doi:10.4149/neo_2011_03_189

Analysis of CD40 expression in breast cancer and its relation to clinicopathological characteristics.

Z. SLOBODOVA[1,2], J. EHRMANN[1,2,3],V. KREJCI[1,2], J. ZAPLETALOVA[4], B. MELICHAR[2,5]

[1]Laboratory of Molecular Pathology, Institute of Pathology, Faculty of Medicine and Dentistry, Palacky University and University Hospital, Hnevotinska 3, 775 15 Olomouc, Czech Republic, e-mail: slobodova.zuzana@seznam.cz; [2]Institute of Molecular and Translation Medicine, Faculty of Medicine and Dentistry, Palacky University and University Hospital, Olomouc; [3]Department of Histology and Embryology, Faculty of Medicine and Dentistry, Palacky University and University Hospital, Olomouc; [4]Department of Medical Biometry, Faculty of Medicine and Dentistry, Palacky University and University Hospital, Olomouc; [5]Department of Oncology, Palacky University and University Hospital, Olomouc, Czech Republic

Received December 21, 2010

Breast cancer is currently the most common cancer in women worldwide. For this reason, new biomarkers for better predicting response to treatment are needed. CD40, described as expressed in haematological and epithelial tumors, is linked to apoptosis and offers promise as a new predictive/ prognostic marker. We evaluated CD40 expression in formalin-fixed, paraffin-embedded samples from 181 breast carcinomas using immunohistochemical staining with CD40 antibody. Samples were divided according to hormone (oestrogen receptor /ER/, progesterone receptor /PR/) and her-2/neu status into groups: 1.Luminal A (ER+PR+her-2/neu-), 2. Luminal B (ER+PR+her-2/neu+), 3.Triple-negative (ER-PR-her-2/neu-) and 4. Her-2/neu (ER-PR-her-2/neu+).

The results of CD40 staining were correlated with clinicopathological data. CD40 was found to be expressed in membrane, cytoplasm and nucleus. Normal ducts expressed cytoplasmic CD40 in 30% of cases, in breast tumor ducts in 53% of cases. CD40 was evaluated as an independent marker and significant positive correlation was found with Bcl-2 (p =0.002), early stage (p =0.016) and preoperative chemotherapy (p =0.043).

There was higher overall survival for patients with cytoplasmic CD40 expression (0.05). Differences in expression of cytoplasmic CD40 between groups with different hormonal and her-2/neu status were statistically highly significant (p=0.00003). In groups with different hormonal status, a positive statistical correlation was found for the luminal A group with relapse (p=0.024) and stage (p=0.006). No correlation was found with age, disease onset, family history of cancer/ breast cancer, patient history, hormonal replacement therapy, menopausal status at onset of disease, adjuvant chemotherapeutic treatment or disease free survival. Nuclear expression of CD40 was found to be unrelated to any clinicopathological data. However, there was higher ratio of positive cases in cancer cases (83%) than in normal tissue (30%).

In conclusion, cytoplasmic expression of CD40 is related to factors connected to better prognosis and suggest that CD40 may have potential as a new prognostic factor in breast cancer.

Key words: breast cancer, CD40 expression, luminal A subtype, Bcl-2.

Breast cancer is the most common cancer diagnosed in European women with a mortality of 131 900/year in 2008 and owing to earlier detection the incidence is increasing (421 000 cases/year) [1]. The traditional classification of breast cancer proved inadequate for current tailored treatments and for this reason a new classification was established. This is based on gene expression profiling and it divides breast carcinomas into luminal subtypes which typically express oestrogen-related genes (oestrogen and progesterone receptor positivity), her-2/neu subtype (her-2/neu positivity) and basal-like subtypes (hormonal receptor-negativity and cytokeratine 5/6 positivity) [2, 3]. These are associated with different prognoses [4, 5, 6, 7, 8, 9].

Chemotherapeutics, anti-hormonal drugs and/or trastuzumab are not always effective and resistance to treatment is common [10]. For this reason, new biomarkers that could identify potentially resistant cases are being investigated. Further, tumors that respond to therapy are prognostically different and hence predictors are of paramount important.

CD40 is the member of the tumor necrosis factor (TNF) receptor family of cell surface proteins, originally described as

a B cell restricted antigen. The importance of this molecule was revealed as an immune response in all type of leukocytes [11, 12] but most promising are studies focused on dendritic cells related to apoptosis and anti-tumor immune response [13, 14].

Expression of CD40 has also been found in several carcinoma cell lines and tissues including breast, pancreas, melanoma [15], urinary bladder [15, 16], ovarian cancer [17], non-small cell lung cancer and cells from squamous epithelial carcinoma of the head and neck [18, 19] and non-tumor cells including fibroblasts [20]. Its expression has been described in membrane, cytoplasm and nucleus [15, 21, 22] and expression in these tumors is related to different behavior and prognosis. Ligation of CD40-ligand (CD40L) with CD40 in epithelial and haematological malignancies was found to produce a direct growth- inhibitory effect through cell cycle blockage and/or apoptotic induction with no overt side-effects on their normal counterparts [19].

Our study focused on the relation of levels of CD40 expression in breast carcinomas correlated with clinicopathological characteristics. Here we present CD40 as a potential independent biomarker related to better prognosis in breast tumors.

Materials and methods

Tissue samples. The 181 breast cancer samples were collected over the period from June 2006 to December 2007 by the Laboratory of Molecular Pathology, Institute of Pathology, Faculty of Medicine and Dentistry, Palacky University and University Hospital Olomouc Czech Republic. Specimens were obtained from core-cut biopsies, excision and ablation. They were fixed in 10% buffered formalin and embedded in paraffin. Pathological examination assessed diagnosis according to the World Health Organization (WHO) classification [23]. Hormonal status (oestrogen receptor and progesterone receptor) and her-2/neu was established according to the guidelines [see ref 24, 25]. The stage was assessed according to the WHO classification [23]. Proliferative activity of carcinoma was measured by expression of proliferating cell nuclear antigen (PCNA).

Patients. Patients included those for whom prospective follow-up and clinical data were available: age, patient history, family history of cancer and breast cancer, hormonal replacement therapy, menopausal status at the time of disease onset, adjuvant preoperative chemotherapy treatment, relapse, overall survival and disease free survival. Information was obtained from the Department of Oncology Palacky University and University Hospital Olomouc Czech Republic. The details are presented in table. 1

Immunohistochemistry. Immunohistochemistry was performed on 4 μm tissue sections. Sections were fixed on slides for 60 minutes at 65°C. The slides were dewaxed in xylene and dehydrated in alcohol. Microwave antigen retrieval was performed in citrate buffer solution (pH 6) at high power at 120°C for 5 minutes and cooled in room temperature for 20 minutes. Endogenous peroxidase activity was inhibited with 6% hydrogen peroxide in methanol for 15 min. Slides were washed twice with Tris and then incubated in Tris/Tweeen for 5 minutes. The samples were then incubated with primary antibody at room temperature for 1 hour. The following primary antibody was used: Rabbit polyclonal antiSanta Cruz Biotechnology, Inc., Unconjugated) in 1:50 dilution. As a secondary antibody we used EnVision™+ Dual Link System-HRP (Dako) incubated at room temperature for 1 hour. The slides were washed twice with Tris and then incubated in Tris/Tweeen for 5 minutes. Colour development was achieved with diaminobenzidine staining (DAB) (Dako) and sections were finally counterstained with haematoxylin. The slides were dehydrated, cleared in xylene and mounted in Canada balsam (Sigma-Aldrich). Negative controls included omission of the primary antibody. As a positive internal control we used expression of CD40 in lymphocytes where we detected membrane expression.

Assessment of immunostaining. Immunostaining was evaluated by two pathologists.An immunohistoscore was used for assessment of CD40. The percentage of strongest positive tumor cells was graded as follows: 0 for less than 1% of tumor cells, 1 for 1-30% of tumor cells, 2 for 31-60% of tumor cells, 3 for more than 60%. The intensity of staining was graded as follows: 0 no staining, 1 weak staining, 2 moderate staining, 3 strong staining. The overall score (range 0 to 9) was obtained by multiplying the percentage by the intensity. Expression with overall score 0 was considered as negative – without expression. An overall score ranging from 1 to 3 described a weak expression, overall score from 4 to 6 described moderate expression and an overall score from 7 to 9 described strong expression.

Statistical analysis. The data were analyzed using the statistical software SPSS version 15 (SPSS Inc., Chicago, USA). Qualitative and categorized quantitative variables were compared using the Fisher's exact test. Differences in quantitative variables were tested by the Wilcoxon rank sum test. Kaplan-Meier's test was used for evaluation of overall and disease free survival. A p value of less than 0.05 was considered statistically significance.

Results

We found CD40 expression in different compartments of the normal and cancer cell. CD40 was expressed in the cytoplasm of normal ducts at low levels in 30% of patients. Expression was present in the luminal cells of normal ducts, basal cells were negative and the intensity was weak. Tumor cells showed cytoplasmic CD40 expression in 53% of cases and the intensity of staining was from weak to strong (Fig. 1). Membrane expression was present in 14 cases (7.7%): In all these cases cytoplasmic staining was also present and intensity of staining was from weak to strong.

Even nuclear expression of CD40 was found. It was present in normal ducts in luminal cells equally as in cytoplasm. Basal cells were negative. The intensity of staining was from moderate to strong. The distribution of different types of CD40 expression is shown in Table 2.

Tumor cells showed weak to strong intensity of CD40 nuclear staining in 147 cases (81.2%). Cancer cells with mem-

Table 1. Characteristics of patient group and significance of Fischer's exact test and Kaplan-Meier's test (*) in different subgroups.

Factor	Number of CD40 cytoplasmic negative patients	Number of CD40 cytoplasmic positive patients	Number of patients	P value of Fischer's exact test (p)
Age (onset of disease)				
≤ 45 years	13 (48,1%)	14 (51,9%)	27	1,000
≥ 45 years	73 (47,4%)	81 (52,6%)	154	
Type of carcinoma				
Ductal	55 (42%)	76 (58%)	131	0,077
Lobular	8 (57,1%)	6 (42,9%)	14	
Ductal carcinoma *in situ*	7 (77,8%)	2 (22,2%)	9	
other	16 (59,3%)	11 (40,7%)	27	
Tumor grade				
G1	13(35,1%)	24 (64,9%)	37	0,267
G2	31 (47%)	35 (53%)	66	
G3	34 (51,5%)	32 (48,5%)	66	
Nodal status				
N0	51 (46,8%)	58 (53,2%)	109	0,755
N1	33 (50%)	33 (50%)	66	
Stage				
Stage 0, I., II.	67 (44,1%)	85 (55,9%)	152	**0,016**
Stage III., IV.	18 (72%)	7 (28%)	25	
Bcl-2 status				
Bcl-2+	29 (69%)	13 (31%)	139	**0,002**
Bcl-2-	57 (41%)	82 (59%)	42	
PCNA				
low	57 (46,3%)	66 (53,7%)	123	0,723
High	21 (50%)	21 (50%)	42	
Adjuvant chemotherapy				
with	42 (50%)	42 (50%)	84	0,537
Without	37 (44,6%)	46 (55,4%)	83	
Preoperative chemotherapy				
with	12 (70,6%)	5 (29,4%)	17	**0,043**
without	68 (43,9%)	87 (56,1%)	155	
Relapse				
with	10 (55,6%)	8 (44,4%)	18	0,619
Without	69 (46,9%)	78 (53,1%)	147	
Menopausal status				
premenopausal	22(52,4%)	20 (47,6%)	42	0,569
postmenopausal	63 (46,3%)	73 (53,7%)	136	
Hormonal replacement therapy				
with				
without	16 (40%)	24 (60%)	40	0,280
	64 (50,4%)	63 (49,6%)	127	
Family history of cancer				
negative	41 (48,2%)	44 (51,8%)	85	0,232
positive	30 (57,7%)	22 (42,3%)	52	
breast	14 (38,9%)	22 (50,9%)	36	
Overall survival				
Death	11 (13,6%)	5 (5,7%)	16	*0,052
Surviving	70 (86,4%)	82 (94,3%)	152	
Disease free survival				
Relapse	10 (55,6%)	8 (44,4%)	18	*0,619
Without relapse	69 (46,9%)	78 (53,1%)	147	

brane staining were always negative for nuclear expression but positive cytoplasmic CD40 staining could be present in cases with nuclear CD40 expression.

Correlation of different types of CD40 expression with clinicopathological characteristics was statistically significant only in cases of cytoplasmic expression. Nuclear expression and membrane staining did not reach statistical significance (Table 3.). When cytoplasmic CD40 was examined as an independent tumor marker we found the following (see Table 1.):

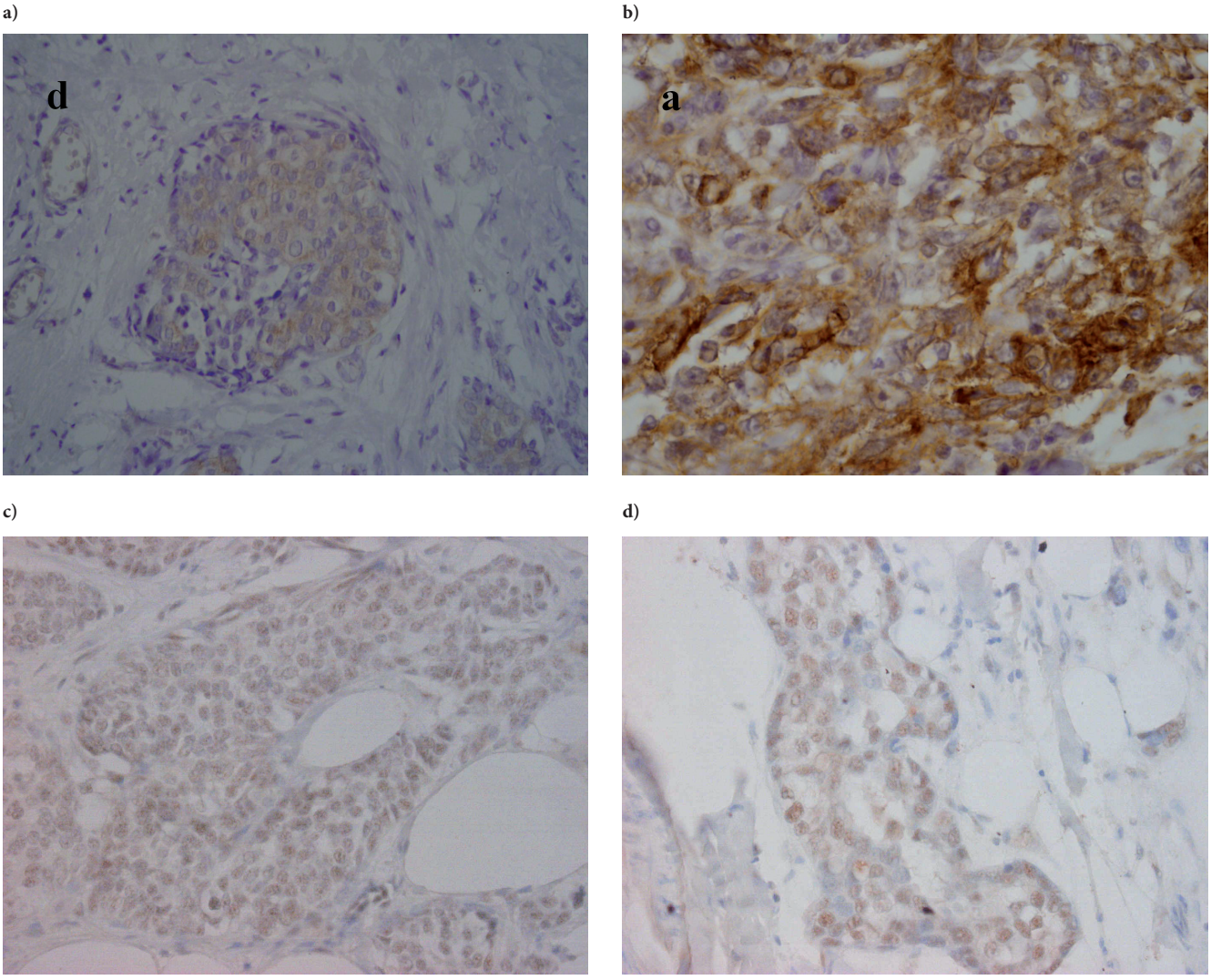


Figure 1. Types of CD40 expression in breast carcinoma: a) b) examples of cytoplasmic expression; c) d) examples of nuclear expression (magnification × 400).

a) positive association between cytoplasmic expression of CD40 and presence of Bcl-2 – Fischer's exact test proved a statistically significant lower ratio of patients expressing CD40 in tumors without Bcl-2 expression compared to higher ratio of patients expressing CD40 in Bcl-2 positive tumors (p =0.002).
b) positive association between cytoplasmic expression of CD40 and early tumor stage – Fischer's exact test showed a statistically significant lower ratio of patients expressing CD40 in advanced stages (III., IV.) compared to higher ratio of patients expressing CD40 in tumors in earlier stages (0, I., II.) (p =0.016).
c) positive association between low cytoplasmic expression of CD40 and presence of preoperative chemotherapy – Fischer's exact test showed a statistically significant lower ratio of patients expressing CD40 in tumors with preoperative chemotherapy compared to higher ratio of patients expressing CD40 in cytoplasm without preoperative chemotherapy (p =0.043).
d) Kaplan-Meier test showed that overall survival was higher (p=0.05) in the group of patients with CD40 expression (Graph 1.)

No correlation was found between CD40 expression as an independent biomarker and grade, PCNA, age, onset of disease, family history of cancer/ breast cancer, patient history, hormonal replacement therapy, menopausal status at onset of disease, adjuvant chemotherapeutic treatment, relapse and disease free survival (see Table 1.).

Next we compared CD40 cytoplasmic expression in 4 subgroups of patients with different prognosis: luminal A (ER+ PR+ her-2/neu-), luminal B (ER+ PR+ her-2/neu+), her-2/neu (ER- PR- her-2/neu+) and triple-negative (ER- PR- her-2/neu).

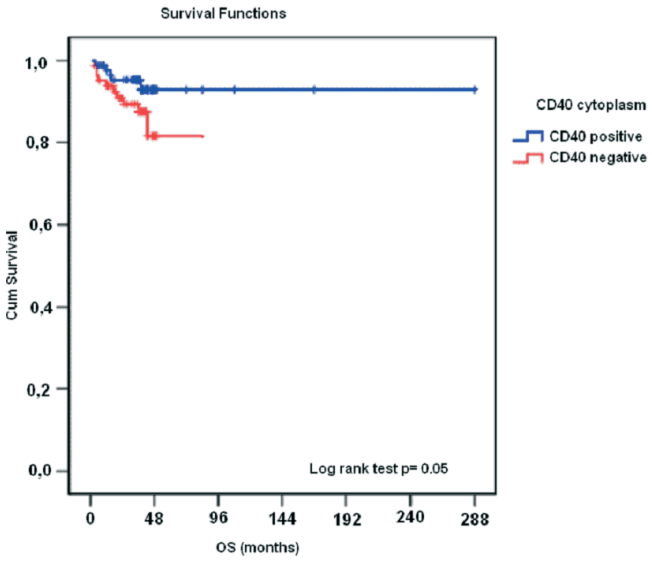
The number of patients and cytoplasmic CD40 expression in different groups are shown in Table 2. Differences between groups were significant (p=0.00003) and the expression of CD40 was significantly higher in groups with expression of hormonal receptors compared to groups without hormonal receptors (Table 4.).

The following results were found for luminal A subgroup:

a) Fischer's exact test showed a significantly lower ratio of patients with cytoplasmic CD40 in tumors in advanced stage (III., IV.) compared to tumors in early stage (0, I., II.) p=0.006.
b) Fischer's exact test showed a significantly lower ratio of patients with cytoplasmic CD40 in tumors with relapse (p=0.024).
c) Lower level of CD40 was found in tumors with preoperative chemotherapy though this was not significant (Table 5).

In other subgroups the differences did not reach statistical significance at the 0.05 level.

We also analyzed the intensity of CD40 expression in relation to clinicopathological characteristics. Membrane



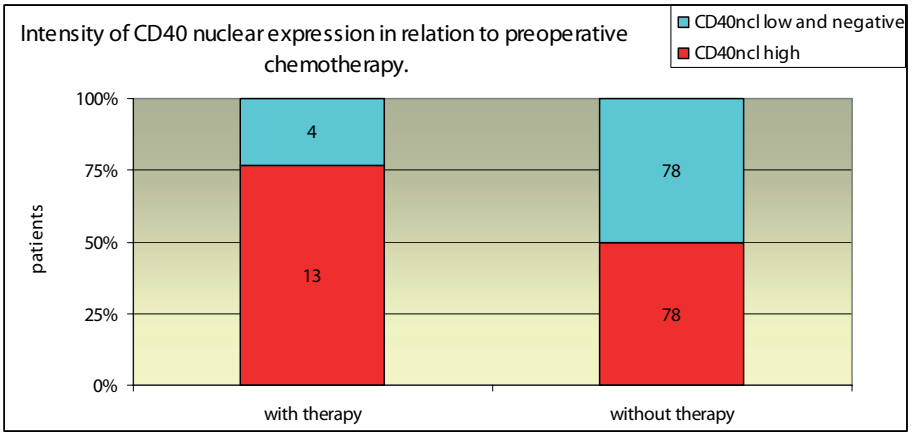
Graph 1. Overall survival in complete group according to cytoplasmic C40 expression.

Table 2. Different types of CD40 expression in groups with different hormonal and her-2/neu status.

Group	Patients in group	CD40 cytoplasmic expression /positive cases (percent) negative cases (percent)	CD40 nuclear expression /positive cases (percent) negative cases (percent)	CD40 membranous expression /positive cases (percent) negative cases (percent)
Luminal A	104	62 (59,6 %) 42 (40,4 %)	88 (84,6 %) 16 (15,4 %)	4 (3,9%) 100 (96,1%)
Triple-negative	36	14 (39 %) 22 (61 %)	28 (77,8 %) 8 (22,2 %)	7 (19,4%) 29 (80,6%)
Her-2/neu	16	1 (12,5 %) 15(87,5 %)	9 (56,3 %) 7 (43,7 %)	0 (0 %) 16 (100%)
Luminal B	25	18 (72 %) 7 (28 %)	22 (88 %) 3 (12 %)	3 (12%) 22 (88%)
Total	181	95 (52,5 %) 86 (47,5 %)	147 (81,2 %) 34 (18,8 %)	14 (7,7%) 167 (92,3%)

Table 3. Values of Fischer's exact test (p) (lines 1.-13.) and values of log rank test in Kaplan-Meier's test (lines 14., 15.) for clinical data in relation to groups with different hormonal and her-2/neu status in nuclear type of CD40 expression.

	Clinical data	Complete group	Luminal A	Triple- negative	Luminal B	Her-2/neu
1.	Type of carcinoma	0,624	0,331	1,000	1,000	0,868
2.	PCNA	0,353	0,196	0,564	0,177	0,302
3.	Bcl-2	0,370	1,000	0,422	0,120	0,550
4.	Grade	0,492	0,363	0,285	0,308	1,000
5.	Stage	0,420	0,329	0,648	1,000	1,000
6.	Nodal status	0,426	0,418	0,644	1,000	1,000
7.	Adjuvant chemotherapy	0,481	0,759	1,000	1,000	1,000
8.	Preoperative chemotherapy	0,741	1,000	0,302	1,000	0,438
9.	Relapse	0,532	0,094	0,300	1,000	0,457
10.	Onset of disease	1,000	0,069	0,555	1,000	0,475
11.	Menopausal status	0,173	0,002	1,000	1,000	0,308
12.	Hormonal replacement therapy	0,643	0,509	0,403	1,000	1,000
13.	Family history of cancer	0,529	0,646	0,261	0,413	0,816
14.	Overall survival	0,451	0,466	0,876	-	0,798
15.	Disease free survival	0,966	0,829	0,985	0,705	0,516



Graph 2. Intensity of CD40 nuclear expression in relation to preoperative chemotherapy.

and cytoplasmic CD40 expression did not reach statistical significance for any category (data not shown). We found a statistically significant positive relation between intensity of nuclear CD40 expression and preoperative chemotherapy (p= 0.038) (Graph 2.).

Table 4. Correlation of cytoplasmic CD40 between groups with different hormonal and her-2/neu status.

Compared group	Compared group	P value of Fischer's exact test
Triple-negative	Luminal B	**0,018**
Triple-negative	Luminal A	**0,035**
Triple-negative	her2/neu	**0,021**
Luminal B	Luminal A	0,359
Luminal B	Her-2/neu	**0,0001**
Luminal A	Her-2/neu	**0,0001**

Discussion

CD40 has been thoroughly investigated in haematological malignancies but its importance in solid tumors remains unclear. CD40 is not expressed in all tumors [26] and its expression has different effects in different tumor types and tissues. It has not been found in prostate adenocarcinoma [26, 27]. In non-small lung cancer cells its appearance correlates with poor prognosis [28] but in most tumors, overexpression relates to a favourable prognosis. The new molecular classification of breast carcinomas describes different behaviour of tumors related to expression of hormonal receptors and her-2/neu expression [4]. We found a statistically significant

Table 5. Values of Fischer's exact test (p) (lines 1.-13.) and values of log rank test in Kaplan-Meier's test (lines 14., 15.) for clinical data in relation to groups with different hormonal and her-2/neu status in cytoplasmic type of CD40 expression.

	Clinical data	Luminal A	Triple- negative	Luminal B	Her-2/neu
1.	Type of carcinoma	0,200	0,230	0,611	1,000
2.	PCNA	0,480	0,666	0,481	1,000
3.	Bcl-2	0,391	0,501	0,280	1,000
4.	Grade	0,964	0,268	0,826	1,000
5.	Stage	**0,006**	0,267	1,000	1,000
6.	Nodal status	0,839	1,000	0,630	1,000
7.	Adjuvant chemotherapy	0,525	0,501	1,000	1,000
8.	Preoperative chemotherapy	0,073	1,000	0,304	1,000
9.	Relapse	**0,024**	1,000	0,272	1,000
10.	Onset of disease	0,418	0,277	0,418	1,000
11.	Menopausal status	0,160	0,221	0,597	0,313
12.	Hormonal replacement therapy	0,630	0,057	0,621	1,000
13.	Family history of cancer	0,351	0,389	0,600	0,188
14.	Overall survival	0,143	0,678	-	-
15.	Disease free survival	0,876	0,605	0,478	-

positive relation of cytoplasmic CD40 expression with groups that express hormonal receptors. *Vice versa* negative statistical significance was found in loss of CD40 expression and tumors that are triple negative and her-2/neu positive.

This could be connected with the involvement of CD40 in the oestrogen receptor signalling pathway in breast cancer. In breast cancer cells, CD40 expression has been described in oestrogen positive cell line MCF-7 [29]. Treatment with the selective oestrogen receptor modulator toremifene was found to increase expression of CD40 in these cells [29]. The involvement of CD40 expression in the oestrogen receptor signalling pathway has also been described in another histogenetic type – endothelial cells [30].

Several preclinical studies from different laboratories have suggested that the CD40 signalling pathway plays a role in the modulation of chemosensitivity in breast cancer cells but no relation with hormonal receptors and her-2/neu in breast carcinoma has yet been established. Esteva et al. [31] showed that patients with breast cancer overexpressing her-2/neu in stages II. to III.A had decreased expression of the genes associated with the CD40 signalling pathway and they were more likely to have residual cancer after preoperative trastuzumab-plus- paclitaxel, 5-fluorouracil, epirubicin, cyclophosphamide chemotherapy (T/FEC). These results suggest that patients without decreased gene expression may be more likely to achieve pathologic complete response (pCR) to trastuzumab-plus-T/FEC therapy. In our study loss of CD40 overexpression positively correlated with breast tumors that received preoperative therapy as an independent marker without relation to hormonal and her-2/neu status. A similar finding though not statistically significant was the cytoplasmic CD40 status of patients in the luminal A group. In this group was also established decreased CD40 expression in correlation with relapse and higher stage of disease. Correlation was found also with stage and CD40 as an independent marker. Similar findings were reported by Pinzon-Charry A [32].

After anti-C40 treatment in cell lines of solid tumors with CD40 expression growth inhibition, cell cycle progression and apoptosis induction have been reported [15].

The importance of CD40-CD40L interaction in breast carcinoma has been described in one study [21], where soluble recombinant human CD40 ligand – srhCD40L induced apoptosis in human breast carcinoma cell lines. These findings were also confirmed in vivo: application of anti-CD40 or srhCD40L MoAb to immunodeficient SCID mice resulted in significant increase in survival of the tumor-bearing mice [21]. Patients with breast cancer have a significantly higher percentage of apoptotic blood dendritic cells. CD40 stimulation protects blood dendritic cells against TDSN-induced apoptosis [32, 33]. Incubation with CD40L produced a significant decrease in apoptosis induced by supernatant derived from tumors compared to levels seen in control cultures [32, 33]. A number of potential new vaccines and immunotherapeutic treatment/drugs focused on CD40-CD40L interaction not only in haematological malignancies have been described [19, 34, 35]. However, it will be many years until the preclinical studies confirm the use of these drugs in solid tumors. Another interesting finding was the relation of CD40 expression to Bcl-2 expression. Bcl-2 protein, a member of Bcl family, is related to apoptosis regulation [ref. 36, 37] and influenced by multiple mechanisms delayed cell death and final common pathway involved in programmed and apoptotic cell death [ref. 38, 39]. Normal breast tissue from healthy women shows lower expression of Bcl-2 protein than normal breast tissue in women with cancer and in cancer tissue [40]. In breast carcinoma, Bcl-2 has been described as an independent prognostic and predictive marker [41, 42, 43] and loss of its expression in high risk breast carcinoma has been related to increased overall mortality, distinct metastasis and locoregional recurrence [44]. In Bcl-2 positive tumors Pinzon-Charry provided evidence of good prognosis related to CD40 expression [32, 33] as we have done in this study. Moreover we found longer overall survival in patients with cytoplasmic CD40 expression.

Nuclear expression of CD40 has been described in B lymphocytes and in B cell lymphomas [22, 45] but not in epithelial cells. Zhou et al [22] showed that the function of CD40 is not limited to signal transduction through plasma membrane into cytoplasm. Similar to receptors for growth factors EGFR and FGFR [46, 47] that can serve as transcription factors, after transport into the nucleus via importin-α a β [48] CD40 interacts with transcription factors and with promotors of transcription factors [45]. It is not the only member of the TNF receptor superfamily with nuclear localization. Nuclear expression has also been described in p75 NGFR where it interacts with cyclin E1 promotor [49], but not with other members of the superfamily. In our study as in the work of Zhou et al we used the same antibody for staining. The antibody reacts with the cytoplasmic C-terminus of the CD40 molecule which is involved in nuclear transport. Membrane and cytoplasmic expression is related to inactivity and dormancy of cells and after activation nuclear localization appears [20, 45],. In aggressive B cell lymphoma nuclear localization leads to mitotic activation and higher proliferation. A similar observation was made for breast cancer. In normal breast ducts, nuclear expression of CD40 was present in luminal cells; basal cells that were in the stage of dormancy were negative. In breast cancer cells, nuclear CD40 expression was found in 83.4%. Although no relation between nuclear CD40 expression in breast cancer and proliferative activity (measured as expression of PCNA) was found, expression in breast cancer was clearly higher than in normal breast ducts (30% to 83.4%). It is also interesting that in 76.5 % of tumors with preoperative chemotherapy, we observed higher intensity of nuclear staining. This could reflect the influence of therapy on the behavior of the CD40 molecule and downstream proteins related to cell proliferation. Nuclear expression of CD40 was not related to expression of hormonal receptors or her-2/neu and there was no relation to other clinicopathological data.

CD40 in breast cancer is expressed in different cellular compartments but for clinical behavior most important is cytoplasmic expression. Expression in breast carcinomas is more

frequently associated with expression of hormonal receptors and in luminal A subgroup it is associated with lower tumor stage. *Vice versa,* loss of expression is more often related to advanced tumor stage, relapse of the disease and loss of Bcl-2 expression which is a negative prognostic marker. Cytoplasmic expression correlates with longer overall survival though this was not statistically significant. Nuclear expression of CD40 is a biological phenomenon related to cell proliferation that is more common in cancer cells than in normal ducts and reaches higher intensity after chemotherapy.

In conclusion, cytoplasmic expression of CD40 was found to correlate with factors connected with better prognosis and these could indicate CD40 as a potential new prognostic factor for breast cancer.

Acknowledgments: The work was supported by grants 80110091, GACR 303/09/H048 and MSM 6198959216 and project Biomedicine for regional development and human resources (BIOMEDREG) C.1.05/2.1.00/01.0030.

References

[1] FERLAY J, PARKIN DM, STELIAROVA-FOUCHER E Estimates of cancer incidence and mortality in Europe in 2008. Eur J Cancer 2010; 46: 765-81. doi:10.1016/j.ejca.2009.12.014

[2] SØRLIE T, PEROU CM, TIBSHIRANI R, AAS T, GEISLER S et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci U S A 2001; 98: 10869-74. doi:10.1073/pnas.191367098

[3] MUÑOZ M, FERNÁNDEZ-ACEÑERO MJ, MARTÍN S, SCHNEIDER J Prognostic significance of molecular classification of breast invasive ductal carcinoma. Arch Gynecol Obstet 2008; 280: 43-8.

[4] BRENTON JD, CAREY LA, AHMED AA, CALDAS C Molecular classification and molecular forecasting of breast cancer: ready for clinical application? J Clin Oncol 2005; 23: 7350-60. doi:10.1200/JCO.2005.03.3845

[5] JANSEN MP, FOEKENS JA, VAN STAVEREN IL, DIRKZWAGER-KIEL MM, RITSTIER K et al. Molecular classification of tamoxifen-resistant breast carcinomas by gene expression profiling. J Clin Oncol 2005; 23: 732-40. doi:10.1200/JCO.2005.05.145

[6] SØRLIE T, WANG Y, XIAO C, JOHNSEN H, NAUME B et al. Distinct molecular mechanisms underlying clinically relevant subtypes of breast cancer: gene expression analyses across three different platforms. BMC Genomics 2006; 7: 127. doi:10.1186/1471-2164-7-127

[7] LOI S Molecular analysis of hormone receptor positive (luminal) breast cancers: what have we learnt? Eur J Cancer 2008; 44: 2813-8. doi:10.1016/j.ejca.2008.09.012

[8] DAWOOD S, HU R, HOMES MD, COLLINS LC, SCHNITT SJ et al. Defining breast cancer prognosis based on molecular phenotypes: results from a large cohort study. Breast Cancer Res Treat. 2010; 10: 1113-7.

[9] CHEANG MC, CHIA SK, VODUC D, GAO D, LEUNG S et al. Ki67 index, HER2 status, and prognosis of patients with luminal B breast cancer. J Natl Cancer Inst. 2009; 101: 736-50. doi:10.1093/jnci/djp082

[10] KATZENELLENBOGEN BS Antiestrogen resistance: mechanisms by which breast cancer cells undermine the effectiveness of endocrine therapy. J Natl Cancer Inst 1991; 83: 1434-5. doi:10.1093/jnci/83.20.1434

[11] ROUSSET F, GARCIA E, BANCHEREAU J Cytokine-induced proliferation and immunoglobulin production of human B lymphocytes triggered through their CD40 antigen. J Exp Med 1991; 173: 705-10. doi:10.1084/jem.173.3.705

[12] ALDERSON MR, ARMITAGE RJ, TOUGH TW, STROCKBINE L, FANSLOW WC et al. CD40 expression by human monocytes: regulation by cytokines and activation of monocytes by the ligand for CD40. J Exp Med 1993; 178: 669-74. doi:10.1084/jem.178.2.669

[13] ZHANG X, KEDL RM, XIANG J CD40 ligation converts TGF-beta-secreting tolerogenic CD4(-)8(-) dendritic cells into IL-12-secreting immunogenic ones. Biochem Biophys Res Commun 2009; 379: 954-8. doi:10.1016/j.bbrc.2008.12.179

[14] BALKOW S, LOSER K, KRUMMEN M, HIGUCHI T, ROTHOEFT T et al. Dendritic cell activation by combined exposure to anti-CD40 plus interleukin (IL)-12 and IL-18 efficiently stimulates anti-tumor immunity. Exp Dermatol 2009; 18: 78-87. doi:10.1111/j.1600-0625.2008.00800.x

[15] ALEXANDROFF AB, JACKSON AM, PATERSON T, HALEY JL, ROSS JA et al. Role for CD40-CD40 ligand interactions in the immune response to solid tumours. Mol Immunol 2000; 37: 515-26. doi:10.1016/S0161-5890(00)00079-1

[16] COOKE PW, JAMES ND, GANESAN R, WALLACE M, BURTON A et al. CD40 expression in bladder cancer. J Pathol 1999; 188: 38-43. doi:10.1002/(SICI)1096-9896(199905)188:1<38::AID-PATH315>3.0.CO;2-B

[17] GHAMANDE S, HYLANDER BL, OFLAZOGLU E, LELE S, FANSLOW W et al. Recombinant CD40 ligand therapy has significant antitumor effects on CD40-positive ovarian tumor xenografts grown in SCID mice and demonstrates an augmented effect with cisplatin. Cancer Res 2001; 61: 7556-62.

[18] POSNER MR, CAVACINI LA, UPTON MP, TILLMAN KC, GORNSTEIN ER et al. Surface membrane-expressed CD40 is present on tumor cells from squamous cell cancer of the head and neck in vitro and in vivo and regulates cell growth in tumor cell lines. Clin Cancer Res 1999; 5: 2261-70.

[19] TONG AW, STONE MJ Prospects for CD40-directed experimental therapy of human cancer. Cancer Gene Ther 2003; 10: 1-13. doi:10.1038/sj.cgt.7700527

[20] BROUTY-BOYÉ D, POTTIN-CLÉMENCEAU C, DOUCET C, JASMIN C, AZZARONE B Chemokines and CD40 expression in human fibroblasts. Eur J Immunol 2000; 30: 914-9. doi:10.1002/1521-4141(200003)30:3<914::AID-IMMU914>3.0.CO;2-D

[21] HIRANO A, LONGO DL, TAUB DD, FERRIS DK, YOUNG LS et al. Inhibition of human breast carcinoma growth by a soluble recombinant human CD40 ligand. Blood 1999; 93: 2999-3007.

[22] LIN-LEE YC, PHAM LV, TAMAYO AT, FU L, ZHOU HJ et al. Nuclear localization in the biology of the CD40 receptor in normal and neoplastic human B lymphocytes. J Biol Chem 2006; 281: 18878-87. doi:10.1074/jbc.M513315200

[23] TAVASSOLI FA, DEVILEE P, editors. World Health Organization Classification of Tumours. Pathology and genetics of tumours of the breast and female genital organs. Lyon: IARC Press, 2003.

[24] WOLFF AC, HAMMOND ME, SCHWARTZ JN et al. American Society of Clinical Oncology/College of American Pathologists Guideline Recommendations for Human Epidermal Growth Factor Receptor 2 Testing in Breast Cancer. Arch Pathol Lab Med 2007; 131: 18.

[25] HARVEY JM, CLARK GM, OSBORNE CK, ALLRED DC Estrogen receptor status by immunohistochemistry is superior to the ligand-binding assay for predicting response to adjuvant endocrine therapy in breast cancer. J Clin Oncol 1999; 17: 1474-81.

[26] PALMER DH, HUSSAIN SA, GANESAN R, COOKE PW, WALLACE DM et al. CD40 expression in prostate cancer: a potential diagnostic and therapeutic molecule. Oncol Rep 2004; 12: 679-82.

[27] MOGHADDAMI M, COHEN P, STAPLETON AM, BROWN MP CD40 is not detected on human prostate cancer cells by immunohistologic techniques. Urology 2001; 57: 573-8. doi:10.1016/S0090-4295(00)01005-0

[28] ISHIKAWA K, MIYAMOTO M, YOSHIOKA T, KATO T, KAJI M et al. Up-regulation of CD40 with juxtacrine activity in human nonsmall lung cancer cells correlates with poor prognosis. Cancer 2008; 113: 530-41. doi:10.1002/cncr.23618

[29] KOMI J, LASSILA O Toremifene increases the expression of intercellular adhesion molecule-1 (ICAM-1) on MCF-7 breast cancer cells and Jurkat cells. Scand J Immunol. 2000; 51: 73-8. doi:10.1046/j.1365-3083.2000.00653.x

[30] GERALDES P, GAGNON S, HADJADJ S, MERHI Y, SIROIS MG et al. Estradiol blocks the induction of CD40 and CD40L expression on endothelial cells and prevents neutrophil adhesion: an ERalpha-mediated pathway. Cardiovasc Res. 2006; 71: 566-73. doi:10.1016/j.cardiores.2006.05.015

[31] ESTEVA FJ, WANG J, LIN F, MEJIA JA, YAN K et al. CD40 signaling predicts response to preoperative trastuzumab and concomitant paclitaxel followed by 5-fluorouracil, epirubicin, and cyclophosphamide in HER-2-overexpressing breast cancer. Breast Cancer Res 2007; 9: R87. doi:10.1186/bcr1836

[32] PINZON-CHARRY A, SCHMIDT CW, LÓPEZ JA The key role of CD40 ligand in overcoming tumor-induced dendritic cell dysfunction. Breast Cancer Res 2006; 8: 402. doi:10.1186/bcr1386

[33] PINZON-CHARRY A, MAXWELL T, MCGUCKIN MA, SCHMIDT C, FURNIVAL C et al. Spontaneous apoptosis of blood dendritic cells in patients with breast cancer. Breast Cancer Res 2006; 8: R5. doi:10.1186/bcr1361

[34] VONDERHEIDE RH Prospect of targeting the CD40 pathway for cancer therapy. Clin Cancer Res 2007; 13: 1083-8. doi:10.1158/1078-0432.CCR-06-1893

[35] BEREZNAYA NM, CHEKHUN VF Expression of CD40 and CD40L on tumor cells the role of their interaction and new approach to immunotherapy. Exp Oncol 2007; 29: 2-12.

[36] VAUX DL Toward an understanding of the molecular mechanisms of physiological cell death. Proc Natl Acad Sci U S A 1993; 90: 786-9. doi:10.1073/pnas.90.3.786

[37] BASU A, HALDAR S The relationship between Bcl2, Bax and p53: consequences for cell cycle progression and cell death. Mol Hum Reprod 1998; 4: 1099-109. doi:10.1093/molehr/4.12.1099

[38] YIP KW, REED JC Bcl-2 family proteins and cancer. Oncogene 2008; 27: 6398-406. doi:10.1038/onc.2008.307

[39] Zamzami N, Brenner C, Marzo I, Susin SA, KROEMER G Subcellular and submitochondrial mode of action of Bcl-2-like oncoproteins. Oncogene 1998; 16: 2265-82. doi:10.1038/sj.onc.1201989

[40] BATCHELDER AJ, GORDON-WEEKS AN, WALKER RA Altered expression of anti-apoptotic proteins in non-involved tissue from cancer-containing breasts. Breast Cancer Res Treat 2009; 114: 63-9. doi:10.1007/s10549-008-9988-2

[41] CALLAGY GM, WEBBER MJ, PHAROAH PD, CALDAS C Meta-analysis confirms BCL2 is an independent prognostic marker in breast cancer. BMC Cancer 2008; 8: 153. doi:10.1186/1471-2407-8-153

[42] CALLAGY GM, PHAROAH PD, PINDER SE, HSU FD, NIELSEN TO et al. Bcl-2 is a prognostic marker in breast cancer independently of the Nottingham Prognostic Index. Clin Cancer Res 2006; 12: 2468-75. doi:10.1158/1078-0432.CCR-05-2719

[43] TRERÉ D, MONTANARO L, CECCARELLI C, BARBIERI S, CAVRINI G et al. Prognostic relevance of a novel semiquantitative classification of Bcl2 immunohistochemical expression in human infiltrating ductal carcinomas of the breast. Ann Oncol 2007; 18: 1004-14. doi:10.1093/annonc/mdm074

[44] KYNDI M, SØRENSEN FB, KNUDSEN H, ALSNER J, OVERGAARD M et al. Impact of BCL2 and p53 on postmastectomy radiotherapy response in high-risk breast cancer. A subgroup analysis of DBCG82 b&c. Acta Oncol 2008; 47: 608-17. doi:10.1080/02841860802050746

[45] ZHOU HJ, PHAM LV, TAMAYO AT, LIN-LEE YC, FU L et al. Nuclear CD40 interacts with c-Rel and enhances proliferation in aggressive B-cell lymphoma. Blood 2007; 110: 2121-7. doi:10.1182/blood-2007-02-073080

[46] LIN SY, MAKINO K, XIA W, MATIN A, WEN Y et al. Nuclear localization of EGF receptor and its potential new role as a transcription factor. Nat Cell Biol 2001; 3: 802-8. doi:10.1038/ncb0901-802

[47] WELLS A, MARTI U Signalling shortcuts: cell-surface receptors in the nucleus? Nat Rev Mol Cell Biol 2002; 3: 697-702. doi:10.1038/nrm905

[48] LANGE A, MILLS RE, LANGE CJ, STEWART M, DEVINE SE et al. Classical nuclear localization signals: definition, function, and interaction with importin alpha. J Biol Chem 2007; 282: 5101-5. doi:10.1074/jbc.R600026200

[49] PARKHURST CN, ZAMPIERI N, CHAO MV Nuclear localization of the p75 neurotrophin receptor intracellular domain. J Biol Chem 2010; 285: 5361-8. doi:10.1074/jbc.M109.045054