

Research article

**THE ANTI-APOPTOTIC PROTEIN LIFEGUARD IS EXPRESSED
IN BREAST CANCER CELLS AND TISSUES**VESNA BUCAN*, KERSTIN REIMERS, CLAUDIA YU CHOI,
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Abstract: Lifeguard (LFG) is an anti-apoptotic protein that inhibits Fas-mediated death in tumour cells. However, the molecular function of human LFG in the carcinogenesis of human breast cells is uncertain. We studied the expression and function of endogenous LFG in four breast cancer cell lines (MCF-7, MDA-MB-231, T-47D and HS 578T), a human breast epithelial cell line (HS 578Bst), and in healthy and cancerous breast tissues. Molecular (Western blot and RT-PCR) and immunohistochemical techniques were used to investigate the LFG expression. To investigate the breast cancer cell proliferation in the presence of Fas, we performed fluorescent cell viability assays. The possible association of Fas with LFG was analyzed by immunofluorescence microscopy. In this paper, we provide convincing evidence that LFG is overexpressed in several human breast cancer cell lines. More importantly, we found that the LFG expression correlates with high tumour grades in primary breast tumours. Finally, we demonstrated that Fas sensitivity is reduced in breast cancer cell lines expressing LFG. Our results indicated that LFG is strongly expressed in breast cancer epithelial cells. Moreover, the overexpression of LFG correlated with tumour grade and reduced Fas sensitivity. Our findings support the idea that LFG may have a role in the downregulation of apoptosis in breast cancer cells.

Key words: Apoptosis, Lifeguard, Fas, Breast cancer

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Abbreviations used: BI-1 – Bax inhibitor-1; DAPI – 4', 6'-diamidino-2-phenylindole; EDTA – ethylenediaminetetraacetic acid; EGF – epidermal growth factor; FCS – fetal calf serum; LFG – Lifeguard; RT-PCR – reverse transcribed polymerase chain reaction; PMSF – phenylmethyl sulfonyl fluoride; PVDF – polyvinylidene fluoride

INTRODUCTION

Programmed cell death (apoptosis) is an evolutionarily conserved pathway that is necessary for embryonic development and tissue homeostasis [1]. Apoptosis is the normal physiological response to many stimuli, including irreparable DNA damage. In cancer, the balance between proliferation and programmed cell death is disturbed, and defects in apoptotic pathways allow cells with genetic abnormalities to survive [2]. Apoptosis is controlled through the expression of many cellular proteins, such as the members of the bcl-2 family, which includes inducers (Bax, Bcl-Xs, Bad, Bak and Bik) and inhibitors (Bcl-2, BAG-1, Bcl-XL and Mcl-1) of apoptosis [3-11]. The potential for defects in apoptosis-regulating genes suggested that they are important in the pathogenesis of human cancer [12-15]. This hypothesis is supported by observations that *p53*, an inducer of apoptosis, is frequently deleted or mutated in various human cancers, whereas Bcl-2, an inhibitor of apoptosis, is overexpressed in a number of cancers, including lung, prostate and breast cancer [16-22].

Lifeguard (LFG) has been identified as a molecule that uniquely inhibits Fas-mediated death [23, 24]. The structure of LFG resembles the anti-apoptotic protein Bax Inhibitor-1 (BI-1). BI-1 is an evolutionarily conserved integral membrane protein containing multiple membrane-spanning segments. It is predominantly localized to intracellular membranes, similarly to the Bcl-2 family proteins. Moreover, BI-1 can interact with Bcl-2 and Bcl-XL but not with Bax or Bak [25, 26]. Reimers *et al.* demonstrated that LFG interacts with Bax and might be localized in the cellular membranes including the ER and plasma membrane [27]. Although the exact mechanism of action of LFG is not clear, it might play a role by interacting with Fas, or at the level of the Fas/FADD complex [24]. A rat homologue, NMP35, was identified as a protein that is up-regulated during the post-natal development of the nervous system [28]. The NMP35 protein is widely expressed throughout the brain and spinal cord, being most prominently localized at post-synaptic sites and in dendrites [29].

To date there has been no published data evaluating the role of LFG in carcinogenesis. In this study, we assessed the expression of LFG in breast cancer cell lines *in vitro* and the expression in human breast cancer tissue samples using real time PCR and immunofluorescence. We tested the functional relationship between Fas and LFG expression by demonstrating the congruency of LFG expression and resistance to cell death stimulation of an agonist Fas antibody.

MATERIALS AND METHODS

Cell lines

A normal human mammary cell line (HS578), derived from normal breast epithelium, and four human breast carcinoma cell lines (MCF-7, MDA-MB-231, T47D and HS 578T) were used in this study. All of the cell lines were obtained from American Type Culture Collection (Manassas, VA). The MCF-7 and MDA-MB-231 cell lines were grown in Dulbecco's modified Eagle's medium

(DMEM, PAA, Cölbe, Germany) supplemented with 10% FCS (Biochrom, Berlin, Germany) and 50 mg/ml penicillin-streptomycin. The T47D cells were grown in RPMI 1640 medium with 0.2 units/ml bovine insulin (10 mg/ml), and the HS578 cell line was cultured in DMEM/F12 containing epidermal growth factor (EGF; 30 ng/ml). All of the cells were maintained at 37°C with 5% carbon dioxide in a humidified atmosphere. The medium was changed every 2 to 3 days, and the cells were subcultured by treatment with 0.25% Trypsin/0.53 mM ethylenediaminetetraacetic acid (EDTA) solution.

Real-time polymerase chain reaction

Total RNA was isolated from the cultured cells using the NucleoSpin RNAII Kit (MN Macherey-Nagel, Duren, Germany) according to the manufacturer's protocol. The RNA concentration was measured by photometry at 260 nm. The quality of the total RNA was verified by assessing the integrity of 18S/28S ribosomal RNA in 1% ethidium bromide-stained agarose gels. Reverse transcription (RT) was performed with 1 µg total RNA using the iScript™ cDNA Kit (Bio-Rad Laboratories, Hercules, CA). The real-time polymerase chain reaction (Q-PCR) was carried out in 20-µl samples with 5 ng cDNA and 10 pmol of each forward and reverse primer using the 2x SYBR green Sensi-Mix DNA Kit (Quantace, London, UK). The relative gene expression was determined from the fluorescence intensity ratio of the target gene to 18S. The primers used in the real-time PCR reaction were designed based on information from the human genomic database. The following primer sequences were used:

human LFG: forward: 5'-gactcatcctggccatcctctctac-3'
reverse: 5'-ggcgtcggttaccatcagc-3';
human Fas: forward: 5'-ggcatctggaccctctacc-3'
reverse: 5'-caggcctccaagttctgagtct-3';
human 18S: forward: 5'-gagcggtcggcgtccccaacttc-3'
reverse: 5'-gcgcgtgcagcccgacatctaa-3'.

The initial denaturation step at 94°C for 4 min was followed by 40 cycles of denaturation for 30 s, annealing for 30 s at 65°C (LFG) or 60°C (Fas), extension at 72°C for 1 min, and a final extension step at 72°C for 10 min. The PCR products were fractionated and visualized on 2% Agarose gels containing ethidium bromide. All of the experiments were carried out in triplicate and repeated at least at three independent times. The specificity of the Q-PCR products was proven using the appropriate melting curves (specific melting temperature).

Western blot analysis

For the Western blot analysis, the cells were lysed in RIPA buffer containing 0.3 M NaCl, 1% sodium desoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1% Triton-X-100, 20 mM Tris-HCL (pH 8), 1 mM EDTA, and 1 mM phenylmethyl sulfonyl fluoride (PMSF). 25 µg of protein were fractionated by 15% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore Corporation, Bedford, USA), then blocked in Odyssey buffer for 1 h. The

protein expression levels were determined by immunoblotting with the polyclonal antibodies anti-hLFG (1:200 dilution; IMGENEX, San Diego, CA) and monoclonal anti-hFAS (at 1:500; purchased from MILIPORE, Temecula, CA) overnight at 4°C. To quantify the protein expression levels, Odyssey 680/800 nm secondary conjugates were used, and the PVDF membranes were analysed using the Odyssey Infra-Red Imaging System and software (Li-Cor BioSciences, Lincoln, Nebraska, USA).

Immunohistochemistry

Breast tissue slides (BIOMAX.US, Rockville, USA) were deparaffinized in xylene, and transferred through two changes of 100% ethanol. For antigen retrieval, the slides were pressure cooked in 6.5 mM sodium citrate (pH 6.0). To reduce the non-specific background staining, the slides were incubated for 30 min in 0.3% bovine serum albumin/1X Tris-buffered saline. The slides were incubated at 4°C overnight with hLFG rabbit primary antibodies (1:100 dilution; IMGENEX, San Diego, CA) and mouse primary cdc6 antibodies (1:100; MoBiTec, Goettingen, Germany). The slides were washed twice for 5 min with phosphate buffered saline (PBS), and incubated for 30 min with goat anti-rabbit Li-Cor-680 and anti-mouse Li-Cor-800CW conjugated secondary antibody. The signals were detected using the Li-Cor Infra-Red Imaging System.

Cell viability assay

Cell proliferation was determined via Cell Titer Blue cell viability assays (Promega, Madison, USA). Briefly, 10^4 cells from breast cancer cell lines and the Jurkat cell line were seeded in each well of a 96-well plate and incubated with 200-12.5 ng/ml of agonistic anti-Fas (Clone CH11) per ml for 24 h. The relative numbers of viable cells were measured in comparison to the untreated control and the solvent control using the fluorimetric, resazurin-based Cell Titer Blue assay (Promega) according to the manufacturer's instructions at 560_{Ex}/590_{Em} nm in a TecanGENios fluorescence plate reader (TECAN, Switzerland).

Immunofluorescence microscopy

MCF-7 cells were fixed in 4% paraformaldehyde for 20 min and permeabilized with 0.1% Triton X-100 (Sigma Aldrich, Steinheim, Germany) for 4 min. The slides were then incubated with rabbit polyclonal anti-hLFG antibody (1:100 dilution) and mouse polyclonal anti-hFas (MILIPORE, Temecula, CA) antibodies at 37°C for 1 h, washed three times in ice-cold PBS, and incubated with Alexa Fluor 488 and Alexa Fluor 680 conjugated goat anti-rabbit and anti-mouse secondary antibody (each at a 1:600 dilution; Invitrogen, USA) at 37°C for 30 min. After three washing steps with PBS, the sample was dried and covered with an antifade reagent containing the DNA-staining dye DAPI (4',6'-diamidino-2-phenylindole) on Vectashield mounting medium (Vector Laboratories, Burlingame, CA). The images were acquired using a ZEISS Axiovert 200 M fluorescence microscope equipped with the appropriate barrier filters.

RESULTS

LFG is highly expressed in human breast cancer cell lines

Taking into account the low sensitivity of breast cancer-derived cell lines to apoptotic stimuli, we used RT-PCR and Western blotting to examine the expression of LFG mRNA and protein in a cell line (Hs 578 Bst) derived from normal breast epithelium and in four breast cancer cell lines (MCF-7, MDA-MB-231, T47D and HS 578T). As a control, we used the non-transformed mouse keratinocyte cell line JB6, which expresses low levels of LFG.

LFG mRNA expression was analyzed in four human breast cancer cell lines with increasing degrees of malignant behaviour. We found a high LFG gene expression in the highly malignant breast cancer cell lines HS 578T and MDA MB 231 and similar levels in the non-invasive breast cancer cell lines MCF-7 and T47D. The expression of LFG mRNA was low in the normal breast cell line Hs 578 Bst and in the JB6 cells (Fig. 1A). We did not find a dependence of invasiveness on LFG expression in the tested breast cancer cell lines.

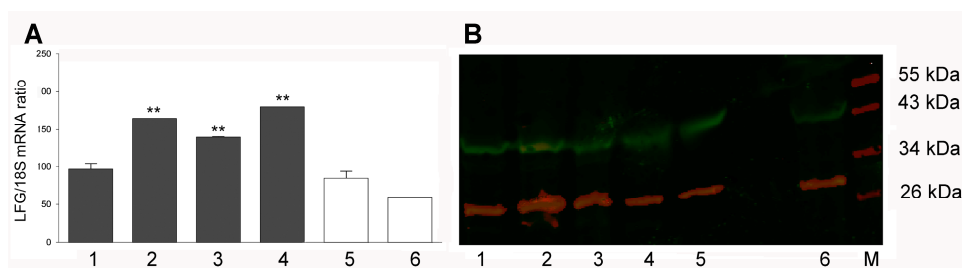


Fig. 1. The expression of LFG in human breast carcinoma cell lines. A – The relative levels of LFG mRNA as determined via real-time polymerase chain reaction (PCR) analysis in normal breast (lane 5: HS578Bst.) and tumour cell lines (lane 1: HS 578T; lane 2: MDA-MB231; lane 3: T47D; lane 4: MCF-7). Control (lane 6: JB6). The data is the means \pm S.D. for triplicate determinations which were repeated in three separate experiments. * $P < 0.05$, ** $P < 0.01$ vs. control. B – Equal amounts of protein (25 μ g) were subjected to 15% SDS-PAGE and electrotransferred onto polyvinylidene fluoride (PVDF) membranes. The signals were detected using anti-human LFG polyclonal antibody. Lane 1: MDA-MB231; lane 2: MCF-7; lane 3: T47D; lane 4: HS 578T; lane 5: HS 578Bst.; lane 6: JB6.

Consistent with the LFG mRNA expression, the protein expression of LFG was low in the normal breast cell line and in the JB6 cells. LFG protein expression was clearly detectable in MCF-7, MDA-MB-231 and T47D. HS 578T expressed relatively high levels of LFG protein, comparable to the LFG protein level in the normal Hs 578 Bst breast cell line (Fig. 1B).

The high expression of LFG protein in breast carcinoma tissues

To test the relevance of LFG protein expression in breast carcinoma cell lines in human breast cancer specimens, we examined its expression in 4 healthy and

22 invasive carcinoma breast tissues (Tab. 1) arranged on a commercially available tissue array slide via near far-red immunofluorescence. Fig. 2A shows an example detected using the Li-Cor system, with the levels of LFG protein expression in the breast carcinoma tissues high compared to those in the control tissues. The patients with grade III tumours (cells appear abnormal and tend to grow and spread more aggressively) had the highest level of LFG expression.

Tab. 1. Multiple breast cancer tissue array with unmatched normal adjacent tissues.

Pos.	Pathology diagnosis
A1	Infiltrating ductal carcinoma not otherwise specified (NOS)
A2	Infiltrating ductal carcinoma not otherwise specified (NOS)
A3	Infiltrating ductal carcinoma not otherwise specified (NOS)
A4	Infiltrating ductal carcinoma not otherwise specified (NOS)
A5	Infiltrating ductal carcinoma not otherwise specified (NOS)
A6	Infiltrating ductal carcinoma not otherwise specified (NOS)
B1	Infiltrating ductal carcinoma not otherwise specified (NOS)
B2	Infiltrating ductal carcinoma not otherwise specified (NOS)
B3	Infiltrating ductal carcinoma not otherwise specified (NOS)
B4	Infiltrating ductal carcinoma not otherwise specified (NOS)
B6	Infiltrating ductal carcinoma not otherwise specified (NOS)
C1	Infiltrating ductal carcinoma not otherwise specified (NOS)
C2	Infiltrating ductal carcinoma not otherwise specified (NOS)
C3	Infiltrating ductal carcinoma not otherwise specified (NOS)
C4	Infiltrating ductal carcinoma not otherwise specified (NOS)
C5	Infiltrating ductal carcinoma not otherwise specified (NOS)
C6	Infiltrating ductal carcinoma not otherwise specified (NOS)
D1	Infiltrating ductal carcinoma not otherwise specified (NOS)
D2	Infiltrating ductal carcinoma not otherwise specified (NOS)
D3	Cancer adjacent normal tissue
D4	Fibrofatty tissue
D5	Cancer adjacent normal tissue
D6	Cancer adjacent normal tissue

The histogram in Fig. 2B shows the intensity levels of LFG expression in the breast tissue with different grades of tumour. The fluorescence emission was measured in an infrared scan and expressed in relative fluorescence units of LFG expression normalised to cdc6 expression.

Fig. 3 shows representative image pairs from tissue samples with different grades of tumour showing the varying levels of the LFG protein expression versus the control staining.

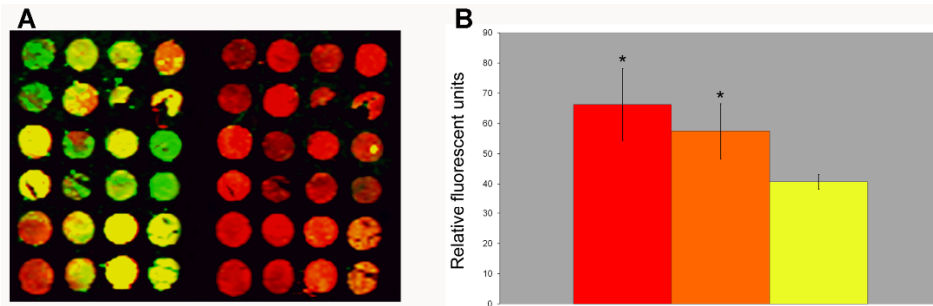


Fig. 2. The expression of LFG in human breast cancer. A – Immunofluorescence analysis of LFG expression in breast tumor tissue specimens arranged on tissue slides. The slides were stained with anti-hLFG followed by secondary antibody 800CW (left slide, pseudocoloured green) or remained without the primary antibody (right slide). As a control, both slides were stained with anti cdc6 followed by Alexa680 (pseudocoloured red). The fluorescence was detected using the Li-Cor system. B – The fluorescence was quantified and normalized to cdc6 expression. The high LFG expression was measured in breast cancer tissue of metastasis grade II (orange) and metastasis grade III (red), and compared to the expression in normal breast tissue (yellow). The data is the means \pm S.D. for the experiments. *P < 0.05, **P < 0.01 vs. control.

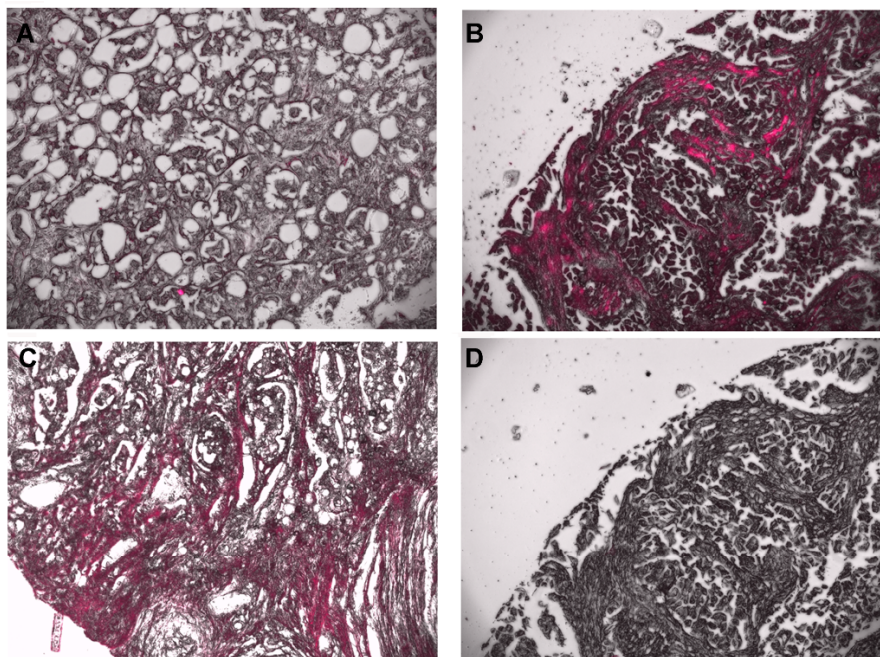


Fig. 3. LFG expression in breast cancer tissue. The images are composed of brightfield microscopy and pseudocoloured immunofluorescence (pink). A – LFG expression in normal breast tissue B – LFG expression in grade II breast cancer tissue. C – High levels of LFG in a grade III breast tumour. D – A grade II breast tumour without the primary antibody. Original magnification: x100.

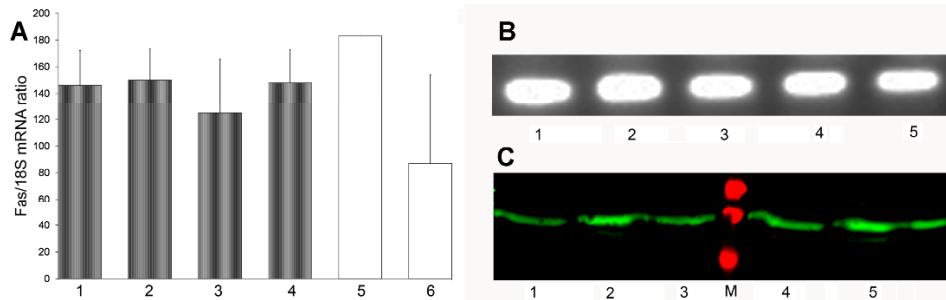


Fig. 4. The expression of Fas in the breast cancer cell lines. A – The mRNA expression levels of Fas were detected by Q-PCR, and the differences in the Fas expression transcript calculated relative to the 18S standards. Experiments were performed in triplicate. The results are the means \pm S.D. for triplicate determinations which were repeated in three separate experiments. B – Q-PCR products were resolved on a 2% Agarose gel and visualised by ethidium bromide staining. C – Western blot analysis of Fas in breast cancer cells using the monoclonal hFas- antibody. The cell lines are: lane 1: MDA-MB231; lane 2: MCF-7; lane 3: T47D; lane 4: HS 578T; lane 5: HS 578Bst; lane 6: JB6.

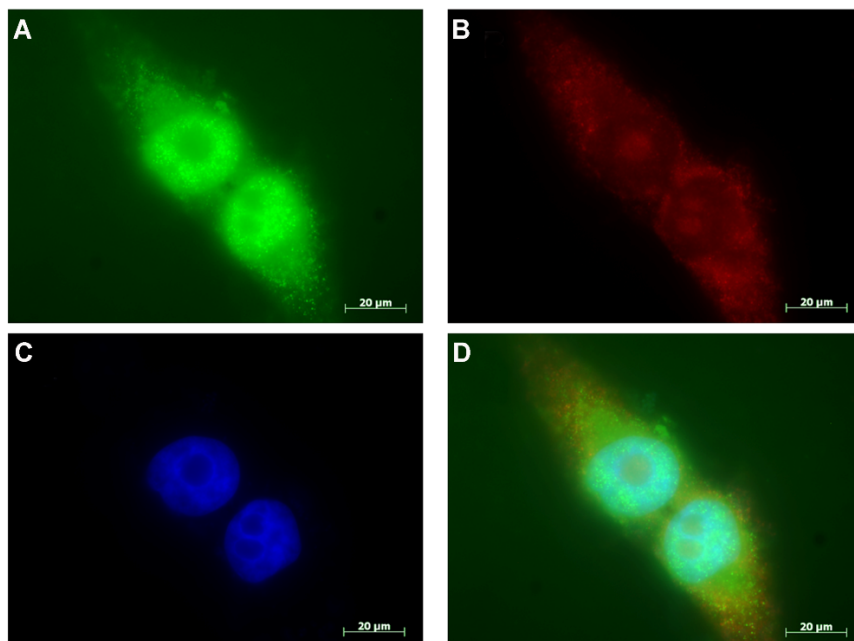


Fig. 5. The co-localization of Fas and LFG in MCF-7 cells. A – MCF-7 cells were stained with mouse anti-Fas (green) and B – with rabbit anti-LFG (red). C – DNA-staining dye DAPI. D. Image composed of pictures A, B and C. Almost no co-localization between LFG and Fas could be detected. Original magnification: x1000.

The detection of Fas in human breast cancer cell lines

Taking into consideration the Fas pathway-blocking activity of LFG (Somia *et al.*) [24], we next addressed the question whether Fas expression and LFG expression coincided in the tested breast cancer cell lines. Using semi-quantitative RT-PCR, we determined Fas mRNA expression in the same cancer and normal breast cell lines (Fig. 4A, B). We did not find significant differences in the Fas expression level between the breast cancer cell lines (MCF-7, MDA-MB-231, T47D and HS 578T) and the normal breast cell line (Hs 578 Bst). Consistent with the mRNA results and as detected by western blot, Fas protein expression was not elevated in the breast cancer cell lines (Fig. 4C).

To study the possible association of Fas with LFG, we analysed the distribution of Fas and LFG in MCF7 breast cancer cells via immunofluorescence (Fig. 5). We showed that Fas predominantly displayed a membranous staining pattern and LFG was mainly not co-localized with Fas.

Anti-proliferative effects in breast cancer cells

To examine the sensitivity to Fas stimulation in human breast cancer, cells from the lines MDA-MB-231 and T47D were treated with increasing concentrations of Fas. Cell viability was measured after 24 h. Fas did not cause growth inhibition nor a significant decrease in the viability of the MDA-MB-231 and T47D cells. The Fas-sensitive Jurkat cells were included as a positive control.

The Jurkat cells exhibited typical morphological features of apoptosis, and their cell vitality decreased in a concentration-dependent manner when treated with Fas, whereas the MDA-MB 231 and T47D cells showed no signs of apoptotic cell death (Fig. 6).

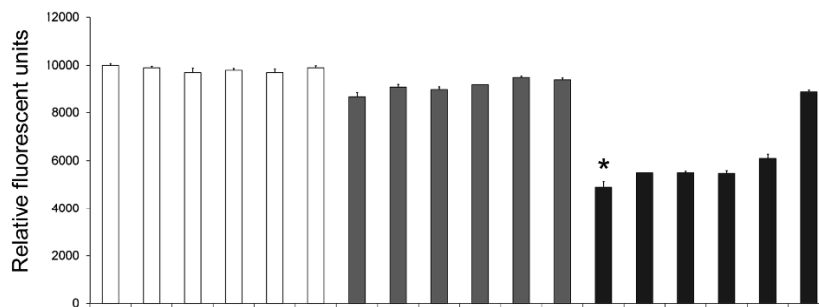


Fig. 6. A viability assay of breast cancer cell lines. Cells of MDA-MB231 (white), T47D (grey) and Jurkat (dark grey) were treated with Fas (Clone CH11) at concentrations of (from left to right) 200 ng, 100 ng, 50 ng, 25 ng, 12.5 ng and 0 ng for 24 h. After the incubation, the cell viability was determined. All of the samples were run in duplicate. The data is the means \pm S.D. for triplicate determinations which were repeated in three separate experiments. *P < 0.05, **P < 0.01 vs. control.

DISCUSSION

Although breast cancer is the leading cause of cancer-associated death for women, the molecular mechanisms for carcinogenesis have not yet been fully understood. A number of observations have indicated that the dysregulation of apoptosis plays an important role in the pathogenesis of human cancers [30, 31]. Inactivation of proapoptotic proteins such as p53 and Bax, and activation of anti-apoptotic proteins such as Bcl-2 and Bcl-XL has been observed in a variety of human cancers [32-34]. Bcl-2 was found to be overexpressed in some breast carcinoma cell lines [35], but its expression level was unchanged or even lower in some other breast cancer cell lines or tissues [36]. Reduced expression of the proapoptotic gene bax was observed in breast cancer cell lines and tissues, and the expression of mRNA for the antiapoptotic genes bcl-2 and bcl-XL was similar in normal and cancerous breast tissues [34, 37]. Heterogeneous expression of Bax was also detected in primary breast tumours [38]. To fully understand the role of apoptosis in the pathogenesis of breast cancer, it is necessary to examine the expression of genes that are involved in apoptosis other than p53, bax, and bcl-2.

In this study, we examined the expression of LFG, a Bax-interacting anti-apoptotic protein, in normal breast cell lines and in breast carcinoma cell lines and tissues using Western blot analysis and real-time PCR. We found convincing evidence that the expression of the LFG protein was increased in breast carcinoma relative normal cell lines and tissues. Moreover, enhanced expression of LFG correlated with the grade of the tumour (grade II/grade III) in primary breast tumours. Unlike with BI-1, for which high expression rates have been demonstrated in several tumour tissues and cancer cell lines [39-42], this is the first time that high LFG expression rates could be phenotypically linked to human cancer [43].

Because the levels of LFG gene expression and protein were closely correlated in breast cell lines, the enhanced expression of LFG protein in these carcinoma cell lines is likely due to an increased production of LFG mRNA. One possible cause for the differential expression of LFG in normal breast and breast carcinoma cell lines versus normal breast and primary breast carcinoma tissues is the change occurring in the cell growth requirements or cell selection during the initial stage or after a prolonged culture of healthy or tumour cells *in vitro*. Additional experiments using a greater number of breast cell lines and tissues are needed to confirm these possibilities.

In a screen for prognostic secreted and transmembrane proteins, BI-1 was detected among other positive clones and found in 41.4% of the samples tested [45]. Although an association with clinicopathological features could not be proved, BI-1 was found to be expressed independently of the tumour grade and steroid receptor expression [40, 44]. However, siRNA-mediated downregulation of BI-1 in several breast cancer cell lines resulted in higher cell death rates only in the estrogen-dependent cell line MDA-MB 231, while the estrogen-dependent

cell lines MCF-7 and T47D remained rather unaffected [40]. Also in our study, high LFG expression was detected in all the tested cell lines independently of their invasiveness and estrogen-dependency. However, interferences with BI-1 expression on a functional level still remain to be determined. By contrast, we found an increased LFG expression with a higher tumour grade in our breast cancer tissue samples. A more detailed view of LFG expression compared to BI-1 expression in breast cancers is needed.

The Fas/Fas ligand system is a key signalling transduction pathway of apoptosis in cells and tissues [45]. Ligation of Fas by its agonistic antibody or its mature ligand induces receptor oligomerization and the formation of a death-inducing signalling complex, followed by the activation of caspase-8, then the further activation of a series of caspase cascades resulting in apoptotic cell death [45, 46]. Defects in the Fas/Fas ligand apoptotic signalling pathway provide a survival advantage to cancer cells and may be implicated in tumourigenesis. Indeed, the expression of the Fas ligand by breast cancer cells is associated with the loss of Fas expression, thus eliminating the possibility of self-induced apoptosis, and is involved in drug resistance [47, 48].

LFG is a protein which inhibits the Fas-dependent killing pathway via an unknown mechanism [24]. Its rat homolog, NMP35, is abundantly expressed in the CNS [28] but has not yet been linked to neuronal apoptosis. In this study, we found that Fas expression remained unchanged independently of an increased LFG expression in breast cancer cells (Fig. 3). This might indicate additional as-yet undiscovered functions independent of Fas signalling.

Using an antibody against LFG and Fas, we detected LFG expression in MCF-7 cells, but no co-localization with Fas (Fig. 5). This is an interesting finding that contradicts the results of Somia *et al.* [24] and Fernandez [23], who found a physical association between LFG and Fas. These differences might be due to the distinct functions of LFG associated with healthy and cancer cells. Further studies concerning the functional role of LFG in breast cancer cells are needed to address this point. In our study, we found that the lines of breast cancer cells with high LFG expression rates (MDA-MB-231 and T47D) displayed a reduced sensitivity against stimulation with an agonistic Fas antibody. We cannot therefore exclude that LFG interfered with Fas signalling in breast cancer cells.

In summary, we have shown that high levels of LFG expression are associated with the grade of the breast tumour. These results suggest that LFG might play an important role in the development of breast cancer. High-level expression of LFG could be used as a predictive marker of a poor prognosis for breast cancer patients. Furthermore, functional studies of differential LFG expression are under way and may clarify their role in the pathogenesis and the development of breast cancer.

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