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Expression of the apoptosis-inducing ligands FasL and TRAIL in malignant and benign human breast tumors

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Abstract Apoptosis-inducing ligands such as Fas ligand (FasL) and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) have been found to play an important role in cell regulation. Different malignant tumors show an altered expression of these ligands and their respective receptors compared to normal tissues. The purpose of this study was therefore to investigate expression of TRAIL, FasL, and its receptor Fas on protein and mRNA levels in breast carcinomas (n=40), fibroadenomas (n=7), and normal breast tissues (n=5). Immunohistochemical reaction demonstrated that FasL was strongly expressed in breast cancer tissues (34/40) while only one fibroadenoma and one normal breast tissue reacted weakly positive for FasL. All fibroadenomas and normal breast tissues as well as the majority of breast cancer tissues expressed Fas on protein level. Quantitative RT-PCR analysis detected high expression of FasL mRNA in breast cancer tissues and fibroadenomas, whereas fibroadenomas showed the highest Fas mRNA copy numbers, followed by breast cancer tissues and normal breast tissues (P<0.05). Compared to FasL expression, TRAIL could be detected in less breast cancer tissues on protein level (21/40) and was found in only one fibroadenoma and none of the normal breast tissues. Thus, it can be concluded that malignant breast tumors show an altered expression of the two apoptosis-inducing ligands FasL and TRAIL.

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Introduction

Apoptosis or programmed cell death plays an important role in normal cell regulation and is regulated by a variety of intracellular and extracellular signals. In recent years, cytokines of the tumor necrosis factor (TNF) family have been identified as taking part in the regulation of apoptosis. Fas ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL) induce apoptosis through their respective receptors thereby activating a cascade of caspases leading to initiation of apoptosis in the nucleus (Nagata 1997; Griffith et al. 1998). While FasL interacts with only the Fas receptor (Fas), a variety of receptors are described for TRAIL. DR-4 and DR-5 contain cytoplasmic death domains and therefore mediate apoptosis (Pan et al. 1997; Sheridan et al. 1997), while DcR1 lacks an intracellular signaling domain and DcR2 bears a truncated death domain, thus lacking the ability to mediate apoptosis upon stimulation (Goldstein 1997). It has been reported that a variety of malignant tumors show an altered expression and function of TRAIL and the FasL-Fas system (O'Connell et al. 1996; Strand et al. 1996; Niehans et al. 1997; Bennett et al. 1998; Lee et al. 1998; Rabinowich et al. 1998; Rieger et al. 1999). Due to upregulation of FasL and TRAIL, downregulation of Fas, or by interruption of the signaling pathway downstream of Fas, malignant tumors evade normal cell regulation by the immune system via induction of apoptosis. This study was designed to analyze expression of the apoptosis-inducing ligands FasL and TRAIL in malignant and benign breast tumors. As a possible mechanism for the tumor's immune escape we tried to identify altered expression of the ligands and the Fas receptor in breast cancer tissue compared to fibroadenomas and normal breast tissue.

Materials and methods

Patients and tissue collection

Tissue samples of 40 unselected primary breast carcinomas, seven fibroadenomas, and five normal breast tissues were collected dur-

ing surgery at the Department of Obstetrics and Gynecology of the University of Rostock between 1996 and 1998. None of the patients had received chemo-, radio-, or immunotherapy prior to surgery. the mean age for breast cancer patients was 59 years (range 33–82 years), for patients with a fibroadenoma 35 years (range 17–48 years), and for patients with normal breast tissue 31 years (range 20–42 years). Eight (20%) patients with breast cancer were premenopausal and 32 (80%) were postmenopausal. Thirty-five tumors (87.5%) were invasive ductal carcinomas, three were ductal carcinomas in situ, and two were medullary carcinomas (WHO criteria). Tumor grading according to the histoprognostic grading of Bloom and Richardson included G1 (n=13; 32.5%), G2 (n=19; 47.5%), and G3 (n=8; 20%). Women with bilateral breast carcinomas or multiple fibroadenomas were excluded. Upon submission of the investigation to the ethics committee of the Rostock Medical Association it was ruled that no approval by the committee was necessary.

Cell culture

Malignant breast cancer cell lines MCF-7 (ECACC number 86012803) and T47D (ECACC number 85012201) were used for immunofluorescence detection of Fas and FasL. Cells were cultured in DMEM, containing 10% FCS, 2 mM glutamine, and 1% penicillin/streptomycin.

Real-time RT-PCR for FasL and Fas

Total RNA was prepared using the acid guanidium thiocyanate-phenol-chloroform protocol. A minimum of 30 mm^3 tissue taken out of macroscopically well-defined areas of the tumors was used for RNA extraction. All specimens were tested by analysis of housekeeping gene expression using conventional RT-PCR. First-trimester placenta (FasL) from women undergoing legal abortion and liver mRNA (Fas) served as positive controls. The primer pairs and probes were designed using the Primer Express 1.0 program (PE Applied Biosystems, Foster City, Calif., USA). Oligonucleotide hybridization probes and primer pairs with the following sequences were synthesized as follows: FasL: TaqMan probe 5'-TCC AAC TCA AGG TCC ATG CCT CTG G, forward primer 5'-AAA GTG GCC CAT TTA ACA GGC, and reverse primer 5'-AAA GCA GGA CAA TTC CAT AGG TG. The corresponding sequences for Fas are: TaqMan probe 5'-AAT CAT CAA GGA ATG CAC ACT CAC CAG CA, forward primer 5'-ACT GTG ACC CTT GCA CCA AAT, and reverse primer 5'-GCC ACC CCA AGT TAG ATC TGG. Primers and probes were obtained from Applied Biosystems (Weiterstadt, Germany). The primers yielded RT-PCR products of 82 (FasL) and 105 (Fas) nucleotides.

The TaqMan EZ RT-PCR kit (PE Applied Biosystems) was used for reverse transcription and amplification of both targets and standards. All RT-PCR reactions were performed in duplicate with a final volume of 25 µl in the ABI PRISM 7700 SDS (PE Applied Biosystems). Reaction conditions were as follows: 2 min at 50°C, 30 min at 60°C, 5 min at 95°C, 35 cycles with 20 s at 94°C, and 1 min at 60°C. Quantification of RNA standards was linear over eight logs and the assay measures as little as 100 copies of FasL or Fas mRNA per tube. Preparation of the RNA standard was done as previously reported (Reimer et al. 2000). All results are expressed as copy numbers per 200 ng total RNA.

Conventional RT-PCR for TRAIL

cDNA was synthesized from 1 µg RNA in a final volume of 20 µl using SuperScript II (Life Technologies, Gaithersburg, N.Y., USA) and oligo(dT) priming (Life Technologies). As previously published (Rieger et al. 1999), PCR conditions were as followed: 35 cycles, 45 s at 95°C, 45 s at 58°C, 45 s at 72°C, forward primer 5'-AGA CCT GCG TGC TGA TCG TG (nucleotides 131–150), and reverse primer 5'-TTA TTT TGC GGC CCA GAG CC (nucleotides 525–544). PCR products of 413 bp were separated in 2% agarose gels and visualized by ethidium bromide. A water control was run in each amplification and placenta cDNA served as positive control. Submission of breast tissue DNA to the protocol did not show amplification, therefore confirming that no contamination of genomic DNA occurred.

Immunohistochemical detection of Fas, FasL, and TRAIL

Paraffin-embedded sections of breast carcinomas, fibroadenomas, and normal breast tissues were deparaffinized in xylene and rehydrated before analysis. Slides were treated with a pepsin solution and endogenous peroxidase was quenched with 0.5% hydrogen peroxide. Slides were washed in PBS, blocked with 5% normal goat serum, and incubated overnight at 4°C with a rabbit polyclonal anti-human Fas-specific IgG (Santa Cruz Biotechnology, Santa Cruz, Calif., USA) at 0.4 µg/ml. A Vectastain ABC detection kit (Vector Laboratories, San Diego, Calif., USA) containing a biotinylated secondary antibody and avidin-conjugated horseradish peroxidase was used to identify antibody binding. Diaminobenzidine was used as substrate for the horseradish peroxidase. Slides were counterstained with hematoxylin. For control staining, the immunizing peptide (Fas, amino acids 316–335; Santa Cruz Biotechnology) was coincubated at 4 µg/ml at primary antibody incubation. In all cases staining was inhibited by the immunizing peptide. FasL and TRAIL detection followed the same protocol except for using a monoclonal antibody for FasL (NOK-1; PharMingen, San Diego, Calif., USA) at a concentration of 40 µg/ml and a polyclonal antibody for TRAIL (K-18 and K-18 immunizing peptide; Santa Cruz Biotechnology) at a concentration of 10 µg/ml. Incubation of the primary antibody was done for 45 min at 37°C. Placental tissue serving as positive control for TRAIL showed a positive reaction. Results were evaluated by the intensity of the immunohistochemical reaction of the different cellular components. Reaction was considered positive when more than 10% of tumor cells showed a positive reaction compared to the negative controls.

Immunofluorescence detection of FasL and Fas

Immunofluorescence staining was performed on cells of the two breast cancer cell lines MCF-7 and T47D. Cells were cultured for 6–8 h on eight chamber cell culture slides and fixed with 5% formalin in PBS-Dulbecco afterwards. Cell membranes were permeabilized with digitonin (6 µg/ml). Slides were washed in PBS 3 times thereafter. Monoclonal primary antibodies (FasL: NOK-1, Fas: G254–274; PharMingen) were incubated at 4°C for 1 h and slides were washed 3 times afterwards. Goat anti-mouse Cy-2 conjugated antibody (Dianova, Hamburg, Germany) was used as secondary antibody. As an anti-fading agent, *p*-phenylenediamine was added to the reaction.

Statistical analysis

RT-PCR results of breast cancer tissues, fibroadenomas, and normal tissues were compared using the Mann-Whitney U-test. The significance level was set at $P<0.05$, and all tests were two-tailed. Correlation of RT-PCR results with tumor grading and lymph node status was performed using the Spearman rank correlation coefficient.

Results

Expression of FasL and Fas

Immunohistochemical staining of Fas showed a strong homogeneous expression in all fibroadenomas and nor-

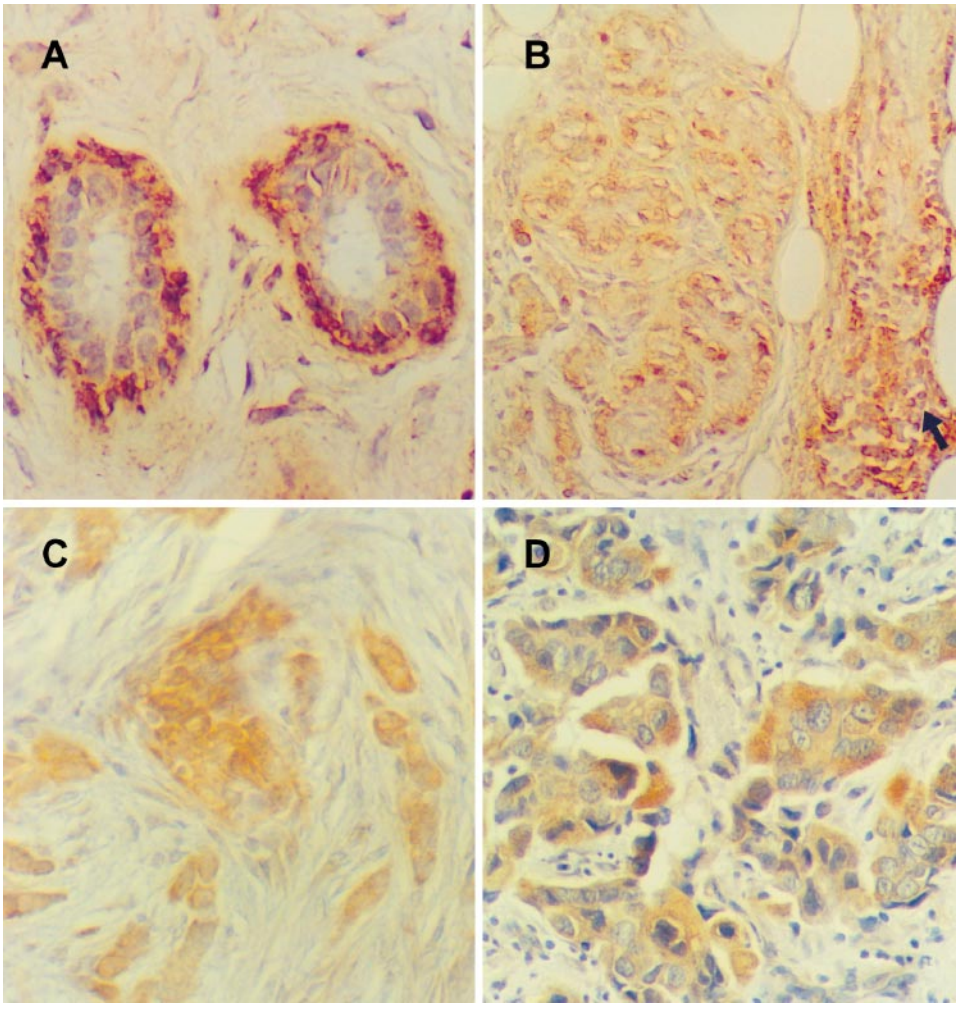

Fig. 1A–D Immunohistochemical detection of Fas ligand (FasL), Fas receptor (Fas), and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). A monoclonal antibody was used for FasL and a polyclonal antibody for Fas and TRAIL detection. An ABC detection kit and diaminobenzidine as substrate for horseradish peroxidase were used for staining. **A** Strong expression of Fas in the basal membrane of the luminal epithelia and surrounding myoepithelia of a fibroadenoma. **B,C** Strong immunohistochemical reaction of Fas (**B**) and FasL (**C**) in a ductal invasive mammary carcinoma. Fas-positive infiltrating lymphocytes can be seen in **B** (*arrow*). **D** Strong immunohistochemical staining of TRAIL in a ductal invasive mammary carcinoma

Table 1 Immunohistochemical results of Fas ligand (FasL), Fas receptor (Fas), and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)

Tissue	Fas positive/tested	FasL positive/tested	TRAIL positive/tested
Breast cancer	35/40	34/40 (10/40: weak reaction)	21/40 (13/40: weak reaction)
Fibroadenoma	7/7	1/7 (weak reaction)	1/7
Normal tissue	5/5	1/5 (weak reaction)	0/5

mal breast tissues on protein level. Fas immunoreactivity was found on the basal cell membrane of the luminal epithelia, in surrounding myoepithelial cells, and in stromal cells (Fig. 1A). Most breast carcinoma cells still expressed Fas (35/40), although expression was less intense than in infiltrating lymphocytes found in the same tumors or in fibroadenomas (Fig. 1B). Fas was predominantly localized on the cell membrane and inhomogeneously distributed throughout the tumor. These results were in agreement with the data of the quantitative analysis of Fas mRNA (TaqMan technology). Fas mRNA copy numbers were significantly higher in fibroadenomas (13786, 95% CI 2409–26607) than in breast cancer tissues (5407, 95% CI 5369–9573) and normal breast tissues (2924, 95% CI 214–14285) ($P<0.05$).

Immunoreactive FasL was detected in 85% of the breast cancer tissue samples (34/40), whereof 10 showed a weak reaction only. FasL expression was found in the cytoplasm of the tumor cells and in a few infiltrating lymphocytes (Fig. 1C). Although staining was distributed inhomogeneously throughout the tumor, only eight tumors showed markedly FasL positive and negative areas within the same tissue sample. A weak reaction for FasL could be detected in the myoepithelial cells of one fibroadenoma (1/7) and of one normal breast tissue sample (1/5). All immunohistochemical results are shown in Table 1.

Although immunohistochemical expression of FasL was found almost exclusively in carcinoma cells, quantitative analysis of FasL mRNA showed the highest copy numbers in fibroadenomas (4558, 95% CI 2165–7481), followed by breast cancer tissues (3671, 95% CI 4661–10731) and normal tissue samples (995, 95% CI 0–2231). Calculation of the FasL/Fas mRNA ratio in

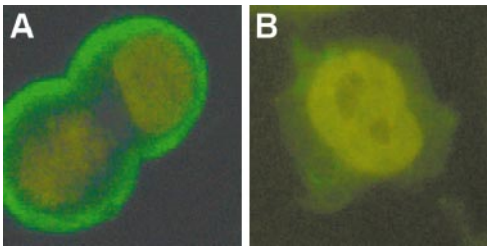

Fig. 2A,B Immunofluorescence detection of Fas and FasL in the breast cancer cell cultures T47D (Fas) and MCF-7 (FasL). A monoclonal primary antibody and an anti-mouse Cy-2-labeled secondary antibody were used for Fas and FasL detection. Fas expression was detected on cell membranes (**A**) while FasL was predominantly found in the cytoplasm (**B**). The anti-fading agent *p*-phenylenediamine was added producing a yellowish-brown reaction in the nucleus

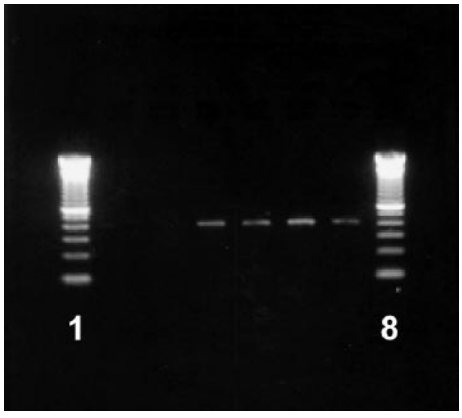

Fig. 3 Expression of TRAIL mRNA in a breast cancer tissue, fibroadenoma, and normal breast tissue (*lanes 5*, *6*, and *7*, respectively). TRAIL mRNA expression was analyzed by RT-PCR. The amplified fragment size for TRAIL was 413 bp. Placental tissue served as a positive control (*lane 4*). DNA of breast cancer tissues (*lane 3*) and a water control (*lane 2*) did not amplify transcripts

each tissue sample revealed that only breast cancer tissues showed a ratio greater than 1 (17/40), indicating a shift toward FasL mRNA expression in breast cancers. FasL mRNA, Fas mRNA, and the ratio did not correlate with tumor grading or lymph node status.

Immunofluorescence staining of Fas and FasL

In order to analyze distribution of Fas and FasL within the tumor cells, immunofluorescence staining was performed on the two breast cancer cell lines MCF-7 and T47D. In both cell lines it could be demonstrated that Fas was expressed on the cell membrane while FasL was located predominantly in the cytoplasm (Fig. 2).

Expression of TRAIL

Compared to FasL expression in breast cancer tissues immunohistochemical staining of TRAIL was less intense. Of breast cancer tissues, 52.2% (21/40) showed a positive reaction for TRAIL (Fig. 1D), whereof 13 expressed TRAIL only weakly throughout the tumor. Only one fibroadenoma showed a weak positive reaction (1/7), whereas tumor-infiltrating lymphocytes and normal breast tissue did not stain positive at all. Carcinoma cells and the luminal epithelia of the fibroadenoma expressed TRAIL in the cytoplasm. Although only 52.5% of the breast cancer tissues expressed TRAIL on protein level, 97.5% (39/40) of the breast cancer tissues as well as all fibroadenomas and normal breast tissues expressed TRAIL mRNA (Fig. 3).

Discussion

The apoptosis-inducing ligands FasL and TRAIL have been shown to play an important factor in the tumor's ability to evade the immune system. In this study it could be demonstrated that breast cancer tissues show an altered expression of FasL and TRAIL compared to fibroadenomas and normal breast tissue. In agreement with O'Connell et al. (1999) we found breast cancers to express high levels of FasL protein in contrast to normal breast tissue. Surprisingly, quantitative mRNA analysis revealed high copy numbers of FasL not only in breast cancer tissues but also in fibroadenomas. Since tissue for quantitative RT-PCR-analysis is taken out of the fibroadenoma as a whole, it is not possible to determine which cells exactly expressed high levels of FasL mRNA but it appears that FasL mRNA is not sufficiently translated into protein to show a positive immunohistochemical signal. In contrast to the benign fibroadenomas, breast cancer tissues seem to have lost this control of translation and express the protein. Comparing results of quantitative RT-PCR results of breast carcinomas and fibroadenomas it was found that 42.5% of the breast cancer tissues showed a FasL/Fas ratio greater than 1 while the ratio of all fibroadenomas and normal breast tissues was below 1. This shift toward FasL expression in carcinoma tissue indicates a difference between malignant and benign tumors on mRNA level.

Fas expression was strong in fibroadenomas and normal breast tissues. In contrast to other malignant tumors such as hepatoma or intestinal-type stomach carcinoma (Strand et al. 1996; Vollmers et al. 1997) many breast cancer tissues still expressed Fas. Different mechanisms could explain how breast carcinomas avoid induction of apoptosis within the tumor by Fas-FasL interaction. One mechanism could be tight control of FasL expression on cell membranes in regions of the tumor where induction of apoptosis is not desired. We therefore performed immunofluorescence staining of the two breast cancer cell lines MCF-7 and T47D. It could be demonstrated that FasL was mainly located in the cytoplasm while Fas was expressed on the cell membrane. Another mechanism avoiding FasL-induced apoptosis in the tumor would be alteration of the signaling pathway downstream of Fas in the tumor cells. It has been reported that MCF-7 Fas-

resistant cell lines became Fas-sensitive upon transfection of the interleukin-1β-converting enzyme or of bax-α cDNA (Bargou et al. 1996; Keane et al. 1996). Both proteins are important regulators of Fas-mediated apoptosis downstream of the receptor.

In some breast cancer tissues, strong infiltration of lymphocytes was identified within the tumor. Most lymphocytes showed a positive reaction for Fas but were negative for FasL. Although it is known that breast cancer tissues show infiltration of lymphocytes, it appears that immune response is inefficient and the tumors cells can spread. It has been shown in vitro that Fas-positive Jurkat T cells undergo apoptosis upon contact with FasL-positive colon cancer cells (O'Connell et al. 1996). It is suggested that the same mechanism is partly responsible for the inefficient in vivo immune response of lymphocytes in breast cancer: FasL expression of the tumor cells induces apoptosis in Fas-positive lymphocytes. Gutierrez et al. (1999) found increased numbers of apoptotic, TUNEL-positive lymphocytes in the proximity of FasL-positive breast carcinoma. In esophageal cancer it has been demonstrated that areas of the tumor which expressed high concentration of FasL showed a reduction of infiltrating lymphocytes and an increased apoptosis of these cells (Bennett et al. 1998). Since only some of the carcinomas investigated in this study showed markedly FasL-positive and -negative areas within the same tissue sample, no significant results on reduction of lymphocytes in FasL-positive areas could be obtained.

Immunohistochemical staining of TRAIL demonstrated a less frequent and strong expression of TRAIL on protein level compared to FasL expression. Conventional RT-PCR analysis showed that all tissues expressed TRAIL mRNA, while only 52.5% of breast cancer tissues, one fibroadenoma, and none of the normal breast tissues reacted positive for immunohistochemical staining of TRAIL. Expression of TRAIL mRNA has been reported in many benign tissues. Similar to FasL mRNA, this study demonstrates that, although TRAIL mRNA is found in the majority of the tissue samples, translation into protein occurs predominantly in the malignant breast tumors. Since expression of TRAIL was detected less frequently in breast cancer tissues compared to FasL and the complex expression of the different TRAIL receptors in these tissues has not been studied so far, it is not clear which role TRAIL plays in the ability of breast cancers to evade the immune system. In contrast, TRAIL has been discussed as a possible anticancer drug inducing apoptosis in cancer cells. While FasL has been discarded as an anticancer drug due to severe hepatotoxicity (Ogasawara et al. 1993), two recent studies by Ashkenazi et al. (1999) and Walczak et al. (1999) showed that TRAIL induced apoptosis in a variety of cancer cell lines and reduced tumor size and incidence in mice bearing colon carcinoma or mammary adenocarcinoma. Since many benign tissues are expressing the decoy receptors DcR1 and DcR2 or intracellular regulators such as FLICE-inhibitory protein, TRAIL did not induce the same severe toxicity as FasL (Wiley et al. 1995; Pitti et al. 1996; Griffith et al. 1998).

In this study it was demonstrated that on protein level apoptosis-inducing ligands FasL and TRAIL are upregulated in breast cancer tissues compared to fibroadenomas and normal breast tissues. Quantitative RT-PCR analysis of FasL and Fas mRNA showed a shift toward expression of FasL mRNA in breast carcinomas. These findings suggest that FasL and to a lesser extent TRAIL take part in the tumor's ability to evade the immune system.

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