MicroRNA signatures associated with immortalization of EBV-transformed lymphoblastoid cell lines and their clinical traits

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Abstract

Objective: MicroRNAs (miRNAs) are negative regulators of gene expression that play important roles in cell processes such as proliferation, development and differentiation. Recently, it has been reported that miRNAs are related to development of carcinogenesis. The aim of this study was to identify miRNAs associated with terminal immortalization of Epstein–Barr virus (EBV)-transformed lymphoblastoid cell line (LCL) and associated clinical traits.

Material and Methods: Hence, we performed miRNA microarray approach with early- (p6) and late-passage (p161) LCLs.

Results and Conclusion: Microarray data showed that nine miRNAs (miR-20b*, miR-28-5p, miR-99a, miR-125b, miR-151-3p, miR-151:9.1, miR-216a, miR-223* and miR-1296) were differentially expressed in most LCLs during long-term culture. In particular, miR-125b was up-regulated in all the tested late-passage LCLs. miR-99a, miR-125b, miR-216a and miR-1296 were putative negative regulators of *RASGRP3*, *GPR160*, *PRKCH* and *XAF1*, respectively, which were found to be differentially expressed in LCLs during long-term culture in a previous study. Linear regression analysis showed that miR-200a and miR-296-3p correlated with triglyceride and HbA1C levels, respectively, suggesting that miRNA signatures of LCLs could provide information on the donor's health. In conclusion, our study suggests that expression changes of specific miRNAs may be required for terminal immortalization of

LCLs. Thus, differentially expressed miRNAs would be a potential marker for completion of cell immortalization during EBV-mediated tumorigenesis.

Introduction

Lymphoblastoid cell line (LCLs) are biological resources that have been used in various research fields related to human genetics, pharmacogenomics and immunology. For example, LCLs from patients with autism spectrum disorder and control subjects have been used for identification of disease-associated genes (1). LCLs from acute lymphoblastic leukemia patients with and without Down syndrome have been used to estimate cytotoxic effects of chemotherapeutic agents (2). Similarly, genomic alteration resulting from glucose deprivation has been tested using LCLs from subjects with schizophrenia and compared to those from non-psychotic relatives (3). Recently, LCLs have been considered to be an important cell-based model for assessment of chemotherapeutic drug response related to individual genetic variation (4,5). However, there is some concern about the widespread use of LCLs as genetic changes can occur during LCL generation, maintenance and immortalization. To contribute to the understanding of LCLs, many scientists have investigated biological and genetic characteristics of these cell lines. For example, we have reported that expression of an oncogene, *STMN1*, increased in LCLs at early passages relative to primary B cells (6), and DNA copy numbers of primary B cells were maintained in LCLs at early passages, except for copy number change in 1p36.33 (7). Recently, it was demonstrated that 16 genes including NF- κ B signalling-related genes (e.g. *PTPN13* and *HERC5*) and carcinogenesis-associated genes (e.g. *XAF1*, *TCL1A*, *PTPN13* and *CD38*) were differently expressed in late-passage (p161) LCLs compared to early-passage (p4) LCLs (8). It has been reported that miR-146a was highly induced by latent membrane protein 1 (LMP1) during Epstein–Barr virus (EBV)-mediated B-cell transformation (9).

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MicroRNAs (miRNAs) are small non-coding RNAs of approximately 22 nucleotides (10,11) that regulate several cell processes (proliferation, differentiation and apoptosis) (12,13). These miRNAs lead to inhibition or degradation of their target mRNAs through direct binding to them (14). Some of them function as oncogenes or tumour suppressors (15–17). For example, *miR-155* is up-regulated in Burkitt's lymphoma (18), and expression of *miR-15* and *miR-16* was lower in samples from patients with chronic lymphocytic leukaemia (compared to normal tissues) (19). Proliferation of HeLa cells decreased when expression of *miR-21* was inhibited (20). However, little remains known about contribution of miRNAs to LCL generation, maintenance or terminal immortalization.

In general, LCLs are considered to be terminally immortalized cell lines when allowed to proliferate past passage number 160. In this study, we compared miRNA microarray data of 17 late-passage (p161) LCLs with those of matched early-passage (p6) LCLs to identify miRNA expression changes during their long-term culture. These microarray data were validated by real-time RT-PCR experiments. Next, we predicted target genes of miRNAs differentially expressed in late-passage LCLs. Finally, clinical trait-associated miRNAs were identified through correlation analysis between miRNA expression levels of 20 early passage LCLs and their donor's clinical traits.

Materials and methods

Cell culture

Long-term subculturing of 20 LCL strains to obtain terminally immortalized specimens was performed as described previously (21). Among these strains, only 17 proliferated past passage number of 160. LCLs are generally considered to be terminally immortalized when proliferated past passage 160. In this study, early- and late-passage cells were cultured in RPMI1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% foetal bovine serum.

miRNA microarrays

Total RNA was isolated from 20 early- (p6) and 17 late-passage (p161) LCLs using Trizol reagent (Invitrogen), according to the manufacturer's instructions. Biotinylated cDNA was prepared from total RNA (2 µg) using high-throughput gene expression profiling by DNA-mediated annealing, selection, extension and ligation (DASL) assays (Illumina Inc., San Diego, CA, USA) (22). Subsequently, fluorescently labelled cDNA was hybridized to Illumina Sentrix BeadChips U1536-16 in accordance with

the manufacturer's protocol. After hybridization, chip images were scanned using Illumina Bead Array Reader Confocal Scanner, and raw data were extracted using the Illumina BeadStudio v3.1.3 (Gene Expression Module v3.3.8). These data were filtered by detection *P*-value of <0.05 and normalized by quantile methods using Array-Assist® 5.5.1 Software (Stratagene, La Jolla, CA, USA). Hierarchical clustering analysis was performed using R statistical language v.2.4.1.

Real-time RT-PCR

cDNA samples were generated from total RNAs from 17 early- (p6) and matched late-passage (p161) LCLs using the TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) and specific RT primer mixtures for miR-20b*, miR-28-5p, miR-99a, miR-125b, miR-151-3p, miR-216a, miR-223* or miR-1296 in TaqMan® MicroRNA Assay (Applied Biosystems), according to the manufacturer's recommendations. Real-time RT-PCR was performed with a mixture (20 µl) containing 1.33 µl of cDNA sample, 10 µl of TaqMan 2× Universal PCR Master Mix (No AmpErase UNG) and 1 µl of 20× PCR Mixture in TaqMan® MicroRNA Assay (Applied Biosystems). PCR reactions were performed using an ABI HT 7900 (ABI), and cycles were as follows: 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Real-time RT-PCR data were represented as the $\Delta\Delta C_t$ value ($\Delta\Delta C_t = (C_t, \text{target gene} - C_t, \text{control gene})_{\text{LCLs at the late passages}} - (C_t, \text{target gene} - C_t, \text{control gene})_{\text{LCLs at the early passages}}$). All PCR reactions were conducted in duplicate in at least two independent experiments. RNU6B was used as an internal control.

Prediction of miRNA target gene

Target genes of miRNAs, which were differentially expressed in late-passage LCLs relative to early-passage LCLs, were predicted using two major online miRNA target prediction algorithms: TargetScan (<http://www.targetscan.org>) and miRDB (<http://mirdb.org/miRDB>).

Identification of miRNAs associated with clinical traits

We have previously identified 46 clinical trait-associated genes (e.g. *FGD4*, *SQMS1*, *RHOF* and *TMSL8*) by correlation analysis between expression phenotypes of 20 early-passage (p4) LCLs and corresponding donors' 23 clinical traits (height, weight, systolic blood pressure, waist size, body mass index and total cholesterol) (8). To identify miRNAs associated with clinical traits, we selected the top five putative miRNA regulators targeting these 46 genes using online miRNA target prediction

algorithms (miRDB), then analysing correlations between expression levels of these miRNAs in 20 early-passage (p6) LCLs and donors' 23 clinical traits. Correlations were assessed by linear regression analysis using SPSS, version 12.0 (SPSS, Chicago, IL, USA).

Results

miRNA microarray data

We performed miRNA microarray experiments for 17 pairs of early- (p6) and late-passage (p161) LCL strains to identify their miRNA expression changes during long-

term culture. Microarray data showed that 46–133 miRNAs per LCL strain were up- or down-regulated with greater than 2-fold change in late-passage LCLs compared to matched early-passage LCLs. Expression of 18–76 miRNAs increased, and expression of 28–73 miRNAs decreased (Fig. 1a, Table 1). Among these, nine miRNAs (miR-20b*, miR-28-5p, miR-99a, miR-125b, miR-151-3p, miR-151:9.1, miR-216a, miR-223* and miR-1296) were differentially expressed in at least 14 of 17 LCL strains: miR-99a, miR-125b and miR-1296 were up-regulated in late-passage LCLs relative to early-passage LCLs, whereas miR-20b*, miR-28-5p, miR-151-3p, miR-151:9.1, miR-216a and miR-223* were down-regulated

Figure 1. Hierarchical clustering of early- (p6) and late- (p161) passage LCL samples. (a) Clustering analysis of miRNAs shows an increase or decrease (>2-fold change) in at least one of 17 LCLs at late passage (p161) relative to early passage (p6). (b) miRNAs differentially expressed in at least 14 late-passage LCLs.

Table 1. Number of miRNAs differentially expressed in each LCL strain

LCL strain	Up ^a	Down ^b	Total ^c
A1	76	57	133
A2	50	36	86
A3	43	41	84
A4	26	53	79
A5	40	48	88
A6	36	73	109
A8	28	39	67
A10	37	41	78
K1	30	43	73
K2	43	44	87
K3	38	30	68
K5	27	34	61
K6	18	28	46
K7	38	48	86
K8	18	40	58
K9	26	27	53
K10	30	45	75

^{a,b,c}Number of miRNAs that were up- or down-regulated at greater than 2-fold change in late-passage LCLs compared to corresponding early-passage LCLs.

(Fig. 1b, Table 2). These results suggest that these nine miRNAs might control maintenance or terminal immortalization of LCLs.

Validation of microarray results

To confirm microarray data, real-time RT-PCR experiments were conducted using specific primers for eight miRNAs (miR-20b*, miR-28-5p, miR-99a, miR-125b, miR-151-3p, miR-216a, miR-223* and miR-1296). As shown in Table 3, all miRNAs showed expression patterns similar to microarray results, except for miR-216a, which was not detected by real-time RT-PCR. In particular, miR-125b increased in all tested late-passage LCLs

compared to LCLs at early passages, suggesting that it may play a role in the terminal immortalization of LCLs.

Target gene prediction of miRNAs

When putative target genes of nine differentially expressed miRNAs (miR-20b*, miR-28-5p, miR-99a, miR-125b, miR-151-3p, miR-151:9.1, miR-216a, miR-223* and miR-1296) were searched with two online algorithms for miRNA target prediction (TargetScan and miRDB), dozens to hundreds of target genes were predicted for each miRNA, except for three of them (miR-20b*, miR-151:9.1 and miR-223*) (data not shown). For example, predicted target genes of miR-28-5p included *THRB* and *BAG1*. Putative target genes of miR-99a, miR-151-3p and miR-1296 included *SMARCA5*, *CLK1* and *ABL2* respectively (Table 3).

In previous studies, we have estimated mRNA signatures of late-passage (p161) LCLs relative to early-passage (p4) ones using cDNA microarrays. Expression of 15 genes (*PRKCH*, *PTPN13*, *CD38*, *CD180*, *FCRL5*, *GPR160*, *HERC5*, *IFIT1*, *OAS3*, *RASGRP3*, *RFTS7H*, *TC2N*, *TCL1A*, *XAF1* and *ZNF382*) increased or decreased in at least 16 of 17 late-passage LCLs relative to early-passage ones and these microarray data were validated through real-time RT-PCR experiments. Interestingly, putative target genes of miRNAs differentially expressed in LCLs during long-term culture included some of these 15 genes (Table 4). For example, putative target genes of miR-28-5p were *CD38*, *FCRL5* and *XAF1*, and putative target gene of miR-99a was *RASGRP3*. The predicted target gene of miR-125b was *GPR160* and that of miR-1296 was *XAF1*. In case of miR-216a, *PRKCH*, *GPR160* and *XAF1* were predicted as target genes. In particular, miR-99a, miR-125b, miR-216a and miR-1296 showed negative regulation of *RASGRP3*, *GPR160*, *PRKCH* and *XAF1* respectively. These results suggest that

miRNA	Up ^a	Down ^b	Mean ^c ± SD	Sequence
miR-20b*	1	14	-7.2 ± 7.6	ACUGUAGUAUGGGCACUCCAG
miR-28-5p	0	15	-10.2 ± 6.1	AAGGAGCUCACAGUCUAUUGAG
miR-99a	14	1	10.5 ± 14.2	AACCCGUAGAUCGACUUGUG
miR-125b	15	1	9.3 ± 10.9	UCCCUGAGACCUAACUUGUGA
miR-151-3p	1	14	-8.9 ± 7.8	CUAGACUGAAGCUCCUUGAGG
miR-151:9.1	1	15	-4.0 ± 3.3	ACUAGACUGAAGCUCCUUGAGG
miR-216a	0	16	-3.3 ± 1.2	UAAUCUCAGCUGGCAACUGUGA
miR-223*	0	15	-5.8 ± 3.6	CGUGUAUUUGACAAGCUGAGUU
miR-1296	15	0	2.9 ± 0.9	UUAGGGCCUGGCUCCAUCUCC

^{a,b}Number of LCL strains in which each miRNA was differentially expressed at greater than 2-fold change during long term-culture.

^cMean value of log₂ (signal intensity of late/early-passage LCL sample) from microarray results of 17 LCLs.

Table 2. Summary of microarray results

Table 3. Validation of miRNA microarray results by real-time RT-PCR and target gene prediction of miRNAs

miRNA	Up ^a	Down ^b	Mean ^c ± SD	Putative targets ^d
miR-20b*	4	9	0.4 ± 2.9	–
miR-28-5p	1	14	4.6 ± 3.4	C18orf24, THRB, KIAA0355, IKBKB, BAG1
miR-99a	13	1	–3.5 ± 2.9	SMARCA5, KBTBD8, LOC100128223, TARDBP, TMPRSS13
miR-125b	17	0	–3.9 ± 2.9	SH3TC2, ZNF543, KCNA7, LACTB, ALPK3
miR-151-3p	1	13	4.3 ± 3.6	UPP2, ZFAND5, CLK1, ZMAT1, KCNH8
miR-216a	–	–	–	SGCD, LOC100134025, SPEF2, FAM44A, TMEM161B
miR-223*	4	11	1.8 ± 3.2	–
miR-1296	11	0	–2.3 ± 1.7	PDZK1IP1, LOC730074, C6orf35, TRIP12, ABL2

^{a,b}Number of LCL strains in which each miRNA was up- or down-regulated at greater than 2-fold change.

^cMean of $\Delta\Delta Ct$ values ($\Delta\Delta Ct$ value = $Ct_1(\text{target gene } Ct - \text{control gene } Ct)_{\text{LCL at the late passages}} - Ct_2(\text{target gene } Ct - \text{control gene } Ct)_{\text{LCL at the early passages}}$).

^dTop five putative targets identified by miRDB.

Table 4. Prediction of miRNA regulating genes related to LCL immortalization

miRNA microarray		cDNA microarray	
miRNA ^a	Expression ^b	Target gene ^c	Expression ^b
miR-28-5p	Down	CD38	Down
		FCRL5	Down
		XAF1	Down
miR-99a	Up	RASGRP3	Down
miR-125b	Up	GPR160	Down
miR-216a	Down	PRKCH	Up
		GPR160	Down
		XAF1	Down
miR-1296	Up	XAF1	Down

^amiRNAs differentially expressed in 17 late-passage LCLs relative to early-passage LCLs.

^bExpression pattern in 17 late-passage LCLs relative to early-passage LCLs.

^cPutative target genes of miRNAs which selected in genes differentially expressed in LCLs during long-term culture.

these miRNAs may control LCL immortalization through target gene regulation.

Identification of miRNAs associated with clinical traits

In a previous study, we had identified 46 genes associated with clinical traits through comparisons between cDNA microarray data of 20 LCL strains and 23 clinical traits of donors. To identify clinical trait-associated miRNAs, we chose the top five miRNAs putatively targeting each of these 46 genes, and then investigated correlations between expression levels of these miRNAs and donors' 23 clinical traits. As shown in Fig. 2, expression of miR-188-3p showed significant negative correlation with systolic blood pressure ($R^2 = 0.202$, $P = 0.047$) and miR-296-3p showed significant positive relationship with HbA1C levels ($R^2 = 0.272$, $P = 0.018$). miR-107 and miR-103

showed a tendency to positive correlation with body weight, while miR-200a showed a tendency to positive correlation with triglyceride level.

Discussion

We have identified miRNA expression changes in LCLs over long-term culture through comparison between microarray data from 17 late-passage (p161) LCLs and matched early-passage (p6) LCLs. Other reports have demonstrated that a few miRNAs including miR-21, miR-23a, miR-24, miR-27a, miR-34a, miR-146b (23), miR-146a (9,23), and miR-155 (23,24) are differentially expressed in EBV type III latency cell lines (e.g. Jy and IB4) compared with EBV type I latency cell lines (e.g. Rael and Mutu), suggesting that type III latency genes (such as EBNA1, EBNA 2, EBNA3 and LMP1) control expression of these miRNAs. In contrast, our study was conducted to answer the question of what would be changed in miRNA expression profiles between early and late passages of LCLs. In this study, expression of nine miRNAs (miR-20b*, miR-28-5p, miR-99a, miR-125b, miR-151-3p, miR-151:9.1, miR-216a, miR-223* and miR-1296) was found to be changed during long-term subculture of LCLs. These differentially expressed miRNAs would provide an miRNA signature involved in the process of terminal immortalization of LCLs rather than in the initial stage of EBV-mediated B-cell transformation. In particular, expression of miR-125b was higher in all late-passage LCLs, suggesting that this could be a novel marker for terminal immortalization of the cells.

It has been reported that miR-99a and miR-125b are involved in carcinogenesis. For example, miR-99a is down-regulated in oral squamous cell carcinoma (25), lung carcinoma (26) and ovarian cancer (27); miR-125b is down-regulated in prostate (28), bladder (29), ovarian cancer (27) and oral squamous cell carcinoma (25).

Figure 2. miRNAs associated with clinical traits. Expression levels of miR-107 (a) and miR-103 (b) were associated with body weight and those of miR-200a (d) were associated with amount of triglyceride present. Expression of miR-188-3p (c) and miR-296-3p (e) correlated significantly with systolic blood pressure and HbA1c levels respectively. (An asterisk indicates P -value < 0.05 .)

However, to date, little is known about function of the other seven miRNAs (miR-20b*, miR-28-5p, miR-151-3p, miR-151:9.1, miR-216a, miR-223* and miR-1296). Among predicted target genes of the differentially expressed miRNAs during long-term culture of LCLs, *THRB* (30), *BAG1* (31), *SMARCA5* (32), *CLK1* (33) and *ABL2* (34) have been previously identified as cancer-associated genes, suggesting that their putative regulators (miR-28-5p, miR-99a, miR-151-3p and miR-1296) may influence cancer development through control of these genes. Conceivably, these seven miRNAs may be also associated with carcinogenesis. Taken together, these find-

ings support the notion that nine miRNAs identified here have roles in the process of terminal immortalization of LCLs.

In addition, our previous microarray study demonstrated that putative target genes (e.g. *RASGRP3*, *GPR160*, *PRKCH* and *XAF1*) of miRNA species (e.g. miR-99a, miR-125b, miR-216a and miR-1296, respectively) were differentially expressed in LCLs during long-term culture. Thus, terminal immortalization of LCLs may be controlled by gene targeting of these miRNAs.

Some reports have demonstrated usefulness of expression phenotypes in human genetic studies when transcript

levels of particular genes were thought of in relation to phenotypes of donors (35,36). In our association study of miRNA expression phenotypes with clinical variables, expression level of miR-296-3p was significantly associated with HbA1C levels, and miR-200a expression showed a tendency to positive correlation with amounts of triglyceride present. Thus, the expression phenotype of miRNAs in LCLs may be used as a tool to help provide a donor's health information. It has already been reported that gene expression patterns correlated with the specific clinical traits. For example, we have previously identified clinical trait-associated genes through comparison cDNA microarray data of 20 LCLs and donors' 59 clinical traits (8). Other reports have shown that 160 genes (e.g. *AIF1*, *MMP19* and *EPIM*) correlated with coronary artery disease index using the microarray approach (37). In addition, it was demonstrated that a few genes (for example, *ACSL4*, *HMGCS2*, *ALAS1*, *S100A8* and *SGK*) are associated with non-alcoholic steatohepatitis (38). Thus, clinical trait-associated miRNAs (such as miR-296-3p and miR-200a) may be new molecular candidate targets for studies of metabolic syndromes and diabetes.

In conclusion, this study suggests that nine miRNAs (miR-20b*, miR-28-5p, miR-99a, miR-125b, miR-151-3p, miR-151:9.1, miR-216a, miR-223* and miR-1296) may regulate immortalization of LCLs. In particular, miR-125b, up-regulated in all tested late-passage LCLs, has a potential as a marker for immortalization of LCLs.

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