RESEARCH ARTICLE

Identifying Differentially Expressed Genes and Screening Small Molecule Drugs for Lapatinib-resistance of Breast Cancer by a Bioinformatics Strategy

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Abstract

Background: Lapatinib, a dual tyrosine kinase inhibitor that interrupts the epidermal growth factor receptor (EGFR) and HER2/neu pathways, has been indicated to have significant efficacy in treating HER2-positive breast cancer. However, acquired drug resistance has become a very serious clinical problem that hampers the use of this agent. In this study, we aimed to screen small molecule drugs that might reverse lapatinib-resistance of breast cancer by exploring differentially expressed genes (DEGs) via a bioinformatics method. Materials and Methods: We downloaded the gene expression profile of BT474-J4 (acquired lapatinib-resistant) and BT474 (lapatinib-sensitive) cell lines from the Gene Expression Omnibus (GEO) database and selected differentially expressed genes (DEGs) using dChip software. Then, gene ontology and pathway enrichment analyses were performed with the DAVID database. Finally, a connectivity map was utilized for predicting potential chemicals that reverse lapatinib-resistance. Results: A total of 1, 657 DEGs were obtained. These DEGs were enriched in 10 pathways, including cell cycling, regulation of actin cytoskeleton and focal adhesion associate examples. In addition, several small molecules were screened as the potential therapeutic agents capable of overcoming lapatinib-resistance. Conclusions: The results of our analysis provided a novel strategy for investigating the mechanism of lapatinib-resistance and identifying potential small molecule drugs for breast cancer treatment.

Keywords: Lapatinib-resistance - differentially expressed genes - dysfunctional pathway - breast cancer - bioinformatics

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Introduction

Breast cancer is the most common invasive cancer in women worldwide. Over-expression of human epidermal growth factor receptor 2 (HER2) occurs in nearly 15%–30% of breast cancers, and is associated with increased disease recurrence, nodal metastasis and worse prognosis (Ba et al., 2014; Schroeder et al., 2014). Use of Lapatinib, an orally active dual EGFR/HER2 tyrosine kinase inhibitor, showed significant improvement in the outcomes of patients with HER2-positive breast cancer, including progression free survival (PFS) and overall survival (OS) (Kacan et al., 2014). However, nearly all responding patients treated with Lapatinib eventually develop drug resistance that markedly restrains its efficacy on breast cancer.

Molecular mechanisms underlying acquired Lapatinib-resistance are still not fully understood. Studies have shown that a variety of HER2 alterations and bypass signaling pathways participated in this process. For examples, aberrant expression and/or activation of various receptors and signaling molecules including PI3K (Brady et al., 2014), RON (Wang et al., 2013), SRC-family kinases (Formisano et al., 2014), PP2A (McDermott et al., 2014), CXCR4 (De Luca et al., 2014) , and NF-κB (Bailey et al., 2014) were responsible for Lapatinib-resistance. Taken together, these data reflect that there are multiple alterations in Lapatinib-resistant cells, which increases the difficulty in further treatment. In recent years, multifarious therapeutic approaches trying to overcome Lapatinib-resistance have been investigated (Bailey et al., 2014). However, because of the undefined pathogenesis, the therapeutic effects are not satisfactory. Actually, most of the published studies focused on any certain target. Traditional single gene studies are underpowered to clarify the mechanisms because cancer is a disease involving multi-genes and multi-steps. Thus, methods related to global gene expression profiling that involved multi-factors might help explore the mechanisms

Microarray is an effective high-throughput measure for detecting global changes in gene expression and has been widely utilized for exploring concrete mechanisms of pathogenesis. Using this tool, studies described the gene expression profile of acquired Lapatinib-resistant breast cancer cells (Liu et al., 2009). Therefore, to develop drugs with better therapeutic effects, it is important to continue the study of gene expression profile related to acquired Lapatinib-resistance.

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In the present study, we utilized microarray data to investigate differentially expressed genes (DEGs) between acquired Lapatinib-resistant and Lapatinib-sensitive breast cancer cells. Then, functional changes of DEGs were investigated by gene annotation and pathway enrichment. In addition, candidate small molecules for reversing Lapatinib-resistance were screened by CMAP, which provided basic works for new therapeutic approaches to treatment of breast cancer.

Materials and Methods

Identification of DEGs from public microarray data

To explore the DEGs in acquired Lapatinib-resistant breast cancer cells compared to Lapatinib-sensitive breast cancer cells, from the Gene Expression Omnibus (GEO) database (http: //www.ncbi.nlm.nih.gov/geo/), the gene expression profile (GSE16179) was downloaded. This dataset, deposited by Liu et al in 2009 (Liu et al., 2009), contains information about HER2 positive breast cancer cell line BT474 (Lapatinib-sensitive) and BT474-J4 (acquired Lapatinib-resistance cell line). Then, the dataset was analyzed by dChip software (v.2011.01) (http: //www.hsph.harvard.edu/). DEGs between BT474-J4 and BT474 cells were identified through T-test, with a threshold of *P*-value <0.05 and fold-change ≥2.

Functional Enrichment Analysis of DEGs

The functional enrichment analysis of the DEGs, including gene ontology (GO) function analysis and Kyoto Encyclopedia of Genes as well as Genomes (KEGG) pathway analysis, was carried out using the Database for Annotation, Visualization and Integrated Discovery (DAVID) database. In the GO categories, including cellular component (CC), biological process (BP), and molecular function (MF) terms, *P*-value <0.01 was regarded as suggesting statistically significant differences. In addition, the enriched KEGG pathways were identified, according to the hypergeometric distribution with a *P*-value < 0.05.

Connectivity Map analysis

CMAP (The Connectivity Map http: //www.broad.mit.edu/cmap/) database contains more than 7,000 expression signatures involving 6100 small molecules as treatment-control pairs. By comparing queried expression signatures, CMAP has previously been used to explore the mechanisms of drug action, as well as to identify new potential drugs (Lamb et al., 2006). The DEGs, classified into up-regulated and down-regulated groups, were submitted to CMAP for analysis. Then, the enrichment scores that ranged from -1 to 1 were calculated. Small molecules with negative enrichment scores, implying the ability of reversing the expression direction of query genes, were selected as potential drugs for treatment of Lapatinib-resistant breast cancer. To further identify the potential agents with the capability of overcoming Lapatinib-resistance, more strict criteria were utilized by limiting the number of repeat experiments to more than 4 times, setting the proportion of effective rate to >50%, and using a threshold of *P*-value <0.05.

Results

Identification of DEGs between Lapatinib-sensitive and acquired Lapatinib resistant breast cancer cells

Based on the publicly available microarray data set GSE16179, the T test in dChip Software was utilized to analyze the gene expression profiles and identify the DEGs between Lapatinib-sensitive BT474 cells and acquired Lapatinib-resistant BT474-J4 cells with the described criteria. As a result, a total of 1675 DEGs were obtained, including 673 up-regulated and 1002 down-regulated DEGs. For examples, the top ten significant up-regulated DEGs were LOC401131, HTT, Col4a2, Six1, NAV1, FAM5C, SYNM, CHCHD6, SOX11, and FOLH1. Accordingly, the top ten significant down-regulated DEGs were RHOT1, TARP, Dars, MYH14, Dld, Csnk1a1,

Table 1. GO Enrichment of DEGs in Cellular Component Ontology (top 20)

Term	Count	*P* value
GO: 0043228~non-membrane-bounded organelle	270	7.60E-10
GO: 0043232~intracellular non-membrane-bounded organelle	270	7.60E-10
GO: 0031981~nuclear lumen	168	2.51E-09
GO: 0031974~membrane-enclosed lumen	200	2.02E-08
GO: 0043233~organelle lumen	195	5.11E-08
GO: 0070013~intracellular organelle lumen	191	6.44E-08
GO: 0005654~nucleoplasm	109	1.23E-07
GO: 0005856~cytoskeleton	154	1.72E-07
GO: 0015630~microtubule cytoskeleton	72	3.03E-06
GO: 0005792~microsome	38	1.35E-05
GO: 0042598~vesicular fraction	38	2.61E-05
GO: 0005829~cytosol	138	4.03E-05
GO: 0005794~Golgi apparatus	97	5.66E-05
GO: 0031252~cell leading edge	25	7.81E-05
GO: 0044451~nucleoplasm part	67	9.60E-05
GO: 0005783~endoplasmic reticulum	103	1.33E-04
GO: 0044430~cytoskeletal part	102	1.53E-04
GO: 0070161~anchoring junction	28	1.75E-04
GO: 0005730~nucleolus	78	3.00E-04
GO: 0005813~centrosome	32	5.97E-04

TMEM189, NUSAP1, AGK, and Ssh3.

Functional annotation and pathway enrichment of DEGs

In order to explore the altered biological function of the DEGs, they were clustered by DAVID, according to the Gene Ontology (GO) with $P < 0.01$. The top 20 enriched GO terms were listed (Table 1-3) and divided into three ontologies, including CC, BP and MF.

In the CC ontology, non-membrane-bounded organelle was listed as the first GO category with 270 genes. Besides, the second enriched GO category was membrane-enclosed lumen (200 genes). Furthermore, the other enriched CC GO terms included cytoskeleton (154 gene), cytosol (138 gene), nucleoplasm (109 gene), endoplasmic reticulum (103 gene), cell leading edge (22 genes) and nucleolus (78 gene). In the BP ontology, the most significant GO categories were protein transport related items such as protein localization (112 genes) and protein transport (100 genes). Meanwhile, enriched items regarding cell proliferation and cell motility included cell cycle (98 genes), cell division (46 genes), cytoskeleton organization (59 genes), and nuclear division (37 genes). In addition, the other enriched categories included regulation of cellular protein metabolic process (62 genes) and macromolecular complex assembly (80 genes). In the MF ontology, the binding-related items were the most significant GO categories, which included nucleotide binding (228 genes), cytoskeletal protein binding (64 genes), transcription factor binding (63 genes), and purine nucleotide binding (181 genes). The second enriched items were related to regulator activity, such as transcription activator activity (50 genes), nucleoside-triphosphatase regulator activity (49 genes) and protein serine/threonine kinase activity (50 genes).

Table 2 GO Enrichment of DEGs in Biological Process Ontology (Top 20)

Term	Count	*P* value
GO: 0045184~establishment of protein localization	103	5.22E-08
GO: 0008104~protein localization	112	2.18E-07
GO: 0015031~protein transport	100	2.28E-07
GO: 0007049~cell cycle	98	1.77E-06
GO: 0046907~intracellular transport	85	3.68E-06
GO: 0051301~cell division	46	1.00E-05
GO: 0000278~mitotic cell cycle	54	1.09E-05
GO: 0000280~nuclear division	37	1.64E-05
GO: 0007067~mitosis	37	1.64E-05
GO: 0006417~regulation of translation	27	1.80E-05
GO: 0070271~protein complex biogenesis	67	2.03E-05
GO: 0006461~protein complex assembly	67	2.03E-05
GO: 0000087~M phase of mitotic cell cycle	37	2.45E-05
GO: 0010608~posttranscriptional regulation of gene expression	35	3.87E-05
GO: 0007010~cytoskeleton organization	59	3.90E-05
GO: 0048285~organelle fission	37	3.98E-05
GO: 0032268~regulation of cellular protein metabolic process	62	6.66E-05
GO: 0008380~RNA splicing	42	8.82E-05
GO: 0065003~macromolecular complex assembly	80	9.64E-05
GO: 0016197~endosome transport	15	1.11E-04

Table 3 GO Enrichment of DEGs in Molecular Function Ontology (Top 20)

Term	Count	*P* value
GO: 0045184~establishment of protein localization	103	5.22E-08
GO: 0008104~protein localization	112	2.18E-07
GO: 0015031~protein transport	100	2.28E-07
GO: 0007049~cell cycle	98	1.77E-06
GO: 0046907~intracellular transport	85	3.68E-06
GO: 0051301~cell division	46	1.00E-05
GO: 0000278~mitotic cell cycle	54	1.09E-05
GO: 0000280~nuclear division	37	1.64E-05
GO: 0007067~mitosis	37	1.64E-05
GO: 0006417~regulation of translation	27	1.80E-05
GO: 0070271~protein complex biogenesis	67	2.03E-05
GO: 0006461~protein complex assembly	67	2.03E-05
GO: 0000087~M phase of mitotic cell cycle	37	2.45E-05
GO: 0010608~posttranscriptional regulation of gene expression	35	3.87E-05
GO: 0007010~cytoskeleton organization	59	3.90E-05
GO: 0048285~organelle fission	37	3.98E-05
GO: 0032268~regulation of cellular protein metabolic process	62	6.66E-05
GO: 0008380~RNA splicing	42	8.82E-05
GO: 0065003~macromolecular complex assembly	80	9.64E-05
GO: 0016197~endosome transport	15	1.11E-04

Table 4 Eleven Therapeutic Small Molecule Agents with Potential Abilities to Overcome Lapatinib-Resistance of Breast Cancer

Cmap name	Mean	N	Enrichment	*P* value	Specificity	Percent non-null
anisomycin	-0.495	4	-0.687	2.06E-02	0.1695	75
lycorine	-0.45	5	-0.683	7.35E-03	0.18	80
N6-methyladenosine	-0.352	4	-0.683	2.22E-02	0.0207	75
progesterone	-0.286	4	-0.656	3.20E-02	0.0537	50
chloroquine	-0.282	4	-0.649	3.57E-02	0.0227	50
puromycin	-0.315	4	-0.639	4.07E-02	0.2164	50
tetrandrine	-0.485	4	-0.637	4.17E-02	0.1019	75
asiaticoside	-0.348	4	-0.634	4.37E-02	0.0327	50
ciclopirox	-0.359	4	-0.625	4.89E-02	0.2095	75
chloropyrazine	-0.484	4	-0.623	5.00E-02	0.0844	75
geldanamycin	-0.323	15	-0.562	8.00E-05	0.0703	53

Moreover, via the KEGG pathway analysis, a total of 10 dysfunctional pathways were enriched, which included cell cycle (hsa04110, 22 genes, p=8.57E-04), pathogenic Escherichia coli infection (hsa05130, 12 genes, p=4.98E-03), endocytosis (hsa04144, 26 genes, p=6.08E-03), regulation of actin cytoskeleton (hsa04810, 29 genes, p=6.87E-03), adherens junction (hsa04520, 14 genes, p=7.72E-03), focal adhesion (hsa04510, 27 genes, p=9.84E-03), glutathione metabolism (hsa00480, 10 genes, p=1.67E-02), prostate cancer (hsa05215, 14 genes, p=2.46E-02), neurotrophin signaling pathway (hsa04722, 17 genes, p=3.89E-02), and Steroid biosynthesis (hsa00100, 5 genes, p=4.24E-02).

Lapatinib-resistant breast cancer cell signature-specific drug screening from CMAP database.

Compared with the BT474 cells (Lapatinib-sensitive), there were 673 up-regulated and 1002 down-regulated genes in BT474-J4 cells (acquired Lapatinib-resistant), which were analyzed by CMAP tool. As results, 675 small molecule drugs with negative enrichment scores were predicted, which showed the potential to reverse the Lapatinib-resistance signature. By further filtering with the described criteria, 11 remained molecules were screened out as the most promising therapeutic small-molecule candidates to overcome Lapatinib-resistance of breast cancer (Table 4). For example, anisomycin, lycorine, progesterone, chloroquine, and tetrandrine were included in the list.

Discussion

Overcoming Lapatinib-resistance is a great challenge for treating patients with breast cancer. Therefore, it is very necessary to investigate the mechanisms of Lapatinib-resistance, and develop effective treatment strategies for it. Through gene expression profiling, important genes related to resistance were found, which could be further utilized to identify novel therapeutic strategies.

In this study, we identified 1657 DEGs between Lapatinib-resistant and -sensitive breast cancer cells. In addition, through GO annotation and pathway enrichment using DAVID analysis, we found that the DEGs were annotated in membrane-enclosed lumen, cytoskeleton, cytosol, protein transport, factor binding and others cellular component, and were enriched in cell cycle, endocytosis, regulation of actin cytoskeleton, focal adhesion, glutathione metabolism and neurotrophin signaling pathway. Finally, small molecule agents that had a potential to reverse Lapatinib-resistance were screened out. These findings not only demonstrated possible mechanisms and candidate drugs for Lapatinib-resistance, but also presented novel strategies for treatment of breast cancer.

The GO is an excellent method for functional annotation of biomolecules. In this study, we found the majority of cellular components, including non-membrane-related and membrane-related items, enriched in Lapatinib-resistance cells. For example, non-membrane-bounded organelle, membrane-enclosed lumen, cytoskeleton, cytosol, nucleoplasm, endoplasmic reticulum, cell leading edge, and nucleolus were invovled. The results reflected that Lapatinib-resistance might occur through complex cellular molecular mechanisms involved in both membrane and non-membrane structure. Previous evidence suggests that actin cytoskeleton (Mithraprabhu et al., 2014) and ion pumps (Eljack et al., 2014) conferred resistance to various tumor cells. In GO category of biological process, our data showed that the most significant items concerned protein transport and localization, and the second significant category was related to cell proliferation and motility, such as cell cycle, cytoskeleton organization and cell division. As we know, numerous genes have been shown to promote drug resistance through participating protein transport and cell cycle, thereby suppressing cell death signals and maintaining proliferation ability under the condition of drug exposure. For example, Six1, a homeodomain-containing transcription factor listed in the top ten up-regulated DEGs, can mediate resistance to paclitaxel in breast cancer cells (Li et al., 2013a) and induces proliferation of pancreatic cancer cells through activation of Cyclin D1 (Li et al., 2013b). In addition, cancer cells with drug resistance phenotype frequently have the enhanced capability of migration and invasion. The DEGs might have influence on migration and invasion by participating in cell motility and cytoskeleton organization. Furthermore, 62 DEGs were enriched in the category of regulation of cellular protein metabolic process, which might facilitate drug resistance by disturbing some certain metabolic process. As we know,

sphingolipid metabolism and glutathione metabolism have been reported to play important roles in multidrug resistance of cancers (Ramsay and Dilda, 2014; Truman et al., 2014). Taken together, the data show that various biological processes may participate in the formation and development of Lapatinib-resistance of breast cancer. In the molecular function portion, abundant DEGs were enriched in the binding-related items, including nucleotide binding and protein binding. These results revealed that the DEGs may affect the binding between Lapatinib and DNA or protein. Accordingly, we hypothesized that the DEGs might also play important roles in Lapatinib-resistance, although which remains unclear. Another group of enriched items are about regulator activity, including transcription activator activity, nucleoside-triphosphatase regulator activity and protein serine/threonine kinase activity, which suggests that Lapatinib-resistance is related to aberrance of activity of numerous regulators such as kinase and enzyme.

Pathway analysis may indicate more precise biological roles and interactions of genes than GO analysis. In the present study, enriched pathways about cell cycle, regulation of actin cytoskeleton, endocytosis, adherens junction, focal adhesion and glutathione metabolism confirmed their concordance with GO analysis, suggesting their important function in researching Lapatinib-resistance. First, a total of 22 DEGs were enriched in cell cycle pathway that was the most significant pathway in the list. Evidence demonstrated that various cyclin and CDK are involved in drug resistance of cancers. Recently, Lapatinib has been reported to induce p27 (Kip1)-dependent G1 arrest (Tang et al., 2013). Another study showed that Lapatinib treatment suppressed RTK-mediated signaling by regulating p53, p27, and cyclin D1 and thus led to cell-cycle arrest in breast cancer cells (Li et al., 2011). Thereby, the DEGs enriched in cell cycle pathway might be involved in the process of Lapatinib-resistance. Meanwhile, the adherens junction and focal adhesion are two important factors related to cancer development (Andl, 2010), especially cell adhesion-mediated drug resistance (CAMDR) and cell adhesion-mediated radioresistance (Eke and Cordes, 2014). In Lapatinib-resistance, currently, the roles of focal adhesion pathway are unclear and deserve further consideration. Then, 29 DEGs enriched in the category of actin cytoskeleton, while abnormity of structure or function of actin cytoskeleton is related to drug resistance of many cancers including breast cancer. Previous reports indicated that the molecular motor recruitment in the microtubule environment was associated with Taxol resistance of breast cancer (Froidevaux-Klipfel et al., 2011), and the actin filament cross-linker L-plastin was related to TNF-alpha resistance in breast cancer (Janji et al., 2010). We speculated that actin cytoskeleton disorder might also play important roles in Lapatinib-resistance, but concrete mechanism is still worth further studying. Moreover, 10 DEGs were shown to be enriched in Glutathione metabolism, which is one of typical mechanisms related to drug resistance. Previous research showed that elevated glutathione S-transferase P1 (GSTP1) contributed to chemoresistance in many cancers (Xu et al., 2013). Glutathione lever increased in Lapatinib-resistant breast cancer cells, and depletion of intracellular glutathione by l-buthionine sulfoximine markedly enhanced Lapatinib cytotoxicity (Hardy et al., 2014). The results indicated that the DEGs enriched in glutathione metabolism might be involved in Lapatinib-resistance by increasing intracellular glutathione. Finally, 17 DEGs were enriched in neurotrophin signaling pathway. This pathway has been reported to confer anoikis-resistance in breast cancer by inducing EMT (Kumar et al., 2011; Howe et al., 2012) that has been proven to promote acquired Lapatinib-resistance in HER2-positive gastric cancer (Kim et al., 2014) and breast cancer (Zhang et al., 2014). Therefore, we hypothesized that neurotrophin signaling pathway might play important roles in Lapatinib-resistance of breast cancer through EMT pathway.

In the present study, several molecule agents with highly significant negative scores were screened out, which may be used for Lapatinib-resistance treatment. Among them, Anisomycin, an antibiotic produced by Streptomyces griseolus, has been demonstrated to partially overcome cisplatin resistance in ovarian cancer cells and glucocorticoid resistance in lymphoblastic leukemia cells via MAPK activation (Liu et al., 2013; Wilson et al., 2013). Meanwhile, Lycorine, a toxic crystalline alkaloid found in various amaryllidaceae species, exhibits anticancer activities against some cancer types, including lung cancer, breast cancer, cervical cancer and ovarian cancer (Wang et al., 2014). Moreover, Chloroquine, a drug used in the malaria treatment and prevention, has been found to overcome primary resistance of trastuzumab in HER2-positive breast cancer (Cufi et al., 2013). Furthermore, Tetrandrine, a bis-benzylisoquinoline alkaloid and a calcium channel blocker, could reverse the resistance of tamoxifen in breast cancer cells (Chen and Chen, 2013) and restrain cancer angiogenesis and metastasis (Gao et al., 2013). Overall, base on the abilities of reversing resistance of a variety of therapeutic drugs in cancers, these above small molecule drugs might have potential for overcoming Lapatinib-resistance, however, which has not been reported so far. Other screened agents, including Progesterone, Puromycin, Asiaticoside, Ciclopirox, and Geldanamycin, also have been reported to show some levels of antitumor activity. Progesterone has been reported to induce apoptosis in hepatoma cells treated with epirubicin (Chang et al., 2014). Puromycin, an antibiotic that is a translation inhibitor, presents strong anticancer activity for various tumors (Ueki et al., 2013). Asiaticoside, a triterpene, could increase apoptosis of tumor cells and enhance anti-tumor activity of vincristine via regulation of cell cycle related proteins (Huang et al., 2004). Geldanamycin, a benzoquinone ansamycin antibiotic that inhibits the function of Hsp90, has shown anticancer effects in HER2 positive breast cancer (Zhao et al., 2014). Ciclopirox, a synthetic antifungal agent for topical dermatologic treatment, could induce autophagy through reactive oxygen species-mediated JNK signaling activation in rhabdomyosarcoma (Zhou et al., 2014) and induce apoptosis by Wnt signaling inhibition in renal cell carcinoma (SA et al., 2014). Nevertheless, it is unclear whether the above chemicals have abilities to reverse

resistance of therapeutic drugs in cancers, especially Lapatinib-resistance in breast cancer. Further research is also needed in the areas.

On the whole, the above small molecules may provide promising targets for future development in new treatment of Lapatinib-resistance. However, these are preliminary findings, and further evaluation for their potential use is still required.

In conclusion, the study provides some clues about the mechanism of Lapatinib-resistance of breast cancer. DEGs of Lapatinib-resistant breast cancer cells were analyzed using computational bioinformatics methods. Meanwhile, the dysfunctional pathways in Lapatinib-resistant cancer cells were identified. Moreover, some small molecule chemicals were screened out for reversing Lapatinib-resistance. This study may suggest an efficient strategy for the treatment of Lapatinib-resistant breast cancer. However, further investigations are still needed to validate our hypothesis.

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