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Molecular melting profile of MDR1 gene in doxorubicin and docetaxel treated MCF-7 and natural MCF-7 cell line

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ABSTRACT

In this study we compared nucleotide and amino acid composition of MDR1 gene in natural MCF-7 cell line (MCF-7/wt) and in MCF-7 cell line treated with combination of cytostatics doxorubicin (DOX) and docetaxel (DOC) (MCF-7/adr). MCF-7 cell line was exposed to cytostatics for six hours. The concentration of cytostatics was 5µg/ml for DOC and 0.15µg/ml for DOX. We wanted to determine some changes of cell viability as well as some changes at the molecular level. For this purpose we performed cell proliferation assay and HRM analysis of purified DNA. We obtained results using the evaluation programs BioEdit Sequence Alignment Editor. We observed the change of cell viability and also some changes in percentage of nucleotides and amino acid of MDR1 gene. We found out whether application of 5µg/ml for DOC and 0.15ug/ml for DOX on MCF-7 cell line for six hours retarded metabolic activity to 96.98% ($p<0.01$). We determined the change in molecular profile of MCF-7. In MCF-7/adr, the number of amino acids was 661 in comparison with MCF-7/wt, where the number of amino acids was 646.

***Key words*:** MCF-7 cell line, Doxorubicin, Docetaxel, MDR1, HRM analysis.

INTRODUCTION

High-resolution melting (HRM) analysis is simple, rapid, and inexpensive, requiring only PCR, a DNA dye, and melting instrumentation. HRM of DNA is a simple solution for genotyping, mutation scanning and sequence matching. The method is real-time PCR, and it can be completed in less than 5 min after completion of PCR.

In a field more relevant to clinical diagnosis, HRM analysis has been shown to be suitable in principle for detection of the breast cancer genes mutations. In the study James et al., 2006 [1] were used three cell lines harbouring different BRCA mutations to assess the HRM methodology. It was found that the melting profiles of the resulting PCR products could be used to distinguish the presence or absence of mutation in the amplicon. More than 400 mutations have been identified in these genes. HRM analysis offers a faster and more convenient method of assessing the presence of mutations and it gives us the result which can be further investigated.

MDR1 gene was first characterized by Endicott and Ling [2] in resistant cancer cells, as the ATP-dependent transporter responsible for the efflux of chemotherapeutic agents. The gene has 28 exons encoding for 170 kDa P-gp (MDR1) protein. Expression of P-gp was detected in various tumours and haematological malignancies, which were also correlated to clinical outcome of patients. P-gp is responsible for the development of resistance to a variety of

anticancer agents. There is a lot of studies dealing with the antitumor activity of the various chemical compounds [3,4]. Some of them could be also doxorubicin and docetaxel [5] *in vivo* and/or *in vitro* effects of which are intensively studied [6]

MATERIALS AND METHODS

Cell Growth Conditions: The MCF-7 cell line was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). It is a model cell line for human mammary carcinoma which exhibits features of differentiated mammary epithelium [7]. This cells were cultured in 10% DMEM medium (Invitrogen, Carlsbad, California, USA) containing foetal bovine serum – FBS (Invitrogen), Penicillin and Streptomycin (Pen Strep) (Invitrogen), 5% CO_2 and 95 % humidication, at 37°C. Cells were harvested at 80-90% confluence using trypsin (Invitrogen). Cells were prepared in pellets. This pellet was used by purified DNA. For analysis, the number of cells in the pellet was approximately 2.10^6. The solutions were used of cell culture grade quality and the equipment was used in cell culture commercially pre-sterilized and disposable.

Cell Harvesting (Passaging): The cells were grown in Petri dish (Sarstedt, Nuembrecht, Germany). When attached cell concentration on flask surface exceeded 80% confluence, they were trypsinized with the enzyme called trypsin – EDTA (Invitrogen). Medium was poured out and the cells, attached to dish surface, were washed with 10% DMEM medium. Trypsin was added to the cells (10 ml for 1 Petri dish). Petri dish was incubated at 37°C. After cells were removed from the dish surface we counted them in Burker´s chamber. Then cells were passed into the new Petri dish, separated for further experiment or frozen.

Drugs: Docetaxel and doxorubicin were kindly provided by Dr. Andrašina (Department of Oncology, L. Pasteur University Hospital, Košice). Docetaxel was stored at room temperature in polysorbate 80 as 40 mg/ml concentration. Doxorubicin was stored as 3.4 mM stock solution in distilled water at room temperature. These solutions were further diluted with the cell culture medium and stored at -20°C. 5µg/ml for DOC and 0.15µg/ml for DOX of initial concentration were directly applied into the culture medium. The medium with cytostatics was removed from the cell culture after 6h and then we added 10% filtered DMEM. The cells were grown in this medium without cytostatics for 24h. Consequently MTT test and isolation of DNA were made.

MTT test: Antiproliferative effect of docetaxel and doxorubicin on parental MCF-7 cells was measured using standard calorimetric test described in detail in the study of Sabo et al. [8]. Shortly, cell suspensions containing 4×10^4 viable cells per vial were cultivated in 96 well tissue culture plates (Sarstedt, Germany) with/without tested drugs in final volume of 100 µl as duplicates. Drugs were added approximately 24 hours after seeding. Cells were allowed to grow for 6 hours after the drug treatment. The optical density of each well was measured spectrophotometrically at 480 nm in an ELISA reader MRX Dynatech (Great Britain). The obtained values were also calculated and expressed as percentage of metabolic activity in comparison with controls considered to have 100% metabolic activity.

DNA samples: We have obtained DNA samples from the MCF-7 cell line. We have isolated DNA from MCF-7/wt (natural cell line) and DNA from MCF-7/adr (DOX and DOC treated cell line). DNA was isolated from cell lines using the kit Genomic DNA from Tissue (Macherey-Nagel, Biotech), according to the manufacturer´s instruction with small modifications.

Determination of MDR1 Amplification State: Two primers were chosen according to the study of Sung-Tsai et al, 2007 [9], where DNA fragment is between exon 14 and intron 14 of MDR1 gene. Forward primer 5´-TGGGGCTTTTAGTGTTGGAC-3´. Backward primer 5´-TGTGGAGAGCTGGATAAAGTCA-3´. HRM analysis was performed on the Line-Gene K (KRD molecular technologies) using FastStart Universal SYBR Green Master (ROX) (Roche Diagnostics) as recommended by the manufacturer. The samples were amplified with a precycling hold at 95°C for 10 min., followed by 80 cycles of denaturation at 95°C for 15s. Melting temperature was 61°C.

High Resolution Melting Analysis: DNA melting curves were obtained on the Line-Gene K (KRD molecular technologies) by measuring the fluorescence of SYBR Green during a linear temperature from 61°C to 95°C at 0.5°C/s. Fluorescence data were converted into melting peaks by the Software of Line-Gene K Fluorescence Quantitative PCR detection to determine DNA binding efficiency of cytostatics.

Sequencing DNA: Sequencing DNA has been done in Sequencing Center, Department of Molecular Biology, Faculty of Sciences in Bratislava, Slovak Republic, where we have sent amplicons of size 180bp.

Statistical Analysis: All results are presented as mean± SD. Comparison between groups were analysed via *t*-test (two-sided). Probability values of $p<0.01$ were considered statistically significant. In addition, MTT test was also analysed using Analysis Software Gen5^{TM}. The results percentage of nucleotide bases and amino acid of MDR1 gene were analysed by evaluation programs BioEdit Sequence Alignment Editor.

RESULT AND DISCUSSION

For our study we have chosen MDR gene because many MDR inhibitors have been identified but any of them was being clinically useful without side effects. The application of anticancer drugs with MDR modulators is accepted in case if the pharmacokinetic interaction between modulator and anticancer drug is synergistic. Initially identified reversal agents had other pharmacological activities than inhibition of MDR since they were not specifically developed for inhibiting MDR. Their affinity was low for MDR pumps and necessitated the use of high doses, resulting in high toxicity, which limited their applications by certain compounds. These agents can include antibiotics, steroid hormones and anti-steroids, calcium channel blockers and others [10,11]. We have evaluated the effect of docetaxel and doxorubicin, on metabolic activity of human breast cancer cell line MCF-7 and we have evaluated changes of the percentage of nucleotide bases and amino acids MDR1 gene. Cell line MCF-7 was exposed to cytostatics for six hours. Cells were taken from a single parental Petri dish and placed into control and exposed plates. Control cultures were maintained during exposure in incubator (temperature, 5% CO_2, humidity). A series of eight experiments was performed. For each experiment, pair of plates with cells MCF-7/wt and MCF-7/adr was placed into the incubator. Cells were allowed 24 hours to grow. After 24h we added medium with cytostatics. Cytostatic drugs were there for 6 hours. Then we changed medium with cytostatic drugs for 10% medium filtered and we have let cells to grow for 24h. The percentage of growth inhibition was determined by the MTT assay. Data were presented as mean±SD from eight independent experiments. Application of cytostatic drugs for 6h on MCF-7 cells retarded metabolic activity to 96.98% ($p<0.01$).

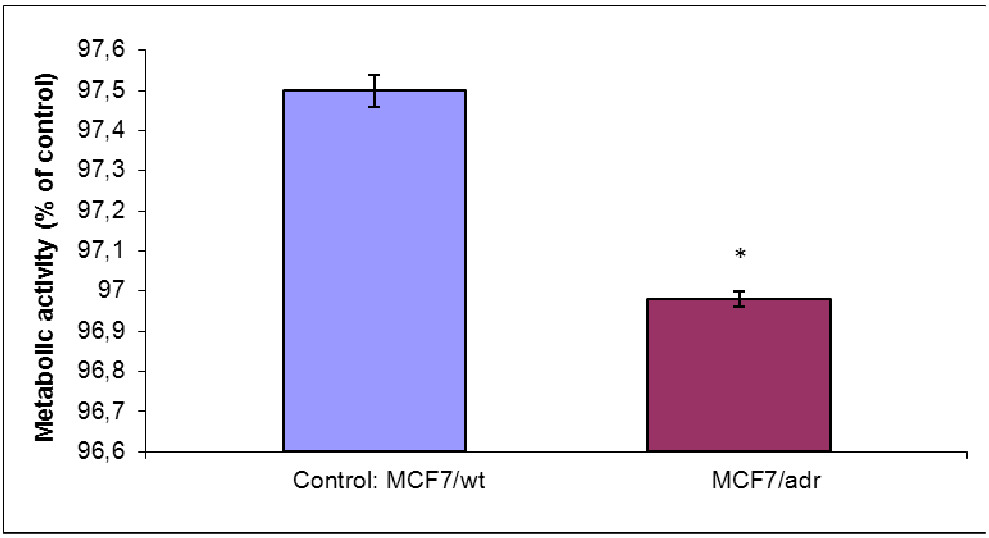

Fig.I. Effect of cytostatic drugs on metabolic activity MCF-7 cell line. Bars represent the mean ± SD of eight independent experiments. The error margins represent standard deviation (SD). Statistical significance is assessed using Analysis Software Gen5^{TM}; For Control: MCF-7/wt is CV 2.549%, for MCF-7/adr is CV 1.528%. Statistical significance was assessed also using *t*-test; * $p<0.01$.

The metabolic activity assay was used to investigating the molecular profile of DNA in MDR1 gene. We have isolated heterozygous DNA from MCF-7/wt and DNA from MCF-7/adr. We have isolated DNA from cell line using the kit Genomic DNA from Tissue (Macherey-Nagel, Biotech), according to the manufacturer´s instruction with small modifications. We have identified the DNA concentration using NanoPhotometer^{TM}, Version 2.0, the concentration of MCF7/wt and MCF7/adr was 33 µg/µl and 32 µg/µl respectively. After successful HRM analysis, samples have been evaluated in Sequencing Center, Department of Molecular Biology, Faculty of Sciences in Bratislava. Finally, we have determined changes in molecular profile of MCF-7 using evaluation program BioEdit Sequence Alignment Editor.

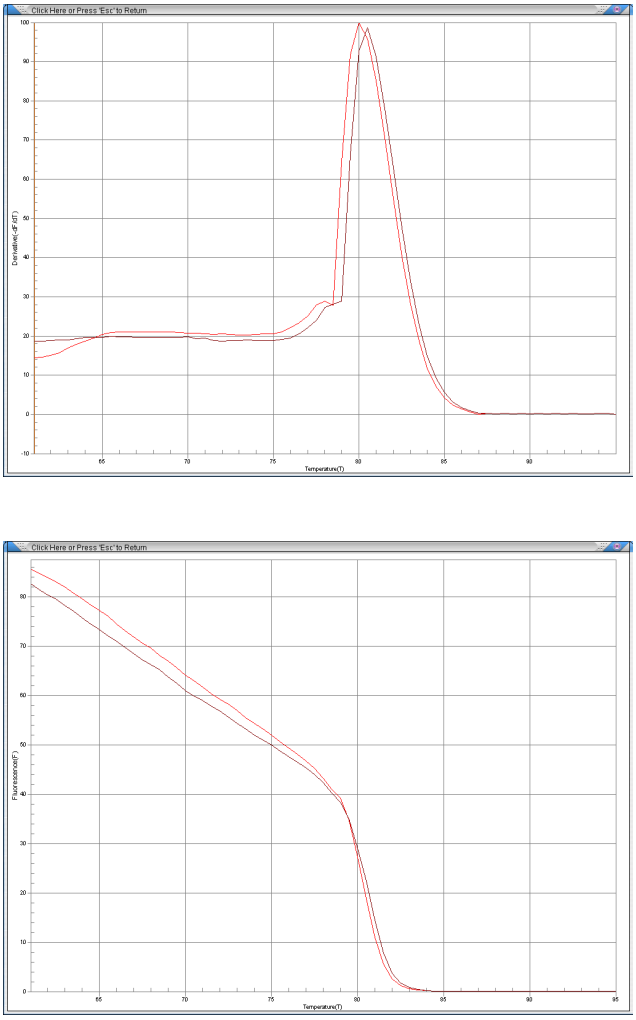

Fig. II, III. HRM analysis: Melting curves

Tab. I. Representation of the nucleotides, * representation of the polymorphisms

Nucleotides	mol % MCF-7/wt	mol% MCF7/adr
A	23,68	20,88
C	23,99	18,15
G	22,76	19,52
T	25,08	21,33
R*	1,86	4,08
Y*	0,62	4,08
W*	0,15	1,51
S*	0,31	1,97
K*	1,24	4,54
M*	0,31	3,93

The careful design of HRM assay could be successfully employed to detection of mutations in genes, which encode the tumor suppressor proteins in clinical samples of breast and ovarian cancer. James et al. in 2006 [1] and Krypuy et al., 2007 [12] show, that changes in the melting profile can be in the form of a shift in the melting temperature or an obvious difference in the shape of the melt curve. Both of these parameters are the functions of the amplicon sequence.

Tab. II. Representation of the amino acid

Amino acid	number, (mol%) MCF7/wt	number, (mol%) MCF7/adr
Ala	153, (23.68)	138, (20,88)
Cys	155, (23.99)	120, (18,15)
Asp	0	0
Glu	0	0
Phe	0	0
Gly	147, (22.76)	129, (19,52)
His	0	0
Ile	0	0
Lys	8, (1,24)	30, (4,54)
Leu	0	0
Met	2, (0,31)	26, (3,93)
Asn	0	0
Pro	0	0
Gln	0	0
Arg	12, (1,86)	27, (4,08)
Ser	2, (0,31)	13, (1,97)
Thr	162, (25,8)	141, (21,33)
Val	0	0
Trp	1, (0,15)	10, (1,51)
Tyr	4, (0,62)	27, (4,08)
Sum	**646**	**661**

CONCLUSION

In our study we confirmed the effects of cytostatics in cell survival. Application of cytostatics drugs for 6h on MCF-7 cells retarded metabolic activity to 96.98% ($p<0.01$). We have determined changes in molecular profile of MCF-7, the GC/AT ratio in MCF7/wt was 46.75%/48.76% in comparison with MCF7/adr, where GC/AT ratio was decreased, 37.67%/42.21%. In MCF-7/adr, the number of amino acids was 661 in comparison with MCF-7/wt, where the number of amino acids was only 646.

Acknowledgements
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