Original Research Article

Molecular features of doxorubicin-resistant development in colorectal cancer CX-1 cell line

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ABSTRACT

Background and aim: Resistance to chemotherapy is the key obstacle to the effective treatment of various cancers. Accumulating evidence suggests significant involvement of the epithelial-to-mesenchymal transition (EMT) in the chemoresistance of most cancer types. This study aimed at analyzing the gene expression profile of doxorubicin (DOX)-resistant colorectal cancer cells CX-1.

Materials and methods: DOX-resistant CX-1 cell sublines were acquired by stepwise increment of DOX concentrations in cell growth media. Global gene expression profiling was performed using human gene expression microarrays. The expression levels of individual genes were assessed by means of quantitative PCR (qPCR), while the DNA methylation pattern of several selected genes was determined by methylation-specific PCR.

Results: Four DOX-resistant CX-1 sublines were established as a valuable tool for cell chemoresistance studies. Altered expression of the EMT, cell adhesion and motility, and chemoresistance-related genes was observed in DOX-resistant cells by genome-wide gene expression analysis. Besides, early and significant upregulation of the key EMT genes ZEB1 (5.8 ×; P < 0.001) and CDH2 (6.2 ×; P = 0.044) was identified by qPCR, with subsequent activation of drug transporter gene ABCC1 (3.3 ×; P = 0.007) and cell stemness gene NANOG (2.4 ×; P = 0.008). Downregulation of TET1 (2.1 ×; P = 0.041) and changes in the methylation status of the p16 gene were also involved in the acquisition of cell resistance to DOX.

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1. Introduction

Colorectal cancer is the third most common cancer worldwide and the fourth leading cause of cancer-related death [1]. In Lithuania about 1700 new cases and 900 deaths of both colon and rectal cancer were registered in 2012 [2]. The metastasis or dissemination of cancer cells from the primary tumor through the blood into other organs is the main cause of high mortality rates in colorectal cancer [3]. Tumor metastasis is a complex phenomenon, which involves such processes as aberrant cancerous cell adhesion, epithelial-to-mesenchymal transition (EMT), degradation of extracellular matrix, invasion, and angiogenesis.

EMT is a cellular process by which epithelial cells lose their cell–cell junctions and undergo changes of cytoskeleton organization, which result in the loss of cell polarity and alteration of the cell shape (from cobble stone-like to elongated and spindle-shaped cells) enabling the cell to migrate and invade [4,5]. At the molecular level, the EMT is characterized by the loss of epithelial markers E-cadherin, cytokeratin, zonula occludens proteins, and upregulation of mesenchymal markers, such as N-cadherin, fibronectin, vimentin [6]. The replacement of E-cadherin by N-cadherin, termed cadherin switch, is a key molecular hallmark of the EMT process, which leads the formation of weaker cell–cell junctions between neighboring cells [5,7]. Such molecular switch is regulated by the key EMT-inducing transcription factors (TFs), classified into 3 families: the Snai family of zinc-finger transcription factors (SNAI1 and SNAI2), the zeb family zinc-finger E-box-binding factors (ZEB1 and ZEB2), and the basic helix–loop–helix factors (TWIST1 and TWIST2). Through interaction with each other these TFs affect multiple promoters and orchestrate most of the EMT-related processes, including cadherin switch [8,9].

The roles of EMT in cancer progression have been long discussed. The traditional approach suggests that EMT facilitates cancer cell spreading and then the cells execute a reverse process known as the mesenchymal-epithelial transition (MET) for clonal outgrowth at metastatic sites [3]. Due to EMT, cancer cells obtain the potency of apoptosis resistance and migration as well as become poised to enter cancer stem cells (CSC)-like state [10-12]. Such cells can be characterized by aberrant expression of stem cells-specific key pluripotency-related TFs (POU5F1, SOX2, NANOG), and they have capabilities of self-renewal, tumor initiation and chemoresistance [11], therefore can be associated with oncogenesis and poor prognosis in various human malignancies, including colon cancer [13]. Besides, upregulated expression of EMT-inducing TFs can stimulate expression of the ABC transporter genes and result in multidrug resistance of tumors [14]. Recent evidences [15,16] suggest slightly different interpretation of the significance of EMT process in cancer, disclosing that EMT can contribute to cancer chemoresistance, as described above, but is not necessary for the metastases formation. Two recent studies [15,16] collectively point to the notion that EMT can be induced by anti-cancer drugs and then can lead to the formation of chemoresistant metastases.

For better characterization of the mechanisms involved in cancer cell resistance to chemotherapy, doxorubicin resistant colon cancer CX-1 cell sublines were established and global gene expression analysis by microarrays was performed. Expression levels of drug transporter genes (ABCB1, ABCG2), key pluripotency (POU5F1, SOX2, OCT4) and EMT (CDH1, CDH2, SNAI1, ZEB1) markers, and epigenetic regulators (TET1, TET2, EZH2) were analyzed. In addition, epigenetic integrity of the cells was evaluated through the assessment of DNA methylation in promoter region of well-known tumor suppressor genes (TSGs) and the genes coding drug transporters.

2. Materials and methods

2.1. Cell cultivation and establishment of resistant sublines

The CX-1 cell line, obtained from the German Cancer Research Center (DKFZ), Heidelberg, Germany, was maintained as an attached monolayer culture in RPMI 1640 medium, supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 U/ml and 100 μg/ml penicillin–streptomycin (all from Biochrom AG, Germany). The cells were grown in cell culture bottles (growth surface 75 cm²) with filter screw caps (Carl Roth, Switzerland) incubating them at 37 °C in an atmosphere comprising 90% humidity and 5% CO₂.

For the development of doxorubicin (DOX)-resistant sublines, stepwise selection method was used. Initially sensitive CX-1 cells were incubated with 10 nM DOX (Teva, UK) and doubling of the drug concentration was performed each time when the treated cells reached the growth rate of the untreated cells, until the final concentration of 80 nM DOX was applied. Accordingly, CX-1 cell sublines resistant to various concentrations of DOX (CX-1/D) were developed.

The morphological changes in DOX-resistant CX-1 cells were observed by inverted fluorescence microscope Motic AE31 (Xiamen, China). The images were captured at a magnification of 200×.

2.2. RNA and DNA extraction

For the isolation of nucleic acids, chemoresistant CX-1 cell sublines grown in DOX supplemented medium were used.
Total RNA was extracted from 2 × 10^6 cells on average with mirVana Kit (Ambion, Life Technologies, Thermo Fisher Scientific, Foster City, CA, USA) according to manufacturer’s protocol (Life Technologies, Forster City, CA, USA). The concentration of RNA was measured spectrophotometrically using NanoDrop 2000 (Thermo Scientific, Thermo Fisher Scientific, Wilmington, NC, USA). RNA integrity number (RIN) was ≥9.4, as evaluated with 2100 Bioanalyzer system using RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, CA, USA).

For genomic DNA extraction, at least 1 × 10^6 cells on average was used and DNA was extracted by digestion with proteinase K followed by purification with Macherey-Nagel NucleoSpin Tissue Kit (Macherey-Nagel, Germany) according to manufacturer’s protocol. The concentration of DNA was measured spectrophotometrically with NanoDrop 2000.

2.3. **Global gene expression profiling**

Microarray hybridization was performed according to manufacturer’s protocol for One-Color Microarray-Based Gene Expression Analysis v6.5 (Agilent Technologies). Briefly, 200 ng of total RNA was labeled and amplified with Low Input Quick Amp Labeling Kit, One Color and using RNA Spike-In Kit, One Color (Agilent Technologies). After the purification with RNeasy Mini Kit (Qiagen, Valencia, CA, USA), samples were hybridized onto Human Gene Expression v2) 8 × 60 K microarrays, design ID 039494 (Agilent Technologies), for 17 h at 65 °C. Microarrays were scanned using SureScan microarray scanner and images were analyzed with Feature Extraction software v10.7 (Agilent Technologies).

GeneSpring software v12.6 (Agilent Technologies) was used for data preprocessing. Dataset of each sample was log2-transformed and 75-percentile normalized without baseline transformation. Non-uniform, saturated or outlier probe signals were removed prior to normalization. Probe annotations were automatically uploaded from the eArray platform (Agilent Technologies).

2.4. **Target gene expression profiling by RT-qPCR**

For reverse transcription, Maxima First Strand cDNA Synthesis Kit for reverse transcription quantitative PCR (RT-qPCR) was used according to manufacturer’s protocol (Thermo Scientific, Thermo Fisher Scientific). Aliquots containing 1 μg of total RNA were used from each sample.

Expression levels of selected genes – ABCB1, ABCC1, POU5F1, NANOG, SOX2, TET1, TET2, EZH2, CDH1, CDH2, SNAI1, ZEB1, and endogenous control HPRT1 – were evaluated in all cell sublines using TaqMan Gene Expression Assays (Hs_00184500_m1, Hs_01561502_m1, Hs_00999632_g1, Hs_04260366_g1, Hs_04260357_g1, Hs_00286756_m1, Hs_00325999_m1, Hs_00544833_m1, Hs_01023895_m1, Hs_00983056_m1, Hs_00195591_m1, Hs_00232783_m1, and Hs02800695_m1 respectively). QPCR was performed in duplicates on ViIA 7 Real Time PCR System (Applied Biosystems, Thermo Fisher Scientific). Reaction volume of 20 μL consisted of 1× Gene Expression Master Mix, 2× TaqMan Gene Expression Assay (both from Applied Biosystems, Thermo Fisher Scientific) and 2 μL of cDNA sample. Thermocycling parameters were 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Two technical replicates of the experiment starting from the RT step were performed. For the analysis of relative changes in gene expression, raw cycle of quantification (Cq) values were normalized to HPRT1 and converted to linear scale.

2.5. **DNA methylation analysis by MSP**

Isolated DNA (400 ng) was modified with sodium bisulfite using EZ DNA Methylation™ Kit (Zymo Research, USA) and standard protocol. For DNA methylation assessment, the pairs of primers in 5′ region of the ABCB1, ABCC1, p16, p14, RARB, RASSF1, DAPKI, GSTP1, MGMT, and TERT genes were designed using Methyl Primer Express v1.0 software (Applied Biosystems, Thermo Fisher Scientific) or selected from publications (Table S1). Methylation-specific PCR (MSP) assays were carried out in a reaction volume of 25 μL, consisting of 1 μL of bisulfite-modified DNA, 1 μM of each primer, and components of Maxima Hot Start Taq DNA polymerase kit (Thermo Scientific, Thermo Fisher Scientific). Bisulfite-modified human leukocyte DNA from healthy donors, treated or untreated with CpG Methylase (M.SssI, Zymo Research), were used as methylated or unmethylated controls, respectively. Non-template (water) controls also were always included in MSP. Reaction products were analyzed in 3% agarose gel, stained with ethidium bromide and visualized under ultraviolet illumination.

2.6. **Statistical analysis**

For the comparison of gene expression profile in DOX-resistant and parental CX-1 cell lines, a fold change (FC) value was calculated. Only genes with an absolute FC of ≥2 were further analyzed. Gene ontology (GO) terms were considered significant if corrected P < 0.05. RT-qPCR data were preprocessed (GenEx v.6.0.1, MultiD Analyses AB, Sweden) and relative quantification method was used to evaluate expression changes of mRNA. The t test was used to calculate the differences in expression levels of the same gene between parental and DOX-resistant cells.

3. **Results**

3.1. **Establishment of DOX-resistant CX-1 cell sublines**

We repeatedly treated parental CX-1 cells with stepwise increasing concentrations of DOX, starting from 10 nM. When the cells were tolerating the particular concentration of the drug, it was doubled. Performing the repeated treatments, four DOX-resistant CX-1 cell sublines were established and named as CX-1/D10, CX-1/D20, CX-1/D40, CX-1/D80. We found that only a small number of cells survived the initial exposure to DOX (10 nM) and every following duplication of the concentration: a small fraction of cells was able to continue proliferation, while others perished. Besides, microscopic images of DOX-resistant CX-1 cells showed morphological alterations consistent with EMT process (Fig. 1A and B). Parental CX-1 cells formed the colonies of tightly packed cancer cells. The DOX-exposed cells started to lose contact with neighboring cells.
Fig. 1 - Characterization of DOX-resistant CX-1 colon cancer cells. Microscopic images of parental (A) and DOX-resistant CX-1 cells (B), magnification 200×. (C) Gene ontology (GO) groups for genes that were deregulated during acquisition of DOX-resistance, obtained by microarray analysis. (D) The list of genes, represented by GO terms like “response to stimulus,” “cellular response to chemical stimulus,” and “cellular response to stimulus.”

(there were more floating cells), became distorted, and acquired elongated spindle-shaped morphology.

3.2. Gene expression profile of DOX resistant CX-1 cells

Gene expression profiling was performed on the maximal (80 nM) DOX concentration-resistant (CX-1/D80) and on DOX-unexposed (control) CX-1 cell lines. Deregulated expression (≥2 fold-change) of 726 genes was identified in CX-1/D80 cells in comparison to DOX-unexposed ones, including 548 up- and 178 down-regulated genes. Functional annotation analysis indicated that deregulated genes were significantly (P < 0.05) represented by gene ontology (GO) terms like “response to stimulus,” “cellular response to chemical stimulus,” and “cellular response to stimulus” (Fig. 1C and Table S2). Among all genes from these GO term-related groups, 73 overlapping genes were identified. A set of these overlapping genes were EMT, cell adhesion and motility-related genes (Fig. 1D). Deregulated expression of cancer stem cell marker KLF4, chemokines, oxidative stress-related and cell-cycle control genes was also observed. Moreover, upregulation of phase I (DHRS2, CYP3A7) and phase II (SULT1A2, SULT1C2) drug metabolizing enzymes was found in CX-1/D80 cells. Hence, these results indicate that EMT activation together with the upregulation of drug metabolizing enzymes possibly contributed to chemoresistance of these cells.

3.3. Expression of EMT, cell stemness and ABC transporter genes

To determine the exact course of changes in DOX-resistant cells, the expression levels of EMT (CDH1, CDH2, SNAI1, ZEB1), cell stemness (POUSF1, SOX2, NANOG) and ABC transporter genes (ABCB1, ABCC1) were assessed in CX-1/D cells resistant to various doses of DOX in comparison to the parental cell line (Table). Parental CX-1 cells were characterized by much higher CDH1 and SNAI1, compared to CDH2 and ZEB1. Upon selection for DOX resistance, expression of CDH2 and ZEB1 significantly increased, however, no considerable changes of SNAI1 expression were observed (Table). The strongest changes of CDH2 (6.2×; P = 0.044) and ZEB1 (5.8×; P < 0.001) were induced by the highest doses (40 and 80 nM) of DOX, and the expression of CDH1 was also slightly increased (2.0×; P = 0.008) in CX-1/D80 subline (Fig. 2A–D).

Additionally, resistance to the maximal DOX concentration was associated with the increased expression of regulators of cell stemness: POUSF1 (1.5×; P = 0.090), SOX2 (3.6×; P = 0.053), and NANOG (2.4×; P = 0.008; Fig. 2E–G). A similar, but statistically insignificant, effect was registered in cells resistant to lower concentrations of DOX, except of NANOG showing considerable changes in expression level already at 20 nM of DOX (Table).

In CX-1 cells the expression levels of ABC transporter genes ABCC1 and ABCB1 were comparably high. During the development of DOX resistance the expression level of the ABCC1 transcript gradually increased, while ABCB1 was downregulated by low doses of DOX (Table). Finally, the cells resistant to 80 nM of DOX showed a significant upregulation of ABCB1 (3.3×; P = 0.007) and a slight increase in ABCB1 level (1.6×; P = 0.143; Fig. 2H and I).

This RT-qPCR-based study revealed an early and marked upregulation of EMT genes during the acquisition of DOX-resistance, followed by a gradual increase in the levels of chemoresistance and cell stemness-related genes, ABCC1 and NANOG respectively.
3.4. Epigenetic changes and the expression of epigenetic regulators

DNA methylation status of several well-known TSGs involved in cell cycle control, drug metabolism, and reparation (p16, p14, DAPK1, RASSF1, RARB, GSTP1, MGMT, and TERT), in addition to multidrug resistance-related genes (ABCB1 and ABCC1), was assessed in parental cells and in DOX-resistant derivatives. Hypermethylation at promoter regions of RARB and p16 was observed in both parental and DOX-resistant cells, but unmethylated p16 promoter-specific MSP product was identified in maximal DOX concentration-resistant cells. Promoters of TERT and ABCB1 were partially methylated, while ABCC1 – unmethylated in all CX-1 sublines (Fig. 3A and B).

In order to correlate the changes in DNA methylation status with the gene expression levels of epigenetic regulators, abundance of transcripts coding methylcytosine dioxygenases TET1 and TET2 as well as histone methyltransferase EZH2 was evaluated (Fig. 3C-E). The expression of TET1 was lower in parental CX-1 cells, compared to TET2, and additional decrease was observed in DOX-resistant sublines (Table). Slight increase of TET2 level was observed in CX-1/D80 cells (1.5×; P = 0.172), but the effect was not statistically significant. No significant changes in expression levels of EZH2 were detected upon cell induction to DOX resistance (Table). To conclude, resistance to DOX was accompanied by downregulation of TET1.

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4. Discussion

Resistance to chemotherapy is the main obstruction to the effective treatment of most cancer types [4]. DOX is an anthraquinone-type anticancer chemotherapeutic drug commonly used for the treatment of a wide variety of cancers including advanced colorectal cancer [17,18]. It is quite effective DNA intercalating agent inhibiting the action of topoisomerase II. However, DOX resistance is the major cause of failure in clinical chemotherapy of colorectal cancer patients. In the present study, we established a range of DOX-resistant colon adenocarcinoma CX-1 cell sublines through exposure to gradually increasing concentrations of DOX. To the best of our knowledge, this is the first effort to establish DOX-resistant CX-1 cell subline and the first report of global gene expression profile of these cells. In addition to genomic analysis, expression levels of the genes important for EMT, cell stemness, drug efflux, and epigenetic regulation were assessed, and DNA methylation status of the major regulatory genes was evaluated in parental cells and DOX-resistant sublines. Our study revealed that only a small number of cells survived after initial exposure to DOX and, after each duplication of the dose, a small fraction of cells was able to continue proliferation, while others perished. In DOX-exposed CX-1 cells quite rapid dose-dependent upregulation of EMT-related transcription factor gene ZEB1 and drug transporter gene ABCC1 was observed, while cell stemness genes tend to be activated only by maximal concentration of DOX.

EMT is a critical process in the development of normal tissues and is also involved in the growth and spread of cancer, including colorectal cancer [19]. Functional importance of the EMT includes acquired cell motility, invasiveness, resistance to apoptosis, and chemoresistance [11,20]. Genome-wide analysis of gene expression changes occurring upon acquisition of DOX-resistance in CX-1 revealed an increased expression of 12 EMT-related genes, namely CTGF, COL6A1, CXCL1, CXCL2, IL8, IL1RAP, DHR52, HBEFG, HMOX1, NRP1, SERPINE1, and TIMP2. Several of these genes have been related to tumor progression, invasion, and metastasis in previous studies [21,22], while for others the connection to colorectal cancer is reported for the first time. Besides, our qPCR-based study showed marked (>5-fold) upregulation of the mesenchymal markers CDH2 and ZEB1 in DOX-resistant cells at 80 nM, while
no significant changes in the expression level of SNAI1 was found. Similar results were obtained with DOX-resistant breast cancer cells previously [23].

Several previous studies [14,24] reported significant ZEB1 contribution to DOX-resistance, and knockdown of ZEB1 was shown to increase the sensitivity of cells to DOX and reverse the drug-induced EMT. Besides, Saxena et al. [14] demonstrated the importance of ZEB1 in the regulation of DOX-mediated induction of several multidrug resistance-related ABC transporters, including ABCC1, in breast cancer cells. Similarly to these studies, increased expression of ABCC1 was observed in CX-1 cells that also showed elevated levels of ZEB1. DOX is a known substrate for ABCC1, and upregulation of this efflux transporter determines the increased resistance of cells to this drug [24]. In previous studies, resistance to DOX has been related to the upregulation of ABCB1 mRNA level [25]. However, in our study, no significant changes in ABCB1 expression were observed. Chemotherapy-induced EMT has a significant effect not only on drug transporters like ABCB1 and ABCC1, but can also change expression of many enzymes related to drug metabolism, including aldehyde dehydrogenases (ALDHs), cytochromes P450 and glutathione metabolism-related enzymes [15]. In agreement with this, an increased expression of several drug metabolizing enzymes (CYP3A7, DHRS2, SULT1A2, and SULT1C2) was identified in CX-1/D80 cells in our genome-wide gene expression analysis.

EMT response also involves upregulation of cell stemness-related genes and transition to the CSC state. The previous study [26] revealed a direct involvement of ZEB1 in these changes. In our study an increased expression of the key

Fig. 2 – Gene expression analysis. Relative expression of EMT (A–D), cell stemness (E–G) and drug efflux (H, I) related genes in parental and DOX-resistant cells estimated by means of RT-qPCR.
pluripotency-related genes was observed in DOX-resistant CX-1 sublines, with the most evident upregulation of NANOG. The over-expression of NANOG is determined in various cancer types and the recent studies suggest that this transcription factor is a core regulator of both EMT process and stemness in colorectal cancer cells [27,28]. Besides, several studies have provided a positive correlation between the expression of NANOG and cell chemoresistance [27,29].

Epigenetic changes together with genetic alterations play an important role in cancer progression and development. During carcinogenesis the TSGs are epigenetically silenced, while oncogenes become activated. Active DNA demethylation is catalyzed by methylcytosine dioxygenases that belong to the ten-eleven translocation (TET) family. Recent studies revealed that TET family members can repress EMT, stemness, and tumor metastasis [30], thus are inactivated during oncogenesis. Loss of TET1 protein and the reduced level of 5-hydroxymethylcytosine have been shown in solid tumors and related to poor cancer prognosis [31,32]. In agreement with the previous study [33], reduced levels of TET1, but not TET2, were observed in DOX-treated CX-1 cells. Besides, hypermethylation at promoter region of several regulatory genes (RARB, p16) was observed in CX-1 cell line, suggesting activity of DNA-methylating enzymes, in addition to TET family members. However, reduction in DNA methylation level at the promoter of p16 gene in maximal DOX concentration-resistant cells points on the possible participation of TETs in the development of chemoresistance. Despite significant advances in cancer treatment, metastasis remains as a key barrier to effective therapy and the main cause of cancer-related death [34]. Accumulating evidences [15,16] suggest significant involvement of the EMT in chemoresistance of various tumors. In the present study we used the series of DOX-resistant CX-1 sublines that allows consecutive reconstruction of the processes involved in the development of DOX resistance. According to our results, under the treatment with DOX a set of CX-1 cells undergoes EMT, activates the expression of the ABC transporter gene (ABCC1) and remains viable. Besides, although the EMT process facilitates the generation of CSC-like cells, the number of such cells in low concentration DOX-resistant cell culture may be too small to be detectable. Higher concentrations of DOX are able to activate NANOG, as a main regulator of cell stemness in this model system. Relatively low concentrations of DOX were used in our study to treat the cells, but this enabled us to detect the molecular changes occurring during early steps of chemoresistance development. Further studies in cell lines resistant to higher doses of DOX might reveal additional mechanisms of chemoresistance in colon cancer.

5. Conclusions

In summary, DOX-resistant CX-1 colon cancer cell sublines can serve as an informative model system for investigation of the development of cancer drug resistance. Our study suggests
possible involvement of the EMT, cell adhesion and motility, and chemoresistance-related genes in DOX-resistant CX-1 cell line, and shows an early activation of the ZEB1 and ABCC1 genes during the development of chemoresistance.

Authors' contributions

R.K. performed the RT-qPCR and MSP experiments and statistical analysis, drafted the manuscript. I.S. was responsible for cell culture. K.D. performed the microarray experiments and conducted bioinformatics analysis. R.D. provided resistant cells, critically revised the manuscript, was responsible for the granting. S.J. conceived and designed the study and drafted the manuscript. All authors read and approved the final manuscript.

Conflict of interest

The authors state no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.medici.2016.09.003.

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