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Inhibition Growth of Multidrug Resistant KB_{V200} Cells by MDR1 Antisense RNA

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Acquisition of resistance to multiple drugs of tumor cell caused by overexpression of the MDR1 gene is one of major obstacles in cancer chemotherapy. We have attempted to reverse the multidrug resistance (MDR) phenotype by treating vincristine (VCR) and adriamycin (ADM) resistant KB_{V200} cells with MDR1 antisense RNA. Retroviral vector expressing the antisense RNA was transfected into KB_{V200}. In the transfected cells, a stable expression of antisense RNA and a reduction of cellular MDR1 mRNA could be detected by RT-PCR, and a reduction of MDR1 specific P-glycoprotein (P-gp) was also detected by Western blot, whereas an increase of the drug concentration in the cells was detected by FACS. The IC_{50} of transfected cells to VCR and ADM was reduced by 65 and 47%. This study demonstrates that antisense RNA can increase the sensitivity of tumor cells to anticancer drug by decreasing the expression of the MDR1 gene. This strategy may be applicable to cure cancer patients with P-gp mediated MDR phenotype.

Mammalian cells can acquire resistance to a wide variety of non-related drugs when they are exposed to chemotherapeutic agents. This phenomenon is called multidrug resistance (MDR) [1,2]. One of the underlying mechanisms of MDR is overproduction of Permeability-glycoprotein (P-gp), a 170-kDa transmember protein acting as a drug efflux pump, which is encoded by a small group of closely related genes termed MDR genes. Only MDR1 is known to confer drug resistance. Overexpression of MDR1 mRNA is the main cause for P-gp related resistance in tumor cells [3-5]. Chemosensitizers may reverse MDR in some patients, but significant side effects are common [6]. In order to develop a highly selective and efficient way to reverse MDR, we adopted antisense RNA technology.

There are several advantages with the technology: (a) antisense RNA only combined with its own target mRNA and inhibited its expression specifically. (b) the templates of antisense RNA can be efficiently delivered into target cells by viral vector, and their active forms can be produced within the cells in a controllable manner by different promoters. (c) antisense RNA could be produced in large scale easily and cheaply by culturing viral vector. These advantages make it develop rapidly in cancer research and anti-cancer gene therapy [7-10].

MATERIALS AND METHODS

Cells and culture conditions. KB_{V200} was obtained from Peking Union Medical College and Chinese Academy of Medical Sciences. These cells were isolated from a human cancer KB cell line by stepwise selection on exposure to increasing doses of vincristine (VCR). The VCR-resistance of KB_{V200} cells was found to be 175 fold higher than that of the original KB cells. Similarly, the adriamycin (ADM)-resistance was 14.5 fold higher than that of the original KB cells. The MDR1 gene was highly expressed in KB_{V200}. KB_{V200} Cells were maintained in an RPMI 1640 medium containing 10% fetal bovine serum and 0.2ug/ml VCR to retain their multidrug resistance in daily culture [11].

Primers. The following DNA primers for PCR and RT-PCR were prepared in this study:

Primers for β-actin (PriACTIN):
upstream: 5′CGAGAAGATGACCCAGATCA3′,
downstream: 5′GATCTTCATGAGGTAGTCAG3′.

Primers for MDR1 (PriMDR1):
upstream 5′CTGGTGTTTGGAGAAATGACAG3′;
downstream 5′CCCAGTGAAAAATGTTGCCATTGAC3′ [12];

Primers for antisense RNA (PriAS)
upstream 5′GGAATTCTGAAACCTGTAAGCAGCAACC3′,
downstream 5′CGGGATCCTCGAGTAGCGGCTCTTCCAAG3′.

Primers for pLXSN (priLXSN)
upstream 5′ACAATCGGCTGCTCTGAT3′,
downsteam 5′CTCGCTCGATGCGATGTT3′.

Construction of antisense RNA expressing plasmid. The retroviral vector plasmid pLXSN [13] was generously provided by Prof. Chun-Hai Li (Institute of Basic Medicine, Beijing). An MDR1 cDNA fragment spanning from nucleotide −134 to nucleotide +402 was ob-

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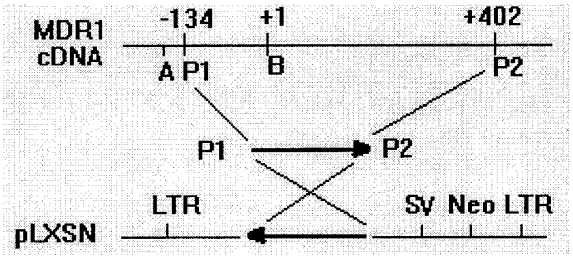

FIG. 1. Construction of pLXSNas. A: major transcription initiation site. B: translation initiation site. P1, P2: the primer complementary sequence. LTR: long terminal repeat. SV: SV40 promoter. Neo: neo gene.

tained by PCR from Plasmid pHaMDR1/A [14]. The cDNA fragment was subcloned to pLXSN in antisense orientation (Fig.1).

DNA transduction. The antisense vector (pLXSNas) was transfected into the KB_{V200} cells by lipofectin-mediated (GIBCO-BRL)transfection [15]. 48hr after transfection, G418 was added into the medium at a concentration of 400μg/ml. 25 days after transfection, individual colonies were picked up and grown in culture for further analysis (termed as KB_{AS} cells). The control vector pLXSN was transfected in the same way. The cells containing control vector pLXSN was designated as KB_{CON}. The KB_{V200} cells, which were maintained in the absence of VCR, was designated as KB_R. The cells KB_R and KB_{CON} were used as controls. If there was a MDR decrease in KB_{AS}, the former would represent to the MDR decrease in KB_R; the latter would stand for that the decrease did not arise from the expression of control vector.

PCR analysis integration of the pLXSNas and pLXSN. Total genomic DNA was extracted from the parental and transfected cells and subjected to PCR analysis by using two pairs of primers, priLXSN and priAS. PCR was performed as the following: single pretreatmeat (95°C, 5 min) and 35 cycles of denaturation (94°C, 30sec), annealing (55°C, 30sec) and extension (72°C, 1min). In the final cycle, a single extension (72°C, 7min) was included.

RT-PCR analysis for expression of antisense RNA and MDR1 mRNA. Total mRNA was extracted from the parental and transduced cells [16], and then subjected to PCR analysis, by using four pairs of primers, priLXSN, priMDR1, priACTIN, and priAS. PCR procedures were as same as above mentioned.

Western blot analysis expression of P-glycoprotein in total amount. Total proteins of cells were prepared as described in Molecular Cloning [17] and then size-fractionated by using SDS-PAGE. The proteins on the gel were transferred to the nitrocellulose filter with electrobloting apparatus (Bio-Rad). The P-gp was identified by incubating with monoclone antibody JSB-1Novocastra Laboratories Ltd. UK.8hr at 4°C, followed by incubation with biotin-labeled goat anti-mouse second antibody 30min at 37°C, and biotin-avidin-labeled horseradish peroxidase 30min at 37°C, and then stained by DAB substrate solution at room temperature.

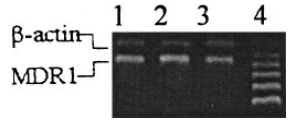

FIG. 3. RT-PCR analysis for the expression of MDR1. Lanes (1-4): cell KB_{CON}, KB_R, KB_{AS}, and DNA markers.

FACS analysis the concentration of ADM in KB_{CON} **and KB_{AS}.** The KB_{CON} and KB_{AS} were cultured in routine medium containing 100ng/ml ADM, and detected the fluorescent intensity in the cells at 50min, 100min, 150min and 200min separately by fluorescence-activated cell sorter (FACSCabibur Becton-Dickinson USA) [18].

MTT analysis drug resistance in KB_R, KB_{CON}, and KB_{AS}. The cells placed in 96 well microplate (1×10^4 cells / 200μl per well) were routinely cultured in a humidified incubator for 24hr. Drugs were added in step-wise concentration, reincubated 72hr. MTT(10 ul, 5 mg/ml, Sigma) was added to every well and reincubated for additional 4hr. DMSO was added to dissolve formed formazan crystals. The plate was then read on a microplate reader at 570nm. Medium was acted as a blank, whereas medium with 1×10^4 cells was used as control of 100% survival .Each test was set up in triplicate [19].

RESULTS AND DISCUSSION

Expression of antisense RNA. There were three levels in detecting MDR1/P-gp: MDR1 mRNA, P-gp and the function of P-gp. The most specific, sensitive and reliable method is RT-PCR to detect the expression of MDR1 mRNA. Therefore in our study PCR method was used instead of the Northern and Southern blot. Genomic DNA and total mRNA were analyzed by PCR and RT-PCR to demonstrate the expression of antisense RNA at DNA and RNA level (Fig.2, A, B). The result showed antisense RNA and retroviral vector pLXSN were all integrated and expressed.

Whether or not the PCR and RT-PCR analysis, the endogenous MDR1 and the exogenous antisense RNA would not interfere with each other. When performed PCR to analyze genomic DNA, because the endogenous MDR1 gene fragment between the two PCR primers is more than 10Kb, containing five introns, which is too long to be amplified by PCR; whereas the PCR amplificating fragment of antisense gene is a cDNA sequence of endogenous MDR1 gene, which is 536bp, could be

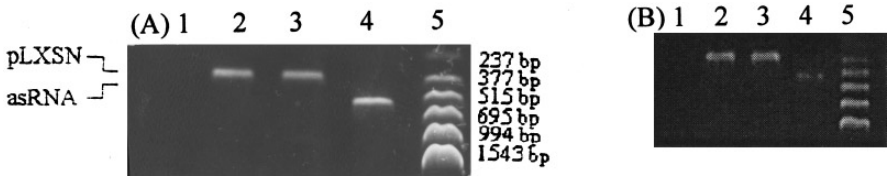

FIG. 2. PCR and RT-PCR analysis for the integration and expression of pLXSN and antisense RNA. (A) PCR result. (B) RT-PCR result. Lanes (1-5): KB_R PriAS, KB_{CON} + PriLXSN, KB_{AS} + PriLXSN, KB_{AS} + PriAS, DNA markers.

FIG. 4. Western blot analysis of P-gp. Lanes (1-3): cell KB_{CON}, KB_R, and KB_{AS}.

amplified by PCR (Fig.2, A). When performed RT-PCR to detect endogenous MDR1mRNA, the downstream primer would be added during reverse transcription; whereas performed RT-PCR to analyze antisense mRNA, the upstream would be added during reverse transcribingFig.2, B. So when detecting MDR1mRNA, the antisense mRNA would not be amplified by RT-PCR, conversely, it was the same.

This result indicated the PCR method, which is more convenient, economic than northern and southern blot. could be used in many cases, especially in detecting the integration and expression of exogenous gene.

Antisense RNA inhibits the expression of MDR1 mRNA. Total mRNA were analyzed by RT-PCR to demonstrate decrease of MDR1 mRNA. at the same time, using -actin as external control. The result showed that there was a reduction of MDR1 mRNA in KB_{AS}, but no significant difference in the expression of -actin. This suggested that the expression of MDR1 was specially inhibited by antisense RNA, without affecting the expression of other gene, such as β-actin gene (Fig. 3).

Antisense RNA inhibits the expression of P-gp. The result of western blot (Fig.4) indicated the P-gp was reduced in total amount.

Antisense RNA enhance the tumor cells' sensitivity to drug. The ADM emitted the red fluorescence, whose intensity represent the ADM intracellular concentration. Because the assay undertook in the normal culture condition, without affecting elements, and the FACS can detected the intercellular fluorescent intensity precisely, we can observe the change of drug concentration in the cells in an accurate and objective way. The result showed the ADM concentration in the KB_{as} was higher than in control cells (Fig. 5). It was the

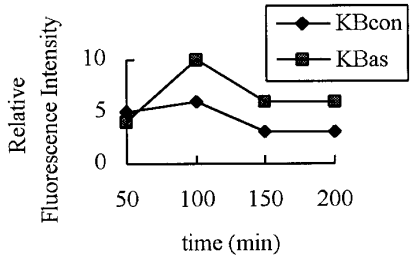

FIG. 5. FACS analysis for the drug concentration in KB_{CON} and KB_{AS}. The results were the averages of triplicate trials.

TABLE 1

Comparison of Drug Resistance of KB_R, KB_{CON}, and KB_{AS}[a]

Anticancer agent	IC_{50}(ng/ml)			Reduction[b]
	KB_R	KB_{CON}	KB_{AS}	
VCR	569 ± 57	535 ± 77	186 ± 20	65%
ADM	54 ± 6	51 ± 2	27 ± 4	47%

[a] All data were analyzed by Statistics Analysis System 6.04 software.

[b] Reduction: $(IC_{50KBR} - IC_{50KBAS})/IC_{50KBR}$.

direct proof to demonstrate that the reduction of the expression of MDR1 gene can increase the tumor cells sensibility to the anticancer drug.

Finally, the MTT. result showed The IC_{50} of transfected cells to VCR and ADM was reduced by 65 and 47%, respectively compared to those of the control (Table 1). It mean tumor cells treated by MDR1 antisense RNA could be inhibited in lower drug concentration.

In a summary, the antisense RNA inhibited the expression of MDR1 gene in two level, MDR1 mRNA and P-gp, which increased the anticancer drug concentration intercellular, and finally enhanced the tumor cells' sensitivity to drug treatment. This antisense strategy may be potentially useful in decreasing the MDR phenotype of tumor cells [20].

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