Expression of CD24 and CD44 in breast cancer cells 
after cytotoxic drug exposure

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ABSTRACT

Background

Each year approximately 1.38 million women are diagnosed with breast cancer and 458,000 die from the disease. Despite increasing knowledge about the risk factors and better therapies the mortality rate remains high and is the leading cause of death from cancer in women. Two cell surface markers, CD24 and CD44, can be used to identify cancer stem cells and have a prognostic value for disease free survival. The aim of present study was to investigate how the subpopulations of CD24+/CD44− and CD24+/CD44+ cells changed when exposed to three widely used cytostatic drugs.

Methods

The cell lines BT-474, MCF-7 and ZR-75-1 were analyzed for HER2 and ER protein expression by Western blot and for gene expression by using qPCR. Further, BT-474 and MCF-7 were exposed to three different cytostatic drugs tamoxifen, trastuzumab and paclitaxel for 72h and then flow cytometry was used to analyze their expression of CD24 and CD44.

Results

ZR-75-1 overexpresses the HER2 receptor but shows a low expression of ER. The cell lines BT-474 and MCF-7 both express the HER2 and ER but with intermediary values. Western blot showed that all the cell lines have proteins corresponding to the ER but ZR-75-1 lacked the HER2 protein, which seems to be an error from the western blot. MCF-7 showed an increased expression of CD24+/CD44+ when treated with paclitaxel and trastuzumab/paclitaxel.

Conclusions

Exposure to cytostatic drugs does not result in a statistically significant change in the expression of the CD24 and CD44 cell surface markers in the breast cancer cell lines.
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<tr>
<td>ADCC</td>
<td>Antibody-dependent cell-mediated cytotoxicity</td>
</tr>
<tr>
<td>ATCC</td>
<td>American type culture collection</td>
</tr>
<tr>
<td>CSC</td>
<td>Cancer stem cell</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen receptor</td>
</tr>
<tr>
<td>HER2</td>
<td>Human epidermal growth factor 2</td>
</tr>
<tr>
<td>MFI</td>
<td>Median fluorescence intensity</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>PR</td>
<td>Progesterone receptor</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative polymerase chain reaction</td>
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</table>
INTRODUCTION

Breast cancer is the most common form of cancer that affects women. Each year, 1.38 million new cases are diagnosed and 485,000 women die due to the disease. The highest incidence rate can be found in Western Europe and lowest in the developing countries [1]. The lifetime risk of getting the disease is 1/8 but only 5 percent of the cases occur before the age of 40 [2]. The origin of breast cancer is not fully known although hereditary factors have shown to affect its development and be the cause of 5-10% of the cases. Breast cancer normally arises from epithelial cells that can be found within the breast, and show a great heterogeneity both at molecular and clinical level, which complicates the choice of treatment [3]. Breast cancer is primarily categorized depending on the localization (lobules or ducts) and the molecular subtype.

Subtypes in breast cancer

To distinguish the different types of breast cancer from each other, five major molecular subtypes, Luminal A, Luminal B, Normal breast-like, Human epidermal growth factor 2 (HER2) and Basal-like have been defined. The subtypes have different clinical outcomes and respond with a great variety to cytostatic therapies. The luminal A and B subtypes show a striking resemblance to the luminal cells in the breast and are both ER positive. Approximately 70% of all cases of breast cancer are ER positive which makes it an important factor when choosing between therapies [4]. Luminal A grows slowly and has high survival rates and lower recurrence rates than luminal B which is more aggressive and also overexpresses the HER2 receptor. The normal breast-like subtype are ER positive and resembles normal breast tissue which have raised the question if this really should be defined as a molecular subtype. The HER2 subtype is ER negative but overexpresses the HER2 receptor. It is highly aggressive and metastases to the brain can often be found. The basal-like subtype is called “triple-negative” due to its absence of overexpressed ER-PR-HER2 receptors. It is aggressive and metastases to the brain and viscera. It can be found in women with BRCA1 mutations and have a similar expression to the myoepithelial cells in the breast with P-cadherin, P63 or laminin [5].

The breast epithelium is constantly exposed to estrogen and a high cumulative exposure increases the risk of breast cancer [4]. Some factors that influence the cumulative exposure are time of menarche, oral contraceptives, pregnancies and time of menopause [6]. Two hypotheses exist to describe how the increased estrogen stimulation can lead to breast cancer.
First, stimulation of the estrogen receptor increases the proliferation of mammary cells, which may lead to replication errors and mutations. Second, the metabolism of estrogen may give rise to genotoxic products that result in mutations by damaging the DNA [7]. The estrogen receptor can be found in two different isoatypes, ERα and ERβ, which are expressed at various levels in different tissues and are encoded by the genes ESR1 and ESR2 respectively. ESR1 can be found on chromosome 6 and ESR2 on chromosome 14 [8]. When the receptor is activated it forms homodimers or heterodimers between the different ERα and ERβ and act as a transcription factor upstream of genes by binding estrogen response elements [9]. ERα is frequently upregulated in breast cancer and used to determine ER status in clinical patients [10].

HER2 overexpression can be found in 20-30% of the breast cancer cases and is associated with a more aggressive disease, decreased survival and high recurrence rate [11]. The HER2 gene can be found on chromosome 17 and codes for a transmembrane protein that have tyrosine kinase activity [12]. HER2 has no known ligand but has the ability to undergo dimerization with other EGF receptors such as EGFR (HER1), HER3 and HER4 and triggers PI3K-Akt, MAPK, PLC-PKC, JAK-STAT –intracellular signaling pathways. The activation of these pathways leads to cell-differentiation, -migration, -proliferation and inhibition of apoptosis [13].

**Treatment**

Tamoxifen is used for the treatment of ER positive breast cancer and also has a preventive effect in high-risk women [14]. It is used in both pre-menopausal women and post-menopausal women with ER positive breast cancer, although post-menopausal women are more often treated with aromatase inhibitors [15]. Tamoxifen is metabolized to 4-hydroxytamoxifen and endoxifen in the liver by cytochrome P450 enzymes (e.g. CYP2D6 and CYP3A4) and the metabolites have a high affinity for the estrogen receptor [16]. 4-hydroxytamoxifen and endoxifen bind the estrogen receptor competitively and acts as an antagonist in the breast [17].

Herceptin (trastuzumab) is one of the most widely used therapies against HER2 positive breast cancer due to its ability to block signaling through the HER2-receptor. It is a monoclonal humanized antibody belonging to the immunoglobulin G1 type and binds the extracellular domain of HER2. According to some theories, the Fc portion of the antibody will activate natural killer cells which lead to antibody-dependent cell-mediated cytotoxicity.
(ADCC) and the death of the cell. Trastuzumab also inhibits signaling by the PI3K pathway which promotes apoptosis and arrest of proliferation [18].

Paclitaxel is a mitotic inhibitor that binds to and stabilizes B-tubulin which in turn inhibits the microtubules instability. This leads to stabilization of the microtubule polymer and protects it from disassembly [19]. The treated cells have defects in chromosome segregation and mitotic spindle assembly and can no longer progress through the mitosis. This will eventually lead to an activation of the mitochondrial pathway of apoptosis and cell death [20].

**Cancer stem cells and the cell surface markers CD24 and CD44**

Cancer stem cells (CSCs) are cells that can differentiate, undergo self-renewal and give rise to all cells in the tumor. During the last decade, more and more research has been done in search for surface markers in CSCs that can help predict initiation, invasion, metastasis, heterogeneity and therapeutic resistance. The expressions of the different markers also have a tendency to change in the tumor during its different stages which makes them hard to find and draw any conclusions from. The cell surface markers CD24 and CD44 are associated with CSCs and several studies have been made to extract these cells from breast cancer and normal breast tissue [21].

CD24 is a small protein molecule that is heavily and distinctly glycosylated in different cells. It has an important role/function in cell-matrix and cell-cell interactions. It is expressed in multiple forms of cancer such as ovarian, prostate, renal, breast and lung cancer. Earlier research have shown that it have an important role in both metastasis and cell adhesion and therefore is a poor prognostic factor for disease free survival [22]. CD24 seems to be able to be a ligand for P-selectin which can be found on platelets and endothelial cells and the interaction facilitate the entry of tumor cells into the bloodstream [23].

CD44 is a transmembrane glycoprotein that is expressed on almost every cell and is a receptor for hyaluronic acid. The binding between the receptor and hyaluronic acid promotes migration and also transmits survival signals which are pro-oncogenic. The glycoprotein has a great variety of functions and can present cytokines and chemokines for their complementary receptor and regulates angiogenesis, differentiation, motility, survival and growth [24]. In different forms of cancer there often occurs alternative splicing’s of CD44 which encodes for different isoforms. One of these is osteopontin which is a chemokine-like protein and promotes metastasis and invasiveness in several cancers [25, 26]. Women in late pregnancy show a decreasing amount of CD44+ stem cells in the breast, which correlate well with the
theories of terminal differentiation caused from pregnancy [27]. This is one of the possible reasons why women with pregnancies early in life might have an increased protection from breast cancer [28].

Research made earlier has shown that the cell surface markers CD24+/CD44+ CSC had a far greater capacity to form tumors than CD24+/CD44− in MCF-7 cell lines. The proportions of CD24+/CD44+ population of cells were also larger than in normal breast tissue [29]. The cell lines BT-474 and MCF-7 have in earlier research shown high amounts of CD24+/CD44+ and low amounts of CD24+/CD44+ cells when these are untreated. But no research about the effect of trastuzumab, tamoxifen and paclitaxel on these subpopulations could be found.

Aim
The aim of this study is to analyze how three widely used cytostatic drugs tamoxifen, trastuzumab and paclitaxel can affect the expression of CD24+/CD44− and CD24+/CD44+ in the MCF-7 and BT-474 cell lines.

MATERIAL AND METHODS

Breast cancer cell lines
All the cell lines were bought from American Type Culture Collection (ATCC) and they all originate from breast cancer epithelium. MCF-7 and ZR-75-1 belongs to luminal A, and BT-474 to luminal B subtype. The cell line MCF-7 was grown in an IMDM-phenol free medium (Life technologies) with the addition of 10% Fetal bovine serum (FBS) and 10µg/ml insulin. The BT-474 and ZR-75-1 cells were grown in RPMI -1640-phenolffree medium (Gibco® Life technologies, Paisley, UK) and supplemented with 2.5% HEPES, 10% FBS and BT-474 also with an additional 10µg/ml insulin. The cell lines were grown in 75cm² cell culture flasks and the medium with its supplements were changed every three days to optimize cell growth. The cells were kept in an incubator with a temperature of 37°C and 5% CO₂.

Ethical considerations
No ethical approval was needed in the study as only cell lines were used. The patients consent for using the cell lines in scientific research cannot be found in any records and in the seventies a written consent were not always taken. Despite this the cells have been used for over 40 years and the question still remains how similar the cells now are when compared to the ones taken originally.
**Extraction of DNA, RNA and protein**

The cells from the cell lines BT-474, ZR-75-1 and MCF-7 were counted with a Bürker chamber and duplicate samples with 1x10^6 cells were taken from each cell line for DNA, RNA and protein extraction with Allprep DNA/RNA/Protein mini kit (Qiagen, Hilden, Germany). The instructions from the manufacturer were followed and the protein dissolved in 5% SDS and stored in -20℃. The DNA was stored at -20℃ and RNA was stored in -80℃ for future use.

**Synthesis of cDNA**

Using a Nanodrop spectrophotometer, the concentration of RNA in the samples was measured from the cell lines. Volumes corresponding to 1µg RNA were taken from each sample and then used with a High capacity cDNA reverse transcription kit (Life technologies) according to the manufacturer’s instructions. The cDNA was synthesized at 25℃ for 10 min, 37℃ for 120 min, 85℃ for 5 min and cooled to 4℃ by using the MJ Research PTC-200 Peltier Thermal Cycler (GMI inc, Ramsey, MI). The cDNA was kept in -20℃ for future use.

**qPCR**

For gene specific qPCR (quantitative polymerase chain reaction), 15µl Taqman® mastermix (Life technologies) 1.5 µl of the primers for ER (Hs00174860_m1), HER2 (Hs01001580_m1), B-actin (Hs99999903_m1), ABL (Hs01104728_m1) and 12µl MilliQ water were mixed. 13.5µl of the solution were then added to a 96-well plate along with 1.5µl (40ng) of cDNA. The plate was then put in a Biorad CFX96 C1000 Touch Thermal Cycler. The plate was run at 2 min at 50℃ and then 95℃ for 20sec, then 40 cycles of 95℃ for 3 sec and 60℃ for 30 sec. The Ct values were acquired and the standard deviation (SD) calculated for the target genes and reference genes.

**Protein concentration**

Each protein sample was diluted with a factor of 2 and 5 before put into a 96 –well plate. As reference, bovine serum albumin (BSA) (Sigma-Aldrich, A9418) was solved in MilliQ water with known concentrations of 0, 0.2, 1.0 and 1.5 mg/ml and added into separate wells. DC protein assay kit (Bio-rad, Copenhagen, Denmark) was used according to manufacturer’s instructions. Absorbance was read at 750nm with multiskan ascent (Thermo labsystem, Helsinki, Finland) and protein concentration calculated for each sample.
Western blot

The protein samples were diluted using MilliQ water and then 50:50 with Laemelli buffer (Sigma Aldrich, #S3401) so they had the same concentration. Two gels (Mini-PROTEAN TGX, Bio-rad) were prepared and 11µg were then added from each sample to different wells in the gels. For reference, 10µl of Precision plus Dual color (Bio-rad) were used. The proteins were separated by electrophoresis with 300V and 30mA/gel for 1.5h. After the separation of the different proteins they were transferred to polyvinylidene difluoride membranes for 1.5h at 80V. The membranes were then blocked for unspecific binding with ECL-block (GE Healthcare, Buckinghamshire,UK) solved in TBS-T for 1h at RT. The membranes were incubated with different primary antibodies solved in ECL-block (1:1000), monoclonal rabbit-ER-antibody #8644 (cell signaling technology) respectively polyclonal rabbit HER2/Neu-antibody #2242 (cell signaling technology) overnight in 4°C using rotation. The membranes were washed with TBS-T and incubated with the secondary HRP-conjugated goat anti-rabbit antibody AS09602 (Agrisera, Vännäs, Sweden) solved in ECL-block (1:50 000) for 1h using rotation. For detection, 5 ml ECL prime (GE healthcare) were added to each membrane and they were incubated for 5 minutes in the dark. Excess ECL prime were removed and the membrane sealed in plastic and kept dark until detection with a CCD-camera.

The membranes were stripped with a strip 2x buffer (11 mM β-mercapto-EtOH, 8% SDS, 183mM Tris pH6.7) that was diluted 1:1 with TBS-T. The buffer was prewarmed to 50°C and 25ml were added to each membrane. The membranes were incubated in 50°C for 30 minutes and then washed in phosphate-buffered saline (PBS) and TBS-T.

The above steps with ECL-block down to detection with a CCD camera were done again for the endogenous control B-actin. The polyclonal rabbit B-actin antibody #ab8227 (Abcam, Cambridge, England) and the HRP-conjugated goat anti-rabbit antibody #AS09602 (Agrisera) were used.

Exposure to cytostatic drug

The cell lines BT-474 and MCF-7 were chosen for future experiments and ZR-75-1 removed due to lack of time and their slow cell growth. BT-474 and MCF-7 were washed with PBS and trypsin was added. The cell culture flasks were incubated for 5 min at 37°C until the cells detached. Medium was added to stop the action from trypsin. 250 000 cells with medium were then seeded into 6-well tissue culture plates and cultured for 24h in 37°C, 5% CO2 before adding the cytostatic.
The cytostatic drugs used were (z)-4-hydroxytamoxifen (Sigma-Aldrich,H7904) with the concentration of $10^6$M, Herceptin (Roche Pharma AG, Grenzach-Wyhlen, Germany) with the concentration of 10µg/ml and paclitaxel (Actavis, Hafnarfjordur, Island) with the concentration of 137µg/ml. The total volume added to each well was 2ml and consisted of medium with supplements and the cytostatic drug. During the trials with a combination of Herceptin and paclitaxel the same final concentration of 10µg/ml and 137µg/ml respectively were achieved. The cells were exposed for the cytostatic drugs for 72h.

**Flow cytometry**

After 72h, the experiment was stopped and the cells were prepared for flow cytometry using the Intrastain, fixation and permeabilization kit for flow cytometry (Dako, Glostrup, Denmark). The old medium including dead cells were saved and the 6-well tissue culture plate cells were washed with PBS. Trypsin was added and the cells incubated for 5 min at 37°C. When the cells detached they were transferred to the old medium. The tubes were centrifuged for 5 min at 300g and the supernatant discarded. 50µl of PBS were added and then 10µl CD44-FITC (Beckman Coulter, Marseille, France), 10µl CD24-APC (Beckman Coulter), 10µl IgG1-FITC (Beckman Coulter) and 10µl IgG1-APC (Beckman Coulter) to the respective tubes. The tubes were kept in the dark for 15 min, added 100µl reagent A and then incubated them again for 15 min in the dark. The tubes were centrifuged for 5 min at 300g and the supernatant discarded. 2ml PBS, 100µl Reagent B and 20µl DAPI viability dye (Beckman Coulter) were added. The tubes were incubated in the dark for 15 min, centrifuged for 5 min at 300g and the supernatant discarded. 500µl PBS was added to each tube before analyzing it. The Gallius™ (Beckman Coulter, Brea, CA, USA) with blue laser (488 nm) for FITC-conjugated, red laser (638 nm) for APC-conjugated and violet laser (405 nm) for DAPI detection were used. The data were acquired with Kaluza Flow Cytometry Analysis v1.2 (Beckman Coulter) and the proportion of CD24+/CD44+ and CD24+/CD44− together with Median Fluorescent Intensity (MFI) measured. MFI was calculated and ± standard deviation (SD) from the trials when untreated (n=6) or when treated with Tamoxifen (n=4), Trastuzumab (n=4), Paclitaxel (n=4) or Paclitaxel/Trastuzumab (n=4).

**Statistics**

Mean and SD for Median Fluorescence Intensity (MFI) and qPCR reference gene/target genes were calculated with graphpad Prism v6.0 software. The statistical significance were calculated with a Mann-Whitney U test.
RESULTS

Basal gene expression

The cell lines BT-474, MCF-7 and ZR-75-1 had their mRNA converted to cDNA and were then used in qPCR (Fig. 1 and 2). The target genes analyzed were the ER and HER2 receptor and for endogenous controls, B-actin and ABL were used. BT-474 shows an intermediary expression of HER2 and high levels of ER. MCF-7 has intermediary expression of ER and HER2 receptor. ZR-75-1 have a high expression of the HER2 receptor and low expression of the ER.

Figure 1. The figure shows the ratio between the reference gene/target gene and with the ± standard deviation (SD) for the three cell lines MCF-7, ZR-75-I and BT-474. The target genes were the estrogen receptor (ER) and the human epidermal growth factor 2 (HER2) receptor. As reference genes, the two endogenous controls B-actin and ABL were used.
Western blot analysis

According to the results from the Western blot analysis, the BT-474 cells express higher levels of ER (Fig.2) and HER2 protein (Fig.3) than the MCF-7 and ZR-75-1 cells. The MCF-7 and ZR-75-1 cells seem to express about the same levels of the ER protein and MCF-7 have a higher level of HER2 protein than the ZR-75-1.

Figure 2. For western blot analysis, the proteins were extracted twice from the three cell lines ZR-75-1, BT-474 and MCF-7. Antibodies directed at the ER protein were used and as control for non-specific binding, antibodies directed at B-actin.

Figure 3. For western blot analysis, the proteins were extracted twice from the three cell lines ZR-75-1, BT-474 and MCF-7. Antibodies directed at the HER2 protein were used and as control for non-specific binding, antibodies directed at B-actin.
Drug exposure alters CD24 and CD44 expression

There was an 29% increase in the CD24+/CD44- subpopulation when the MCF-7 cell line were exposed to paclitaxel and trastuzumab/paclitaxel (Table 1). A small increase could also be seen when BT-474 were exposed to paclitaxel alone. Only minor changes (±1%) could otherwise be seen in the CD24 and CD44 expression.

Table 1. The MCF-7 cell line was exposed to the three cytostatic drugs tamoxifen, trastuzumab, paclitaxel and the combination of paclitaxel/trastuzumab. The proportion of CD24+/CD44- and CD24+/CD44+ cells were measured with a flow cytometer before and after exposure. Median fluorescence intensity (MFI) and its standard deviation (SD) were calculated.

<table>
<thead>
<tr>
<th>MCF-7 cell line</th>
<th>CD24+/CD44-</th>
<th>%</th>
<th>MFI ± SD</th>
<th>%</th>
<th>MFI ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td></td>
<td>88.29</td>
<td>386±68</td>
<td>11.61</td>
<td>564±84</td>
</tr>
<tr>
<td>Tamoxifen</td>
<td></td>
<td>88.95</td>
<td>603±43</td>
<td>10.99</td>
<td>731±45</td>
</tr>
<tr>
<td>Trastuzumab</td>
<td></td>
<td>88.25</td>
<td>535±114</td>
<td>11.68</td>
<td>765±156</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td></td>
<td>58.72</td>
<td>661±97</td>
<td>40.96</td>
<td>876±95</td>
</tr>
<tr>
<td>Paclitaxel/Trastuzumab</td>
<td></td>
<td>58.81</td>
<td>769±184</td>
<td>40.98</td>
<td>915±156</td>
</tr>
</tbody>
</table>

Table 2. The BT-474 cell line was exposed to the three cytostatic drugs tamoxifen, trastuzumab, paclitaxel and the combination of paclitaxel/trastuzumab. The proportion of CD24+/CD44- and CD24+/CD44+ cells were measured with a flow cytometer before and after exposure. Median fluorescence intensity (MFI) and its standard deviation (SD) were calculated.

<table>
<thead>
<tr>
<th>BT-474 cell line</th>
<th>CD24+/CD44-</th>
<th>%</th>
<th>MFI ± SD</th>
<th>CD24+/CD44+</th>
<th>%</th>
<th>MFI ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td></td>
<td>98.43</td>
<td>412±134</td>
<td>1.51</td>
<td>511±170</td>
<td></td>
</tr>
<tr>
<td>Tamoxifen</td>
<td></td>
<td>98.40</td>
<td>433±32</td>
<td>1.55</td>
<td>527±29</td>
<td></td>
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<tr>
<td>Trastuzumab</td>
<td></td>
<td>98.32</td>
<td>864±137</td>
<td>1.50</td>
<td>924±115</td>
<td></td>
</tr>
<tr>
<td>Paclitaxel</td>
<td></td>
<td>95.10</td>
<td>616±141</td>
<td>4.55</td>
<td>843±201</td>
<td></td>
</tr>
<tr>
<td>Paclitaxel/Trastuzumab</td>
<td></td>
<td>98.20</td>
<td>939±105</td>
<td>1.64</td>
<td>1017±0.4</td>
<td></td>
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</table>

11
Changes in cell cycle phase distribution

The proportion of cells in the G0/G1 and S/G2/M phase changed when the MCF-7 cell line was exposed to paclitaxel and trastuzumab/paclitaxel (Fig. 4). There was a 34% increase of cells in the S/G2/M phase when treated with paclitaxel and a 30% increase when trastuzumab/paclitaxel was used. Only minor changes (±5%) could be seen with tamoxifen and trastuzumab. For the BT-474 cell line, only paclitaxel (Fig. 5) had any substantial effect with a 24% increase of the cells in the S/G2/M phase. Tamoxifen, trastuzumab and trastuzumab/paclitaxel only showed minor changes (±5%).

![Figure 4. The figure shows the proportion of the subpopulations G0/G1 and S/G2/M after exposure to cytostatic drugs for 72h in the MCF-7 cell line. The drugs used were tamoxifen, trastuzumab, paclitaxel and a combination of trastuzumab/paclitaxel.](image)
Figure 5. The figure shows the proportion of the subpopulations G0/G1 and S/G2/M after exposure to cytostatic drugs for 72h in the BT 474 cell line. The drugs used were tamoxifen, trastuzumab, paclitaxel and a combination of trastuzumab/paclitaxel.
DISCUSSION

The cancer stem cell markers CD24 and CD44 can be found in different forms of breast cancer and their expression is important for the breast cancers survival and invasiveness. In our trial we used three breast cancer cell lines and analyzed their basal gene expression of HER2 and ER and then exposed these to three different cytostatic drugs for 72h. We then used flow cytometry to see if there were any difference in their expression of CD24 and CD44.

The cell lines MCF-7 and BT-474 have in our and earlier studies shown high amounts (table 1) of the CD24+/CD44- subpopulation and lower for CD24+/CD44+ when the cells are untreated[32]. And after the treatment with the different cytostatic drugs we could only see a major change in MCF-7 that was treated with paclitaxel or trastuzumab/paclitaxel. The amount of cells that co-expressed the CD24 and CD44 cell surface markers had gone up with roughly 30% and the MFI increased. This may seem as a bad prognostic factor according to earlier research, but some studies have proven that CD44 can in some cases slow down tumor progression through inducing cell differentiation and apoptosis [33]. CD44 also has the ability to activate the Ras-MAPK and PI3K/AKT intracellular signaling pathways so this might actually be one of the reasons why we see an increased CD44 expression in response to paclitaxel exposure [34]. This theory has its flaws due to the fact that we cannot see increased expression in the BT-474 cell line and due to the low level of trials (n=4), we had no possibility to see if the results for the cell lines were statistically significant.

The three cell lines MCF-7, BT-474 and ZR-75-1 are epithelial cells that were extracted from different women in the 1970’s. Since then, these cell lines have undergone extensive research and their genomes have been mapped. We could see in our protein analysis with Western blot that MCF-7, ZR-75-1 and BT-474 express the ER and the HER2 receptor (Fig.2 and 3) and BT-474 overexpress the HER2 receptor. In the ZR-75-1 sample B we could detect proteins but not in sample A. This is probably due to some error in the loading of the protein into the gel before electrophoresis, which can have a variety of different reasons [30]. We also performed qPCR (Fig. 1) which show that BT-474 has high amounts of HER2 mRNA and lower for ER. MCF-7 and ZR-75-1 both express ER and HER2 mRNA at intermediary levels. The qPCR test requires small volumes and minor mistakes can lead to great changes in the acquired results. But with earlier research in mind, it seems more likely that something went wrong while performing the western blot [31].
The proportion of cells in the G0/G1 and S/G2/M showed small changes in the cell lines that were treated with tamoxifen or trastuzumab alone (Fig. 4 and 5). When treated with paclitaxel or the combination of trastuzumab/paclitaxel we can see a 30% increase in the S/G2/M population as expected. The effect is due to the fact that paclitaxel can work as a mitotic inhibitor which makes the cells stop in the S/G2/M phase and cannot undergo cell division [20]. The trials with a combination of trastuzumab and paclitaxel resulted in an intermediary sized population of S/G2/M because trastuzumab blocks the HER2 receptor and make the cells less likely to start cell division [18]. Due to the few number of samples (n=4) we could not make any statistic calculations and find any statistic significance. Despite CSCs has been studied for over a decade there has never been any proof for their existence in vivo until just recently [35]. All the trials have been done in vitro and this has some crucial limitations that can also be found in this paper. For example, trastuzumab with its HER2 inhibiting function can possibly work as an activator of ADCC and lead to cell death in vivo[18]. To improve this work in vitro, more samples, cytostatic drug concentrations, different exposure times and cell lines are needed. And there still exist some question marks to why only MCF-7 upregulated its CD44 expression and not BT-474. In the future there will hopefully be some way to do the tests in vivo so you can get more accurate results and easier see the possibility for better cancer treatment in humans.

CONCLUSION

No statistically significant change in the proportion of the subpopulations for CD24+/CD44- and CD24+/CD44+ cells could be seen after treatment with tamoxifen, trastuzumab, paclitaxel and trastuzumab/paclitaxel for 72h.

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