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Research paper

Elevated expression of S100P, CAPL and MAGE 3 in doxorubicin-resistant cell lines: comparison of mRNA differential display reverse transcription-polymerase chain reaction and subtractive suppressive hybridization for the analysis of differential gene expression

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Subtractive suppressive hybridization (SSH) and mRNA differential display reverse transcription-polymerase chain reaction (DDRT-PCR) were compared for their ability to detect the expression of drug-resistance associated genes in a doxorubicin-resistant and -sensitive colon carcinoma cell line (LoVo H67P). The expression pattern of more than 9000 bands obtained by DDRT-PCR were identical in both cell lines by more than 95%. Of the remaining differentially expressed DDRT-PCR products, 21 cDNA fragments were further analyzed after cloning. A total of 210 clones were sequenced resulting in 40 different sequences of which only five were differentially expressed as revealed by Northern blot analysis. SSH, on the other hand, resulted in 30 different sequences of 37 clones analyzed. Thirteen of 30 sequences (43%) could be identified by databank analysis (excluding expressed sequence tags) in contrast to nine of 40 clones (23%) obtained by DDRT-PCR. Of the clones identified by SSH, 60% exhibited a differential expression comparing the doxorubicin-resistant and -sensitive cell line, respectively, as compared to only 13% of the DDRT-PCR derived clones. The application of SSH resulted in the identification of differentially expressed genes in three doxorubin-resistant cell lines (LoVo DxR, ARH D60 and KB-V1) as compared to the sensitive parental cell lines. A significant higher expression of S100P, a protein involved in calcium metabolism, as well as MAGE 3 (melanoma antigen gene) was found in the resistant cell lines using this methodology. The expression of CAPL, a second protein involved in calcium metabolism, was only moderately elevated in the doxorubicin-resistant cells. We found that subtractive suppressive hybridization proved to be a more rapid and reliable method for the detection of differentially expressed mRNAs in our system. [© 1998 Lippincott-Raven Publishers.]

Key words: CAPL, doxorubicin, MAGE 3, RT-PCR, S100P, SSH.

Introduction

Differential display reverse transcription-polymerase chain reaction (DDRT-PCR) has rapidly become a popular procedure for the detection of differentially expressed genes[1-6] and was successfully applied for the identification of different genes which are important for cellular regulation. The basic strategy of differential display is a RT of the cellular mRNA pool followed by an amplification with a set of anchored oligo(dT) primers and arbitrary decamers[7,8] or a non-anchored PCR using two arbitrary decamers.[9] Subtractive suppressive hybridization (SSH), on the other hand, combines the well-established method of subtractive hybridization with the sensitivity of the PCR reaction, thereby improving the detection of differentially expressed rare transcripts.[10]

Little is known about the complexity of the development of cross-resistance during chemotherapy, and the putative relation between drug resistance and cellular stress response. Therefore, the aim of our investigation was the identification of differentially expressed genes which may be involved in the network involved in cellular drug resistance and stress response. The rationale for the experiments was the assumption that cellular exposure towards cytotoxic drugs represents a general stress signal for the cells which also may

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involve the cellular stress response armentarium. A better understanding of this regulatory network, and the possible link between the cellular stress response repertoire and the development of cellular drug resistance may be helpful in elucidating common steps or pathways resulting in the evolution of drug-resistant tumor clones in cancer chemotherapy. Based on a better knowledge of these adaptive regulations it might be possible to define new therapeutic targets to avoid or to overcome the development of drug-resistant subclones during chemotherapy.

An essential prerequisite for this approach are reliable and feasable methods to compare the gene expression profile, e.g. the mRNA expression status of drug-resistant and -sensitive cell lines. In this study we applied and compared the utility of DDRT-PCR[11] as well as SSH[10] for the identification of differentially expressed genes in a doxorubicin-resistant colon cancer cell line.

Materials and methods

Cell culture

Doxorubicin-sensitive and -resistant colon carcinoma cell line LoVo H67P and LoVo Dx^R, respectively, as well as cisplatin-resistant LoVo cPt^R, vincristine-resistant LoVo Vin^R, and actinomycin-resistant LoVo dAc^R were used throughout this study. In addition ARH 77, doxorubicin-resistant ARH D60 (gift from Dr Bellamy, Tucson, AZ), KB 3-1, KB V1 (doxorubicin resistant) as well as dexamethasone-, doxorubicin- and cadmium chloride-treated LoVo cells were investigated.

Cells were cultured at 37°C in CLICKS/RPMI medium containing 10% heat-inactivated fetal calf serum (FCS). For differential display analysis or subtractive suppressive hybridization 5×10^6 cells were seeded in 80 cm^2 cell culture flasks and grown for 18 h.

RNA isolation, differential mRNA display and reamplification of differentially expressed RT-PCR products

Total RNA was isolated from doxorubicin-sensitive, doxorubicin-resistant as well as stress-treated sensitive LoVo H67P cells according to Chirgwin *et al.*[12] For stress treatment doxorubicin-sensitive cells were exposed either to 43°C for 1 h, to 0.5 μg/ml doxorubicin for 1 h, to 200 μM cadmium chloride for 1 h or to 1 μM dexamethasone for 3 h.[13-15] For cDNA synthesis, 0.2 μg of RNA was subjected to a RT reaction using 2.5 μM of one of the 12 $T_{11}MN$ (M: A, C or G; N: A, C, G or T) anchored primers,[11] 20 μM dNTPs and 200 U MMLV at 37°C for 60 min. The subsequent PCR reaction was performed using 2.5 μM of a combination of two arbitrary primers published by Bauer *et al.*[8] in a buffer containing 70 μM dNTPs, 1.5 mM $MgCl_2$ and 1 U Tfl DNA polymerase for 40 cycles (94°C, 30 s; 40°C, 30 s; 72°C, 30 s) in a 30 μl reaction volume.

Differentially expressed cDNA fragments were directly excised from the silver stained gels,[16] vacuum dried, resuspended in 50 μl PCR buffer [50 mM Tris, pH 9.0, 20 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$] and heated for 30 min at 94°C. The isolated cDNA fragments were reamplified according to the first PCR protocol with the respective primer combination at 150 μM and at an annealing temperature of 42°C instead of 40°C. Reamplification products were analyzed either on 2% agarose gels stained with ethidium bromide or on 6% polyacrylamide gels followed by silver staining. Subtractive suppressive hybridization was performed using the PCR-Select™ cDNA subtraction kit supplied by Clontech (Heidelberg, Germany) according to the manufacturer's instructions.

Slot blot and Northern blot analysis

Probes were generated by cDNA synthesis from the respective cell lines using 5 μg total RNA and an oligo(dT) primer.[8] cDNA probes were labeled with DIG-dUTP according to the manufacturer's instructions (DIG DNA labeling kit; Boehringer, Mannheim, Germany). After the slot blot procedure membranes (HybondN$^+$; Amersham, Amersham, UK) were hybridized in 5 × SSC, 5 × Denhardts, 1% SDS at 65°C for 12 h with one of the labeled cDNAs. Prehybridization was performed in the same solution without the probe. After hybridization membranes were washed in 2 × SSC, 0.1% SDS and subsequently in 0.1 × SSC, 0.1% SDS at 65°C. Detection was done by chemiluminescence with CDP-star as a substrate following the Boehringer protocol (BM chemiluninescence detection kit; Boehringer). Membranes were exposed for between 2 and 8 h to Kodak XAR films.

For Northern blot analysis total RNA was isolated by cesium chloride gradient centrifugation. Total RNA (25 μg) was separated on denaturating agarose gels,[17] transferred to nylon membranes and used for Northern blot analysis with the differentially expressed fragment cDNAs after a preceding confirmation by slot blot analysis.

Cloning and sequencing of cDNAs

After reamplification of excised differential RT-PCR products, cDNA fragments were ligated into the vector

PCR™II using the TA cloning kit according to the instructions of the manufacturer (Invitrogen, Heidelberg, Germany). For the ligation into the above-mentioned vector adenosine residues were added at the 3'ends of the PCR products by incubation in PCR buffer in the presence of Taq DNA polymerase at 70°C for 10 min. Ten randomly chosen bacterial colonies out of each transformation assay were cultured. Plasmid preparations were performed using the Magic Wizzard plasmid preparation kit (Promega, Heidelberg, Germany). After insert control plasmids were sequenced using the cycle sequencing method on an automated sequencer (Applied Biosystems). Cycle sequencing was done according to the manufacturer's instructions. The obtained sequences were analyzed by sequence alignement with the NCBI GenBank.

SSH

SSH was performed using the cDNA Subtraction kit™ (Clontech); mRNA was isolated from total RNA of the doxorubicin-sensitve cell line LoVo H67P as well as of the doxorubicin-resistant variant LoVo Dx^R using dT_{25}-coated magnetic beads (Dynal, Hamburg, Germany) according to the manufacturer's instructions. cDNA synthesis was done using 2 μg of the isolated mRNA. After digestion of the double-stranded cDNA with *RsaI* the adaptors supplied with the kit were ligated to the cDNA of the doxorubicin-resistant LoVo Dx^R cell line (tester). Subtractive hybridization with the cDNA of the sensitive cell line (driver, without adaptors) was performed for at least 24 h. The subsequent PCR was run with 2 U Qia Taq DNA polymerase (Qiagen, Hilden, Germany) for 30 cycles (94°C, 30 s; 58°C, 30 s; 72°C, 1.5 min) using primers 1 and 2 supplied with the kit. For a nested PCR the nested primers were used for 16 cycles with essentially the same PCR conditions, changing only the annealing temperature from 58 to 60°C. For further details refer to the user manual for the cDNA subtraction kit. PCR products were cloned into the PCR™II vector using the TA cloning kit (Invitrogen) following the manufacturer's instructions.

Results

DDRT-PCR

cDNA synthesis using anchored oligo(dT) (T_{11}) primers with two additional bases (MN; M: A, G or C and N: A, C, G or T) at the 3'-termini of the primers to provide specificity was done and resulted in 12 cDNA pools. In a subsequent PCR reaction with an additional 15 arbitrary primer combinations a set of approximately 9000 cDNA fragments was generated and compared visually after separation on non-denaturing polyacrylamide gels and subsequent silver staining.

Stringency of the PCR reactions was adjusted empirically to obtain an average of 50 amplifications per primer combination. PCR products separated on polyacrylamide gels varied in size from 50 to 1500 bp. Banding patterns of the doxorubicin-resistant, -sensitive and the stressed-sensitive cells were more than 95% identical as estimated from PAGE.

For the identification of differentially expressed genes in doxorubicin-resistant and -sensitive cells, cDNA fragments generated during the first PCR reaction were isolated from the polyacrylamide gel, reamplified with the original primer combination and analyzed either on agarose or polyacrylamide gels. A total of 21 cDNA fragments (Table 1) were cloned and sequenced (10 clones were analyzed per cloned PCR fragment) resulting in 40 different sequences. Clones 9 and 19 both showed homology to *Mus musculus* (clone DE-5), only differing in the length of the PCR product obtained. The higher number of sequences (40) in relation to only 21 cloned PCR fragments was due to the fact that presumably isolated PCR products of identical or near identical size (after analysis on polyacryamide gels) resulted in 12 of 21 cases in more than one sequence after cloning. However, these clones contained the expected primer sequences coresponding to the primer combination used for reamplification (data not shown). Only five of these 40 sequences corresponding to 13% of total clones analyzed showed a differential expression as confirmed by Northern blotting. Out of these five clones three sequences were identical to human sequences, two of them with around 90% homology. A product with 96% homology to the only recently discovered oncogene PTI-1[2] and the second product with 87% homology to the human ribosomal protein L4[18] were identified.

SSH

The CapFinder™ PCR cDNA synthesis kit (Clontech) was used to preamplify mRNA from the doxorubicin-resistant and the respective -sensitive human colon carcinoma cell line. cDNA subtraction was performed using the cDNA of the resistant cell line as tester and cDNA of the sensitive cell line as driver. After cloning of the subtracted cDNA 37 randomly selected clones were analyzed. The sequences of 20 clones with an insert length between 120 and 400 nucleotides could not be found in the NCBI GenBank database, four clones were negative in the insert control. Of the

remaining 13 clones, 10 revealed homologies to known human sequences (Table 2).

In addition to the doxorubicin-resistant and -sensitive LoVo H67P cell line we tested three LoVo variants resistant to actinomycin D, cisplatin and vincristine, and two additional couples of cell lines resistant and sensitive to doxorubicin (ARH 77 and ARH D60 as well as KB 3-1 and KB-V1) to further analyze our findings of the SSH screening in the doxorubicin-resistant and -sensitive LoVo H67P cell line. Using semi-quantitative RT-PCR to verify differential expression of five putative genes involved in drug resistance we investigated the expression of RNA polymerase subunit hBRP 33 and the insulin growth factor binding protein, which are not differentially expressed (data not shown), as well as S100P, CAPL and MAGE 3.

Expression of CAPL could be detected in all cell lines analyzed; however, expression in the doxorubi-

Table 1. Homology of cDNA fragments obtained by DDRT-PCR to known sequences and Northern blot analysis for differential expression

Clone	Insert length (nt)	No. of different sequences out of 10	Homology to databank	Differential expression (Northern blot)
1	200	1	no	–
2	200	1	no	–
3	200	2	no	–
4	200	1	no	–
5	800	2	no	–
6	800	2	no	–
7	100	3	no	–
8	180	2	no	n
9	550	4	*M. musculus* (clone DE-5)[a]	+
10	950	2	HSU 22491 human G protein coupled receptor (GPR 7)	+
11	220	2	HSC 2 MH 121 partial human cDNA sequence, clone c2-mhl 2	–
12	150	4	PTI-1, EF-1α	+
13	330	2	no	–
14	220	1	no	n
15	200	3	no	n
16	180	1	no	
17	250	1	ribosomal protein L4	+
18	400	1	HUMEB 101 human G protein coupled receptor (EBI 1) gene	–
19	480	3	*M. musculus* (clone DE 5)[a]	+
20	210	1	human cDNA clone 242660	–
21	220	1	mouse mRNA for NDPP-1 protein, complete cDNA	+

[a]Homology of two different PCR products to the same homolog.

Table 2. Homology of cDNA fragments obtained by SSH to known human sequences

Clone	Homology to	Homology (%)	Insert length (nt)
1	RNA polymerase subunit hRPB 33	95	300
2, 3	aldose reductase	95	294
4	S 100 P mRNA (Ca-binding protein)	99	124
5	insulin-like-growth factor	96	279
6–8	MAGE 3 (melanoma antigene gene)	83	231
9	CAPL mRNA	98	177
10	F1-ATPase β subunit	98	171
11	thyroid hormone receptor interacting protein (trip-1)	97	200
12	iduronate sulfatase	83	135
13	ribosomal protein L5	92	275

cin-resistant cell lines was approximately twice as high as in the respective sensitive parental lines (Figure 1a, lanes 1, 2, 6 and 7, respectively).

S100P shows a weak expression in the doxorubicin-sensitive cell lines LoVo H67P and KB 3-1, and in the doxorubicin-resistant cell line ARH D60 but a higher expression in the doxorubicin-resistant cell lines LoVo Dx^R and KB V1. No expression could be detected in the resistant cell lines LoVo cPT^R, LoVo dAc^R, LoVo Vin^R and in the doxorubicin-sensitive cell line ARH 77 as well as in the stress treated LoVo cell line (Figure 1).

Interestingly, MAGE 3 was found at a high expression level only in the doxorubicin-resistant cell lines LoVo Dx^R, ARH D60 and KB-V1. Weak expression of MAGE 3 was found in the sensitive cell line ARH 77 (Figure 1c).

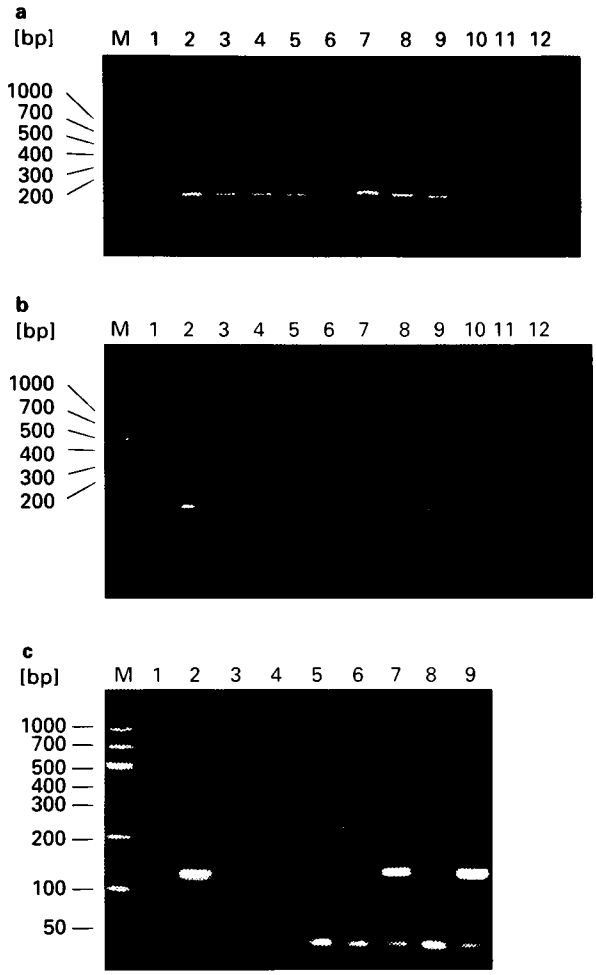

Figure 1. Semi-quantitative RT-PCR analysis of the expression of (a) CAPL (mts1), (b) S100P and, (c) MAGE 3 antigen gene in doxorubicin-sensitive and -resistant tumor cell lines from different tissues. PCR conditions were as follows (94°C, 5 min; 94°C, 30 s; 56°C 30 s, 72°C, 30 s, 26 cycles, 72°C, 5 min) using the respective primers as outlined below. S100P I: 5′-CAGGCTCAGCCTACGGGAAT-3′; S100P II: 5′-AGGCAGGACTCAAATGATGC-3′; CAPL I: 5′-TCTTGGAAGTCCACCTCGTT-3′; CAPL II: 5′-GGCAAAGAGGGTGACAAGTT-3′; MAGE 3 I: 5′-GGTACTCCAGGTAGTGTTCC-3′; MAGE 3 II: 5′-CCACGAGGAGAAAATCTGGGA-3′. Lane 1, LoVo; 2, LoVo Dx^R; 3, LoVo cPt^R; 4, LoVo Vin^R; 5, LoVo dAc^R; 6, ARH 77; 7, ARH D60; 8, KB 3-1; 9, KB V1; 10, LoVo dexamethasone treated; 11, LoVo doxorubicin treated; 12, cadmium chloride treated; M, PCR marker.

Discussion

In this study we compared two relatively new methods used in the analysis of differential gene expression: differential display RT-PCR and SSH. Of 210 clones which were analyzed after DDRT-PCR cloning only nine of 40 (23%) different sequences (homology for the *M. musculus* clone DE-5 was found twice) coding for a known RNA could be found in a database. Only five of these 40 sequences were differentially expressed, corresponding to 13% of the originally identified different clones. On the other hand, using SSH the amount of informative sequences was 43% (13 of 30), 60% of the clones analyzed exhibited a differential expression.

Still, DDRT-PCR compares favorably with SSH in that a smaller amount of material is required for the analysis and the possibility to compare a great number of different tissues or cell lines in the same assay. Thereby it is possible to eliminate putative differentially expressed cDNA fragments which are not related to the topic of interest and only appear due to minor differences of culture conditions.

Limitations of the DDRT-PCR, however, are (i) an adequate adjustement of the DDRT-PCR conditions to assure reproducibility and detection of putative differentially expressed PCR products and (ii) the appearance of multiple PCR products only slightly differing in length. The latter problem requires a high resolution electrophoresis after reamplification of the PCR products, and subsequent cloning and sequencing. Only this procedure presents a reliable way to obtain clonal probes for the Northern blot control of differential expression to avoid false negative results

Table 3. Comparison of DDRT-PCR and SSH

	DDRT-PCR	SSH
No. of clones	210	37
No. of sequences	40	30
Informative sequences	9/40 (23%)	13/30 (43%)
Differential expression	5/40 (13%)	3/5 (60%)

due to other contaminating sequences which are not differentially expressed.

Taken together the time required to run the necessary PCR reactions and the limitations of the DDRT-PCR at least to our experience favor SSH for a rapid and reliable analysis of the mRNA expression status of different cell lines, e.g. drug-resistant and -sensitive cell lines. However, a higher amount of RNA is required for SSH, which is a limitation of this method.

The genes identified in this study by SSH (Table 2) are mostly promising candidates for a role in the development of cellular drug resistance. Recently, Zhang *et al.*[19] could demonstrate a role for insulin in the cellular distribution of the P-glycoprotein, the most prominent component of the MDR phenotype. Increasing evidence is presented by different groups[20-23] for a role of calcium-binding proteins in drug resistance. Involvement of alternative RNA polymerase subunits as well as a regulation at the level of mRNA translation is considered as a mechanism of drug resistance.[24-26] Even proteins involved in alternative metabolic pathways (aldose reductase), protein degradation in the proteasome (F1-ATPase β subunit) or detoxification (iduronate sulfatase) have been discussed in relation to their role in the development of drug resistance.[27,28] Hormone receptors (thyroid hormone receptor interacting protein, trip 1) as well as tumor-associated antigenes are other new candidates which may contribute to drug resistance and/or proliferation, and an increased metastatic potential of tumor clones.[29-32]

To our knowledge, genes of the MAGE family were not correlated with drug resistance so far. Expression of these genes was detected in tumor cells derived from skin (melanoma), neck, lung, colon and lymphatic leukemia. Expression of MAGE genes is increased in normal tissue during wound healing, inflamatory processes and spermatogenesis.[33,34] Further analysis has to evaluate the possible role of MAGE genes in the development of a drug-resistant phenotype.

Taken together, the genes which were identified during this study using SSH are interesting candidates to analyze their potential involvement in the development of drug resistance. Comparing mRNA DDRT-PCR and SSH (Table 3), the presented system favors the latter method to discover differentially expressed genes.

The data obtained in the present study demonstrate that cellular stress response and evolution of drug resistance is correlated with the differential expression of a variety of different genes. Although these data do not directly support a common mechanism causing drug resistance, most of the identified genes might fit in a general stress response network. Therefore, it is important to identify the relevant 'bottle necks' in this adaptive cellular response to interfere with the development of a therapy-induced, drug-resistant phenotype.

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