recovered any of these sequences in our analysis, but we cannot exclude a possibility that our sequencing reactions were not sensitive enough. Our results are in agreement with the previously reported computational analysis which predicted that VZV is unlikely to produce miRNAs in infected cells (Pfeffer et al., 2005). It is puzzling why, in contrast to other herpesviruses, varicella viruses apparently have not evolved to encode miRNAs, but this might reflect the nature of their latency program during which a number of proteins is expressed. Nonetheless, additional analyses are required to unequivocally confirm that hypothesis. Of note, while our manuscript was in the revision, Depledge et al. have published a discovery of a well-conserved spliced latency-associated VZV transcripts (VLT) expressed antisense to the viral transactivator gene 61 (ORF61), which was present in latently infected ganglia (Depledge et al., 2018). These transcripts share several features with LATs of other alphaherpesviruses, however, in contrast to HSV-1, it encodes a protein during lytic infection and authors have not found any evidence for VLT encoding miRNAs (Depledge et al., 2018).

Taken together, we detected a set of HSV-1 encoded miRNAs in human tissues latently infected with HSV-1. We are aware that our analysis might not be sufficiently sensitive to detect all possible miRNAs, nonetheless, we reason that miRNAs detected in our analysis (miR-H2 – miR-H8) represent the fingerprint of a genuine latent infection in humans, and that deeper sequencing might reveal additional miRNAs present in the samples but the proportion and the importance of the most prevalent miRNAs will not change. Based on the current knowledge, these miRNAs likely limit the expression of important virus genes ICP0, ICP4 and ICP34.5, probably by preventing the reactivation and, at the same time, keeping virus poised for reactivation when the balance between miRNAs and its target is disrupted.

Furthermore, in our analysis, we show that the sequences of recovered miRNAs do not exactly match the currently available information in the miRNA DATA base

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(MiRBase) or the previously reported miRNAs found in human samples. The knowledge of the exact sequences of miRNAs is crucial for the target predictions and the design of functional and detection assays (RT-qPCR).

Investigating HSV-1 miRNAs is experimentally very challenging due to several reasons. First, relevant human samples are difficult to obtain, and latency is established in only a small number of cells of the entire sample. Animal models are well-accepted alternative; however, we have observed that the pattern of HSV-1 miRNA expression does not entirely match the human analysis. For example, miR-H6, which was repeatedly found in latent neurons in mice (Du et al., 2011; Jurak et al., 2010; Kramer et al., 2011), but barely detectable in human samples ((Umbach et al., 2009) and this study) might represent only a reminiscent of the productive infection or reactivation in ganglia; i.e. it might represent a miRNA with roles in productive infection like miR-H1. On the other hand, *in vitro* latency models are difficult to establish and not well characterized yet. In conclusion, our limited study indicates that miRNAs are conserved among HSV-1 strains, however more basic investigations are needed, particularly comparative analysis of clinical samples, to completely understand the fundamentals of HSV-1 miRNA expression.

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Figure legends

Figure 1. HSV-1 and VZV DNA detected in human TGs.

Viral genes gD and ORF38 were amplified by PCR to detect HSV and VZV genomes, respectively in DNA samples extracted from the human TGs (sample 1 and 2), MOCK infected HEK-293 cells in culture (-ctrl), HEK-293 cells infected with HSV-1 (+ctrl) or clinical samples previously shown to contain HSV-2 or VZV genomes (+ctrl). The PCR products were analyzed by agarose gel electrophoresis and the size of DNA fragments indicated to the right of the panel.

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Table 1. Clinical description of tissue donors

Subject	Gender	Age (yr)	Cause of death	Time (h) from death to autopsy
1	M	51	Coronary occlusion	19h
2	M	55	Myocardial infarction	13h

Table 2. HSV-1 miRNA detected in human TGs latently infected with HSV-1

HSV-1 miRNA	Frequency			
	Subject 1		Subject 2	
	5p	3p	5p	3p
miR-H2	1	25	1	22
miR-H3	6	14	4	4
miR-H4	4	55	6	44
miR-H6	0	1	0	0
miR-H7	1	7	1	2

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Table 3. Sequence variations in HSV-1 miRNAs in human ganglia latently infected with HSV-1

HSV-1 miRNA		Sequence	Number of reads isomiR/miRNA[1]
miR-H2-3p	**miRBase**	CCUGAGCCAGGGACGAGUGCGACU	
	sample 1	CCUGAGCCAGGGACGAGUGCGACU	9/25
	sample 2	CCUGAGCCAGGGACGAGUGCGACU	10/22
	Umbach et al.	CUGAGCCAGGGACGAGUGCGACU	
miR-H2-5p	**miRBase**	UCGCACGCGCCCGGCACAGACU	
	sample 1	UCGCACGCGCCCGGCACAGACU	1/1
	sample 2	UCGCACGCGCCCGGCACAGACU	1/1
	Umbach et al.	NO READS[3]	
miR-H3-3p	**miRBase**	CUGGGACUGUGCGGUUGGGAC	
	sample 1	CUGGGACUGUGCGGUUGGGAC*C*[2]	10/14
	sample2	CUGGGACUGUGCGGUUGGGAC*C*[2]	2/4
	Umbach eU al.	CUGGGACUGUGCGGUUGG	
miR-H3-5p	**miRBase**	CUCCUGACCGCGGGUUCCGAGU	
	sample 1	CUCCUGACCGCGGGUUCCGAGU	6/6
	sample 2	CUCCUGACCGCGGGUUCCGAGU	2/4
	Umbach et al.	NO READS[3]	
miR-H4-3p	**miRBase**	CUUGCCUGUCUAACUCGCUAGU	
	sample 1	CUUGCCUGUCUAACUCGCUAGU	47/55
	sample 2	CUUGCCUGUCUAACUCGCUAGU	37/44
	Umbach et al.	NO READS[3]	
miR-H4-5p	**miRBase**	GGUAGAGUUUGACAGGCAAGCA	
	sample 1	GUAGAGUUUGACAGGCAAGCA	2/4
	sample 2	GUAGAGUUUGACAGGCAAGC	4/6
	Umbach et al.	GGUAGAGUUUGACAGGCAAGCA	
miR-H6-3p	**miRBase**	CACUUCCCGUCCUUCCAUCCC	
	sample 1	CACUUCCCGUCCUUCCAUCCC*U*[2]	1/1
	sample 2	NO READS	
	Umbach et al.	CACUUCCCGUCCUUCCAUCCC	
miR-H7-3p	**miRBase**	UUUGGAUCCCGACCCCUCUUC	
	sample 1	UUUGGAUCCCGACCCCUCUUC	5/7
	sample 2	UUUGGGUCCCGACCCCUCUUCU	1/2
	Umbach et al.	NO READS[3]	

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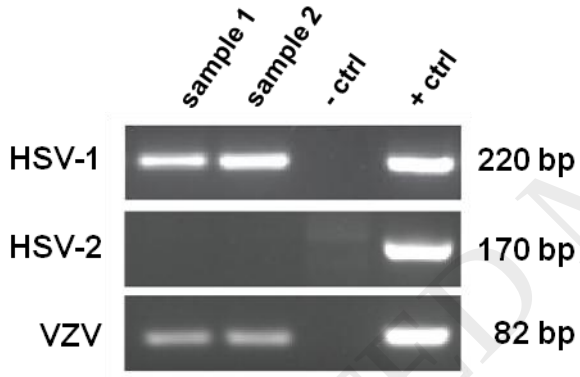
miR-H7-5p	**miRBase**	AAAGGGGUCUGCAACCAAAGG	
	sample 1	GAAAGGGGUCUGCAACCAAAGG	1/1
	sample 2	AAAGGGGUCUGCAACCAAAGG	1/1
	Umbach et al.	AAAGGGGUCUGCAACCAAAGG	

[1]number of reads for most dominat isomiR/total number fo reads assigent to miRNA; [2]nucleotides in italics - non-templated addition according to HSV-1 strain 17 (NC_001806.2); [3]Reads were detected but sequences not available.

Figure 1. HSV-1 and VZV DNA detected in human TGs.



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