Leukemia Research 38 (2014) 983–987

Contents lists available at ScienceDirect

Leukemia Research

journal homepage: www.elsevier.com/locate/leukres

Changes in gene expression profile in two multidrug resistant cell lines derived from a same drug sensitive cell line

Miguel Angelo Martins Moreira[a,*,1], Carolina Bagni[a,b,1], Marcos Barcelos de Pinho[a], Thaís Messias Mac-Cormick[a,b], Mateus dos Santos Mota[a], Flávio Eduardo Pinto-Silva[c], Nathalia Daflon-Yunes[c], Vivian Mary Rumjanek[c]

[a] Genetics Program, Instituto Nacional de Câncer, Rio de Janeiro, Brazil
[b] Genetics Department, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil
[c] Instituto de Bioquímica Médica, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil

ARTICLE INFO

Article history:
Received 12 May 2014
Accepted 1 June 2014
Available online 12 June 2014

Keywords:
MDR cell lines
Multidrug resistance
ABCB transporters
ABCB1
Methylation
Cancer

ABSTRACT

Resistance to chemotherapy is one of the most relevant aspects of treatment failure in cancer. Cell lines are used as models to study resistance. We analyzed the transcriptional profile of two multidrug resistant (MDR) cell lines (Lucena 1 and FEPS) derived from the same drug-sensitive cell K562. Microarray data identified 130 differentially expressed genes (DEG) between K562 *vs.* Lucena 1, 1932 between K562 *vs.* FEPS, and 1211 between Lucena 1 *versus* FEPS. The NOTCH pathway was affected in FEPS with overexpression of *NOTCH2* and *HEY1*. The highly overexpressed gene in MDR cell lines was *ABCB1*, and both presented the *ABCB1* promoter unmethylated.

1. Introduction

Resistance to chemotherapy is one of the most relevant aspects of treatment failure in cancer and it is frequently associated to over-expression of ABC-transporter proteins in tumors treated with systemic drugs or target-specific drugs [1]. Over-expression of the ABCB1 transporter (formerly Pgp, or MDR1) [2] is the most recurrent phenomenon associated to drug resistance in cancer and multidrug resistance (MDR) phenotype; its activity and expression levels are considered as an independent risk factor for treatment strategies [3–5]. Besides ABCB1, ABC-transporters ABCC1 and ABCG2 have also been associated to drug resistance [6,7] and all of them display wide substrate specificities [8].

Notwithstanding the major role of ABC-transporters in drug resistance, other pathways are also affected in cells or tumors with the MDR phenotype. These comprise changes in expression of apoptosis related genes, control of cell cycle, cell adhesion, cell detoxification, and genes associated with stem cell characteristics [9–12].

Cell lines are frequently used models to study resistance to chemotherapeutic agents and cell lines resistant to multiple drugs can be obtained through a gradual selective process against a specific drug [13–16]. In the present work, we analyze the transcriptional profile of two multi-drug resistant cell lines, which were independently derived from the same drug-sensitive cell line K562 (originated from a patient with chronic myeloid leukemia) [17] by selection with increasing concentrations of vincristine [14] or daunorubicin [16]. The MDR cell line selected with vincristine, named Lucena 1, was used as model for understanding resistance against specific drugs and involvement of different pathways in chemoresistance (reviewed in Rumjanek et al. [18]). These include the Hedgehog pathway [19], Wnt/β-catenin on *ABCB1* transcription [20], Low molecular weight protein tyrosine phosphatases (LMW-PTP) as targets for reverting chemoresistance [21] and induction of apoptosis by pomolic acid [22]. The MDR cell line selected with daunorubicin, named FEPS, showed different characteristics from Lucena 1 although chemoresistance of both cell lines were mainly associated to *ABCB1* overexpression [16].

We compared gene expression profiles of K562, Lucena 1 and FEPS, describing the alterations and potential cellular pathways affected by selective processes resulting in each MDR cell line.

* Corresponding author at: Genetics Program, Instituto Nacional de Câncer, André Cavalcanti 37, Rio de Janeiro 20231-050, Brazil. Tel.: +55 21 3207 6586; fax: +55 21 3207 6553.
E-mail address: miguelm@inca.gov.br (M.A.M. Moreira).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.leukres.2014.06.001
0145-2126/

2. Materials and methods

2.1. Cell lines, DNA and RNA isolation

Gene expression profiles were analyzed for (1) K562, a chronic myeloid leukemia cell line [17], (2) Lucena 1 [14], derived from K562 following selection by vincristine and presenting the multidrug resistance phenotype, and (3) FEPS [16], also derived from K562 following selection by daunorubicin and presenting the multidrug resistance phenotype. All cells were maintained in RMPI 1640 medium with 10% FBS and 5% CO_2 at 37 °C, with addition of 60 nM vincristine (VCR) for Lucena 1, and 466 nM daunorubicin (DNR) for FEPS. RNA isolation was carried out from Lucena 1 and FEPS grown with their respective drugs and without them during seven days.

Genomic DNA was isolated using the QIAmp DNA Mini Kit (Qiagen, Valencia, CA, USA) and total RNA using RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. RNAs were quantified and stored at −80 °C.

2.2. Microarray analyses

Gene expression profiles were obtained with the GeneChip® Human Genome U133 Plus 2.0 Array (Affymetrix, Singapore) according to the manufacturer' instructions; all experiments were carried out in duplicate. The following samples were analyzed: K562 RNA from cells grown without drugs; Lucena 1 RNA from cells grown in medium with vincristine (Lucena 1 + VCR); Lucena 1 RNA from cells grown in medium without drugs (Lucena 1 – VCR); FEPS RNA from cells grown in medium with daunorubicin (FEPS + DNR), and FEPS RNA from cells grown in medium without drugs (FEPS – DNR). Data are available from the Gene Expression Omnibus (www.ncbi.nlm.nih.giv/geo/) under accession number GSE57470.

Data were analyzed with Bioconductor (R version 2.12.0; www.bioconductor.org) with the following packages: AFFY [23], GENEFILTER [24], GCRMA [25], LIMMA [26], ANNAFFY [27] (version 1.16.0) and SPIA [28]. Quality control microarray assays comprised visual inspection of several diagnostic plots, mainly box plots of transcript intensities, image plots of arrays, and MA plots of raw data. In addition, Affymetrix software parameters were also evaluated. RLE (relative log expression) and NUSE (normalized unscaled standard error) plots were constructed after hybridization, and normalization was carried out. Data pre-processing, background correction, normalization and expression estimates were carried out with the GCRMA package. In order to find differentially expressed genes, data were analyzed with a GENFILTER for removing all genes exhibiting low variance across samples, genes with duplicate entries, genes without a corresponding "entrez gene tag" and all Affymetrix control probes.

In order to identify differentially expressed genes a statistical model was selected from the LIMMA package for comparing gene expression of different cell lines grown with their respective drugs. The moderated *t*-statistics method was used for analyzing the significance and the false discovery rate (FDR) controlled by the Benjamini and Hochberg (BH) method [29]. The cutoff criteria for comparing gene expression accounted for $p < 0.01$ (after BH correction) and absolute expression difference (fold change) $\geq 4\times$.

Genes identified as differentially expressed were classified by Gene Ontology (GO) and analyzed for biological functions with Ingenuity Pathways Analysis (Ingenuity® Systems, www.ingenuity.com). The Right-tailed Fisher's Exact Test was used for estimating the probability that each biological function assigned to datasets was due to chance. Threshold, <0.05 *p*-values, following application of Benjamini and Hochberg method of multiple testing corrections, were considered indicative of differences in biological functions.

2.3. Quantitative PCR (qPCR)

qPCR was used for validating differences in gene expression for 16 selected genes: *ABCB1*, *ABCC1*, *ABCG2*, *ACRBP*, *ARHGDIB*, *CAV2*, *CD36*, *GRK5*, *HES1*, *HEY1*, *LAMB1*, *MGAT4A*, *NOTCH2*, *NT5CD4*, *SCIN*, and *VAMP8*. This was carried out with RNAs from each cell line grown without drugs and treated with RQ1® RNase-Free DNase (PROMEGA). RNAs were retro-transcribed with SuperScript® II Reverse Transcriptase kit (Life-Technologies) and cDNAs were used as templates for quantification by qPCR with Power SYBR green (Life-Technologies) or Go-Taq® qPCR Master Mix (PROMEGA). Experiments were carried out thrice, each time in triplicate. The $2^{-\Delta\Delta Ct}$ method [30] was used for estimating differences in gene transcription between cell lines. *B2M* (Beta-2 Microglobulin) was used as reference gene for normalization. Primers used for qPCR are described in supplemental Table 1.

2.4. Methylation pattern of the ABCB1 promoter region

Genomic DNA from each cell line, grown without VCR or DNR, was treated with sodium bisulfite using the "Fast DNA Modification CpGenome Kit" (Chemicon, Tamecula, CA, USA). The *ABCB1* promoter region was amplified by nested-PCR (primers for the first PCR being: F1 5′-GAAATGTTTTTAATGATTTAGTTGATG-3′ and R1 5′-AAACTTACCAAAAAACTTCACACTATC-3′; primers for the nested PCR being: F2 5′-GGAAGTTAGAATATTTTTTTTGGAAAT-3′ and R2 5′-CTATCCCATAATAACTCCCAACTTTAC-3′). PCR products were cloned into plasmids PMOS-Blue (GE-Heathcare) or TOPO pCR 2.1(Life-Technologies) and 10–14 clones were sequenced for each cell line in an ABI3130-XL platform (Life-Technologies). A region comprising a total of 350 bp was analyzed, encompassing 19 CpG sites from −141 to +144 of the *ABCB1* major transcription start site [31].

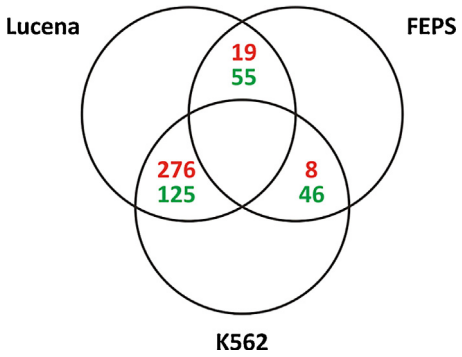

Fig. 1. Venn diagram showing the number of genes differentially expressed shared between the cell lines analyzed. Number of genes overexpressed in red. Number of genes underexpressed in green. (For interpretation of the references to color in this figure caption, the reader is referred to the web version of the article.)

3. Results

3.1. Differentially expressed gene profiles between cell lines

Comparisons of microarray expression data were carried out between K562 *vs.* Lucena 1 grown in medium without VCR (Lucena 1 – VCR); K562 *vs.* FEPS grown in medium without DNR (FEPS – DNR); and Lucena 1 – VCR *vs.* FEPS – DNR. Additionally, we compared expression profiles of each resistant cell line grown in different conditions: Lucena 1 – VCR *vs.* Lucena 1 + VCR, and FEPS – DNR *vs.* FEPS + DNR. Only genes meeting the cutoff criteria were considered.

Comparisons between K562 *vs.* Lucena 1 – VCR identified a total of 130 differentially expressed genes, 65 of which with overexpression and 65 with underexpression in Lucena 1. When comparing expression profiles between K562 *vs.* FEPS – DNR, a total of 932 genes were differentially expressed, 288 overexpressed and 644 underexpressed in FEPS. Comparisons of expression profiles between Lucena 1 – VCR *vs.* FEPS – DNR showed 1211 differentially expressed genes, 459 overexpressed and 752 underexpressed in FEPS (supplemental files 1–3). Table 1 shows the ten genes with the highest differences in expression in each comparison. *ABCB1* showed the highest difference in gene expression, being overexpressed in both MDR cell lines with respect to K562.

Several genes (see supplemental file 4) showed similar expression profiles when comparing K562 *vs.* Lucena 1 – VCR, K562 *vs.* FEPS – DNR, and Lucena 1 – VCR *vs.* FEPS–DNR; their number is shown in a Venn diagram (Fig. 1).

Comparisons of expression profiles of resistant cell lines grown in different conditions (Lucena 1 – VCR *vs.* Lucena 1 + VCR, and FEPS – DNR *vs.* FEPS + DNR) did not show differences. However, with a relaxed cut-off (p-value < 0.05), a total of 30 differentially expressed genes were identified between FEPS – DNR *vs.* FEPS + DNR, 28 of which with overexpression and 2 with underexpression in FEPS + DNR (supplemental file 5). With the same relaxed cut-off for Lucena 1 – VCR *vs.* Lucena 1 + VCR, differential expression was not observed.

The differentially expressed genes between K562 *vs.* Lucena 1 – VCR, K562 *vs.* FEPS – DNR, and Lucena 1 – VCR *vs.* FEPS – DNR, classified by gene ontology and grouped by Ingenuity Pathway Analysis (IPA), pointing to significantly affected biological functions are listed in Table 2. This analysis identified changes in 20 biological pathways in Lucena 1 respective with K562, 5 pathways between FEPS and K562, and 7 pathways between Lucena 1 and FEPS.