Abstract

The general objective of presented research was to investigate molecular mechanisms underlying the development of resistance to trastuzumab - the first targeted therapy approved as a first line treatment for breast cancers characterized by over-expression of HER2 receptor.

To achieve this goal we have designed a study based on two main approaches using SKBR3 and BT474 cell lines as two independent biological models. Firstly, we implemented a novel approach aiming to investigate changes throughout the duration of trastuzumab exposure assuming that resistance development most likely is a complicated multi-step process rather than a single event. Secondly, in order to get information about the expression changes of a broad spectrum of genes and microRNAs related with the process, the method of choice was a high-throughput microarray technique. Genes and microRNAs expression data has been subjected to comprehensive statistical analysis, following by careful study of mechanisms, processes, networks and pathways using bioinformatic tools, i.e. GO (Gene Ontology) Terms enrichment analysis, KEGG Pathway deep study using Kyoto Encyclopedia of Genes and Genomes and PPI (Protein-Protein Interaction) analysis.

The study has revealed 8 874 and 13 892 genes differentially expressed during the process of trastuzumab resistance development in BT474 and SKBR3 cell lines, respectively. 5 675 of them were common between both cell lines. The majority of genes ranked as 25 most significant ones in one biological model was found in top 200 in the other cell line, highlighting the strong overlap between models and supporting the idea that they may share at least one pattern of a drug resistance development. Importantly, four genes has been found in top 25 most significant transcripts in both cell lines, these are: BIRC5, E2F1,USP1 and TFRC. BIRC5 and E2F1 are reported to be involved in HER2 downstream signaling and to interact with many genes associated with trastuzumab resistance, whereas USP1 and TFRC are novel, putative Herceptin resistance contributors. In addition, several other molecular players have been found within the top 25 most significant genes in at least one of the biological models in this study. These are: IGF2BP1, GSTM3, RASD1, KLK11, GSTP1, YWHAH, DTL, DOLK, NACC2, DDIT and DNAJA3. Their potential drug resistance contribution is supported by previously reported functional characteristics. The quality and relevance of our study have been verified positively by finding a correlation between following genes: BIRC5, E2F1, BRCA1, RB1, ERBB2, EPHA2, IGFBP3, ADAM10, FOXM1, RAC1, MYC, CCND1, PTEN, TP53, MAP2K4 and PI3KCA and trastuzumab resistance development, which is in line with the current literature. However, a great number of transcripts (*loci)*

of long non coding RNAs and proteins with no or limited information available were found to be significant in the studied process opening variety of possibilities for further investigation regarding their molecular role in the Herceptin resistance development.

Using Protein-Protein Interaction analysis we demonstrated that the great majority of top 25 most significant gene products in both cell lines were involved in molecular interaction networks of proteins already reported to have role in Herceptin resistance development. Among them BIRC5, E2F1 and RB1 are placed in the center of these networks forming hubs where many protein-protein interactions takes place.

Gene Ontology enrichment analysis have shown, that in total, 193 molecular function (MF) GO terms were significantly enriched in SKBR3, and 103 overrepresented in case of BT474 cell line. Main molecular functions involved in trastuzumab resistance development were related to: receptor binding, protein kinase activities, GTPase related activities, ATP/ATPase associated mechanisms, transferase activities, RNA processing, DNA replication and organization as well as p53 binding. Regarding biological processes (BP), 600 and 354 GO terms have been indicated as significant for trastuzumab resistance in SKBR3 and BT474 biological models respectively. The most important concern: cell cycle regulatory mechanisms, mitochondrial functioning, apoptosis, microRNA activity, stress response, viral infection, microtubules organization and DNA damage repair.

In the KEGG pathway enrichment analysis we indicated 9 and 75 molecular networks significantly affected during trastuzumab resistance development in BT474 and SKBR3 cell lines respectively. Among them eight pathways were common for both independent experimental models, suggesting their importance in the process. These pathways are: cell cycle pathway, p53 signaling pathway, Fanconi anemia pathway, cellular senescence, DNA replication, EBV infection and two cancer-related pathways (bladder and colorectal cancer pathways).

This study revealed, that the mechanisms of acquired resistance may involve both HER2-dependent and HER2-independent ways of action. We have confirmed that truncated protein or receptor mediated endocytosis influence on therapy resistance, however hyperactivation of HER2 downstream signaling might also contribute. In contradiction with some scientific reports, this study did not confirm epitope masking mechanism of resistance, neither involvement of other ERBB receptors in overcoming Herceptin therapeutic effect.

Interestingly, this research revealed higher complexity of mechanisms involved in trastuzumab resistance development for SKBR3 in comparison to BT474. This was found

to be associated with the disequilibrium in number of significant genes, GO terms and pathways as well as PPI interactions. We suggest, that this characteristics may be related with the different stage of cancer (primary vs. metastatic tissue), from which both cell lines has been derived.

MicroRNAs data analysis surprisingly revealed, that over 99% of known human microRNAs have been found significant in Herceptin resistance development. Many of them have been reported in the literature as related with the process. Similarly, as in case of genes, four microRNAs has been found in top 25 most significant ones in both cell lines: hsa-miR-574-3p, hsa-miR-4530, hsa-miR-8485 and hsa-miR-197-3p. Additionally, several others cell line-specific highly important miRs have been identified. These are: hsa-miR-6886-3p, hsa-miR-4254, hsa-miR-4701-5p, hsa-miR-3151-3p, hsa-miR-6834-3p, hsa-miR-4281, hsa-miR-4649-3p, hsa-miR-3162-3p, hsa-miR-6826-5p and hsa-miR-6869-5p.

The results obtained in this study contribute a lot to basic research-based knowledge regarding trastuzumab resistance development. Revealing novel significant molecular contributors, processes, genetic pathways and proteins networks involved in the process provides a solid basis for indicating the direction of detailed and functional studies in the field of human cancer genetics and molecular medicine. As a consequence, important applied research studies may significantly impact pharmacy and medicine leading to the concept of adjuvant therapy that would significantly mute, slow down, inhibit or overcome the development of resistance to trastuzumab.