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Genome-wide transcriptomic variations of human lymphoblastoid cell lines: insights from pairwise gene-expression correlations

Aims: Human lymphoblastoid cell lines (LCLs) are a rich resource of information on human interindividual genomic, transcriptomic, proteomic and phenomic variations, and are therefore gaining popularity for pharmacogenomic studies. In the present study we demonstrate that genome-wide transcriptomic data from a small LCL panel from unrelated individuals is sufficient for detecting pairs of genes that exhibit highly correlated expression levels and may thus convey insights about coregulated genes. **Materials & methods:** RNA samples were prepared from LCLs representing 12 unrelated healthy adult female Caucasian donors. Transcript levels were determined with the Affymetrix Human Gene arrays. Expression-level correlations were searched using Partek® Genomics Suite™ and the R environment. Sequences of detected correlated gene pairs were compared for shared conserved 3'-UTR miRNA binding. **Results:** Most of the approximately 33,000 transcripts covered by the Affymetrix arrays showed closely similar expression levels in LCLs from unrelated donors. However, the expression levels of some transcripts showed large inter-individual variations. When comparing the expression levels of each of the top 1000 genes showing the largest interindividual expression variations against the others, two sets containing 156 and 4438 correlated gene pairs with false-discovery rates of 0.01 and 0.05 were detected, respectively. Similar analysis of another gene-expression data set from LCLs (GSE11582) indicated that 61 and 39% of identified pairs matched the pairs detected from our transcriptomic data, respectively. Shared conserved 3'-UTR miRNA binding sites were noted for 14–17% of the top 100 gene pairs, suggesting that regulation by miRNA may contribute to their coordinated expression. **Conclusion:** Probing genome-wide transcriptomic data sets of LCLs from unrelated individuals may detect coregulated genes, adding insights on cellular regulation by miRNAs.

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KEYWORDS: Affymetrix gene-expression arrays ■ bioinformatics ■ Epstein–Barr virus ■ *GUSB* ■ human lymphoblastoid cell lines ■ LCLs ■ miRNA ■ *RPL5* ■ *RPL10* ■ *RPL18* ■ transcriptomics

Human lymphoblastoid cell lines (LCLs) immortalized by the Epstein–Barr virus (EBV) are gaining increased popularity as a research tool for the study of human genomic, epigenomic and phenomic variations and are therefore often utilized for pharmacogenomic studies [1–5]. EBV-immortalized LCLs are utilized by the HapMap Project [3] and other international collaborative projects on human genome variation, and many biobanks include large LCL repositories from healthy or diseased individuals as a primary biomedical research tool [1–5].

LCLs are generated from peripheral blood B lymphocytes and retain most of their phenotypic properties, including the production of antibodies. Moreover, the EBV genome does not incorporate with nuclear DNA meaning that the nuclear genomic sequence remains intact, faithfully representing the donor's sequence. The transcriptomes of

LCLs encompass a large portion of human genes from diverse cellular pathways [6] and have been valuable for studying genome-wide individual differences in transcriptomes [7] and in alternative mRNA splicing [8]. Thus, human LCL transcriptomes faithfully reflect heterogeneity specific to the individuals from whom the cells have originated. LCLs have also proved valuable for studies on pharmacogenomic gene-expression markers, for example, for the cancer drugs cisplatin, etoposide, *N*-methyl-*N*-nitro-*N*-nitrosoguanidine, gemcitabine and cytosine arabinoside [9–14], as well as for sensitivity to radiation [15]. Of note, Choy and coworkers have identified batch effect and cellular growth rates as major confounders for some of these findings [16]. Therefore, transcriptomic studies on drug effects in LCLs should be viewed as preliminary until their validation with clinical samples.

Marc Vincent¹,
Keren Oved^{2,3},
Ayelet Morag²,
Metsada Pasmanik-
Chor⁴, Varda Oron-
Karni⁴, Noam Shomron³
& David Gurwitz^{*2}

¹Université Paris Descartes, INSERM UMRS775, Paris, France

²Department of Human Molecular Genetics & Biochemistry, Sackler Faculty of Medicine, Tel-Aviv University, Israel

³Department of Cell & Developmental Biology, Sackler Faculty of Medicine, Tel-Aviv University, Israel

⁴Bioinformatics Unit, George Wise Faculty of Life Sciences, Tel-Aviv University, Israel

*Author for correspondence:

Tel.: +972 3640 7611

Fax: +972 3640 5168

gurwitz@post.tau.ac.il

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Recently, we have employed LCLs from unrelated healthy individuals as tools for discerning shared drug pathways [17], searching for drug-response biomarkers for antidepressant serotonin-selective reuptake-inhibitor drugs [18,19] and for studying sex differences in adverse drug events [20]. LCLs have also been constructive for discovering genes and metabolic pathways implicated in severe diseases such as autism [21].

Studies of gene-expression correlations have proven to be a robust tool for reconstructing gene network pathways [22–24]. Eisen and coworkers have introduced the cluster analysis of genome-wide expression patterns and demonstrated that the method can be applied for gaining insights about the function of poorly characterized genes according to their expression correlations with well-known genes [22]. Numerous studies have subsequently used similar clustering approaches for studying gene ontology and gene-expression changes in various pathological states. In the present article, we describe the potential of genome-wide transcriptomic data sets from LCLs representing unrelated healthy donors for exploring pairwise correlations of gene-expression levels.

Materials & methods

■ Cells

LCLs from 12 consenting healthy adult female donors of Caucasian ancestry were obtained from the National Laboratory for the Genetics of Israeli Populations at Tel-Aviv University (Israel) [101] as previously described [17–20]. The cell lines were immortalized from lymphocytes of healthy adult donors using the same stocks of B-95-derived EBV and with the same immortalization protocol. Cells were maintained in RPMI medium, supplemented with 10% fetal bovine serum and antibiotics (100 U/ml penicillin; 100 µg/ml streptomycin), and kept at a temperature of 37°C, with 6% CO₂ and 100% humidity. All cell culture reagents were purchased from Biological Industries (Israel).

■ RNA extraction

RNA extraction was performed from cells incubated in T-25 flasks under optimal growth conditions (exponential growth). Cells were centrifuged and then lysed using Tri-reagent (T9424®, Sigma-Aldrich, MO, USA), followed by RNA separation using chloroform and precipitation using isopropanol, total RNA purification was achieved using phenol-chloroform extraction and Phase Lock Gel™ tubes (light 2 ml; Fisher Scientific, NH, USA) [25]. RNA quality was

checked using RNase-free, 1% agarose gel, and was quantified using a nanodrop spectrophotometer (ND-1000; Fisher Scientific). The spectrophotometric absorbance parameters of the samples were: 260/280 nm > 2.0 and 260/230 nm > 2.0.

■ Microarray experiment & microarray data analysis

Twelve Affymetrix (CA, USA) GeneChip® Human Gene 1.0 ST arrays were used for gene-expression analysis according to the instruction manual, as described in the Affymetrix website [102]. Human Genome Organisation (HUGO)-compliant probe set annotation was retrieved from the Affymetrix website (latest release 32). Microarray analysis was performed on CEL files using Partek® Genomics Suite™ (Partek Inc., MO, USA) [103]. Batch effect removal was applied for the different samples, to remove individual variations. All expression units are displayed as raw expression data (Affymetrix arbitrary units in log₂ scale). Gene–gene correlations and corresponding plots were obtained with the R environment using linear least-square fitting. First, we selected the 1000 probe sets that showed the highest gene-expression-level variation. We then considered the set of 499,500 pairs that could be generated from these 1000 probe sets. From this set, we kept only the pairs where each of the probe sets annotated with coding genes unambiguously attributed to the same gene family (i.e., annotation of each of the probe sets included one gene family only; this step was essential for removing probe sets whose annotations were ambiguous). Since our goal was to find novel unreported gene pairs, we proceeded to crude filtering of probe set pairs that we considered obvious or uninformative, as both pair members belong to the same gene family. Our criterion to decide *a-priori* if a probe set pair was uninformative was to check whether the probe sets targeted the same genes or genes belonging to the same family. Gene families were retrieved from HUGO gene annotations using mapping files available for download at the HUGO gene nomenclature website [104]. We added supplementary mapping rules to regroup immunoglobulin genes in a corresponding superfamily.

At the end of the filtering procedures we obtained 410,557 probe set pairs corresponding to 275,189 gene pairs produced from 887 genes. We proceeded to analysis with these remaining pairs, and their expression signals in the 12 LCLs were compared pairwise. The correlation of two probe sets was assessed using a two-sided

statistical test based on the Pearson correlation coefficient, as available in the 'cor.test' routine from the 'stats' package in the R environment. The statistical significance of the correlations was characterized using the t-statistic and computing the corresponding p-value. In order to select sets of pairs to be further studied, we chose a level of significance α to be used with p-values that were corrected for multiple testing using appropriate procedures. Namely, to obtain two large sets with small expected false discovery rate (FDR) we used the Benjamini and Hochberg step-up FDR control procedure [26]. To obtain a small list of gene pairs with low probability of containing false positives we used a Bonferroni control procedure. We denote by α the level at which we control either the FDR or the family-wise error rate.

■ Common miRNA binding sites for paired genes

Sequences of paired genes showing transcriptome correlations were analyzed for the presence of shared evolutionally-conserved 3'-UTR miRNA binding sites. For each gene pair we first predicted miRNAs that target each gene using DIANA microT v3.0 software [27,28,105] and TargetScanHuman 5.2 [106]. The DIANA-microT 3.0 and TargetScanHuman 5.2 algorithms are based on parameters that are calculated individually for each miRNA, and for each miRNA recognition element, depending on binding and conservation levels. The total predicted score is calculated as the weighted sum of conserved and nonconserved miRNA recognition elements on the 3'-UTR of a gene [27,28]. For DIANA microT v3.0, a higher score corresponds to higher possibility of targeting the gene; in our predictions we used score threshold of 7.3, which is the software default [105]. We subsequently compared between the miRNAs predicted to target one gene of each pair with the miRNAs predicted to target the other pair member, and searched for miRNAs targeting both pair members. Lastly, data were filtered by analyzing shared miRNA binding sites with either software [105,106].

■ Comparison with web resources of human transcriptomics data

Data from our microarray experiments were compared with data from the Gene Expression Omnibus on the NCBI server [107]. Top-expressed genes and those showing the highest expression level standard deviations among individual LCLs were searched using the Geo Data Set browser interface [107]. The search was

limited to expression data derived from LCLs from healthy donors and using the Affymetrix or Illumina (CA, USA) arrays. One data set (GSE11582; [108]) from the HapMap Project corresponding to cell lines from 269 unrelated CEPH individuals was found to be suitable for comparison. From this data set, we used the 83 LCLs corresponding to donors of European ancestry. This data set is based on Affymetrix Human Genome U133A arrays. The comparison was performed using the CEL files available from the website, and analysis by Partek Genomics Suite [103] as previously described [18,19]. In order to proceed with the comparison, we selected the gene pairs associated with probe sets pairs showing a correlation with a Benjamini and Hochberg (BH)-FDR-corrected p-value either under level $\alpha = 0.05$ (BH-FDR large set) or $\alpha = 0.01$ (BH-FDR small set) when using data from the 12 LCLs. We then found the corresponding genes and probe sets pairs in the GSE11582 data set using Affymetrix's latest annotation files for the U133A arrays and performed a correlation analysis using the GSE11582 expression data. For each original gene pair we then obtained a raw $p\text{-Val}_{\min}$ measure equal to the minimum p-value observed over all corresponding probe set pairs. Equivalently, we obtained $r^2_{\max}$ measures for the Pearson correlation coefficients. These aggregate measures were necessary owing to the large numbers of relationships between gene pairs and probe set pairs after filtering. In order to assess the significance of these aggregated p-values and the Pearson correlation coefficients, we sampled pairs of unrelated genes from the GSE11582 data set and measured their respective $p\text{-Val}_{\min}$ and $r^2_{\max}$. We repeated the sampling ten times for $n\alpha$ gene pairs, where $n\alpha$ is the number of gene pairs we had previously retrieved in the GSE11582 data set at given level.

■ Novelty check using interaction databases

The 4438 gene pairs that were found using the gene-expression data sets from our 12 LCLs following the filtration steps were programmatically searched in two protein interactions databases, iRefIndex [29,109] and STRING [30,31,110]. For the iRefIndex search, the HUGO gene annotations were converted to uniprot annotations using mapping files available from the HUGO website [104]. Of 666 initial genes that are present in the large list of gene pairs (SUPPLEMENTARY TABLE 1; see www.futuremedicine.com/doi/suppl/10.2217/pgs.12.179), 628 were found to have corresponding uniprot identifiers

and could be examined in these protein interactions databases. The iRefIndex database was then downloaded and queried using the iRefR package from the R environment. To recover interactions from the STRING database we used the STRING web API [110]. As a first step, we queried the API to obtain STRING identifiers from the HUGO annotations. Next, 640 of the 666 initial genes were found to have corresponding entries in STRING. A second query, again employing the STRING database, was carried out using the obtained identifiers in order to retrieve interactions of interest. For each database, the set of interactions was constrained to represent human proteins.

Results & discussion

■ Gene pairs & their tentative coregulation by miRNAs

When analyzing the basal transcriptomes of individual LCLs representing 12 unrelated female donors, most of the approximately 33,000 transcripts covered by the Affymetrix arrays showed closely similar expression levels. However, 125 transcripts coding for known genes showed variations of more than twofold (>1 Affymetrix raw expression units in $\log_2$ scale), as determined with the Affymetrix Human Gene 1.0 ST arrays. TABLE 1 presents the mean expression levels and standard deviations for the top 25 genes with the highest interindividual expression levels variations. As shown, for some genes the expression levels vary by over 50-fold between LCLs with high- and low-expression levels. Notably, five of the ten top genes in this list (*IGHD*, *IGKV3D-11*, *IGKC*, *VSIG6* and *IGHM*) code for immunoglobulin family proteins. The correlations in their individual expression levels are not surprising as they reflect inherent recombination during B-cell maturation; nonetheless, these correlations demonstrate the power of our computational approach. For a full list of transcript level variations within the same LCLs panel see SUPPLEMENTARY TABLE 2.

The 15 most notable correlations found between paired genes are shown in TABLE 2 & FIGURE 1. This list was constructed by comparing the expression levels of each of the 1000 most variable transcripts in each of the 12 unrelated individuals with each other, followed by filtration as described under 'Materials & methods'. It is evident that some expression level correlations for paired genes are very high, as also illustrated by the examples presented in FIGURE 2. All 15 pairs presented have a Bonferroni corrected $p < 0.05$.

The examples shown in FIGURE 2 demonstrate

that high expression level correlations were also observed for genes located on different chromosomes – apparently reflecting coregulation by the same transcription factor or miRNAs. A search for possible shared miRNAs which may bind to conserved 3'-UTR regions of both genes in each of the top 100 gene pairs was performed using the DIANA-microT 3.0 algorithm [27,28,105] and TargetScanHuman 5.2 [106] software (see 'Materials & methods' section). These searches yielded 17 and 14 pairs, respectively, where the same miRNAs were predicted to bind and apparently regulate the expression of both pair members, as presented in TABLE 3. That is, our search has detected common miRNAs binding sites for 17 and 14% of the top 100 gene pairs, using DIANA and TargetScan software, respectively. The two software tools agreed for 11 of these 100 top gene pairs (TABLE 3). There is a continuous debate in the miRNA field about the reliability and the methods used for target-gene predictors. While this is beyond the scope of this study, we found it appropriate to investigate the shared miRNA binding sites for the correlated expressed pairs of genes. We did not want to rely on merely one target predictor as this might bias us to one method for target selection. Therefore, we chose two of the most popular ones (out of many others available). The sometimes low overlap in the targets between the two methods indicates the different features each method uses to detect target genes. For example, one tool might rely on conserved targets while the other on nonconserved ones. Thus, combining the output of the two sets of target miRNA allows the reader to gain insight into two approaches to identify these sets and does not necessarily weaken any of the conclusions regarding these overlaps. If one wants to be on the cautious side then one should take only the overlapping miRNA for further studies, and for this purpose these are also shown in TABLE 3.

Of note, over half of human genes are estimated to be regulated by miRNAs, and each miRNA is assumed to regulate the expression of several genes [32,33]. Interestingly, in the context of pharmacogenomics, miRNAs were suggested to be more commonly implicated in the regulation of genes relevant for drug pharmacodynamics as compared with drug pharmacokinetics [34].

The list of gene pairs with highly correlated transcript levels yielded some surprising gene pairs not known to share a biological pathway. For example, a striking correlation with $p = 4 \times 10^{-9}$ and Pearson correlation coefficient $r^2 = 0.98$ was noted for the expression of the orphan G-protein-coupled receptor *GPRC5D*

(located on chromosome 12p13.3) and *AKAP6* (*PRKA*; located on chromosome 14q12). Likewise, a correlation with $p = 7 \times 10^{-8}$ and Pearson correlation coefficient $r^2 = 0.97$ was observed for the expression of *CHL1* (located on chromosome 3p26.1) and *CRIM1* (located on chromosome 2p21); while the proteins coded by the latter two genes are both implicated in tissue development [35,36], they are not known to share a cellular pathway.

Further examples of gene pairs exhibiting high expression level correlations (small and large BH-FDR sets with FDR controlled at level $\alpha = 0.01$ $\alpha = 0.05$) in LCLs from 12 unrelated individuals are presented in SUPPLEMENTARY TABLE 1 & SUPPLEMENTARY TABLE 3 containing 156 and 4438 gene pairs, respectively. It is noteworthy that the expression levels are positively correlated for the majority of such gene pairs: from the top 100 correlations (by r^2 value) only six gene pairs (6%) show negative expression level correlations. This observation suggests that these top correlations are unlikely to represent chance findings, in which case one would expect to observe equal numbers for positive and negative expression level correlations for gene pairs. This conclusion is further supported by the finding that when examining the large BH-FDR set of 4438 gene pairs, the proportion of negative correlations rises from only 6 to 25% of all gene pairs. Moreover, the high representation of positive correlations suggests that the transcription of most detected gene pairs is coregulated by shared transcription factors and/or miRNAs.

■ Novelty of the detected gene pairs

With the large BH-FDR list of 4438 gene pairs we proceeded to query protein interaction databases to examine whether these pairs were novel when compared with protein interactions included in the interaction databases iRefIndex and STRING (see 'Materials & methods'). In the iRefIndex database, no known interactions were found for any of these 4438 gene pairs, while only 29 (0.63%) gene pairs had corresponding interactions in the STRING database. These results are better interpreted keeping in mind that the two protein-centric databases include additional interaction types besides direct physical protein–protein interactions. More specifically, they both include known genetic interactions and the STRING database also includes functional associations inferred from scientific literature text-mining (both co-citations and natural language processing), as well as coexpression analysis [30, 31]. From this

Table 1. Genes exhibiting high expression level variations among human lymphoblastoid cell lines from unrelated individuals.

Gene	Average [†]	SD	Range (fold difference)
<i>IGHD</i>	10.60	2.53	5.17–12.41 (104)
<i>UGT2B7*</i>	7.58	2.40	2.69–10.12 (172.4)
<i>IGKV3D-11</i>	6.25	2.11	3.02–10.3 (155.4)
<i>PLOD2</i>	6.14	1.95	3.41–8.85 (43.4)
<i>IGKC</i>	6.5	1.92	3.28–9.43 (71)
<i>NRN1</i>	6.94	1.88	4.29–10.0 (52.3)
<i>VSIG6</i>	6.84	1.86	4.95–10.72 (54.6)
<i>IGHM</i>	9.67	1.86	5.74–11.59 (57.7)
<i>HLA-DQA1</i>	9.55	1.85	6.2–11.38 (36.2)
<i>RGS13</i>	7.09	1.84	3.69–9.46 (54.6)
<i>UGT2B15</i>	6.60	1.82	3.24–8.60 (41.1)
<i>IGLJ3</i>	10.43	1.82	7.14–12.36 (37.3)
<i>IGHA1</i>	10.56	1.79	7.32–12.53 (37)
<i>NLRP2</i>	4.92	1.75	2.82–7.33 (22.8)
<i>LEPREL1</i>	5.5	1.69	4.01–8.7 (25.8)
<i>IGK</i>	6.36	1.65	3.98–9.35 (41.3)
<i>APP</i>	6.66	1.64	3.53–8.84 (39.7)
<i>FAM169A</i>	5.60	1.63	3.71–7.98 (19.3)
<i>MMP7</i>	5.02	1.60	3.01–7.61 (24.3)
<i>CHI3L2</i>	8.95	1.59	6.21–10.87 (25.3)
<i>TC2N</i>	5.33	1.56	3.0–7.86 (29)
<i>PLD1</i>	7.31	1.55	4.11–9.12 (32.2)
<i>KIAA1324L</i>	4.86	1.51	3.0–6.69 (12.9)
<i>CHL1</i>	6.99	1.50	4.32–8.76 (21.7)
<i>GUCY1B3</i>	7.86	1.49	4.3–9.57 (38.6)

Mean and SD expression values are presented for the top 25 genes whose expression varied the most (highest SD) among the 12 individual human lymphoblastoid cell lines from unrelated female donors.

[†]Expression values are shown in arbitrary Affymetrix units in $\log_2$ scale (see text). See SUPPLEMENTARY TABLE 2 for the entire data set.

*This entry represents transcript #8100758 on the Affymetrix Human Gene 1.0 ST microarrays, which, according to the Affymetrix website, detects UGT2B7. However, according to our analysis this annotation seems to be incomplete, as the probes used for this transcript also perfectly match the sequences of UGT2B10, UGT2B11, UGT2B15 and UGT2B17 (all are closely similar human genes). The Affymetrix support team has been notified about this mistake.

SD: Standard deviation.

we infer that most of the gene pairs detected in our study represent novel associations compared with known interactions involving human genes and their protein products.

■ Confirmation with an independent human LCLs gene-expression data set

Considering the small number of transcriptomic data sets in the present study (12 arrays), and in order to confirm the significance of the detected gene pairs, we quantified to what extent their expression levels were correlated in an independent and by far larger LCLs genome-wide

Table 2. Gene pairs exhibiting the highest correlations of expression levels in human lymphoblastoid cell lines from unrelated donors.

First gene	Second gene	r ²	Raw p (pVal _{min} in GSE11582†)
AKAP6	GPRC5D	0.9724	3.92 × 10 ⁻⁹ (5 × 10 ⁻³)
BEND4	IFI44L	0.9665	1.05 × 10 ⁻⁸
HNF1B	EPS8	0.9661	1.12 × 10 ⁻⁸ (~0)
CPNE4	CYP27A1	0.9629	1.76 × 10 ⁻⁸
SGPP2	IFI44	0.9581	3.21 × 10 ⁻⁸
CREB5	NCKAP5	0.9542	5.04 × 10 ⁻⁸
SLC39A10	MYO1F	0.9541	5.09 × 10 ⁻⁸
CREB5	IFIH1	0.9538	5.27 × 10 ⁻⁸ (6 × 10 ⁻⁹)
BEND4	SGPP2	0.9528	5.82 × 10 ⁻⁸
LRRK2	LMO3	0.9523	6.19 × 10 ⁻⁸
CHL1	CRIM1	0.9515	6.71 × 10 ⁻⁸ (4 × 10 ⁻¹⁵)
IFI44L	SGPP2	0.9506	7.35 × 10 ⁻⁸
SAMD9L	OAS2	0.9492	8.50 × 10 ⁻⁸
OGFRL1	XYLT1	0.9492	8.50 × 10 ⁻⁸ (6 × 10 ⁻⁵)
CYP27A1	LIPN	0.9484	9.12 × 10 ⁻⁸

The expression levels of the top 1000 genes whose transcripts exhibited the highest variations in human lymphoblastoid cell lines from 12 unrelated Caucasian female donors (after removal of nameless transcripts) were compared pairwise and r² and p-values were calculated. Filtering was performed for removing spurious signals as described in the 'Materials & methods' section. The top 15 gene pairs with family-wise error rate Bonferroni corrected p < 0.05 are presented. The p-values of five of these gene pairs that were retrieved in GSE11582 are also displayed. The 92 and 4466 pairs whose expression yielded correlations with a BH-FDR controlled p-value at 0.01 and 0.05 for the 12 human lymphoblastoid cell lines are presented in SUPPLEMENTARY TABLE 1 & SUPPLEMENTARY TABLE 3. An example is presented in FIGURE 2 for the correlation between the expression levels of CHL1 and CRIM1 in 87 human LCLs from the GSE11582 data set.

†When available.

expression data set (GSE11582, see 'Materials and methods' section). When querying the GSE11582 human LCLs data set, and comparing with our two BH-FDR sets (FDR controlled at level $\alpha = 0.01$ and $\alpha = 0.05$, respectively), we retrieved 85 of our 156 pairs (54%) and 2307 of our 4438 pairs (52%; SUPPLEMENTARY TABLE 4), respectively. These numbers are consistent with the fact that the GSE11582 data set was obtained using an earlier Affymetrix chip that covers approximately half of the transcriptome (Affymetrix GeneChip® Human Genome U133A), while our study used a later chip aimed at covering the whole transcriptome (Affymetrix GeneChip® Human Gene 1.0 ST). In these two BH-FDR sets 61 and 39% of the pairs, respectively, had pVal_{min} < 0.05 (adjusted for multiple testing using the Bonferroni procedure). The median pVal_{min} of these retrieved pairs were 7.30 × 10⁻⁶ and 2.33 × 10⁻⁴ while their median r²_{max} values were 0.47 and 0.39, respectively. This comparison clearly showed a correlation enrichment when compared with ten random samples of n_α pairs from the same data

set (with n_{0.05} = 85 and n_{0.01} = 2307), showing for $\alpha = 0.05$ a median pVal_{min} of 0.18 ± 0.02 (inter-sample standard deviation) and a median r²_{max} of 0.2 ± 0.005. For $\alpha = 0.01$, as expected, the samples showed roughly similar median pVal_{min} of 0.14 ± 0.04 and r²_{max} of 0.22 ± 0.01. The same enrichment was observed when examining the proportion of pairs showing a pVal_{min} under the corrected significance level 0.05 in the ten random samples, which was only 1 ± 0.5% ($\alpha = 0.05$) and 4 ± 0.02% ($\alpha = 0.01$). Thus, our examination of an unrelated human LCL genome-wide expression data set indicated that approximately half the gene pairs detected in our data using this approach are most likely true examples of human gene pairs with highly correlated expression levels. Gene pairs not confirmed in the independent larger data set may represent false hits, or pairs missed in the larger data set (GSE11582) because it represents CEPH cell lines prepared by several laboratories, unlike our data set, thus introducing batch effect [16].

Future perspective

The data presented here should be considered as preliminary. Definitely, more robust data on gene pairs with correlated transcript levels, and possibly more structured gene networks, may be derived from larger panels of LCLs or other tissues collected from larger numbers of unrelated individuals. In addition, analyzing genome-wide expression data for larger gene sets (e.g., triads) is desirable, but beyond the scope of the present manuscript. For the time being, the following points should be considered in favor or using the data as a preliminary reference for studies on gene-expression variations among LCLs from unrelated individuals. First, the 12 human LCLs used for preparing the RNA samples and estimating the genome-wide expression profiles all represent female donors of Caucasian ancestry; the cell lines were all immortalized by the same laboratory [101] and using the same stock of EBV; these are key considerations when studying drug-response phenotypes and related transcriptomes in human LCLs, as demonstrated by Choy and coworkers [16]. Inclusion of transcriptomics data from female donors only may be crucial, as variations were noted between the male and female human transcriptomes [37]. However, as shown above, approximately half the gene pairs detected in our small female LCL cohort were also detected in the GSE11582 data set, which includes both male and female donors. In future, it would

be useful to compare the current data with genome-wide expression profiles of LCLs from larger numbers of male and female donors. Second, our LCLs were all grown in parallel using similar growth conditions and the RNA samples were prepared and assayed in parallel with similar protocols. Such similar growth and assay conditions assure high reproducibility of the transcriptomics data and enhance its quality for comparisons between LCLs from individual donors.

Two additional considerations support the validity of the recorded observations. First, for most genes the standard deviations for measurements of expression levels in the RNA samples representing the 12 unrelated female donors are small ($SD > 1$ were observed only for 139 of 33,297 transcripts). Second, in a recent study, the raw expression data from the same microarrays were employed for finding 12 tentative gene-expression biomarkers for LCL sensitivity to growth inhibition by the antidepressant drug paroxetine, and in that study the Affymetrix array data for these 12 genes were validated by real-time PCR and found to be in close correlation with the raw microarray expression data [18].

The concept of cluster analysis using genome-wide expression profiling from microarray experiments performed in different cell lines, or in a given cell line studied in different physiological conditions, is well established [22–24]. However, we would like to point out that the pairwise expression correlation approach as applied in the present report allows certain advantages compared with cluster analysis. First, while the primary input of pairwise analysis and cluster analysis are both pairwise relations (correlation/distance) between gene-expression probes, in cluster analysis these relations are aggregated to yield clusters. This implies that when attempting validation we either have to consider each cluster as a whole or to consider the separate pairwise relations that constitute them. In the former case it may not always be possible to find the exact same cluster composition using the validation data set, while the latter case reduces the validation to pairwise analysis. Moreover, the choice of the aggregation technique used (e.g., single-link hierarchical clustering) may add a bias on the choice of the clusters found and therefore complicate the interpretation of the results. Therefore, examining single pairs of gene-expression levels instead of clusters simplifies the definition, interpretation and validation of

the relationships found between genes. However, hierarchical clustering of gene-expression data sets definitely has other advantages over pairwise analysis, as it allows the construction of elaborate interactome maps and may help uncover their underlying structure [22–24].

Data on the whole-genome transcriptomes of human LCLs representing unrelated donors are openly available in several web databases, such as Gene Expression Omnibus on the NCBI server [107]. However, to our knowledge, such data have not been discussed in the scientific literature for the purpose of showing pairwise gene-expression patterns of human genes, as presented in this article. We hope that this concept will be further applied for reconstructing gene networks, using LCL transcriptomic data sets. It would also be of interest to examine if unique gene pairs are detected when performing such analysis for female versus male donors, young versus older donors, or donors of different ethnic backgrounds.

Our data may in addition be of use for selecting sets of genes with high expression levels and minimal variations in LCLs representing unrelated donors. Examples for ten such genes are shown in TABLE 4. Of note, three of them

Figure 1. Pearson correlation plot between the expression levels of *CHL1* and *CRIM1* as determined in human lymphoblastoid cell lines from 87 unrelated individuals from the GSE11582 data set.

Figure 2. Pearson correlation plots for the expression levels of four pairs of genes in human lymphoblastoid cell lines from 12 unrelated Caucasian female donors. Gene names are followed by their chromosomal locations; note that pair members in these examples are located on different chromosomes **(A)** *CHL1* (3p26.1) and *CRIM1* (2p21). **(B)** *CREB5* (7p15.1) and *IFIH1* (2q24). **(C)** *SLC39A10* (2q32.3) and *MYO1F* (19p13.3). **(D)** *CPNE4* (3q22.1) and *CYP27A1* (2q33-qter)

code for ribosomal proteins; by comparison, the expression levels for *GUSB*, which has been proposed as the most appropriate reference gene for expression studies in LCLs [38], show in our data set slightly higher variations with mean $\pm$ SD values of 7.73 ± 0.16 (SUPPLEMENTARY TABLE 2). We therefore propose that the ribosomal protein genes *RPL5*, *RPL10* and *RPL18* should also be considered as reference for expression studies in LCLs.

Microarray gene-expression data have been used for constructing gene interaction networks in *Drosophila melanogaster* [39] and *Saccharomyces cerevisiae* [40]. These studies employed time-course or single cell data from microarray experiments. However, to our knowledge, genome-wide microarray expression data sets from cell lines representing unrelated individuals have not been employed for exploring pairwise gene-expression correlations and the possible involvement of miRNAs in

Table 3. Pairs of genes with correlated expression levels and shared miRNA binding sites.

First gene	Second gene	r^2	Shared miRNAs		
			DIANA	TargetScan	DIANA and TargetScan
<i>HNFB1</i>	<i>EPS8</i>	0.9829	miR-92b, miR-92a, miR-200c, miR-200b, miR-32	miR-200b, miR-200c, miR-429, miR-25, miR-32, miR-92a, miR-92b, miR-363, miR-367	miR-92a, miR-92b, miR-200b, miR-200c, miR-32
<i>NCKAP5</i>	<i>CREB5</i>	0.9768		miR-29a, miR-29b, miR-29c, miR-130a, miR-130b, miR-301a, miR-301b, miR-454, miR-4295, miR-3666, miR-19a, miR-19b, miR-17, miR-20a, miR-20b, miR-93, miR-106a, miR-106b, miR-519d	
<i>LMO3</i>	<i>LRRK2</i>	0.9759	miR-181c, miR-181a, miR-181b, miR-181d	miR-181a, miR-181b, miR-181c, miR-181d, miR-4262	miR-181a, miR-181b, miR-181c, miR-181d
<i>CRIM1</i>	<i>CHL1</i>	0.9755	miR-369–3p, miR-570	miR-28–5p, miR-708, miR-3139, miR-570	miR-570
<i>PXDN</i>	<i>RPS6KA6</i>	0.9681		miR-9, miR-488	
<i>BEND4</i>	<i>ATP1B1</i>	0.9673	miR-9, miR-101, miR-181a, miR-181b, miR-181c, miR-181d, miR-522	miR-221, miR-222, miR-9, miR-543, miR-141, miR-200a, miR-101	miR-9, miR-101
<i>NOTCH2</i>	<i>CAMK2D</i>	0.9662	miR-30a, miR-30b, miR-30c, miR-30d, miR-30e, miR-186, miR-211, miR-204	miR-214, miR-761, miR-3619–5p, miR-204, miR-211, miR-539	miR-204, miR-211
<i>DLGAP1</i>	<i>BTBD3</i>	0.9659	miR-181a, miR-181b, miR-181c, miR-181d, let-7e, let-7a, let-7b, let-7c, let-7g, let-7i, let-7f, miR-148b, miR-148a, miR-152, miR-98, miR-524–5p, miR-520d–5p, miR-543	let-7a, let-7e, let-7b, let-7c, let-7d, let-7g, let-7i, let-7f, miR-98, miR-4458, miR-4500, miR-148a, miR-148b, miR-152	let-7a, let-7e, let-7b, let-7c, let-7g, let-7i, let-7f, miR-98, miR-148a, miR-148b, miR-152
<i>TNFRSF21</i>	<i>PLD1</i>	0.9651	miR-519b–3p, miR-519a, miR-519c–3p, miR-9		
<i>HOXB2</i>	<i>DDX60</i>	0.9626	miR-340		
<i>HOOK1</i>	<i>PLD1</i>	0.9615	miR-203		
<i>IFIT5</i>	<i>FGL2</i>	0.9601	miR-590–3p, miR-340		
<i>IL7</i>	<i>EPS8</i>	0.9587	miR-137, miR-506		
<i>MED13L</i>	<i>LMO3</i>	0.9584	miR-300, miR-381, miR-495	miR-302a, miR-302b, miR-302c, miR-302d, miR-302e, miR-372, miR-373, miR-520b, miR-520e, miR-520a–3p, miR-520c–3p, miR-520d–3p, miR-590–3p, miR-300, miR-381	miR-300, miR-381
<i>SLC39A10</i>	<i>IPCEF1</i>	0.9575	miR-23a, miR-23b, miR-495, miR-543	miR-23a, miR-23b, miR-23c	miR-23a, miR-23b
<i>CPEB3</i>	<i>HNRPLL</i>	0.9568	miR-340		
<i>CLEC4D</i>	<i>TEAD1</i>	0.9551		miR-590–3p	
<i>IFIT5</i>	<i>BEND4</i>	0.9538	miR-590–3p, miR-374a	miR-374a, miR-374b	miR-374a
<i>XYLT1</i>	<i>ACTN1</i>	0.9528	miR-19b, miR-19a	miR-19a, miR-19b	miR-19a, miR-19b
<i>MBOAT2</i>	<i>CREB5</i>	0.9527	miR-579, miR-181b, miR-181d, miR-181a, miR-181c, miR-137, miR-338–5p, miR-200b, miR-200c	miR-200b, miR-200c, miR-429, miR-143, miR-4770, miR-137, miR-33a, miR-33b	miR-137, miR-200b, miR-200c

Shared human miRNAs were analyzed with DIANA-microT 3.0 [23, 24, 105] and the TargetScanHuman 5.2 algorithms for the top 100 gene pairs shown in SUPPLEMENTARY TABLE 3. Data are presented by decreasing r^2 values (as shown in SUPPLEMENTARY TABLE 3). Blank cells indicate that no shared miRNAs were detected.

Table 4. Genes with high expression levels and extremely low variation among individual human lymphoblastoid cell lines.

Gene	Mean [†]	SD
GDPH	12.68	0.060
EEF1A1	12.54	0.038
ENO1	12.34	0.078
RPS4X	12.26	0.061
RPL5	12.21	0.067
PTMA	12.18	0.095
RPL10	12.18	0.080
HLA-A	12.05	0.072
RN7SK	12.00	0.068
RPL18	12.00	0.067

Means and SD expression values are presented for genes with highest expression levels and smallest variations among the 12 examined individual human lymphoblastoid cell lines. Such genes may be useful as reference genes for real-time PCR studies.

[†]Expression values are shown in arbitrary Affymetrix units in log₂ scale (see text). For further genes with high expression levels see SUPPLEMENTARY TABLE 2.
SD: Standard deviation.

such regulatory events. Further research is warranted on the potential use of LCLs microarray transcript data sets for studying gene networks and their intricate regulation by miRNAs.

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Executive summary

Background

- Human lymphoblastoid cell lines (LCLs) are a rich resource for information on human interindividual variations, including transcriptomic variations, and are therefore commonly used in pharmacogenomic studies. In this article we demonstrate that genome-wide transcriptome data of LCLs from a relatively small set of unrelated healthy individuals may detect pairs of genes that exhibit highly correlated expression levels and may thus convey insights about coregulated genes and thereby about cellular pathways.

Materials & methods

- RNA samples were prepared from LCLs representing 12 unrelated healthy adult female Caucasian donors and grown in parallel under optimal conditions. Transcript levels were determined with the Affymetrix GeneChip® Human Gene 1.0 ST arrays. Data were normalized and analyzed for expression levels correlations, and the variations of each of approximately 33,000 transcripts in these 12 LCLs were calculated.

Results & discussion

- When comparing the expression levels of each of the top 1000 genes exhibiting the largest interindividual expression-level variations against each of the other top 999 genes, two sets of highly correlated pairs with false-discovery rates controlled at $p < 0.01$ and $p < 0.05$ were detected, containing 157 and 4438 gene pairs, respectively. Performing the same analysis for another gene-expression data set obtained from human LCLs (GSE11582) detected 61 and 39% of the same pairs as identified from our LCLs gene-expression data ($p < 0.01$ and $p < 0.05$, respectively), indicating high reproducibility between this and the previous data sets.
- Sequences of detected gene pairs were compared for the presence of conserved 3'-UTR microRNA binding sites which may in part explain their correlated expression levels. Shared conserved 3'-UTR miRNA binding sites were noted for 14–17% of the top 100 pairs of strongly correlated genes in our data sets, suggesting that coregulation by miRNAs may contribute to their coordinated expression levels.
- Our data suggest that the ribosomal protein genes *RPL5*, *RPL10* and *RPL18* should be considered as reference for expression studies in human LCLs.
- Transcriptomic data sets from LCLs representing unrelated healthy individuals may be useful for identifying coregulated genes, as well as clues for their coregulation by shared miRNAs. Such data sets may therefore add insights on cellular pathway regulation by miRNAs and on the implications of interindividual transcriptomic variations in pharmacogenomic differences between individuals.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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