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The Establishment of Two Paclitaxel-Resistant Prostate Cancer Cell Lines and the Mechanisms of Paclitaxel Resistance withTwo Cell Lines

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BACKGROUND. Although paclitaxel is used for hormone-resistant prostate cancer, relapse definitely occurs later. Details of the molecular mechanism responsible for paclitaxel- resistance remain unclear.
METHODS. We established paclitaxel-resistant cells, DU145-TxR and PC-3-TxR from parent DU145 and PC-3. To characterize these cells, we examined cross-resistance to other anticancer drugs. Expression of several potential genes that had been related to drug-resistance was compared with parent cells by RT-PCR and Western blotting. Methylation analysis of multiple drug resistance (MDR1) promoter was carried out using bisulfite-modified DNA from cell lines. Knockdown experiments using small interfering RNA (siRNA) were also performed to confirm responsibility of drug-resistance. Finally, cDNA microarray was performed to quantify gene expression in PC-3 and PC-3-TxR cells.
RESULTS. The IC_{50} for paclitaxel in DU145-TxR and PC-3-TxR was 34.0- and 43.4-fold higher than that in both parent cells, respectively. Both cells showed cross-resistance to some drugs, but not to VP-16 and cisplatin. Methylation analysis revealed that methylated CpG sites of MDR1 promoter in DU145 and PC-3 cells were demethylated in DU145-TxR cells, but not in PC-3-TxR cells. Knockdown of P-glycoprotein (P-gp), which was up-regulated in resistant cells, by MDR-1 siRNA restored paclitaxel sensitivity in DU145-TxR but not in PC-3-TxR, indicating that up-regulation of P-gp was not always main cause of paclitaxel-resistance. Microarray analysis identified 201 (1.34%) up-regulated genes and 218 (1.45%) out of screened genes in PC-3-TxR.
CONCLUSIONS. Our data will provide molecular mechanisms of paclitaxel-resistance and be useful for screening target genes to diagnose paclitaxel sensitivity. *Prostate 67: 955–967, 2007.*

KEY WORDS: **prostate cancer; paclitaxel resistance; MDR-1; cDNA microarray**

INTRODUCTION

Prostate cancer (PCa) is the most common malignancy and the second most frequent cause of cancer-related death of men in the United States [1]. Androgen deprivation treatment is very effective for more than 80% of advanced PCa. More than half of those cases of advanced PCa become resistant to deprivation treatment after several years and then several other

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palliative treatments, such as estramustine phosphate (EMP), steroids, are employed for these patients. However, the results are very disappointing because a half of those cases lead to death within a year or 2 years.

Recently, the taxanes [paclitaxel or docetaxel (DTX)] with other agents, such as EMP or predonisone have been used for hormone-resistant prostate cancer (HRPC) and have shown good response [2–5]. Paclitaxel, which is purified from *Taxus brevifolia*, stabilize microtubule and causes apoptosis [6]. The response rates of taxane-based combination therapies are better than combination therapies with other anticancer agents. However, even HRPC treated with paclitaxel-based chemotherapy also relapses as occurred using other anticancer agents. Then the prognosis of the patients after the relapse is extremely poor.

In order to investigate the mechanisms of paclitaxel-resistance, several paclitaxel-resistance cell lines have been generated in ovarian cancer, breast cancer, and lung cancer [7,8]. Some of major mechanisms of taxane-resistance are overexpression of multiple drug resistance (MDR1), and multidrug resistance protein (MRP) family [9]. Especially accumulation of P-glycoprotein (P-gp) encoded from MDR1 might cause resistance of several drugs in some cancers. The microtubule dynamics may also be important for paclitaxel-resistance because the target of paclitaxel is the microtubule [10]. As for the role of bcl-2 as a modulator of paclitaxel sensitivity remains controversial. In human paclitaxel-resistant hepatocellular carcinoma cells bcl-2 was overexpressed [11]. Whereas bcl-2 expression was consistently down-regulated in T47-D breast cancer cells [12]. In PCa, although Bcl-2/Bcl-xL bispecific antisense oligonucleotide also enhanced paclitaxel chemosensitivity in PC-3 and LNCaP cells [13,14], involvement to paclitaxel-resistance of Bcl-2/Bcl-xL in PCa is not clear. Recently, cDNA microarray analyses were performed in order to reveal the key genes that are related with paclitaxel resistance. Not only MDR-1 gene but also Rho guanine dinucleotide phosphate dissociation inhibitor beta (RhoGDI) and insulin-like growth factor-binding protein 3 (IGFBP-3) were up-regulated in paclitaxel-resistant ovarian cancer cell lines [15]. Villeneuve et al.[16]described that 1.9% of 1,728 genes were regulated in paclitaxel-resistant MCF-7 breast cancer cells. Thus it is very important to know the mechanisms of paclitaxel-resistance in PCa.

In the present study, we established two paclitaxel-resistant cell lines from androgen-independent DU145 and PC-3 PCa cell lines by increasing concentration of paclitaxel gradually. Although both cell lines showed resistance to paclitaxel over 30 times more than parents cells and cross-resistance to other anticancer drugs, the mechanism of resistance was different.

MATERIALS AND METHODS

Cell Culture and Cell Proliferation Assay

DU145 and PC-3 cells purchased from American type culture collection were cultured in Dulbecco's modified Eagle medium (DMEM) and RPMI1640 containing 5% fetal calf serum (FCS) and penicillin/streptomycin (Invitrogen, CA, USA). Cell growth inhibition assay was preformed by plating 1×10^5 cells on 6-well plates. Twenty-four hours later, cells were treated with the indicated concentration of anticancer agents, and cultured for an additional 48 hr. At the end of the culture period, the cells were trypsinized and counted with a hemocytometer.

Establishment of Paclitaxel-resistant DU145 and PC-3 Cell Lines

Paclitaxel-resistant cancer cells were obtained by stepwise increased concentrations of paclitaxel. DU145 and PC-3 cells maintained as described above were incubated with 10 nM paclitaxel for 2 days. Then the medium was changed to fresh one without paclitaxel and cells were cultured cells grow well. Whenever we subcultured, the cells were incubated with gradual increasing concentration of paclitaxel for 2 days and cultured without paclitaxel until cells grow well. Some aliquots of the cells were stored whenever we subcultured it. When cells were killed by increased paclitaxel, the aliquots were subcultured again and lower concentration of paclitaxel was used for treatment. Cells that grew at the maximum concentration of paclitaxel were stored for further analyses. For maintenance of paclitaxel-resistant cells, 10 nM paclitaxel was added into the normal medium every time.

RNA extraction and RT-PCR. Twenty-four hours after plating of 1×10^6 DU145 or PC-3 cells, total RNA was purified with RNeasy mini kit (Qiagen, Maryland, USA). Complementary DNA (cDNA) was made by reverse-transcription (RT) of 1 μg each total RNA using ThermoScript RT-PCR system (Invitrogen). Each cDNA sample was amplified with ExTaq (Takara, Japan). PCR reactions for indicated genes were carried out using the following forward (F) and reverse (R) in Table I. Each of the amplified PCR products was determined by electrophoresis on an 1.5% agarose gel.

Western blot analysis. Twenty-four hours after plating 1×10^6 DU145, DU145-TxR or PC-3, and PC-3-TxR cells on 6 cm dishes in DMEM-5% FBS, the cells were lysed with 200 μl hypotonic buffer (20 mM Tris-HCl (pH 7.6), 10 mM NaCl, 1 mM $MgCl_2$, and 0.5% NP-40) and the membrane and cytosol fractions were collected by centrifugation as described previously [17]. To

TABLE I. The Primers Used for RT-PCR Analysis

Gene	Forward	Reverse
GAPDH	5′-GACCACAGTCCATGCCATCA-3′	5′-TCCACCACCCTGTTGCTGTA-3′
MDR-1	5′-ATGCTCTGGCCTTCTGG ATG GGA-3′	5′-ATGGCGATCCTCTGCTTCTGCCCA C-3′
MRP-1	5′-GCATGA TCCCTGAAGACGA-3′	5′-TAGAGCTGG CCCTTGTACTC-3′
MRP2	5′-TAGAGCTGGCCCTTGTACTC-3′	5′-TCAACTTCCCAGACATCCTC-3′
MRP-3	5′-CGCCTGTTTTTCTGGTGGTT-3′	5′-TCCCCCAGTCACAAAGATG -3′
MRP-4	5′-GCTGAGAATGACGCACAGAA-3′	5′-TCCCAGCAAGGCACGATATT-3′
MRP-5	5′-GTCCTGGGTATAGAAGTGTG-3′	5′-CAGAAGATCCACACAACCCT-3′
MRP-6	5′-TTGGATTCGCCCTCATAGTC-3′	5′-TCTTTTGGTCTCAGTGGCCT-3′
MRP-7	5′-CTCCCACTGGATCTCTCAGC-3′	5′-TCGCATACACGGTGAGGTAG-3′
Fas	5′-CAGGCTAACCCCACTCTATG-3′	5′-TGGGGGTGCATTAGGCCATT-3′
Caspase-8	5′-ACTTCAGACACCAGGCAGGGC T-3′	5′-GCCCCTGCATCCAAGTGTGTTC-3′
Bcl-2	5′-ATGTCCAGCCAGCTGCACCTGAC-3′	5′-GCAGAGTCTTCAGAGACAGCCAGG-3′
Bax	5′- GCTTCAGGGTTTCATCCAGG-3′	5′-AAAGTAGGAGAGGAGGCCGT-3′
c-jun	5′- GGAAA GACCTTCTATGACGATGC -3′	5′-GAACCCCTCCTGCTCATCTGT CAC-3′
YB-1	5′-GACTGCCATAGAGAATAACCCCAG-3′	5′-CTCTCTAGGCTGTTTTGGGCGAGGA-3′
Sp-1	5′-GCTGCCGCTCCCAACTTACA-3′	5′-ATCGTGACTGCCTGAGAGCT-3′

extract nuclear protein, the centrifuged pellet after separating cytosol fraction was lysed with 50 μl hypertonic buffer (20 mM Tris-HCl (pH 7.6), 0.42 M NaCl, 1 mM EDTA, and 0.5% NP-40) and nuclear fraction were collected by centrifugation. To extract whole cell protein, cells were lysed with hypertonic buffer directly. Fifty micrograms of cytosol protein, 50 μg of whole cell protein, or 10 μg of nuclear protein was loaded in each lane of 7.5 or 12.5% Ready Gel J (Bio-Rad, NY), subjected to electrophoresis, then electrotransferred to a PVDF membrane (Bio-Rad). The immobilized proteins were incubated with primary antibody, P-gp (rabbit polyclonal IgG, 200-fold dilution; Santa Cruz, CA), YB-1 (goat polyclonal IgG, 200-fold dilution; Santa Cruz), or GAPDH (rabbit polyclonal IgG, 1,000-fold dilution; Trevigen, MD). The presence of primary antibody was visualized by Super signal west pico luminol/enhancer solution (Pearce, IL).

Methylation analysis of MDR1 promoter. Genomic DNA from PC-3, PC-3-TxR, DU145, and DU145-TxR was purified using Blood and cell culture DNA mini kit (Quiagen) 24 hr after 5×10^5 cells were plated on 6 cm dish. One microgram of DNA was subjected to sodium bisulfite modification kit (BisulFast DNA Modification Kit, Toyobo, Osaka, Japan). MDR-1 (223 bp) promoter region (−183 to +40 of transcription initiation site) was amplified from bisulfite-modified DNA as described by Enokida et al. [18,19]. The amplified DNA was further amplified using methylation-specific primer (MSP) or unmethylation-specific primer (USP) after 100-fold dilution of the amplified DNA [19]. PCR reaction was modified to 94°C 15 s, 70°C 30 s, 72°C and 20 cycles for MSP primers and 94°C 15 s, 68°C 30 s, 72°C and 20 cycles for USP primers. Then DNA sequence analysis was also carried out using the amplified 223 bp PCR products.

Small interfering RNA transfection. MDR-1 small interfering RNA (siRNA), lamina/C siRNA, non-targeting siRNA were purchased from Dharmacon (Lafayette, CO). After 3×10^4 DU145-TxR and PC-3-TxR cells or 3×10^5 those cells were cultured on 24-well plates or in 6-well plates for total RNA purification or for protein extraction, respectively, cells were transfected with 0, 10, 20, or 30 nM MDR-1 siRNA, 30 nM lamina/C siRNA, and 30 nM non-targeting siRNA by X-treme GENE siRNA Transfection Reagent (Roche). Forty-eight hours after transfection, total RNA and protein was extracted. In order to see the effect of siRNA on drug resistance, cells were transfected with 30 nM MDR-1 siRNA or non-targeting siRNA 24 hr after plating on 24-well plates. Twenty-four hours later cells were treated with 0, 1, 3, 10, 30, 100, 300, and 1,000 nM paclitaxel and cultured for 48 hr. Then the cells were trypsinized and counted with a hemocytometer.

cDNA Microarray Analysis

Twenty-four hours after plating of 5×10^5 PC-3 cells, total RNA was purified with RNeasy mini kit (Qiagen,). RNA samples were sent to Hokkaido system science (Sapporo, Japan) and analyzed by Agilent technologies (human 1A microarray kit).

RESULTS

Establishment of Paclitaxel-resistant Cell Lines

When we examined the sensitivity for paclitaxel of parent DU145 and PC-3 cells, IC_{50} values of these cells were 11.3 and 5.0 nM, respectively

(Tables II and III). We established paclitaxel-resistant DU145 (DU145-TxR) and PC-3 (PC-3-TxR) cells by stepwise exposure method (from 10 nM paclitaxel) for 9 and 15 months, respectively. Cell growth inhibition assay demonstrated that these DU145-TxR and PC-3-TxR cells become 34.0-fold (IC_{50}: 384.2 nM) and 43.4-fold (IC_{50}: 217.1 nM) more paclitaxel resistant than parent cells (Tables II and III and Fig. 1). We also compared the cross-resistance to other anticancer drugs [EMP, vinblastin (VBL), doxorubicin (DOX), DTX, VP-16, and cisplatin] between parent and paclitaxel-resistant cells (Figs. 2 and 3, Tables II and III). Both of DU145-TxR and PC-3-TxR cells showed almost same cross-resistance to EMP, VBL, DOX, and DTX. However, cross-resistance to cisplatin and VP-16 was hardly observed.

Expression of Several Potential Chemoresistant Genes

Cellular mechanisms of drug resistance include in decreasing intracellular drug concentrations by increased efflux or decreased influx. The drug distribution in an organism is highly dependent on transporters which play a role in absorption and elimination. P-gp and MRP which belong to the ATP-binding cassettes (ABC) family are well-known typical transporters. We evaluated the expression of MDR-1 and MRP1 to MRP7 of DU145-TxR and PC-3-TxR cells by RT-PCR. Only MDR-1 mRNA was overexpressed in both cells (Fig. 4A). Since MDR-1 mRNA was overexpressed in both cells, we confirmed the expression of P-gp which was encoded from MDR-1 mRNA. P-gp as well as MDR-1 mRNA was overexpressed in DU145-TxR and PC-3-TxR cells but not in parent cells (Fig. 4B). Moreover, the level of P-gp in DU145-TxR cells was more expressed than PC-3 cells. Since the cell death by paclitaxel is associated with apoptosis, we also compared the expression of major apoptosis-related genes, Bcl-2, Bax, Fas, and Capase-8 in these cells. However, expression level of all of these genes was not changed between parent and resistant cells (Fig. 4C).

Mechanisms of MDR1 Overexpresssion in DU145-TxR and PC-3-TxR Cells

One of mechanisms by which of MDR-1 is overexpressed in paclitaxel-resistant cells is the induction by Y-box-binding protein 1 (YB-1). YB-1 is mainly located in the cytoplasm [20]. Once cells are exposed to UV irradiation and anticancer drugs, such as paclitaxel, YB-1 tanslocates into nucleus, bind to a *cis*-acting element of the MDR-1 promoter, and induce MDR-1 mRNA expression [21]. In order to see the nuclear localization of YB-1 protein, we performed Western blot analysis. The YB-1 protein level in nucleus was about three times higher in DU145-TxR cells than in DU145 cells and it was almost at the same level between PC-3 and PC-3-TxR cells (Fig. 5A). Nuclear localization of YB-1 was less dramatic compared to the MDR-1 expression in paclitaxel-resistant cells.

Next, we investigated methylation status of CpG sites at the MDR1 promoter region because some

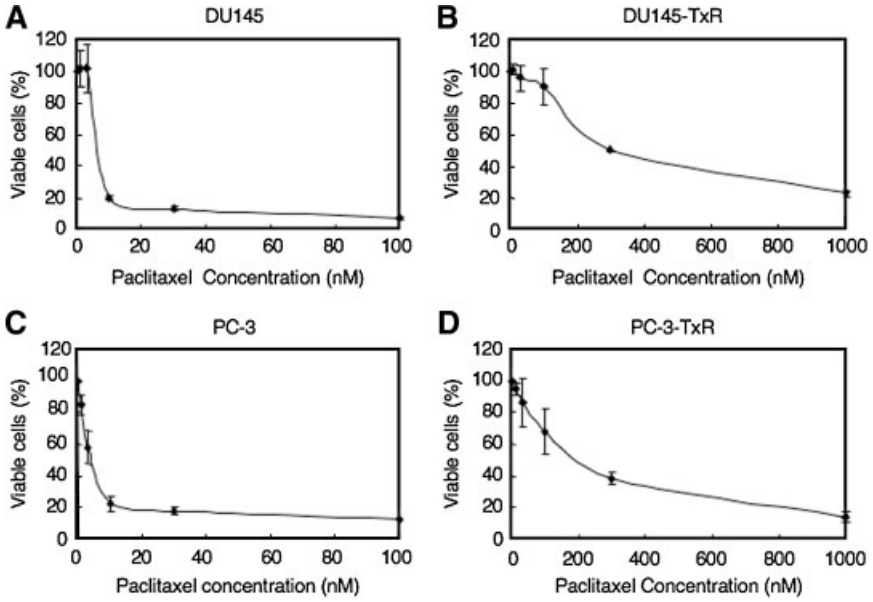

Fig. 1. Establishment of paclitaxel-treated cell lines. DU145 (**A**), paclitaxel-resistant DU145-TxR (**B**), PC-3 (**C**), and paclitaxel-resistant PC-3-TxR (**D**) cells were exposed with indicated concentrations of paclitaxel for 24 hr and counted 2 days after exposure.

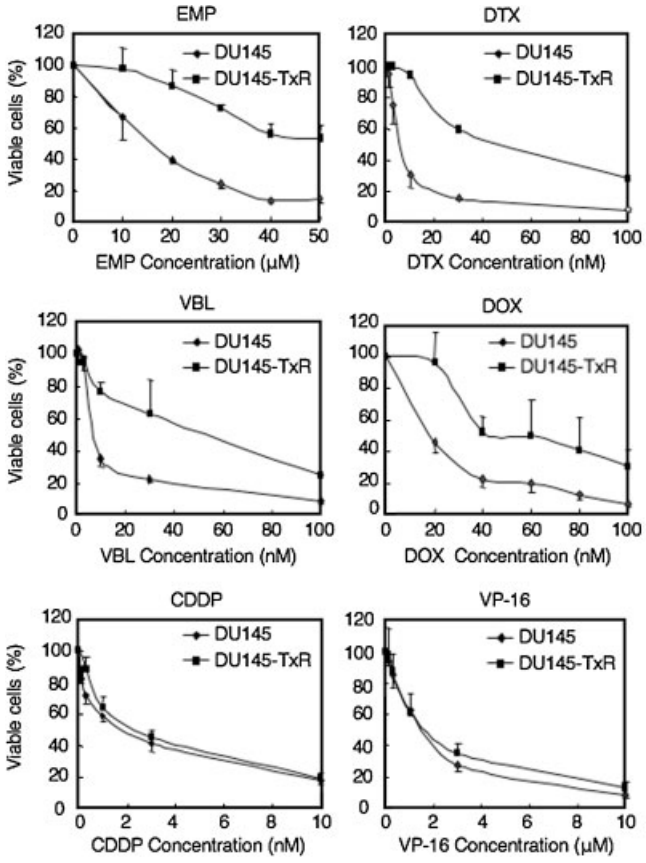

Fig. 2. Cross-resistance of DU145 and DU145-TxR cells. DU145 and DU145-TxR cells were exposed with indicated concentrations of EMP, docetaxel (DTX), vinblastin (VBL), doxorubicin (DOX), cisplatin (CDDP), and etoposide (VP-16) for 24 hr and counted 2 days after exposure.

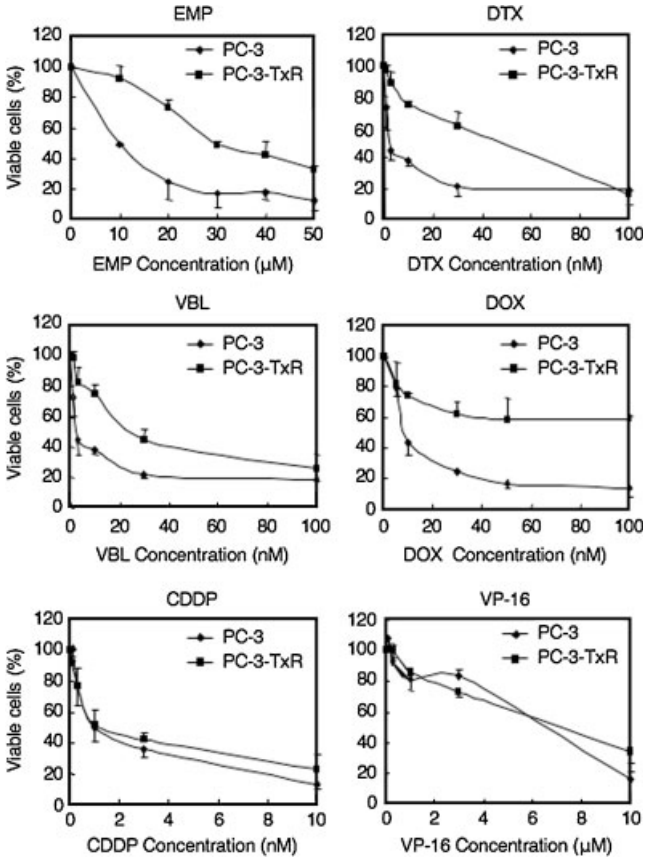

Fig. 3. Cross-resistance of PC-3and PC-3-TxR cells. PC-3 and PC-3-TxR cells were exposed with indicated concentrations of EMP, docetaxel (DTX), vinblastin (VBL), doxorubicin (DOX), cisplatin (CDDP), and etoposide (VP-16) for 24 hr and counted 2 days after exposure.

groups reported inverse correlation between methylation and MDR1 expression in [19,22,23]. Since DU145-TxR and PC-3-TxR cells overexpressed MDR1 mRNA compared to parent cells, we expected that paclitaxel-resistance might cause demethylation of CpG sites at MDR1 promoter. Although MSP published by Enokida et al. detected PCR products from bisulfite-modified DNA in both parent cells and paclitaxel-resistant cells, USP detected stronger PCR band in DU145-TxR cells than in DU145 cells, suggesting that MDR1 promoter in DU145-TxR cells is less methylated than in DU145 cells. However, USP did not detect PCR band in PC-3-TxR cells compared to PC-3 (Fig. 5B). To further confirm the methylated CpG site at the MDR1 promoter, we performed DNA sequence analysis using bisulfite-modified DNA. The MDR1 promoter region of DU145 cells was methylated at the CpG sites of −134, −105, −59, −56, −51, −34, and −29 of the transcription initiation site. The MDR1 promoter region of DU145-TxR cells was methylated only at the CpG site of -105

TABLE II. IC_{50} Value of DU145 and DU145-TxR Cells

Drug	DU145	DU145-TxR	Fold difference
PTX (nM)	11.3	384.2	34.0
EMP (μM)	15.1	49.6	3.28
DTX (nM)	8.30	55.6	6.70
VBL (nM)	14.1	40.8	2.89
DOX (nM)	17.5	61.1	3.49
VP-16 (μM)	0.83	1.10	1.33
CDDP (μM)	1.32	1.97	1.49

TABLE III. IC_{50} Value of PC-3 and PC-3-TxR Cells

Drug	PC-3	PC-3-TxR	Fold difference
PTX (nM)	5.00	217.1	43.4
EMP (μM)	8.57	33.0	3.85
DTX (nM)	3.67	28.2	7.68
VBL (nM)	8.00	27.4	2.43
DOX (nM)	121.3	1,218.2	10.0
VP-16 (μM)	4.40	5.95	1.35
CDDP (μM)	1.47	1.66	1.13

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