PSMB7 is associated with doxorubicin resistance and is a prognostic biomarker in breast cancer

Ph.D. theses

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1. INTRODUCTION

The major cause of cancer therapy failure is either primary **drug resistance** or the development of secondary resistance against currently available antineoplastic agents. Many different mechanisms of drug resistance have been identified, including

1. decreased intracellular drug concentration,
2. the loss of programmed cell death,
3. alterations in cell cycle checkpoints or in the repair of damaged cellular targets

In my thesis, I contrasted the **expression profiles** of four pairs of different human tumor cell lines and of their counterparts resistant to doxorubicin. Overexpression of *PSMB7* was observed in resistant cell lines and further validated for a possible a role in resistance.

The ***PSMB7* gene** encodes a catalytic subunit of the proteasome that degrades damaged and needless proteins in each eukaryotic cell. The proteasome is a multicatalytic proteinase complex with a highly ordered ring-shaped 20S core structure. This is composed of 4 rings of 28 non-identical subunits; 2 rings are composed of 7 alpha subunits and 2 rings are composed of 7 beta subunits.

Proteasome inhibitors are drugs blocking the action of cellular complexes that break down proteins. Despite the wide knowledge about proteasome, we still don't know if there is a

relationship between proteasome and doxorubicin-resistance and whether there is a member of proteasome subunits that could have prognostic characteristics.

During my PhD work to examine the role of *PSMB7* gene in chemoresistance, I silenced the gene by RNA interference, and then doxorubicin treatment was added. RNA interference is a type of posttranscriptional gene silencing, in which short RNA molecules suppress the function of mRNAs and block gene expression. Simultaneously to this silencing, doxorubicin-treatment was added to the resistant cells so examining the role of the gene in resistance.

2. AIMS

My aim was to identify gene expression signatures obtained from resistant cells in long-term culture, untreated sensitive parental cells, and parental cells after short-term drug administration. Based on relevance in clinical samples, I selected one proteasome subunit, the proteasome unit beta-type 7 (PSMB7) for further examination using RNA interference. In this, I validated its role in doxorubicin-resistance. Finally, microarrays data from 1592 patients were used to examine the clinical relevance of *PSMB7*.

3. METHODS

1. Cell lines

The doxorubicin/daunorubicin-resistant derivatives of human gastric carcinoma cell line EPG85-257P, pancreatic carcinoma cell line EPP85-181P, colon carcinoma cell line HT-29 and breast cancer cell line MCF-7 were established. The cell culture media of resistant cell lines were supplemented with daunorubicin/doxorubicin. The 24h treatment of drug-sensitive cells was performed at identical drug concentrations. MTT cell proliferation assay was used to monitor sensitivity of doxorubicin-resistant cell lines. Protocol was done as directed by the manufacturer.

2. RNA isolation

RNA was isolated from $1x10^7$ cells in logarithmic growth phase using the Qiagen RNeasy Mini Kit following the manufacturer's protocol. RNA samples were stored at –80°C.

3. Hybridization protocol and data analysis

For each cell line triplicate (sensitive, resistant and treated) hybridizations were made, thus altogether 12 hybridizations were made according to the protocols provided by Affymetrix. The microarray data can be downloaded from GEO (accession no. GSE3926). We have applied RMA for the normalization of hybridization intensities. We compared drug-sensitive parental,

doxorubicin/daunorubicin-treated parental and resistant cell lines. For identifying discriminating genes, the Prediction Analysis for Mircorarrays (PAM v.1.23) package was used. We decided to pick the top 100 genes from each comparison for the selection of the best discriminatory group of genes. Then, best genes were identified which discriminate between parental and treated parental cell lines (reflecting effect of the drug treatment) and resistant and parental cell lines (reflecting genes of resistance).

4. Oligos for RNA interference

Target of siRNA oligos for *PSMB7* were designed using siRNA Target Finder (http://www.ambion.com) and siDESIGN Center (http://www.dharmacon.com) softwares. DNA oligonucleotide templates for 3 target mRNAs were (targeting the gene at the 200th, the 548th and at the 562th nucleotides) then were synthesized by Silencer siRNA Construction Kit using the manufacturer's instruction. Synthesized siRNA duplex concentrations were measured by NanoDrop ND-1000 spectrophotometer.

5. SiRNA transfection of MCF-7 cell lines

Transfection was carried out with 5 µl SiPORT *NeoFX* Transfection Reagent in 6-well plates. 230.000 cells were plated into each well. The final concentration of siRNA was 10 nM in 2.5 ml serum and antibiotic-free medium. After twenty-four hours of incubation medium was washed twice with sterile

PBS then normal growth medium was added. Total RNA was extracted at the 48th hour after transfection as described previously. cDNA synthesis and DNA amplification were carried out by OneStep RT-PCR Kit with gene-specific PCR primers. GAPDH was used as internal control. PCR primers were designed by Primer3 software and their binding site was verified by NCBI BLAST. Out of 3 synthesized siRNA oligos one silenced effectively *PSMB7* effectively better than 80%.

6. Combination of RNAi and chemotherapy treatment

To investigate the role of selected gene in chemoresistance, I combined RNAi and drug treatment in *PSMB7* overexpressing doxorubicin-resistant MCF-7 cell lines (MCF-7-RAdr). 24 hours after transfection the reaction was stopped by replacing the medium with normal growth medium containing drug in 0,2µg/ml concentration. Cells were trypsinized on the 72th hour and counted by a CASY DT Cell Counter. We used negative siRNA-treated MCF-7-RAdr cells, siRNA-untreated MCF-7-RAdr cells and siRNA-untreated MCF-7 cells for controls to their doxorubicin-treated analogue. For statistical analysis, the number of living cells was counted in each well. Experiments were carried out three times, and each well was measured three times in each experiment. T-test was used for analysis of difference between groups. Significance-level was set at $p=0.05$.

7. Validation on clinical samples

For the *in silico* validation of the genes, a database containing processed GEO microarray samples was established. Datasets include GSE12276, GSE16391, GSE12093, GSE11121, GSE9195, GSE7390, GSE6532, GSE5327, GSE4922, GSE3494, GSE2990, GSE2034 and GSE1456. Kaplan-Meier plots were drawn to illustrate the effect on survival in these patients.

4. RESULTS

1. Identification of differentially regulated genes

The complete data set comprising raw data and Affymetrix .CEL files is available in the GEO database using the GEO accession number GSE3926. The prediction analysis of microarrays was performed to pick the top 100 genes differentially expressed in each sample set independent of tissue origin. To visualize discriminatory expression changes, we clustered the top genes. In these, probe set representing proteasome subunit *PSMB7* was expressed stronger in doxorubicin-resistant cell lines but not in doxorubicin-treated parental cells.

2. Prognostic potential of proteasome subunits

The normalized gene expression of the GEO-downloaded 1592 samples was filtered to include only the

proteasome subunits present on the HGU133A microarrays. The prognostic potential for all 62 probe sets was computed by dividing the patients into over- and under-expressed group as compared to the median (thus, 531 patients were in each group). The analysis resulted in 12 significant genes below an FDR of 0.05. Of these, only one was also detected by the microarray analysis (*PSMB7*) which was further investigated. Immunocytochemistry confirmed the overexpression of *PSMB7* as observed on the microarrays.

3. Survival of cells after gene silencing and treatment with doxorubicin

To examine the role of the *PSMB7* gene in doxorubicin resistance, we combined gene silencing with drug treatment to assess its effect on cell survival. Viability of cells without doxorubicin-treatment was better in both siRNA-untreated and siRNA-treated wells compared to cells with doxorubicin treatment. A specific cytotoxic effect of negative control siRNA was negligible as compared to the effect of gene-specific siRNA.

The normalizated results are presented in Figure 1. 79.8%±13.3% of resistance cells survived after doxorubicin treatment. Combined with gene silencing, only 31.8%±6.4% of the MCF-7-RAdr cells survived. The significance between siRNA-treated and siRNA-untreated MCF-7-RAdr cells after doxorubicin treatment was $p>0.001$. After doxorubicin treatment, 48.3±8.1% of sensitive cells survived. 73.3% of negative control siRNA-treated cells survived.

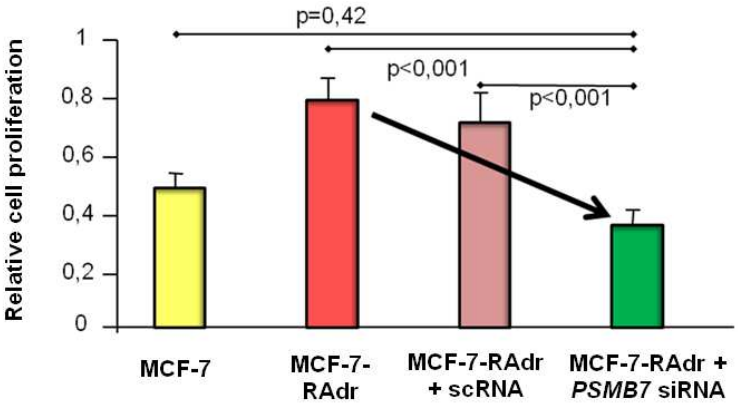

Figure 1. *The effect of PSMB7 silencing on doxorubicin resistance in MCF-7 cells. Difference was significant between survival of resistant and gene-silenced resistant cells (p<0,001), but not between survival of sensitive and resistant cells (p=0,42).*

4. Biomarker potential of *PSMB7* in clinical samples.

To assess the prognostic power of *PSMB7* gene in resistance, clinical samples were used to validate the results of the cell culture experiments. We grouped 1592 breast cancer patients on the basis of the expression of *PSMB7* using the Affymetrix HGU133A probe set 200786_at. Patients above the median had a significantly shorter survival than patients below the median (p<0.001) (Figure 2.).

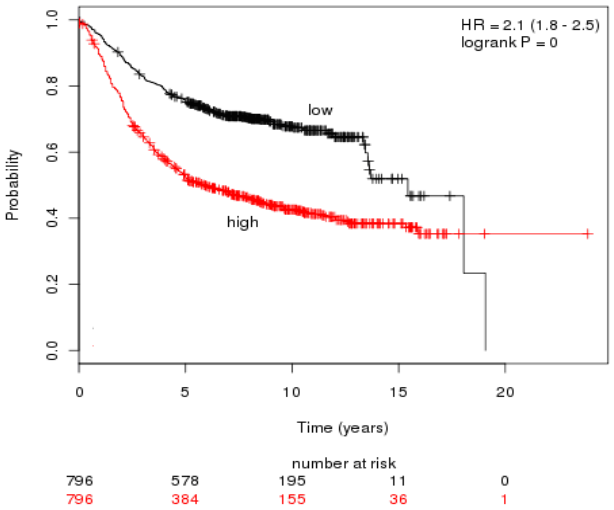

Figure 2. *Kaplan-Meier survival plot of 1592 breast cancer patients grouped by the expression of PSMB7 (200786_at probe set) above or below the median. Patients overexpressing PSMB7 gene have significantly shorter survival than those with downregulated gene (p<0,001).*

5. SUMMARY

In this study I examined the role of the proteasome subunit *PSMB7* gene on drug resistance in a breast cancer cell line and in breast cancer patients. In a cell culture model with a combination of RNA interference and drug treatment we validated the causative role of this gene in chemoresistance. The examination of 1592 breast cancer patients demonstrated

that overexpression of this gene is associated with poor prognostic outcome.

At the preliminary phase of this study we contrasted the expression profiles of four pairs of different human tumor cell lines of gastric, pancreatic, colon and breast origin. Using Affymetrix HG-U133A microarrays, we identified the top transcripts associated with resistance. We have found genes like proteasome subunits coding *PSMB7* playing role in the mechanism of resistance that previously have not been described.

Function of *PSMB7* was further investigated by RNA interference in MCF7 breast cancer cell lines. For this experiment, I designed and synthesized 3 siRNA oligos for *PSMB7*, but only one was able to effectively silence the gene.

After silencing of the *PSMB7* gene in a resistant cell line, cells were treated by doxorubicin. Survival of resistant cells was decreased significantly compared to non-silenced cells ($p<0,001$, Figure 1.). *According to this result resistant cells can be sensitized by silencing of the PSMB7 gene.* Thus, overexpression of *PSMB7* could play a role in resistance against doxorubicin.

After in vitro inspection we further validated the discriminating role of differential *PSMB7* expression in 1592 publicly available microarrays. By dividing the patients as having over- or underexpressed *PSMB7* as compared to the

median we achieved high significance ($p<0,001$, Figure 2.). *According to this result, overexpression of PSMB7 gene can be a prognostic marker in breast cancer.*

6. PUBLICATION

Publications which underlie and are related to this Ph.D. work:

***Munkácsy Gy**, Abdul-Ghani R, Mihály Zs, Tegze B, Tchernitsa O, Surowiak P, Schäfer R, Győrffy B: PSMB7* is associated with anthracycline resistance and is a prognostic biomarker in breast cancer. (Br J Canc. 2010 Jan 19;102(2):361-8. IF: 4,831)

*Fekete T, Rásó E, Pete I, Tegze B, Liko I, **Munkácsy Gy**, Sipos N, Rigó J., Győrffy B:* Meta-analysis of gene expression profiles associated with histological classification and survival in 829 ovarian cancer samples. (2011, in press, IF: 4.926*)*

*Győrffy A, Z Baranyai, A Cseh, **Gy Munkacsy**, F Jakab, Z Tulassay, B Gyorffy*: Promoter analysis suggest the implication of NFkB/C-REL transcription factors in biliary atresia. Hepato-Gastroenterology (IF: 0.68)

***Munkácsy Gy**, Tulassay Zs, Győrffy B:* RNS interferencia és klinikai alkalmazása. (Orv Hetil. 2007 Nov 25; 148 (47): 2235-40.)

Additional publication

*Győrffy B, Rosivall L, Prohászka Z, Falus A, Füst Gy, **Munkácsy Gy**, Tulassay T:* Danubian Biobank Konzorcium: a Duna-menti egyetemek „biobanking" tevékenységének az összehangolása. (Orv Hetil. 2007 Oct 21;148(42): 1999-2002.)

∑IF: 10,437