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Assessment of transcriptomal analysis of varicella-zoster-virus gene expression in patients with and without post-herpetic neuralgia

G. H. Ashrafi · Esther Grinfeld · Paul Montague · Thorsten Forster · Alan Ross · Peter Ghazal · Fiona Scott · Judith Breuer · Roslyn Goodwin · Peter G. E. Kennedy

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Abstract Varicella-Zoster virus (VZV) is a human herpes virus that reactivates from a latent state in human trigeminal and dorsal root ganglia to cause herpes zoster (shingles) which is a painful vesicular dermatomal skin eruption. The major complication of herpes zoster is post-herpetic neuralgia (PHN) which is a serious condition occurring especially in individuals over 50 years. PHN is extremely painful, may be permanent, and is frequently very refractory to all treatment. The ability to identify those patients with herpes zoster who are likely to develop PHN would be highly beneficial as it would allow pre-emptive anti-viral therapy. We have assessed the potential of using long oligonucleotide VZV microarrays to determine whether MeWo cells infected with VZV isolates obtained from 13 patients with zoster who had subsequently developed PHN showed significant transcriptomal differences from MeWo cells infected with viruses isolated from ten zoster patients who had not developed PHN. We found that viral gene expression from sample to sample within a group (PHN patients or non-PHN patients) varied as much, or more, than the viral gene expression between those groups. Quantitative real-time polymerase chain reaction studies carried out on 11 open reading frames on four representative viral infected MeWo cell lines (two from each group) confirmed the transcriptomal heterogeneity between the two groups. Growth curve analyses of ten representative infected cell lines (five from each group) showed that PHN and non-PHN-associated viruses replicated equally efficiently. Taken together, these findings suggest that viral microarray-based transcriptomal measurements are unlikely to prove of clinical utility in predicting the incidence of PHN.

Keywords Varicella-zoster virus · Transcriptome · Microarray · Post-herpetic neuralgia · Herpes zoster

G.H. Ashrafi and Esther Grinfeld contributed equally to this study.

G. H. Ashrafi · E. Grinfeld · P. Montague · R. Goodwin · P. G. E. Kennedy (✉)
Department of Neurology, Division of Clinical Neurosciences, Institute of Neurological Sciences, Southern General Hospital, Glasgow G51 4TF, Scotland, UK
e-mail: P.G.Kennedy@clinmed.gla.ac.uk

G. H. Ashrafi · P. Montague · P. G. E. Kennedy
Institute of Comparative Medicine, University of Glasgow, Glasgow G61 1QH, UK

T. Forster · A. Ross · P. Ghazal
Division of Pathway Medicine, University of Edinburgh Medical School, The Chancellor's Building, Little France Crescent, Edinburgh EH16 4SB, Scotland, UK

F. Scott · J. Breuer
Centre for Infectious Disease, Barts and the London School of Medicine and Dentistry, 4 Newark St, E1 2AT London, England, UK

Present Address:
G. H. Ashrafi
Kingston University London, Kingston Upon Thames, London KT I 2EE, UK

Introduction

Varicella-Zoster virus (VZV) is a pathogenic human herpes virus which causes varicella (chickenpox) as a primary infection following which it becomes latent in neurons within human trigeminal and dorsal root ganglia [1–5]. The virus may then reactivate, either spontaneously or following specific triggering events, to cause herpes zoster (shingles) which may result, especially in the elderly and

immunocompromised, in post-herpetic neuralgia (PHN) and other neurological complications [6, 7]. PHN is a serious condition producing often severe dermatomal pain that can be permanent and extremely refractory to all treatment [8].

While host factors such as age over 50 years, female gender, the presence of a prodrome, a severe disseminated rash, severe pain at presentation and polymerase chain reaction (PCR)-detectable VZV viraemia are all known to be important risk factors for the development of PHN [7, 8], it is also possible that variation in the genotype and/or phenotype of the virus itself may play a role in the development of PHN. If patients with zoster going onto develop PHN could be identified at an early stage then this would be clinically useful and allow pre-emptive anti-viral therapy to prevent or ameliorate PHN. Previously, we have noted viral transcriptomal profile differences amongst various vaccine and laboratory isolates [9]. These observations raise the issue of whether there are 'signature genes' in VZV obtained from patients with herpes zoster that can allow the early identification of those patients who are likely to develop PHN. In order to achieve this, we used previously validated long oligonucleotide VZV microarrays [10] to analyse and compare the transcriptomes of MeWo cell lines infected with a panel of VZV isolates from patients with herpes zoster who either did or did not subsequently develop PHN. Here, we show that that the viral gene expression from sample to sample within a group (PHN or non-PHN patients) varies as much, or more, than the expression between those groups.

Materials and methods

MeWo cell line infection

For in vitro analysis, MeWo cells (Dumas strain) were used [9]. About 100–200 µl of a clinical isolate was absorbed onto of MRC5 or HEL cells in a 25 cm^2 flask at 50–60% confluence for 2 h at 37°C. Following absorption, the sample was aspirated and replaced with minimal MEM medium and incubated at 37°C. Cells were monitored every day for the presence of a cytopathic effect (cpe). Once cpe reached >50%, the cells were removed and absorbed onto MeWo cells as above. When the cpe was >75% by visual inspection, usually several days later, the cells were mixed with an equal number of uninfected MeWo cells, split one in five and incubated for 72 h.

VZV isolates

From an initial cohort of 75 isolates obtained by JB in London (Table 1), we successfully cultured (<5 passages) a total of 23 different isolates (13 PHN and 10 non-PHN). PHN in patients was defined as dermatomal pain that had persisted for at least 3 months after the zoster rash. Since the samples were studied blind and consecutively, it was not possible to closely age and sex match the samples prior to analysis. However, the mean age of the 13 PHN subjects (6 male and 7 female) was 58 years and that of the non-PHN (all male) subjects was 49 years, this reflecting the older age distribution of PHN. Ethical permission for the study was granted by the Local Research Ethics Committee.

Table 1 PHN-associated and non-PHN associated viruses used in study

Sample	PHN	Age	Gender
S29	No	71	M
SUK57	No	40	M
S69	No	55	M
Z202	No	28	M
Z208	No	24	M
S10	No	83	M
S22	No	61	M
S45	No	64	M
S70[a]	No	39	M
Z228	No	25	M
Z132	Yes	39	M
ZAP198	Yes	60	M
Z201	Yes	85	F
Z226	Yes	72	F
S66	Yes	45	M
Z134	Yes	51	F
Z143	Yes	68	M
Z204	Yes	69	F
Z210[b]	Yes	70	F
Z213[c]	Yes	42	F
Z246[c]	Yes	70	F
Z281	Yes	46	M
Z214	Yes	39	M

The sample reference numbers, age and sex of the patients are indicated

[a] HIV positive

[b] On corticosteroids

[c] Immunosuppressed and on corticosteroids

RNA preparation

Total cellular RNA earmarked for microarray and quantitative real-time PCR (qRT-PCR) usage was extracted from infected MeWo cells using a RNeasy kit (Qiagen) and DNase treated. The resultant RNA was purified further and concentrated using a RNeasy Mini-Elute kit (Qiagen). The absence of contaminating genomic DNA was confirmed by

standard PCR using β actin primers The quality of the RNA was assessed electrophoretically on a 2100 Agilent Bioanalyser. Total cellular RNA for use in cDNA synthesis was extracted from infected MeWo cell lines by homogenization in RNABee (ams Biotechnology) according to manufacturer's guidelines and subjected to DNase treatment.

Construction of microarray platform

The detailed construction of the VZV microarrays, array hybridisation, data processing and analysis has been previously described by us in detail [9]. In brief, the VZV array platform comprised 82 open reading frames (ORFs) (71 are unique, others have 2 or 3 probe sequence variants). Arrays were spotted using an ArrayJet (SuperMarathon) robotic jet printer.

Two different array designs were used for this study. Set 1 arrays were one array per slide and 15 on-array print replicates per probe. For Set 2, each slide contained eight full arrays, with three on-array print replicates per probe.

Array hybridisation

Array hybridisation and analysis followed standard operating procedures. After scanning and processing [10], all samples were blinded or anonymised with respect to sample class (PHN, non-PHN) in order to avoid bias throughout the entire array procedure. The code was broken only after all the samples had been studied and analysed on the arrays.

Data processing

Background noise in the image quantification was corrected for as previously described [10] *Quality control* identified several samples that were classed as outliers and could not be used for analysis. Sample-to-sample differences in data distributions before normalisation were very pronounced, much more so in Set 2 than in Set 1. Correlations between replicate samples in Set 2 were generally better ($r \sim 0.8$) than correlation to other patient samples.

Data normalisation was carried out in two stages due to the presence of two distinct sets of data. In stage 1, each of the two sets was normalised independently. In brief, based on the assumption of global inter-sample similarity of ORFs the median across all ORF probes for an array was calculated on the $\log_2$-transformed data. Each array median was then matched to a common reference value, the average of all array medians. For each array (sample), an estimate for expression level for a given ORF was then calculated as the median value of all 15 (or 3 in Set 2) on-chip print replicates of that ORF, thus making use of probe replication and providing some robustness against outlier values. In stage 2, this process was repeated across both sets of data with the values obtained in stage 1. This stage is aimed at integrating the two separate studies (albeit almost identical microarray design and sample types) for combined analysis.

Data analysis consisted of statistical and explorative methods as previously described (10) and was based on the null hypothesis (for each ORF) of no difference in average expression levels between PHN and non-PHN samples. In order to test for this, a general linear model moderated by pooling variance estimates across multiple probes 'empirical Bayes' was applied. Both differential and absolute expression values are given in addition to statistical results for Tables 2 and 3. A value of 0 means no differential expression, a value of 1.5 would mean 2.83-fold ($2^{1.5}$) up regulation in condition 2 with respect to condition 1. A value of -1.5 would correspondingly and symmetrically mean a 2.83-fold down regulation. Absolute expression values for a condition are represented as mean $\log_2$ expression values across all samples in that condition.

Quantitative real-time polymerase chain reaction

Oligonucleotide primers and probes were designed using the Primer Express programme (Table 2a). Using manufacturer's guidelines, qRT-PCR was performed using the TaqMan® One-Step RT-PCR Master Mix Reagents Kit (Applied Biosystems, UK). 25 µl reactions were performed in triplicate comprising 100 ng of total cellular RNA, 45 µM of each forward and reverse primer, and 5 µM of probe. Primers and probe sequences for β-actin amplification were commercially obtained (Applied Biosystems). Reactions were performed using an ABI prism 7,500 sequence detector. Individual ORF gene expression in each of the four-infected cell lines was normalised according to endogenous β-actin gene expression using the comparative C_T method which affords an opportunity to present data in a 'fold change' in expression format. Relative individual ORF expression levels were calculated using the $2^{-\Delta\Delta CT}$ formula with either MeWo cells or ORF7 as the calibrator.

Standard reverse transcription polymerase chain reaction

Random hexamer (Invitrogen) primed cDNA synthesis was performed on 2 µg of total cellular RNA using SuperScript III™ (Invitrogen) reverse transcriptase following supplier's guidelines. PCRs using a range of ORF primers (Table 2b) were carried out on 5 ng of cDNA using REDTaq ReadyMix PCR Reaction Mix (Sigma). Routine thermal cycling comprised an initial denaturation step of 94°C/5 min, a core cycle comprising (94°C/1 min–55°C/1 min–72°C/1 min) for 25

Table 2 Oligonucleotide sequences

(a) qRT-PCR

	Forward primer	Probe	Reverse primer
ORF7	5′-ACACGCACCCCCAAGGA-3′	5′-CGCCCTGCTCCGCCTCCA-3′	5′-ATCCATTCACAGCGGTTAAAGC-3′
ORF11	5′-TGCCGGATTACCACGAGAAG-3′	5′-CGTACCCGACGTACTCCAAGTGACACAG-3′	5′-CCCGATTTGGCAAAACCA-3′
ORF15	5′-ATGGCCGTGAATGGTGAAA-3′	5′-AGCTGTCCATGATGAAAACCTGGGTGTG-3′	5′-AGCGCGGATTAATTCTCTGTC-3′
ORF16	5′-GAATTGCATGCGGGTTTTG-3′	5′-CGCCCCAGAAATCGCCAGAGC-3′	5′-ACCAGTAAGCGTTTTTTCCGTT-3′
ORF18	5′-TTAAAATCCCATGCACCCAAA-3′	5′- CCCGTTTGGTGAACGTCGATGCA-3′	5′-CCGCGCTGAATTTCACGTAT-3′
ORF21	5′-TGTTGGCATTGCCGTTGA-3′	5′-CTGCTTCCCCAGCACGTCCGTC-3′	5′-ATAGAAGGACGGTCAGGAACCA-3′
ORF41	5′-TGGGAACTCGCGCAATTT-3′	5′-ACGCGTCTGTGTGTTTGATGGACCC-3′	5′-CGGTCTCCCGGATGAACA-3′
ORF42	5′-TCATGCACACCACGCATTTT-3′	5′-TTTCATCGGTGCTATTGGGCGATCA-3′	5′-CGCAGAAAGCAGTCAACTCTGT-3′
ORF46	5′-CCACAATACCGACGGACATG-3′	5′-ACCAAATGCAACCGCAGCCGA-3′	5′-TGGTGGATTCTCGTTCTTGCT-3′
ORF56	5′-GGGTAAACAGACCTGACGATCAG-3′	5′-CCACACCAACACCAACCCCGC-3′	5′-CCGCCTTTGCGATGTATACTG-3′
ORF63	5′-CCGGGTTGGGAGATCCA-3′	5′-CCGGCCAGGCTCGTTGAGGA-3′	5′-GCAATACATAAAAAAACACGGTTGA-3′

(b) Semi-quantitative RT-PCR

	Forward primer	Reverse primer	Product length (bp)
ORF7	5′-CCTCTAACGTTAGAAGACGT-3′	5′-ACATCAAACGATGCCTGAGG-3′	374
ORF11	5′-GTCGAGACGCGGATGAGGTT-3′	5′-CGCGGGCCGTATTTATGTGT-3′	454
ORF15	5′-GCGGCATCGACAATTGAAAC-3′	5′-CAGCATCGCGGTTGTTTCTC-3′	493
ORF16	5′-GTCGCTGTAAACGGGTGAAT-3′	5′-CGTACAGACGATGCTTGGA-3′	550
ORF18	5′-AATCGTTTATCACTGTGCCCGC-3′	5′-CAAGAGTGCATCGAGGTTGTCCA-3′	643
ORF21	5′-GGTCACTCCCACTTGTATTTCC-3′	5′-GGTCACTCCCACTTGTATTTCC-3′	372
ORF41	5′-TGCGTCATCGAGCTTCACTG-3′	5′-CGTGCAACCACTTTGGCGAT-3′	358
ORF42	5′-GAGTAACGCAACGCCCTATC-3′	5′-GGATCGTCGGATCAGTTGTT-3′	487
ORF46	5′-AAAGAACGGACCCTCGATCT-3′	5′-GGTTCATGTCCGTCGGTATT-3′	454
ORF56	5′-TTAGCGATCACATTCTTGCCG-3′	5′-TTAGCAGGGTCACTGTATGGGT-3′	337
ORF63	5′-GTTTTGACTCCGGGTTG-3′	5′-TTACATCCGATGGCGTAG-3′	386
CYC	5′-ACCCCACCGTGTTCTTCGAC-3′	5′-CATTTGCCATGGACAAGATG-3′	500

Table 3 Statistical results for comparison of PHN versus non-PHN samples (using empirical Bayes moderated *t* test)

	Log_2 fold change PHN–Non-PHN	*P* value	FDR *P*
ORF61	−0.29	0.0974	0.9627
ORF1a	−0.36	0.1232	0.9627
ORF17	−0.28	0.1268	0.9627
ORF15	0.25	0.1415	0.9627
ORF41	0.29	0.1501	0.9627
ORF11a	−0.32	0.1693	0.9627
ORF62b	−0.48	0.1931	0.9627
ORF29	0.22	0.2129	0.9627
ORF38	−0.24	0.2377	0.9627
ORF21	0.14	0.2457	0.9627
ORF8	0.15	0.2526	0.9627
ORF42	0.19	0.2636	0.9627
ORF4b	−0.20	0.2673	0.9627
ORF57	−0.37	0.2734	0.9627
ORF46b	−0.35	0.2887	0.9627
ORF22	0.20	0.2957	0.9627
ORF46a	−0.38	0.3031	0.9627
ORF52	−0.10	0.3059	0.9627
ORF50a	−0.33	0.3295	0.9627
ORF49	−0.34	0.3337	0.9627
ORF16	−0.19	0.3395	0.9627
ORF14	−0.24	0.3574	0.9627
ORF39	0.30	0.4304	0.9627
ORF2a	−0.10	0.4341	0.9627
ORF3	−0.13	0.4388	0.9627
ORF56	−0.15	0.4435	0.9627
ORF45	−0.25	0.4590	0.9627
ORF53	−0.19	0.4775	0.9627
ORF10	0.09	0.4794	0.9627
ORF11b	−0.16	0.4886	0.9627
ORF31	−0.17	0.4888	0.9627
ORF9	−0.19	0.5227	0.9627
ORF65	−0.08	0.5464	0.9627
ORF37	−0.13	0.5480	0.9627
ORF25a	0.14	0.5481	0.9627
ORF48b	−0.17	0.5546	0.9627
ORF64	−0.21	0.5562	0.9627
ORF4a	−0.14	0.5579	0.9627
ORF63	−0.16	0.5580	0.9627
ORF13a	0.12	0.5616	0.9627
ORF68	0.13	0.5655	0.9627
ORF62a	0.19	0.5680	0.9627
ORF70	−0.15	0.5867	0.9627
ORF51	−0.21	0.6009	0.9627
ORF69	−0.18	0.6010	0.9627
ORF44	0.08	0.6030	0.9627
ORF60	−0.13	0.6234	0.9627
ORF20	−0.08	0.6272	0.9627
ORF13b	−0.07	0.6385	0.9627
ORF11c	−0.14	0.6414	0.9627
ORF34	0.08	0.6437	0.9627
ORF7	−0.09	0.6626	0.9627
ORF36	−0.11	0.6831	0.9627
ORF1b	−0.17	0.6880	0.9627
ORF5	0.10	0.7103	0.9627
ORF30	−0.11	0.7240	0.9627
ORF27	−0.09	0.7243	0.9627
ORF2b	−0.05	0.7324	0.9627
ORF67	−0.09	0.7327	0.9627
ORF59	−0.06	0.7343	0.9627
ORF12	−0.08	0.7352	0.9627
ORF6	0.08	0.7376	0.9627
ORF28	0.06	0.7396	0.9627
ORF23	−0.03	0.8012	0.9940
ORF35	0.10	0.8128	0.9940
ORF71	−0.08	0.8303	0.9940
ORF25b	−0.06	0.8400	0.9940
ORF18	0.03	0.8648	0.9940
ORF48a	0.04	0.8775	0.9940
ORF19	−0.04	0.8897	0.9940
ORF55	0.01	0.8952	0.9940
ORF66	0.04	0.8989	0.9940
ORF40	−0.03	0.9133	0.9940
ORF50b	−0.02	0.9253	0.9940
ORF26	0.03	0.9298	0.9940
ORF32	−0.01	0.9583	0.9940
ORF54	−0.02	0.9629	0.9940
ORF33	0.01	0.9745	0.9940
ORF24	−0.01	0.9789	0.9940
ORF58	0.00	0.9852	0.9940
ORF47	0.00	0.9922	0.9940
ORF43	0.00	0.9940	0.9940

Here are shown, for each ORF, the differential expression between PHN and non-PHN samples, the statistical significance of this mean difference and statistical significance once the *P* values are corrected for testing of multiple variables (ORFs) simultaneously. Differential expression is shown on a log_2 scale, and calculated as the average log_2 scale expression in PHN samples minus average log_2 scale expression in non-PHN samples. A value of 0 means there is no difference in expression, a negative value means down regulation with respect to non-PHN and a positive value means up regulation with respect to non-PHN. Values can be anti-logged to a normal reference frame by using 2 as base and the value shown as exponent (e.g. $2^{-2.9}$ is a 0.82-fold change) Statistical test consists of a *t* statistic moderated through empirical Bayes. Sorted by significance; FDR *P* is the *P* value adjusted for multiple testing

cycles followed by a final extension of 72°C/10 min. *Cyclophilin* was chosen as an internal control for invariant gene expression to normalise for variable amounts of RNA in different samples. *Cyclophilin* PCR products were visualised on ethidium bromide stained 2% agarose gels, and the images digitized on an UVIdocD55XD system (Uvitec, UK) providing gel loading adjustment volumes for the ORF PCR samples.

Growth curve analysis of PHN versus non-PHN virus isolates

Growth curve experiments were also performed to compare the cytopathic effects between cell lines infected with PHN and non-PHN isolates. Standard plaque forming unit (PFU) assays were carried out on ten viral isolates (5 PHN and 5 non-PHN) grown in MeWo cells at days 0–5 post-infection (PI).

Results

Microarray analysis of PHN and non-PHN-associated VZV isolates

The key summary of the microarray data is shown in Tables 3 and 4. The statistical analysis shows no significant differences in regulation of any ORFs between PHN and non-PHN VZV samples, even before adjusting for multiple testing. Averaged across samples of the respective group, the measured fold changes of expression between the two groups are also very small (<twofold up or down). This means that the expression from sample to sample within a group (PHN patients or non-PHN patients) varies as much, or more, than the expression between those groups. With the given number of samples, we cannot know from an analytical perspective if the patient-to-patient variation is large by nature (or procedure) or if theoretically, atypical patients were sampled (the latter is considered most unlikely), or if there are viral expression patterns that differ for currently unrecognized patient subgroups. Power calculations (data not shown) on data from Set 1 suggest that the ability to detect fourfold expression changes for all ORFs with 80% power at a significance level of $\alpha = 0.05$ requires around nine biologically independent samples per group, Towards the ability to detect twofold changes the required sample size rises to around 27. Therefore, we would expect to be able to identify real changes within these two limits in our expanded study. More subtle average expression changes as measured in our study can still be statistically significant but would require much larger sample sizes to achieve sufficient power and would be contraindicated in a search for easily detectable marker transcripts.

The results obtained with the larger dataset (in conjunction with Set 1) confirm the original analysis performed in the first experiment (Set 1). Having re-performed the analysis with a larger input dataset, it appears that the large patient differences (independent of clinical phenotype) are an inherent feature of the biology, the sampling of patients from the population, currently unknown patient subgroups or the further processing stages of patient-derived samples.

Instead of pooling data variance across ORFs (which assumes that all ORFs have similar variance and follow a log-normal distribution) and taking correlation structure between technical replicates into account, we also considered a non-parametric permutation test that estimates statistical significance of an ORF compared to randomly shuffled data of the same ORF. This does not rely on unproven assumptions, but has its own shortcomings in terms of considering technical replicates as equal in importance to biological replicates. These data are shown in Table 4. Using this method, eight ORFs were identified (Table 4) as having different average expression values between PHN and non-PHN samples: ORFs 11b, 46b, 21, 41, 56, 16, 42, 15 (in that order).

Quantitative real-time PCR analysis

Although the microarray data indicated that there was no significant difference in VZV ORF expression between the two patient groups (Table 3), the application of an additional statistical permutation test identified a restricted number of ORFs (see above) whose expression may be altered (Table 4). In order to address this apparent discrepancy, the more sensitive qRT-PCR technique was employed. TaqMan assays were performed on these candidate ORFs in addition to the control grouping comprising ORFs 7, 18 and 63 whose expression is predicted from the microarray data to be invariant. When the ORF comparative C_T data were calculated using MeWo cells as the calibrator, no pattern emerged between the groups. As depicted in Fig. 1, expression levels of all the candidate and control ORFs were significantly higher in the non-PHN line S10 while the other non-PHN line S29 had the lowest level. This trend most likely reflects differences in the VZV genomic copy number among the four-infected cell lines. In order to track expression of each candidate ORF independent of copy number in the four cell lines, the comparative C_T data were expressed relative to one of the control genes viz ORF7. Examination of Table 5 suggests there are no significant differences in gene expression of the candidate or control ORFs between the PHN and non-PHN cell lines.

These differences in message levels among the four cell lines (Fig. 1) were reproducible even when expression was measured by the less sensitive standard semi-quantitative

Table 4 Statistical significance and adjusted significance if using a permutation Welch *t* test instead of empirical Bayes

	P value
ORF11b	0.0030
ORF46b	0.0096
ORF21	0.0116
ORF41	0.0166
ORF56	0.0174
ORF16	0.0266
ORF42	0.0307
ORF15	0.0312
ORF46a	0.0570
ORF10	0.0621
ORF48a	0.0644
ORF17	0.0649
ORF2a	0.0686
ORF29	0.0775
ORF31	0.0830
ORF1a	0.0838
ORF52	0.0994
ORF45	0.1695
ORF1b	0.1739
ORF8	0.1787
ORF61	0.1856
ORF33	0.2117
ORF50b	0.2250
ORF68	0.2391
ORF60	0.2399
ORF14	0.2500
ORF51	0.2511
ORF38	0.2513
ORF12	0.2766
ORF30	0.2817
ORF13a	0.2945
ORF4b	0.3070
ORF62a	0.3237
ORF19	0.3302
ORF32	0.3330
ORF62b	0.3636
ORF26	0.3961
ORF43	0.3987
ORF4a	0.4001
ORF65	0.4065
ORF3	0.4243
ORF49	0.4362
ORF22	0.4401
ORF57	0.4525
ORF71	0.4556
ORF50a	0.4794
ORF54	0.4812
ORF2b	0.5135
ORF47	0.5448
ORF40	0.5847
ORF55	0.6110
ORF53	0.6336
ORF11a	0.6547
ORF58	0.6770
ORF48b	0.7022
ORF37	0.7303
ORF11c	0.7435
ORF44	0.7644
ORF23	0.7645
ORF39	0.7860
ORF28	0.7916
ORF64	0.8044
ORF25b	0.8046
ORF59	0.8061
ORF70	0.8080
ORF36	0.8560
ORF24	0.8690
ORF25a	0.8699
ORF5	0.8889
ORF69	0.9040
ORF9	0.9303
ORF13b	0.9318
ORF6	0.9434
ORF34	0.9465
ORF27	0.9475
ORF35	0.9479
ORF66	0.9563
ORF7	0.9657
ORF20	0.9825
ORF67	0.9842
ORF18	0.9860
ORF63	0.9887

Table 4 continued

For each ORF, Westfall and Young's step-down multiple testing algorithm is applied to statistically test for expression differences between PHN and non-PHN samples. In this approach to statistical testing, actual observed results of a Welch *t* test are compared to Welch *t* test results for all possible sets of randomly permuted data for that ORF. This permutation approach is non-parametric and inherently adjusts for multiple simultaneous testing of hyptotheses (ORFs) as it compares test statistics to an empirical rather than theoretical distribution. Sorted by significance; fold-change is identical to Table 1, once the different sort order is accounted for

RT-PCR method as depicted in Fig. 2. The combined PCR-based studies establish a correlation between ORF expression levels and each individual cell line independent of PHN status. The standard reverse transcription polymerase chain reaction method was used to analyse a further two non-PHN and two PHN cell lines but failed to identify any candidate ORF breaking the relationship between copy number and expression levels in any of the newly tested cell lines (data not shown).

Growth curve experiments in patient isolates

The results of the growth curve experiments for the five PHN and five non-PHN VZV isolates are shown in Fig. 3. It can be seen that they are very similar for all ten isolates

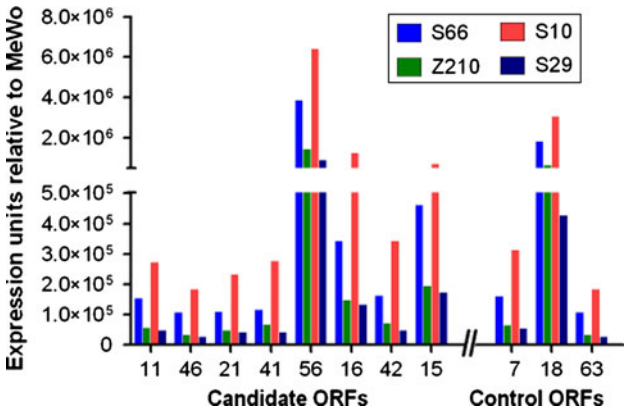

Fig. 1 qRT-PCR derived message abundance relative to MeWo cells of candidate and control ORFs in non-PHN (S10 and S29), and PHN cell lines (S66 and Z210) showing a distinct pattern between the cell lines (NB due to range of expression between the ORFs the X-axis is scaled in a discontinuous format)

Table 5 qRT-PCR data depicting fold change in ORF expression in individual cell lines relative to ORF7

	Non-PHN cell lines		PHN cell lines	
	S10	S29	S66	Z210
Candidate ORFs				
ORF7	1	1	1	1
ORF11	4.646	4.585	4.193	4.535
ORF46	2.105	2.455	1.836	2.369
ORF21	5.176	5.002	5.692	6.268
ORF41	1.782	1.026	1.018	1.467
ORF56	3.562	4.435	3.025	3.215
ORF16	1.85	1.486	1.679	1.422
ORF42	9.883	12.46	10.688	9.613
ORF15	12.527	9.151	10.077	9.487
Control ORFs				
ORF18	37.32	47.242	32.424	36.733
ORF63	2.105	2.455	1.836	2.369

indicating that the viruses associated with PHN are not simply replicating more efficiently in culture than those not associated with PHN.

Discussion

In this study, we used long oligonucleotide VZV microarrays to compare the transcriptomes of PHN and non-PHN-associated viral isolates following infection of MeWo cells, a study that, while of considerable clinical relevance, has to the best of our knowledge not been previously attempted. The key and somewhat unexpected finding following a rigorous and varied statistical analysis was that viral gene expression from sample to sample within a

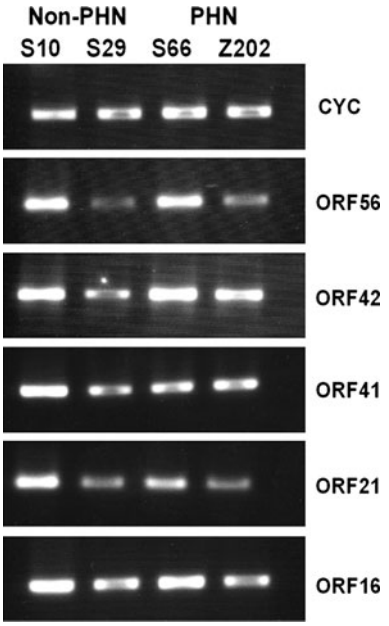

Fig. 2 Standard semi-quantitative RT-PCR depicting expression of a representative number of ORFs relative to the message levels corresponding to the housekeeping gene *Cyclophilin* in non-PHN (S10 and S29) and PHN cell lines (S66 and Z210)

group comprising 13 PHN and 10 non-PHN patients varied as much, or more than between the two groups. The clear inference from this data is that this particular stratagem using viral gene microarray analysis found no sizeable transcriptomal differences that would allow a pre-emptive diagnosis of PHN in an individual patient with herpes zoster. Although we initially obtained 75 individual VZV patient isolates, only about a third of these could be cultured. Undoubtedly, the study would have benefited from a larger sample number in order to identify much smaller expression changes and potential patient subgroupings, and thus might have yielded different results. We consider it very unlikely that the cultured samples would have somehow been selected for producing equivalent transcriptomes in the two patient groups. In addition, the original study plan was not geared towards statistical testing of equivalence in conditions most notably copy number differences between the isolates.

However, the additional permutational analysis on the array data suggested that there might well be differences in mRNA levels corresponding to ORFs 11b, 46b, 21, 41, 56, 16, 42 and 15. In order to confirm or otherwise qRT-PCR was performed on two non-PHN and two PHN representative cell lines. However, when the message levels of these eight ORFs were expressed relative to ORF7 in each of these cell lines there was no pattern difference in gene expression between the groups. Thus, the data obtained from the more sensitive qRT-PCR technique were inconsistent from the findings of the microarray permutational

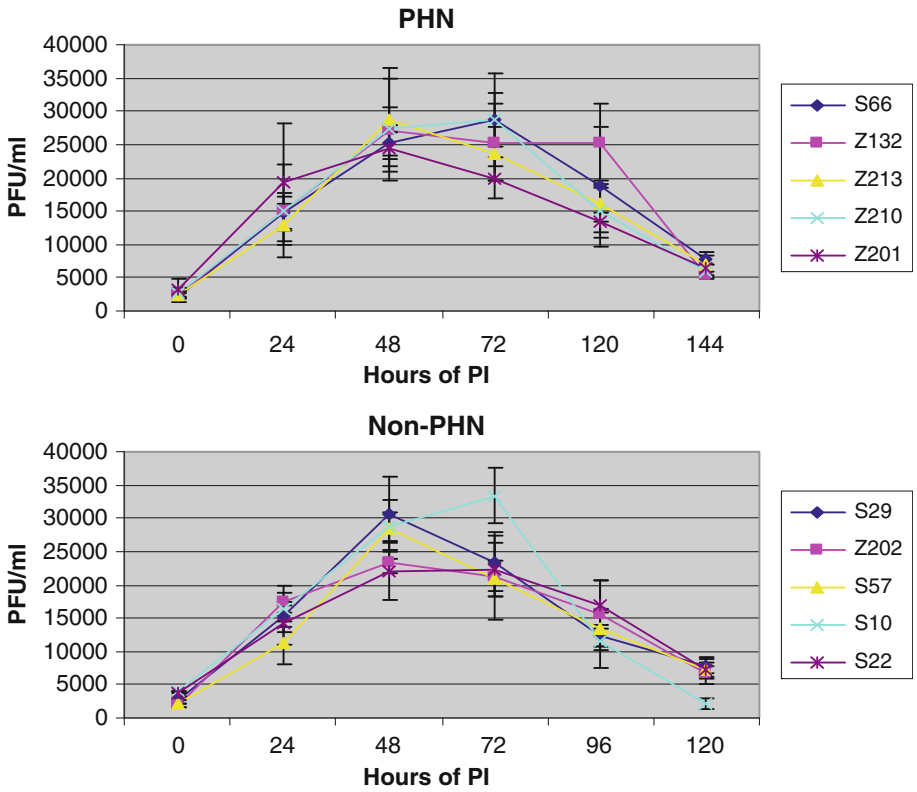

Fig. 3 Growth curve analysis in PHN and in non-PHN isolates. Standard PFU assays were performed on ten viral isolates (5 PHN and 5 non-PHN) grew in MeWo cells at days 0–5 PI. The growth curve patterns are very similar for all ten isolates

analysis thus compromising the candidacy of these ORFs as gene markers to distinguish between the PHN and non-PHN condition. However, the qRT-PCR results which were corroborated with semi-quantitative standard PCR (Fig. 2) were consistent with the general findings of the microarray data (Table 3) concluding that transcriptional fold changes between the two groups were not significant.

It was also important to determine whether PHN-associated VZV might replicate more efficiently than viruses not associated with PHN since it is known that different viruses may show similar growth in culture despite having different viral transcriptomes as we have recently shown using P-Oka and VZV vaccine strains [9]. The growth curve experiments carried out on ten different infected cell lines (five from each group) confirmed that both types of virus replicated with equivalent efficiency in culture.

Taken together, these findings suggest that viral microarray based transcriptomal measurements are unlikely to prove of clinical utility in predicting the incidence of PHN. It is possible, however, that relevant differences in gene transcription in the different isolates could be too small to be detected by our study design which was geared to find twofold or greater changes. Further, we cannot exclude the possibility that there may be an unknown number of patient subgroups with distinct expression profiles that our techniques were unable to detect. The in vitro approach adopted here cannot, of course, be directly extrapolated to the in vivo situation, so it is possible that such transciptomal data reported here may differ from that which might pertain in animal models of VZV. Notwithstanding this conclusion, we consider it important to continue to study viral phenotype heterogeneity in patients with herpes zoster, as well as the possible influence of viral genotype on PHN development, and also to consider revisiting the issue of viral transcriptomal differences. A possible stratagem worth consideration would be to initially select PHN and non-PHN MeWo cell lines with similar VZV copy numbers before comparing their transcriptional profiles, studies that might also be performed in the SVG [10] cell line which may have much relevance to VZV strain-specific expression.

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