Chinese Journal of Cancer Research 10(3):187–190,1998.

MOLECULAR BIOLOGICAL EVIDENCES FOR THE GENETIC STABILITY OF DOXORUBICIN RESISTANT CELL LINE S-180R *IN VIVO*

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Objective: In order to assess the genetic stability of doxorubicin resistance sarcoma S-180R cell line *in vivo*. Methods: The drug resistant genes and molecules were examined by flow cytometry, Southern blot, Northern blot and RT-PCR. Results: The results showed that drug-efflux in S-180R increased nearly 100-folds, as compared with its parent cells, the rate of half peak width resistant cell/peak high decreased from 0.56 to 0.23 measured by flow cytometry after two years. The mdr1 gene amplified and overexpressed significantly in S-180R and the expression of topoisomerase II α gene decreased remarkably in S-180R. There was no significant different of the MRP expression between S-180R and S-180. Conclusion: These results indicated that drug resistance of S-180R was maintained and also increased. The major mechanism of drug resistance is the amplification and overexpression of mdr1 gene, the decreased expression of topoisomerase II α also contributed to it. So, S-180R is an ideal experimental model for the study of doxorubicin resistance and its reversion *in vivo*.

Key words: Multidrug resistance, Doxorubicin, S-180R, Topo II.

The drug resistant researches *in vivo* are the best way to understand the complexity of clinical drug resistance. S-180R is murine ascites tumor cell *in vivo* with high level of doxorubicin resistance. Previous studies had suggested that S-180R was resistant to doxorubicin for 66-folds, to VP-16 for 9-folds and overexpression of multidrug resistant gene products was demonstrated by *immunohistochemistry*.[1] The apoptosis response to doxorubicin was quite different between S-180R and S-180 cell lines.[2] The purpose of this study was to confirm the drug resistant ability and genetic stability of drug resistance with multidrug resistance (mdr1), Topoisomerase II α (Topo II α) and multidrug resistance associated protein (MRP), to provide the better and more useful model for drug resistant research.

Accepted April 23, 1998

MATERIALS AND METHODS

Agents

The Rh-123 was purchased from Sigma CO,. The plasmid of cDNA probe pHDR 5A was kindly provided from Harvard University and the plasmid of cDNA probe Topo II α from Dr. Tan KB. [α-32P] dCTP was purchased from Beijing Furui CO,. The nylon membrane of hybridization was purchased from AMERSHAM CO,. The total RNA kit was purchased from Gibcol CO,. The nick translation kit, the enzyme of AMV, Taq were purchased from Promega CO,. The internal control of β-actin and others agents was purchased from Huamei CO, Chian.

The Primer of mdr1[3]

5'-CCCAT CATTG CAATA GCAGG-3', 5'-GTTCA AACTT CTGCT CCTAG-3'; The primer of Topo II α:[4] 5'-CTTGT ACTGC AGACC CACA-3', 5'-ATAAT AGAAT CAAGG GAATT CCCAA ACTCGA-3'; The primer of MRP:[5] 5'-TCTCT CCCGA CATGA CCGAGG-3', 5'-CCAGG AATAT GCCCC GACTTC-3'; The primer of β-$_2$M:[5] 5'-ACCCC CACTG AAAAA GATGA-3', 5'ATCTT CAAAC CTCCA TGATG-3'; All of primers were produced from high technique laboratory of Beijing Institute for Cancer Research.

Flow Cytometry

Flow cytometer FACS 420 was used in Basic Research Institute of Traditional Chinese Medicine. The method of flow cytometer was the same as previous works.[1] PCR 95A was purchased from Genetics Institute of Chinese Academy of Sciences.

Southern Blot[6]

DNA was extracted from exponential growth stage cells. High molecular weight genomic DNA were extracted by standard techniques, briefly, cells in proteinase K buffer solution to digest cellular protein, followed by RNase digestion to remove RNA. After phenol chloroform extracted, DNA was recovered by ethanol precipitation, stored in TE buffer at 4ºC. Genomic DNA were digested with *Eco*R I, fractionated in a 0.8% agarose gel and then transferred to nylon filter membranes. The filters were baked at 80ºC and then pre-hybridized at 42ºC for 12 h in 50% formamide, 5×SSC, 0.2% SDS, 5×Denhardt solution, 50 mM sodium phosphate, 250 μg/ml of salmon-sperm DNA. Hybridizations were carried out at 42ºC for 24 h with denatured mdr1 or Topo II α probes in the presence of 10% dextran sulphate. The final wash of filters was carried out at 65ºC in 0.1×SSC and 0.1% SDS, and autoradiography at -70ºC on Koda films. Rehybridization of the same filter with β-actin probe was carried out to control variation in loading.

Northern Blot[6]

RNA was extracted by using the total RAN kit. The process according the manufacturer protocols and stored in 0.5% SDS solution at -70ºC. Each of mRNA preparation was fractionated on denaturing 1% agarose gels and transferred to nylon membranes. Hybridization conditions and final wash of filters were as described for Southern blots.

RT-PCR[3,5]

cDNA was synthesized with 1 μg of total cellular RNA and random primer 0.5 μg, RNasin 10 unites, dNTP, 10×buffer, AM 30 units. After 2 h at 42ºC, cDNA 2 μg, 3'primer and 5'primer, dNTP, 10×buffer, 4 min at 94ºC, Ampli Taq Polymerase 5 units. Each cycle of PCR included 30 sec of denaturation at 94ºC, 1 min of primer annealing at 55ºC, and 2 min of extension/synthesis at 37ºC. PCR products were separated on 1.5% agarose gels and stained with ethidium bromide.

Control

The cell lines of MCF$^{mdr}_{7}$ and BGC-823/DOX, both doxorubicin resistant, were selected as control groups.[7]

RESULTS

Flow Cytometry

The drug-efflux of S-180R increased nearly 100-folds, as compared with its parent cells, the two peaks of S-180R and S-180 cells line were separated clearly. The rate of half peak width resistant cell/peak high decreased from 0.56 to 0.23, measured by flow cytometry, as showed in Figure 1.

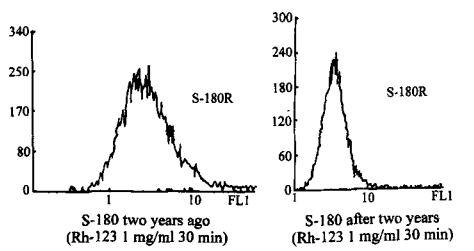

Fig. 1. The change of Rh-123 in S-180R and S-180 cells

Southern Blot

Although loading of S-180R DNA was less than that of S-180, which confirmed by internal control of

β-actin hybridization, the amplification of mdr1 gene was observed remarkably in S-180R, as compared with positive control, high level of doxorubicin resistant cells MCF^{mdr}_{7} (Figure 2). No amplification of the Topo II α gene was detected between the S-180R and S-180 from Southern blot, but BGC-823/DOX and BGC-823, control groups, were positive (Figure 3).

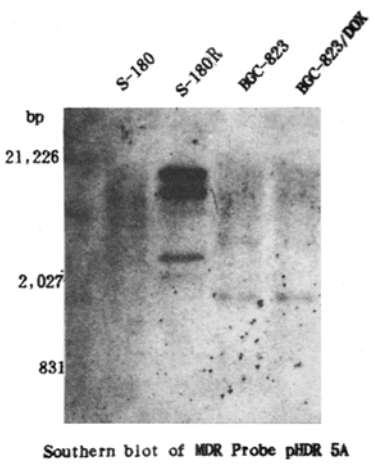

Fig. 2. Southern blot of mdr1 gene

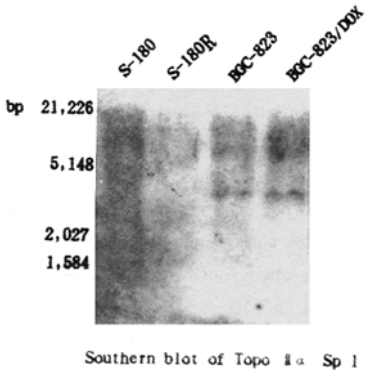

Fig. 3. Southern blot of Topo II α

Northern Blot and RT-PCR

The mdr1 gene expression in S-180R was significant higher than that of S-180 in Northern blot and RT-PCR (Figure 4). The mRNA expression of Topo II α in S-180R was negative, but S-180 was overxpression in mRNA level. This suggested that expression of Topo II α in S-180R was reduced (Figure 5). The levels of MRP were no different between the S-180R and S-180 in RT-PCR and the control groups of MCF^{adr}_{7} and BGC-823/DOX were also no different in MRP expression.

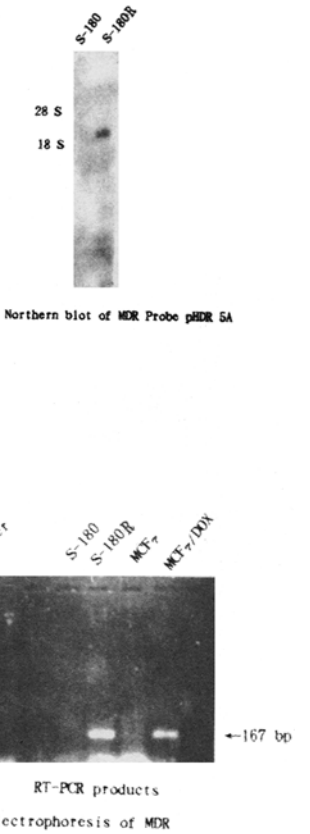

Fig. 4. Northern blot and RT-PCR of mdr1 gene

DISCUSSION

The doxorubicin resistant sarcoma S-180R cell line *in vivo* was developed in our laboratory two years ago. Previous studies had identified that p-glycoprotein, products of mdr1 gene were overexpression on protein level in S-180R, and drug resistance appeared to result from reduced accumulation of the drugs involved by flow cytometry, which suggested that mdr1 gene may be a key factor in it.[1]

The flow cytometry testes showed that drug efflux ability of S-180R cells increased, from 89.3-folds to the near 100-folds, as compared with the parent cells. Moreover, the rate of half peak width resistant cell/peak high decreased from 0.56 to 0.23, which suggested that S-180R cells were more allogeneous than before. This meant that most of resistant cells had the same ability of drug efflux, and separated peaks of S-180R and S-180 cell line are a beneficial marker to research suitable treatment to reverse the multidrug resistance. So, S-180A is a very desirable model in research of drug resistance *in vivo*.

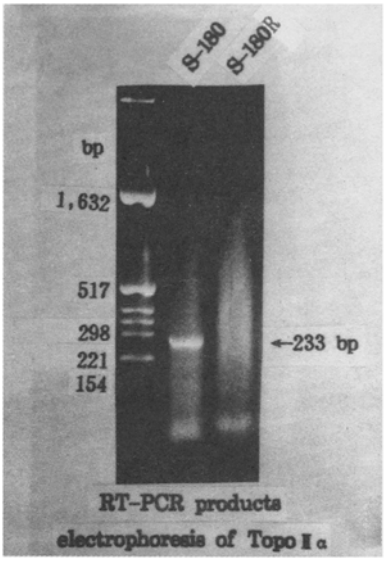

Fig. 5. Expression of Topo II α in RT-PCR

Drug resistance is a multi-factor involved process. Based on previous studies, we further examined genetic stability and drug resistant mechanism of S-180R with genes related drug resistant by modern molecular techniques. The mdr1 gene amplified and overexpresed significantly in cell DNA and mRNA of S-180R, as compared with that of S-180. There were no evidences about amplification of Topo II α gene in S-180R and S-180. The expression of Topo II α gene was remarkably reduced in S-180R cells and the expression of MRP was no different between two groups.

The above findings suggest that S-180R is high level doxorubicin resistant cell line and major mechanism of drug resistance was multidrug resistance, the Topo II α gene also played a part in it. Those evidences provided better molecular background of S-180R cell line *in vivo* in doxorubicin resistant research and S-180R cell line was a desirable model of drug resistance *in vivo*.

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