Differential expression of cellular microRNAs in HPV-11 transfected cells. An analysis by three different array platforms and qRT-PCR.

Anita Dreher [a*], Maria Rossing [b*], Bogumil Kaczkowski [c], Finn Cilius Nielsen [b] and Bodil Norrild [a**].

[a] Institute of Cellular and Molecular Medicine, DNA Tumor Virus Laboratory, University of Copenhagen, Panum Institute, Blegdamsvej 3, DK-2200 Denmark.

[b] Department of Clinical Biochemistry, Copenhagen University Hospital, Blegdamsvej 5, DK-2100 Copenhagen.

[c] The Bioinformatics Centre, Department of Biology and Biomedical Research and Innovation Centre, Copenhagen University, Ole Maaloes Vej 5, DK-2200, Denmark

[*] The two first authors contributed equally to this publication

** Corresponding author:

Professor Dr. Science Bodil Norrild

Panum Institute

Blegdamsvej 3c, Building 22.3

2200 Copenhagen N

Denmark

Phone: +45 2875 7337

Fax: +45 3536 0116

E-mail: bnorrild@sund.ku.dk

ABSTRACT

Human papillomavirus type 11 (HPV-11) infects the genital and the respiratory tract leading to condylomas and respiratory papillomatosis. HPV infections are restricted to epithelial tissue and the progression through the virus lifecycle is tightly coordinated to the differentiation of the host cell. The changes of cellular microRNAs by HPV-11 gene expression were investigated in a cell culture model of HaCaT cells transfected with HPV-11, with the goal of understanding which cellular processes were affected by the virus. Human microRNA profiling was conducted on three different array platform systems and because very few microRNAs (miR-663, -638, -149* and -92b*) were consistently found in all three array data sets we performed extensive statistical analyses of the array data and the qRT-PCR validation. We assume that the most reliable differentially expressed microRNAs are the ones identified by more than one array platform. We also show that TaqMan® qRT-PCR validation is of limited use for less abundant microRNAs.

Key words: miRNA, microarray, profiling, human papillomavirus, HPV-11

INTRODUCTION

HPV plays an important role in the development of both malignant and benign diseases of which genital condylomas, oral- and laryngeal papillomas are the most prevalent. These highly proliferative infections are most often caused by HPV-11 but when developed into malignant lesions the most prevalent HPV type is HPV-16 sometimes combined with HPV-11 [1-3]. The HPV life-cycle is tightly coordinated to the differentiation program of the epithelium and the primary infection occurs in the basal and parabasal cell layers of the squamous- or mucous epithelium. Replication of the viral genome and transcription takes place in more differentiated cells in the mid- or upper spinous cells. The genome encodes seven early proteins and two late structural proteins which form the viral capsids. The viral proteins E6 and E7 are involved in cell cycle deregulation and prevention of apoptosis whereas the E1 and E2 proteins are of importance for genome replication. The two E5 proteins E5a and E5b influence the signal transduction pathways and therefore contribute to the deregulation of cell growth [2;4]. The virus particles are assembled in fully differentiated cells and infectious virus particles are released from the surface of the tissue [4]. It is hypothesized that regulation of viral progression during the normal life cycle and the tight cooperation with the differentiation of the host cell is driven by changes in cellular microRNAs (miRNAs) [5;6]. MiRNAs regulate gene expression through binding to the 5'- or 3'-noncoding regions of mRNAs and hereby translation of proteins is inhibited [7;8]. Several miRNAs are known to regulate cell differentiation of stem cells [9-11] as well as being differentially expressed in tumor cells and tissue [12-15].

To understand if cellular miRNAs influence HPV-11 gene expression - and because no HPV-11 positive cell lines are available - we developed a model system where the full genome of HPV-11 was transfected into the HPV negative human keratinocyte cell line, HaCaT, together with a neomycin resistance marker. RNA was isolated from selected cells containing HPV-11 and control cells, respectively and subsequently analyzed by microarray analysis. In order to obtain robust information we used three commercial array platforms for the analysis. A large number of

differentially expressed miRNAs was found in all three platforms after applying stringent statistics. However, each array platform detected a different subset of differentially expressed miRNAs with only a small overlap between platforms. A recent study conducted by Git et al observed similar discrepancies [16].

In the present study we discuss the changes in miRNA expression derived by HPV-11 transfection. We focus on miRNAs shared by more than one array platform (Invitrogen, Exiqon and Affymetrix) and analyze the agreement between the expression values derived from either microarrays or quantitative reverse transcription PCR (qRT-PCR).

MATERIALS & METHODS

Cell culture and stable transfections

The HPV-negative human keratinocyte cell line HaCaT, originally established from a human skin biopsy, was cultivated in Dulbecco's Modified Eagle Medium (DMEM 1965, Invitrogen) supplemented with 10% Fetal Bovine Serum, 1% Penicillin/Streptomycin, 1% L-glutamate and 1% of 0.1M sodium pyruvate. Cells were grown under standard conditions at 37°C and 5% CO_2 and all experiments were done in triplicates. Cells were either transfected with 6 µg of circularized full genome HPV-11 DNA and 2 µg of selection vector pSV2-neo or with only 2 µg of pSV2-neo. Transfections were done using jetPEI™ transfection reagent (Polyplus Transfections) following manufacturer's instructions. The neomycin resistant cells were maintained by adding 500 µg per ml G418 sulfate (Invitrogen) to the growth medium 24 hours post-transfection. In order to standardize, cells were stringently transfected and harvested in the same passage.

DNA/RNA extraction

Transfected cells were grown with and without antibiotic selection and HPV-11 and pSV2-neo transfected dishes were harvested at three time points (48 hours, 2 weeks and 3 weeks). DNA was purified using the Invisorb Spin Tissue Mini Kit (Invetek) according to manufacturer's instructions. Triplicate of transfected cells were harvested at ~80% confluence and totalRNA was extracted using TriZOL reagent (Invitrogen) following the manufacturer's protocol.

HPV-11 DNA quantitation by PCR

250 ng of DNA were amplified by PCR using primers for HPV-11 non-coding region (NCR) resulting in a fragment of 755 nucleotides. GAPDH was used as an internal control. The primers used were the following: NCR: Forward primer 5'- TACGTACCCGGGTATATGTGTGTCAGTGTG and the reverse primer 5' - TATGCTAAGCTTACTTTCCATAATGCCTCG. The GAPDH forward primer was 5' - TGCACCACCAACTGCTTAG and the reverse primer 5'-

GATGCAGGGATGATGTTC. The PCR program held an annealing temperature of 60°C for HPV-11 and 50°C for GAPDH and was initiated upon 15 min. at 95°C, followed by 35 repeated cycles (1 min.95°C, 1 min.60°C/ or 50°C, 2 min.72°C), ending with 10 min. at 72°C.

RNA labeling, hybridization and microarray platforms

Affymetrix platform: In a one-color labeling system, 1000 ng of totalRNA was labeled using FlashTag™ Biotin RNA Labeling Kits from Genishere and hybridized to Affymetrix GeneChip® miRNA Array according to manufacturer's instructions. Affymetrix miRNA-array contains probes for 847 human miRNAs; all cataloged in the miRBase Sequence Database (Release 11.0, http://microrna.sanger.ac.uk/).

Exiqon platform: In a two-color labeling system, 1000 ng of totalRNA was labeled with fluorescent Hy3™(sample)/Hy5™(reference-sample) dye from the miRCURY LNA™microRNA Array Power Labeling Kit (Exiqon, Vedbaek, Denmark) according to manufacturer's instructions. Using a TECAN hybridization station, labeled samples were hybridized overnight to pre-printed miRCURY LNA™ microRNA Array, v.11.0 (Exiqon), containing probes for 841 human miRNAs, cataloged in the miRBase Sequence Database. Arrays were scanned in an Agilent DNA Microarray Scanner, (Agilent Technologies, Santa Clara, CA) and resulting images analyzed with Genepix Pro 6.0 software (Molecular Devices, Sunnyvale, CA).

Invitrogen platform: Also in a two-color labeling system, miRNA expression levels were determined by microarray analysis. Around 2000 ng of totalRNA was labeled with fluorescent Alexa Fluor dyes using the NCode™ Rapid miRNA Labeling System (Invitrogen). The samples were labeled with Cy5 and a mirVana™ miRNA Reference Panel v9.1 (Ambion) with Cy3, and hybridized overnight in a TECAN hybridization station to NCode™Human miRNA Microarrays V3 (Invitrogen), which contains probes corresponding to 710 miRNAs, cataloged in the miRBase Sequence Database, Release 10.0. Arrays were subsequently handled as described for the Exiqon platform.

Microarray data pre-processing

Affymetrix: Microarray data generated by Affymetrix miRNA Array v1.0 platform were normalized by means of miRNA QCTool version 1.0.33.0, available from affymetrix.com. Default parameters were used. Normalized expression values (log2, single channel intensities) were exported into R environment [17] for subsequent analysis. The data set contained 847 probes and was filtered to exclude probes showing little or no variance (standard deviation < 0.2). The filtered data set contained 488 probes.

Exiqon and Invitrogen: The importing and pre-processing of the Exiqon and Invitrogen data were performed using LIMMA package [18] of Bioconductor project [19].

Data from each platform was processed separately. The mean spot pixel intensities were taken to estimate the foreground and median intensities of surrounding pixels were used for local background estimation. Spots with a quality Flags-value less than -99 were excluded from the analysis. The “normexp”, plus offset=50 method [20] was used for background correction. Subsequently, the log2 Green/Red ratios were computed and normalized by LOESS normalization [21]. Finally, log2 ratios of intra-slide replicate spots were averaged. There were three intra-slide replicate spots for each probe in Invitrogen and four in Exiqon. The Exiqon data sets contained 834 probes and after filtering to exclude probes showing standard deviation below 0.15, the data set was reduced to 381 probes. The Invitrogen data set of 705 probes was filtered using standard deviation threshold of 0.05 resulting in 304 probes in the final data set.

Statistical analysis

The differentially expressed miRNAs were identified by means of empirical Bayes moderated t-statistics as implemented in LIMMA [18]. The p-values were adjusted for multiple testing by Benjamini and Hochberg False Discovery Rate method and significance level of 0.05 was used for statistical testing.

Reverse transcription PCR

CDNA was prepared from 100 ng totalRNA from cultured cells using TaqMan® MicroRNA Reverse Transcription Kit and TaqMan® MicroRNA Assays containing predesigned primers were added. MiR-191, RNU6B, and RNU48 were used for endogenous control [22;23]. RT-PCR reaction was performed using TaqMan® Universal PCR Master Mix No AmpEras® UNG, according to manufacturer's instructions, all from Applied Biosystems. Each amplification reaction was performed in triplicates and median value of the three cycle threshold was used for further analysis. For calculations of fold changes we used the $2^{-\Delta C}{}_{T}$ method [24].

RESULTS

Cell cultures

The HPV negative keratinocyte cell line HaCaT was selected in order to study the HPV mediated changes in the miRNA profile under controlled growth conditions. This made it possible to extract RNA from HPV-11 and control transfected cells grown in parallel and to analyze the differentially expressed miRNA as a result of HPV-11 gene expression. HPV DNA replication is not expected and subsequent analysis of HPV-11 DNA maintenance was necessary. The amount of HPV-11 DNA per µg of total DNA decreased with time but HPV-11 was still abundant after three weeks of selection emphasizing that the cell cultures maintain the HPV-11 genome, hereby verifying that the model mimics infected cells. Furthermore, the transcription of the viral genomes was active as demonstrated by the presence of HPV-11 E7 mRNA (data not shown).

Differentially expressed miRNAs

Expression of cellular miRNAs in HPV-11 transfected HaCaT cells was measured on three different array platforms developed by Invitrogen, Exiqon, and Affymetrix, for latter platform, see Figure 1 for Heatmap of differentially expressed miRNAs. We compared the lists of differentially expressed miRNAs from each platform to determine the agreement between microarray platforms. A total of 33 miRNAs were differentially expressed by the Invitrogen-, 50 by the Affymetrix-, and 92 by the Exiqon platform with a significance level of $p<0.05$. P-values were adjusted for multiple testing by Benjamini & Hochberg method. Surprisingly, there were few differentially expressed miRNAs in common between the three platforms and only four miRNAs (miR-149*, -638, -663, and -92b*) were confirmed on all platforms. Additionally there were 10 miRNAs differentially expressed on two out of three platforms. Only one miRNA was shared between the Invitrogen and the Affymetrix platforms, five miRNAs were shared between the Invitrogen and the Exiqon platforms, and four miRNAs between the Affymetrix and the Exiqon platforms, see Figure 2 for Venn diagram.

Lists of differentially expressed miRNAs for each microarray platform are available in supplementary Tables S1A, -1B, and -1C.

Validation of differentially expressed miRNAs by qRT-PCR

Fold Changes (FC) of differentially expressed miRNAs ranged from +3.3 to -10.3. Comparison of FC values derived from the different platforms correlated with only one exception – FC of miR-584 showed downregulation in Invitrogen and upregulation in Exiqon (Table 1). MiRNAs which showed differential expression on at least two platforms were validated by qRT-PCR. All qRT-PCR analysis was done relative to three internal controls, RNU6b, RNU48 and miR-191. Results showed variation according to choice of internal control, where the most consistent findings were obtained for miR-149*, -7, -1228*, and -886-3p. For the latter miRNAs up- or downregulation did not vary according to internal controls (Table 1). MiR-663 and miR-608 were not validated, as no commercial primers for the TaqMan® system were available and introducing an additional design of primers did not seem favorable with respect to comparability. MiR-654-5p and -185* gave poor results with cycling-threshold (CT) above 39 by qRT-PCR although they obtained high array intensities and were differentially expressed (supplementary material Table S-1B).

In addition to the miRNAs analyzed by qRT-PCR and listed in Table 1, a few miRNAs were selected among the miRNAs differentially expressed in only one of the data sets from either Invitrogen, Affymetrix or Exiqon platform, respectively. These unique miRNAs were selected among the miRNAs expressed in relatively low- (< 4), intermediate- (5-6), or high- (> 6) level according to array intensities. The qRT-PCR data showed relatively low FC for the selected miRNAs and results are only shown with miR-191 as reference, since these results showed the highest correlation with array based FC (Tables S-1A, -1B, -1C). It was only possible to obtain consistent CT values for miRNAs with high array intensities and thus qRT-PCR results for miRNAs with intermediate or low array intensities were not viable. Because of the discrepancy between the array FC values and the qRT-PCR results we conducted a more in depth correlation analysis as described below.

Correlations between microarray results and qRT-PCR validation

For the 9 miRNAs shown to be differentially expressed by at least 2 array platforms and where qRT-PCR results were obtained, we calculated Pearson correlation coefficient (R) to assess the agreement between expression values generated by the different methods used. We compared the correlations a) between three array platforms, b) between CT values and internal controls, and c) between array intensities and CT values (Table 2). Based on Table 2, the results show higher correlations between the different array platforms than between different internal controls in qRT-PCR – analysis. It is evident that there is only little or no correlation between array and qRT-PCR data. Only miR-886-3p shows high correlation both between the three different internal controls in qRT-PCR, between Exiqon and Affymetrix, and between array platforms and qRT-PCR. Even when correlating with Invitrogen results, which gave the poorest correlations of miR-886-3p (R = 0.50-0.78) we still found acceptable correlations.

DISCUSSION

MiRNA profiling of HPV-11 transfected HaCaT cells on three different array platforms showed very few differentially expressed miRNAs in common between all three data sets. We performed an extensive validation and statistical analyses of the data, hereby revealing discrepancies of results based on the different internal controls when analyzing with qRT-PCR (Table 1). The disagreement between internal controls presents a general problem as the true expression of miRNAs is unknown and therefore reliability of the qRT-PCR results has to be questioned. Is qRT-PCR analysis the best method of validating array data since internal controls are not always consistently expressed? We observed cases where two internal controls derived opposite results (Table 1), and this might lead to unsupported conclusions.

QRT-PCR validation does not confirm miRNAs with a low FC based on array data although validated miRNAs are in agreement with more than one array platform. MiRNAs with a FC <2 tend to have random signature in qRT-PCR validation and disagreeing with direction of changes. QRT-PCR validation of miR-886-3p was in agreement with array data and internal controls, all with FC <2. However, microarray derived data confirm numeric values of differential expressed miRNAs (see Tables 2) also in cases where FC were relatively low (~2.5). This result is consistent with the fold changes described before, where miR-886-3p was shown to be downregulated upon HPV-11 transfection by Exiqon and Affymetrix platforms and when using all qRT-PCR internal controls. MiR-886-3p shows a FC of -10 (Affymetrix), which is the highest numeric FC observed. This suggests that greater miRNA alterations are easier confirmed by other methods. The problem occurs when only subtle alterations need to be confirmed.

Our results conclude that microarrays are more sensitive and reproducible than TaqMan® qRT-PCR when changes in miRNA expression are small. The results are restricted to a subset of miRNAs which were chosen based on array result, so more comprehensive selection of miRNAs should be used to draw a more global conclusion. The fact that qRT-PCR may be superior to array technologies for mRNA profiling in terms of specificity and sensitivity does not mean that this is

also true for miRNAs. The short length of miRNAs (~21nt) may present problems for probe design as well as consistent expression of internal controls.

Furthermore we show that reliable and reproducible results can be obtained combining power of two or more microarray platforms, especially when miRNA expressions can only be partially confirmed by qRT-PCR. In our settings, results obtained from a combination of two platforms would be better validated by a third platform than by qRT-PCRs, e.g. four of five miRNAs differentially expressed by the combination of Invitrogen and Affymetrix would be confirmed by Exiqon array analysis (Fig.2). Half of the miRNAs differentially expressed by the combination of Exiqon and Affymetrix were confirmed by Invitrogen array analysis which was also the case for miRNAs shared between Exiqon and Invitrogen and confirmed by Affymetrix array.

In conclusion, our analysis identified 14 miRNAs which showed differential expression on more than one array platform and miR-886-3p was additionally confirmed by qRT-PCR profiling. We believe that these 14 miRNAs are truly altered by HPV-11 transfection and consequently these miRNAs are being further analyzed to reveal their involvement in HPV-11 induced cellular response.

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List of Tables

Table 1: Significantly changed miRNAs shared between two or more platforms. Fold Change derived from array platforms and qRT-PCR validation with different miRNAs used as internal control (RNU6B, RNU48, miR-191) are shown. *nd = not done, **nv = not valid.

Table 2: Correlations between array intensities and qRT-PCR derived cycling threshold (CT). The table depicts Pearson correlations coefficient (R) between a) array platforms, b) CT values and internal controls, and c) array intensities and CT values for the 9 miRNAs shared between two or more platforms and validated by qRT-PCR. Highlighted values indicate R > 0.80 which reflects acceptable correlation.

List of Figures

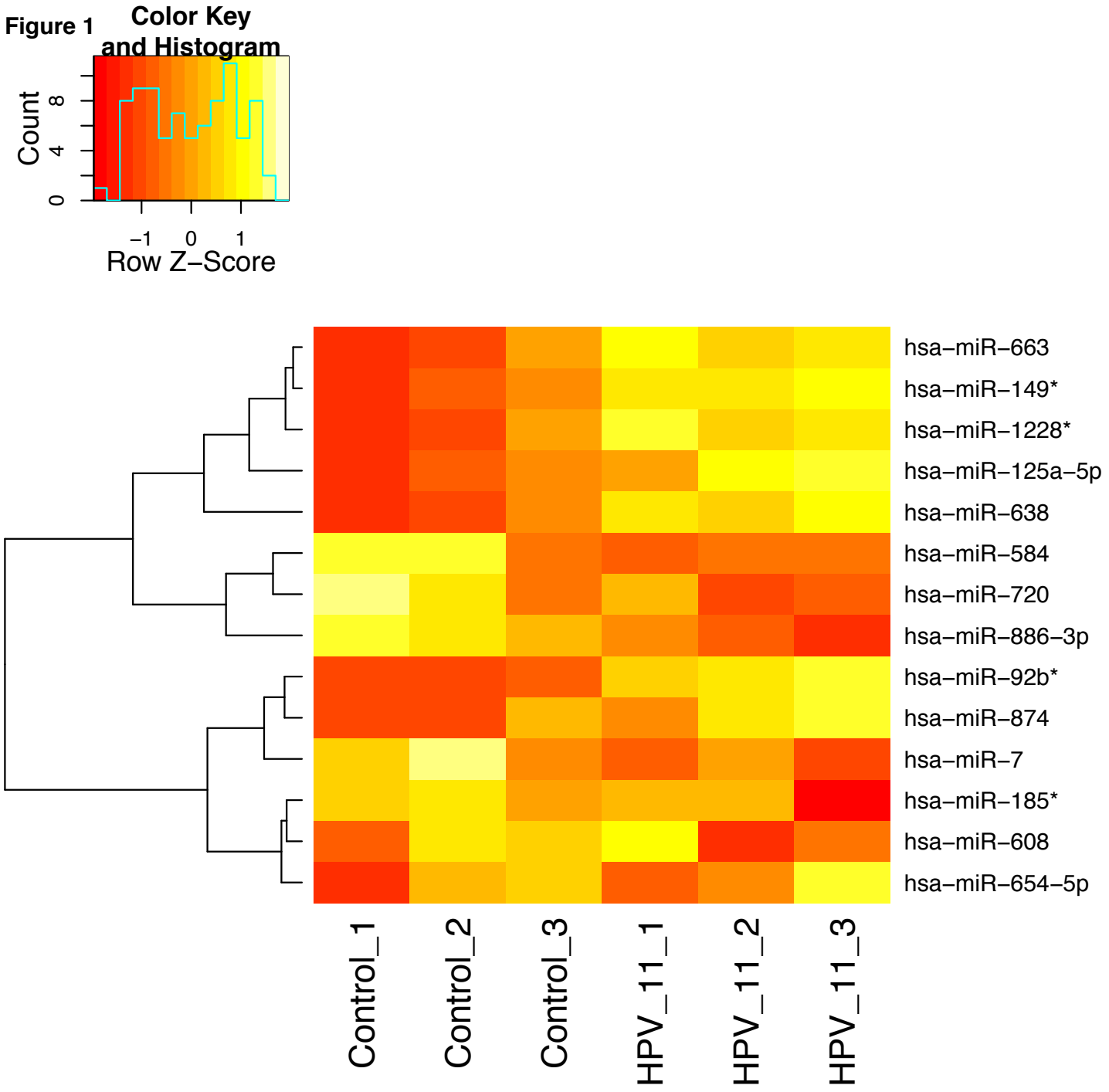
Table 1

Shared plarforms	ID	Fold Change microarray			Fold Change qRT-PCR		
		Invitrogen	Exiqon	Affymetrix	RNU6B	RNU48	miR-191
Affymetrix/ Exiqon/ Invitrogen	miR-663	1.7	1.8	2.5	nd*	nd*	nd*
	miR-638	1.6	2.6	2.6	-1.2	-1.3	1.2
	miR-149*	1.2	1.3	2.6	-1.6	-1.6	-1.1
	miR-92b*	1.2	1.3	2.4	-1.8	-1.3	1.1
Exiqon/ Invitrogen	miR-654-5p	1.4	1.8		nv**	nv**	nv**
	miR-608	1.2	1.5		nd*	nd*	nd*
	miR-584	-1.2	2.6		1.7	-1.2	-1.3
	miR-185*	1.2	1.4		nv**	nv**	nv**
	miR-7	-1.5	-1.9		-1.3	-1.1	-1.1
Invitrogen/ Affymetrix	miR-125a-5p	1.2		3.3	-1.4	-1.1	1.3
Affymetrix/ Exiqon	miR-1228*		1.4	2.7	1.1	1.2	1.8
	miR-886-3p		-2.2	-10.3	-2.7	-7.3	-2.1
	miR-874		2.0	1.9	nv**	nv**	nv**
	miR-720		-1.4	-1.8	-1.1	1.1	1.7

Table 2

	Affymetrix/Exiqon/Invitrogen			Exiqon/Invitrogen		Invitrogen/ Affymetrix	Affymetrix/Exiqon		
	miR-638	miR-149*	miR-92b*	miR-584	miR-7	miR-125a-5p	miR-1228*	miR-886-3p	miR-720
a) Correlations between array platforms									
Exiqon/Affymetrix	0.99	0.89	0.84	-0.85	0.82	0.33	0.77	0.96	0.72
Exiqon/Invitrogen	0.98	0.83	0.93	-0.65	0.99	0.77	NA	0.70	NA
Affymetrix/Invitrogen	0.98	0.95	0.91	0.51	0.81	0.72	NA	0.50	NA
b) Correlations between CT values and internal controls									
RNU6B	0.74	0.71	0.39	0.91	0.84	0.00	0.95	0.91	0.87
RNU48	0.40	0.47	0.51	0.69	0.79	-0.78	0.66	0.97	0.33
miR-191	0.51	0.60	0.67	0.88	0.58	-0.52	0.64	0.97	0.19
c) Correlations between array intensities and CT values									
Exiqon/RNU6B	-0.17	-0.44	-0.16	0.06	0.48	0.22	0.31	0.95	0.18
Exiqon/RNU48	-0.54	-0.79	-0.53	-0.17	0.53	-0.21	0.02	0.97	0.25
Exiqon/miR-191	0.02	-0.39	0.22	-0.28	0.53	-0.03	0.29	0.94	-0.19
Affymetrix/RNU6B	-0.19	-0.52	-0.35	0.35	0.32	-0.11	0.02	0.87	-0.43
Affymetrix/RNU48	-0.52	-0.84	-0.82	0.65	0.83	-0.68	-0.03	0.93	0.04
Affymetrix/miR-191	0.05	-0.39	0.13	0.71	0.64	0.68	0.22	0.82	-0.43
Invitrogen/RNU6B	-0.17	-0.75	-0.36	0.19	0.44	-0.19	NA	0.78	NA
Invitrogen/RNU48	-0.48	-0.87	-0.77	0.13	0.48	-0.27	NA	0.66	NA
Invitrogen/miR-191	-0.02	-0.49	-0.01	0.26	0.49	0.50	NA	0.78	NA

Figure 1


Color Key
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0
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hsa−miR−663
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hsa−miR−125a−5p
hsa−miR−638
hsa−miR−584
hsa−miR−720
hsa−miR−886−3p
hsa−miR−92b*
hsa−miR−874
hsa−miR−7
hsa−miR−185*
hsa−miR−608
hsa−miR−654−5p
Control_1
Control_2
Control_3
HPV_11_1
HPV_11_2
HPV_11_3

Figure 2