New ways to optimize breast cancer treatment

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Death receptors in primary breast cancer

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ABSTRACT

Introduction: Death receptors Fas (receptor for Fas Ligand, FasL), and DR4 and DR5 (receptors for TNF-Related Apoptosis Inducing Ligand, TRAIL) in primary breast tumors, are likely related to apoptosis induction. They may be of interest for breast cancer treatment. Therefore, the presence of death receptors (Fas, DR4 and DR5), and Fas Ligand (FasL) was evaluated using immunostaining. Proliferation (Ki-67), apoptotic index, and apoptosis inhibition (Bcl-2) were evaluated using immunostaining. In addition, since death receptors may be up-regulated by estrogen deprivation, these parameters were evaluated in a series of tumors after pre-operative anti-estrogen therapy. Patients and methods: Primary breast tumors from 35 pre-menopausal, progesterone receptor (PR) positive, breast cancer patients were obtained. Nineteen patients had not received pre-operative treatment; 16 patients had received pre-operative tamoxifen (40 mg p.o., daily for 7-10 days), and LH-RH agonist gosereline (3.6 mg s.c. injection, once). Tumors were stained for Fas, FasL, DR4, DR5, Ki-67 and Bcl-2, immunohistochemically. Apoptotic cell counts were studied microscopically and expressed as apoptotic index (% of apoptotic cells). In five normal breast samples, Fas, FasL, DR4 and DR5 staining was assessed. Results: Tumors were positive for Fas (38%), DR4 (71%), DR5 (91%), or for combinations; all tumors were positive for at least one of these receptors. DR4 or DR5 staining was present in 97% of tumors. In all tumors, DR4 staining was positively correlated with DR5 staining ($p=0.006$), and Bcl-2 staining ($p=0.018$); FasL and apoptotic index were inversely correlated ($p=0.008$). No differences in the evaluated parameters were observed in tumors with or without anti-estrogen treatment. Normal breast samples were positive for Fas (100%), negative for DR4 (100%) and mostly negative for FasL and DR5 (both 75%). Conclusion: Death receptors, DR4 and DR5 in particular, are abundantly present immunohistochemically in primary breast tumors of PR+ pre-menopausal patients, while they are mostly absent in normal breast tissue. Short-

term anti-estrogen treatment does not further increase this. These results indicate that TRAIL could possibly be a tumor-specific future treatment for PR+ breast cancer.

INTRODUCTION

The role of the tumor necrosis factor (TNF) family in inducing programmed cell death or apoptosis in breast cancer cells has gained increasing interest in recent studies. Both Fas Ligand (FasL) and TNF-Related Apoptosis Inducing Ligand (TRAIL) were shown to have increased expressions in malignant breast tissue compared to benign counterparts (1-7). The expression of Fas (the receptor of FasL) was found to be decreased in many malignant (compared to benign) breast tumors (8), which was associated with a worse clinical outcome (2). A high FasL:Fas mRNA ratio was related to reduced disease-free survival and increased mortality in breast cancer patients (9). Upregulation of FasL or TRAIL may prevent tumor cell death by evading the immune system (3, 4, 10). Down-regulation of the Fas receptor, which upon binding with FasL induces apoptosis, may have a similar effect. Whether TRAIL receptors DR4 and DR5 are also affected in the course of malignant progression, has not been described yet in primary breast tumors. It might be suggested that the expression of death receptors and their ligands play an important role in breast cancer cells. The presence of death receptors in primary breast tumors is highly interesting, in view of the potential treatment possibilities for breast cancer. Treatment with TRAIL is of particular interest, as TRAIL induced apoptosis presumably takes place in a fairly tumor specific fashion (11-13), with potentially less systemic toxic effect in-vivo compared to FasL (14). The sensitivity for apoptosis induced by the anti-Fas antibody or TRAIL can be further enhanced by treatment with chemotherapeutic agents (15-17) in-vitro, as well as with radiation (14) in an animal model with a tumor from the human breast cancer cell line MCF-7. Also in response to deprivation of estrogen (the primary stimulant for breast cell proliferation (18, 19) up-regulation of death receptors Fas (20-22), and possibly DR5 (23, 24) was described in an animal model (20) or in-vitro (21-24). In pre-menopausal women with breast cancer, a combination of the (partial) estrogen

antagonist tamoxifen and the luteinizing hormone-releasing hormone (LH-RH) agonist gosereline induces estrogen deprivation comparable to castrate levels (25). This estrogen blockade has recently gained increasing interest (26), as the optimal result of adjuvant treatment in pre-menopausal hormone receptor positive breast cancer patients can be obtained by a combination of chemotherapy and estrogen deprivation (27).

In this light, the current study was performed to evaluate the presence of death receptors Fas, DR4 and DR5, as well as FasL in primary progesterone receptor positive breast tumors, using immunohistochemical techniques. Results were related to expression of the anti-apoptotic protein Bcl-2, and apoptotic- and proliferative index (Ki-67 staining with the antibody MIB-1). In addition, we examined whether the above mentioned parameters were affected by estrogen deprivation in a separate group of primary breast tumors, after pre-operative estrogen blockade (effected by partial estrogen antagonist tamoxifen and luteinizing hormone releasing hormone (LH-RH) agonist gosereline). To our knowledge, this is the first report including immunohistochemical assessment of death receptors DR4 and DR5 in primary breast cancer.

PATIENTS, MATERIALS AND METHODS

Patients

Archival primary breast cancer tissue was retrieved from pre-menopausal, progesterone-receptor (PR) positive, breast cancer patients. Staging of patients was performed according to the TNM system (Union Internationale Contre le Cancer, 1997). Samples from patients without pre-operative treatment were obtained from the Isala Clinics, Zwolle (the Netherlands), and the University Hospital Groningen (The Netherlands). Surgical treatment of these patients was performed in 1994 (Isala Clinics), or between April 1997 and August 1999 (University Hospital Groningen). From these time intervals, samples from all eligible patients (e.g.: pre-menopausal, PR+) were included. This group will be referred to as group I.

In addition, archival primary breast cancer tissue was retrieved from pre-menopausal, PR+ breast cancer patients who had received pre-operative treatment with 40 mg oral tamoxifen daily for 7-10 days (the interval between diagnosis and surgery), and a subcutaneous gosereline 3.6 mg injection, once. From all eligible patients (e.g.: pre-menopausal, PR+) who had received this treatment between February 1990 and May 1996 (Isala Clinics, Zwolle), samples were included. This group will be referred to as group II.

Control archival normal breast samples (n=5) were obtained from pre-menopausal women, operated upon between 1996 and 1999 (University Hospital Groningen). Four samples were derived from patients after prophylactic ablation of the breast, and one sample was obtained after breast reduction; this sample was not found to contain epithelial hypertrophy.

Immunohistochemistry

All primary tumor samples were fixed in 4% formalin and paraffin embedded; 4 µm sections were prepared. The presence of carcinoma in the sections was checked using standard hematoxylin-eosin (H&E) staining on parallel sections.

Prior to staining, slides were deparaffinized and dehydrated. Air-drying was performed for at least 15 min prior to antigen-retrieval, except prior to DR4 staining. Antigen retrieval was performed by means of autoclave treatment with heating 3 times for 5 min at 115 ºC in blocking reagent (2% block + 0.2% SDS in maleic acid, pH=6.0, Roche, Mannheim, Germany) for Fas, FasL, Bcl-2 and Ki-67 (MIB-1 antibody) staining; or by means of adding slides to 10mM citric acid monohydrate (Merck, Darmstadt, Germany) solution in demineralized water, pH=6.0, and subsequent microwave treatment at 100°C for 8 min at 700W for DR5 staining.

Endogenous peroxidase activity was blocked by treatment of slides with 1% peroxide in phosphate buffered saline solution (PBS, 0.14 M NaCl, 2.7 mM KCl, 6.4 mM $Na_2HPO_4.2H_2O$, 1.5 mM KH_2PO_4, pH 7.8) for 30 min. Slides were then washed twice with PBS. Slides were pre-incubated with 1% AB serum, 1% bovine serum albumin (BSA, Life Technologies, Breda, The Netherlands) in PBS, for 30 min. For DR5 staining, slides were additionally pre-incubated with avidin and biotin blocking reagent (Vector laboratories, Burlingame, CA).

All antibodies were supplied by DAKO (Glostrup, Denmark) except if indicated differently, and they were diluted in PBS, 1% BSA. Slides were then stained for 60 min with anti-FasL antibody (Transduction Laboratories, Lexington KY), diluted 1:160; anti-Fas antibody (CH11, Upstate Biothechnology, Waltham, MA), diluted 1: 100; anti-DR4 antibody (Santa Cruz, Santa Cruz, CA), diluted 1: 50; anti-DR5 antibody (Oncogene, Cambridge, MA), diluted 1:100; anti-Bcl-2 antibody, diluted 1:50, or anti-Ki67 antibody (MIB-1, Immonotech, Marseille, France) diluted 1:400. For the negative control, no antibody was added to the PBS. Positive controls were (tumor) tissue

samples, found on previous occasions to stain positive: liver tissue for Fas, FasL and DR5; colon cancer tissue for DR4 and Ki-67; cervical cancer tissue for Bcl-2.

Slides were washed with PBS three times, and then incubated for 30 min with the secondary antibody Rabbit Anti Mouse-bio, diluted 1:300 (for Fas, FasL and DR4), Rabbit Anti Mouse-peroxidase diluted 1:300 (for Ki-67 and Bcl-2) or Swine Anti Rabbit-bio diluted 1:300 (for DR5), followed by washing in PBS 3 times. Slides were then incubated for 15 min with streptavidin, diluted 1:300 (for Fas, FasL, DR4 and DR5), or Goat Anti Rabbit-peroxidase streptavidin, diluted 1:300 (for Ki-67 and Bcl-2), followed by washing in PBS 3 times. For the peroxidase reaction, di-aminobenzidine (DAB: Sigma Cemical CO., St. Louis, MO), 25 mg was dissolved in 50 mL imidazol solution (1 mg/mL PBS), to which 50 µL 30% peroxide was added prior to use. After this staining, slides were washed with demineralized water. Counter-staining was performed with hematoxylin for 2 min, after which slides were washed with regular water for 5 min. Slides were subsequently dehydrated, air-dried, and embedded in mounting medium.

Staining was scored by two individual examiners: in line with Mottolese et al. (2) for Fas and FasL: <10% of tumor cells staining positive was regarded negative; 10-50% of cells staining positive was regarded heterogeneous and > 50% of cells staining positive was regarded positive. The DR4 staining was scored in a similar way as Fas and FasL. Ki-67 staining with MIB-1 was scored in percentage of stained nuclei (in categories of 10%); Bcl-2 and DR5 staining was scored as no staining (0), weak staining (1), moderate staining (2) and strong staining (3). Apoptotic index was determined in H&E slides as the percentage of cells with nuclei showing clumping of chromatin. Of each tumor, at least 3 times 200 cells were scored.

Staining for PR and estrogen receptor (ER) was performed (for confirmation of progesterone and estrogen receptor status) in all samples with a standardized method. After deparaffinization, antigen retrieval was performed as described above for Fas and

FasL staining. An automated immunostainer was used (Ventana Medical Systems Inc., Tucson, AZ), with the anti-PR or anti-ER antibody (Ventana Medical Systems Inc., Tucson, AZ). Slides were subsequently dehydrated, air-dried, and embedded in mounting medium. Samples were scored according to a standard protocol, accounting for the proportion of stained cells as well as the intensity of staining, and samples were considered positive when the overall score was ≥ 3 (28). The positive or negative distinction was used for further analyses.

Statistics

Statistics were performed with SPSS software. The Mann-Whitney U-test was used for comparing immunohistochemical staining of Fas, FasL, DR4, DR5, Ki-67, Bcl-2 and apoptotic index, between group I and group II. Age of patients was compared between group I and group II by means of the Student's t-test. Correlations between Fas, FasL, DR4, DR5, Ki-67, Bcl-2 staining and apoptotic index, and tumor stage, were determined with the Pearson correlation test. P-values ≤ 0.05 were considered statistically significant.

RESULTS

Patients

Patient characteristics are shown in table 1.

Immunohistochemistry

Immunohistochemical staining and apoptotic index are shown in table 2. All stainings were found to be cytoplasmatic, except with the Ki-67 staining with the MIB-1 antibody (nuclear). An example of DR4 and DR5 staining is shown in figure 1. For Fas, FasL and DR4, intensity of staining within the areas comprising malignant tumor, was heterogeneous. Therefore, for scoring these stainings, the percentage of malignant tumor that stained positively was used. The intracellular FasL staining was found to be vesicle-like. The DR5 and Bcl-2 staining was usually homogeneous, allowing scoring for intensity. Ki-67 staining with MIB-1 was nuclear and vesicle-like. Both DR4 and DR5 staining were usually also detected in the normal tissue, surrounding the tumor. This staining was restricted to the epithelial cells, and was less intense than in the neighboring tumor tissue. A similar staining pattern was found with FasL, but with an outspoken distinction between normal surrounding tissue (negative) and malignant parts (positive). In contrast, the Fas staining was generally present in normal epithelial cells, while staining intensity was decreased in the malignant parts. One tumor sample, containing a small part of ductal carcinoma in situ (DCIS) next to an invasive tumor, was found to be positively stained in the normal part and the DCIS part, but was negative in the invasive tissue. Control normal breast samples were generally found Fas positive (5 of 5 samples), FasL negative (3 of 4 samples negative, one sample not assessable), DR4 negative (5 of 5 samples) and DR5 negative (3 of 4 samples negative, 1 sample weakly positive, one sample not assessable).

Between group I and II (with or without anti-estrogen treatment), no statistical differences were found for any of the parameters. This indicates that the investigated parameters were unchanged by the short term administration of anti-estrogen treatment. When both groups were combined, 38% of tumors were Fas-positive and 80% was FasL-positive. Positivity for DR4 (71% of tumors) and DR5 (91% of tumors) was considerable, as was the presence of combinations of death receptors (DR4 and DR5: 68% of tumors; DR4 and DR5 and Fas: 24% of tumors). Nearly all (97%) tumors were positive for either DR4 or DR5. All tumors were positive for at least one death receptor. The occurrence of combinations of these death receptors is shown in table 3. As no difference regarding the stainings was found between the two groups, both groups were shown combined.

Concomitant DR4- and DR5-staining in the combined groups, was found to be positively correlated to apoptotic index ($r=0.344$, $p=0.046$), but the combination of DR4 and DR5 and Fas, or Fas and FasL was not correlated to apoptotic index. A positive correlation was observed between DR4- and DR5-staining ($r=0.465$, $p=0.006$, reflected in figure 2), and between DR4- and Bcl-2 immunoreactivity ($r=0.402$, $p=0.018$, reflected in figure 3). A negative correlation was observed between FasL staining and apoptotic index ($r=-0.449$, $p=0.008$, figure 4).

DISCUSSION

An increasing number of studies indicate a role for death receptors and their ligands in breast cancer. For Fas and FasL in particular, changes in expression have been described, in the course of malignant progression. Fas was found to be decreased and FasL increased, compared to benign counterparts (1-3, 5-9). In most studies (1-4, 6-9), but not all (5), these changes have been attributed to the advantages they create for tumor cells to evade immune responses. TRAIL expression in tumor cells may also present an immunologic advantage (10). While one study reported the presence of the ligand TRAIL in primary breast tumors (7), the status of the death receptors DR4 and DR5 in primary breast tumors was unknown so far. Assessment of these receptors in tumors is particularly interesting in view of the potential use of TRAIL in the treatment of breast cancer. TRAIL induced apoptosis presumably takes place in a fairly tumor specific fashion, through a family of agonistic (DR4 and DR5) and antagonistic receptors (DcR1 and DcR2) (11-13). This aspect renders the clinical use of recombinant human rhTRAIL of potential interest. Furthermore, rhTRAIL does not appear to have the systemic toxic effect in-vivo as treatment with FasL (14), although in-vitro work suggests that hepatoxicity by a polyhistidine-tagged recombinant TRAIL in humans may occur (29). This aspect needs further attention (30). The recent finding that native-sequence (non-tagged), clinical grade rhTRAIL had minimal toxicity towards human hepatocytes and absence of hepatotoxicity in cynomolgus monkeys following repeated administration of intravenous properly folded rhTRIAL is reassuring (31). It may well be that clinical-grade rhTRAIL is suitable for investigation in cancer patients (31). In view of the potential use of rhTRAIL to induce apoptosis in breast cancer, means to upregulate the TRAIL receptors DR4 and DR5 are of interest. Possibly, estrogen deprivation may be one of those means (23, 24). Other TNF receptor family members Fas (20-22) and TNFR1 (32) were also shown to be up-regulated in

breast cancer cells by treatment with some anti-estrogens (20-22, 32), but not with others (32, 33). The current immunohistochemical study was performed to gain more information on the presence of death receptors Fas, DR4 and DR5 and Fas ligand in primary breast tumor samples. Also, proliferation and apoptosis were examined. In addition, the effects of estrogen blockade by means of tamoxifen and gosereline on these parameters was examined.

In line with other studies (1-7), we found a high percentage of FasL positive tumors (80%), and a lower percentage (38%) of Fas positive tumors. Most Fas positive tumors were also positive for FasL (12/13). DR4 and DR5 staining was found present in the vast majority of tumors. Most tumors were DR4 positive (71%), while other tumors showed a more heterogenous staining pattern, or were negative. A large majority of tumors was DR5 positive (91%), and strong staining was found in a number of these samples. In addition, the majority of tumors were actually positive for both DR4 and DR5 (68%), and these stainings were correlated. The simultaneous presence of DR4 and DR5 was also found to be correlated to apoptotic index. Nearly all tumors (97%) were positive for either DR4 or DR5. These results indicate that DR4 and DR5 are abundantly present in primary breast tumors and could serve as a possible target for TRAIL induced apoptosis. In-vitro data have indicated the presence of these receptors in breast cancer cell lines (16), but sofar this has not been confirmed in primary breast cancer. In normal pre-menopausal breast samples, we found no samples positive for DR4, while one sample was weakly positive for DR5. Although the number of samples was small, this could possibly be an indication of the tumor specificity of these receptors, which may increase the clinical feasibility of rhTRAIL based therapy in breast cancer. However, the role of their antagonistic receptors (DcR1 and DcR2) in primary breast tumors in this respect, remains to be clarified. We also found correlations between FasL and apoptosis, and DR4 and Bcl-2 staining. The correlation between FasL staining and apoptotic index was negative, and

may therefore support the notion that increased FasL expression can possibly protect breast cancer tissue from apoptosis induced by Fas bearing immune cells (1, 3, 4, 6, 7). Since TRAIL induced apoptosis can be inhibited by increasing Bcl-2 levels (34), a positive correlation between DR4 and Bcl-2 staining may suggest a protective role for Bcl-2 in breast cancer. The need for such protection may follow from the fact that the majority of breast cancers are DR4 positive (this study), but may also be TRAIL positive (7): this could possibly imply the functional, or membrane related, DR4 receptor in our samples.

In this study, short-term anti-estrogen treatment with gosereline and tamoxifen was not shown to induce clear effects on the examined parameters: no indications were found for an acute increase of apoptosis due to this treatment. The fact that not all tumors in the anti-estrogen treatment group were ER+ (in spite of being all PR+) was presumably induced by the prior treatment itself (35). The Bcl-2 staining intensity tended towards a slight decrease after anti-estrogen treatment in our samples, but this was not significant. Anti-estrogen treatment induced a swift Bcl-2 decrease in-vitro (36-38), and maximal apoptosis after 48 h in an animal model (39). However, in the human in-vivo setting, effects of neo-adjuvant anti-estrogen treatment on Bcl-2 and apoptosis were shown after 14 days to 3 months (35, 40-42). It may be suggested that the duration of tamoxifen administration in our study (7 to 10 days) in combination with gosereline, was too short for detecting effects on proliferation and apoptosis. In addition, it has been described that the combination of tamoxifen and gosereline may actually induce an increase in serum estradiol levels, prior to a reduction of hormones to castrate levels after 3 weeks (25). This hormonal fluctuation could have affected parameters such as Bcl-2 staining, in the time-interval of the present study. Furthermore, this might also be related to the fact that no inhibition of FasL by tamoxifen was found in our in-vivo setting, in contrast to recent in-vitro data (43). In light of this, the in-vivo effects of anti-estrogen treatment on apoptosis

markers and death receptors, and its time-dependency in particular, remain to be clarified. To this end, preferably pre-and post-treatment samples will have to be used in larger studies. Particularly in view of the increasing interest for anti-estrogen treatment in pre-menopausal breast cancer patients (26, 27), evaluation of in-vivo effects on apoptosis and death receptors may help improve breast cancer treatment.

In conclusion, death receptors, and DR4 and DR5 in particular, are abundantly present in the majority of primary breast tumors, while they are mostly absent from normal breast tissue. Short-term anti-estrogen treatment did not increase this further. These results indicate that rhTRAIL treatment could possibly be a tumor-specific treatment in hormone receptor positive breast tumors.

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TABLE 1: **Patient characteristics**

		Group I (n=19) no treatment	**Group II** (n=16) anti-estrogen treatment
Mean age in years (range)		44 (34-52)	46 (32-52)
Disease stage	I	7	0
	II	11	13
	III	0	2
	IV	1	1
PR (n)	positive	19	16
ER (n)	positive	19	13
Tumor type	ductal	18	13
	lobular	1	2
	DCIS	-	1

TABLE 2: **Immunohistochemical staining and apoptosis**

		Group I (n=19) No treatment (tumor no./total no.; percentage)		**Group II** (n=16) Anti-estrogen treatment (tumor no. /total no.; percentag		
Fas	positive	7/18	(39 %)	6/16	(38 %)	
	negative/ heterogeneous	11/18	(61 %)	10/16	(62 %)	
	n.a.*	1/19				
Fas L	positive	16/19	(84 %)	12/16	(75 %)	
	negative/ heterogeneous	3/19	(16 %)	4/16	(25 %)	
DR4	positive	14/19	(74 %)	11/16	(69 %)	
	negative/ heterogeneous	5/19	(26 %)	5/16	(31 %)	
DR5	strong	3/18	(17%)	1/16	(6%)	
	moderate	8/18	(44%)	4/16	(25%)	
	weak	7/18	(39%)	8/16	(50%)	
	negative			3/16	(19%)	
	n.a.	1/19				
Bcl-2	strong	5/18	(28%)	2/16	(13%)	
	moderate	8/18	(44%)	5/16	(31%)	
	weak	5/18	(28%)	5/16	(31%)	
	negative			4/16	(25%)	p=0.09
	n.a.	1/19				
Ki-67	% (range)	mean 23 (0-60)		mean 16 (0-50)		
Apoptosis	% (range)	mean 1.3 (0-6)		mean 1.7 (0-5)		

* n.a.: not assessable

TABLE 3: **Combinations of death receptors (group I and II)**

(in 34 assessable tumors*)

		No. of tumors (percentage)	
Fas and FasL	Positive	12	(35%)
DR4 and DR5	Positive	23	(68%)
DR4 or DR5	Positive	33	(97%)
DR4, DR5 and Fas	Positive	8	(24%)
All death rec.	Negative	0	

***** combinations of death receptors could be determined in 34 tumors, as Fas and DR5 staining was not assessable in one tumor (see: table 2).

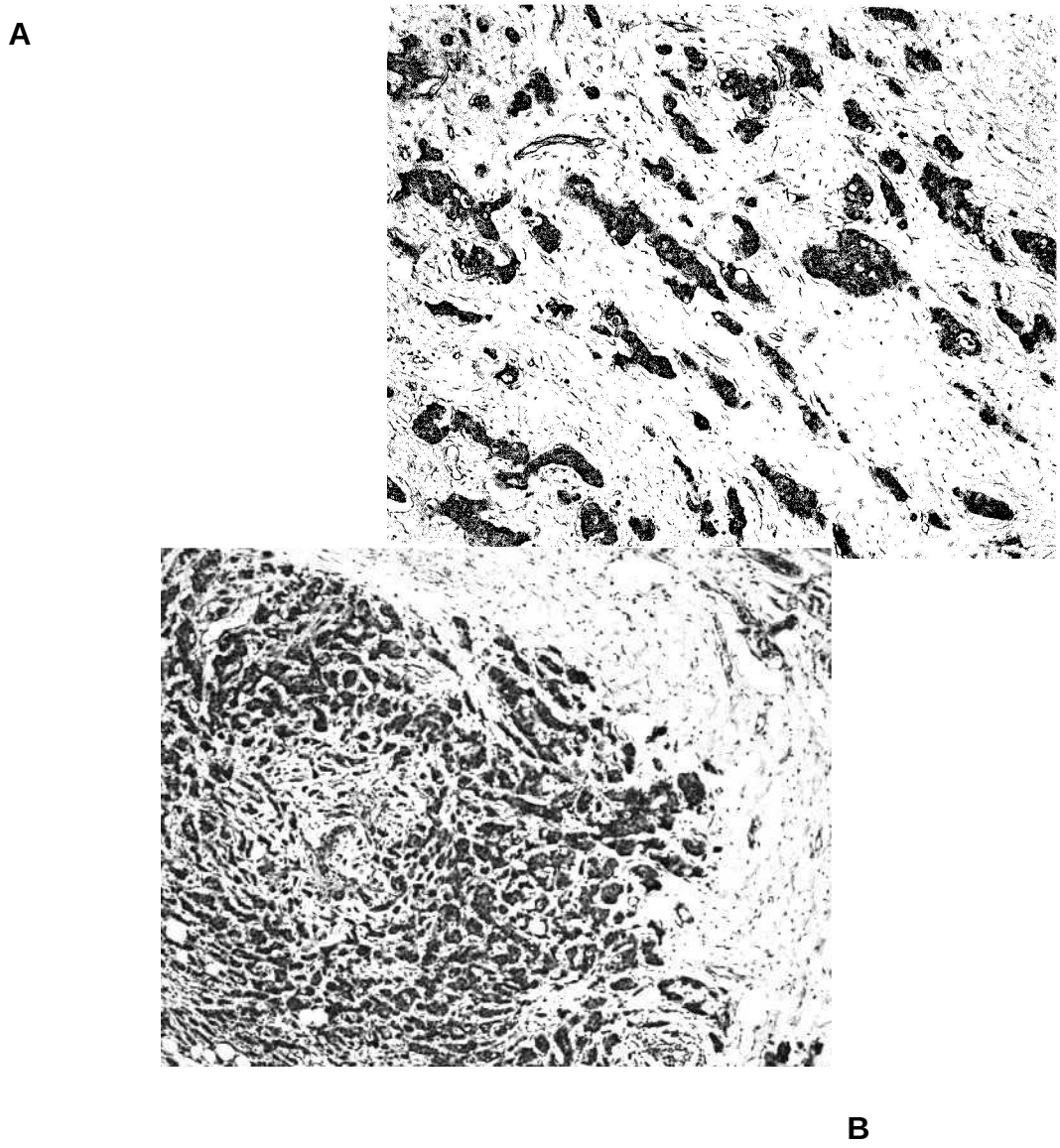

Figure 1: DR4 and DR5 staining
A: example of DR4 staining, magnification 10x10;
B: example of DR5 staining, magnification 10x10.

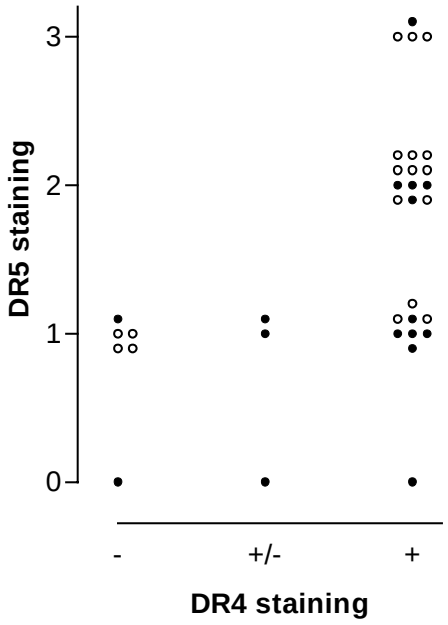

Figure 2: **relation of DR4 and DR5 staining**
X-axis: DR4 staining (negative, heterogeneous and positive); Y-axis: DR5 staining (0: negative, 1: weak, 2: moderate, and 3: strong). Open dot: no anti-estrogen treatment; solid dot: anti-estrogen treatment. DR4 and DR5 are positively correlated for samples of both groups: r=0.465, p=0.006. No difference in either DR4 or DR5 staining is observed between both groups.

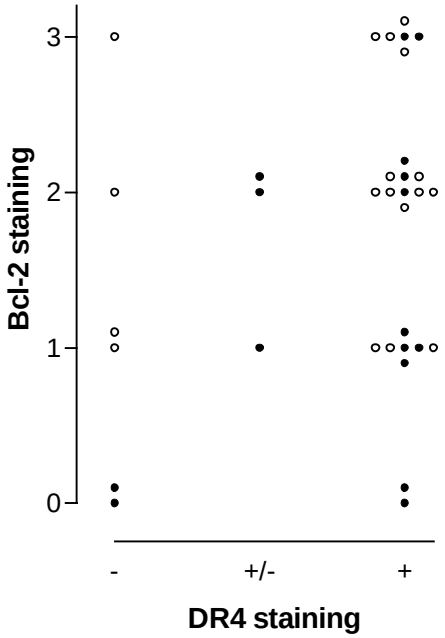

Figure 3: relation of DR4 and Bcl-2 staining
X-axis: DR4 staining (negative, heterogeneous and positive); Y-axis: Bcl-2 staining (0: negative, 1: weak, 2: moderate, and 3: strong). Open dot: no anti-estrogen treatment; solid dot: anti-estrogen treatment. DR4 and Bcl-2 are positively correlated for samples of both groups: r=0.402, p=0.018. No difference in either DR4 or Bcl-2 staining is observed between both groups.

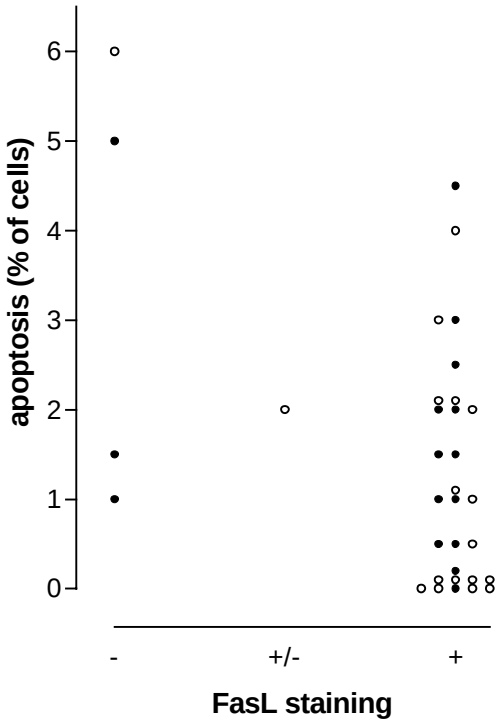

Figure 4: relation of FasL staining and apoptotic index
X-axis: FasL staining (negative, heterogeneous and positive); Y-axis: percentage of apoptotic cells (apoptotic index). Open dot: no anti-estrogen treatment; solid dot: anti-estrogen treatment. FasL and apoptosis are negatively correlated for samples of both groups: r=-0.449, p=0.008. No difference in either FasL or apoptotic index is observed between both groups.

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