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Local expression of complement factor I in breast cancer cells correlates with poor survival and recurrence.

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Abbreviations: ATCC - American Type Culture Collection; CDCC – complement dependent cell cytotoxicity; CI, confidence interval; CSS, cancer-specific survival; C4BP – C4b binding protein; ER, estrogen receptor; FH – complement factor H; FI - complement factor I; Her2, epidermal growth factor receptor 2; HR, hazard ratio; MOPS - 3-(N-morpholino)propanesulfonic acid; NHG – Nottingham histological grade; NSCLC – non-small cell lung cancer; PR, progesterone receptor; PVDF - polyvinylidene difluoride; RFS, recurrence free survival; SDS-PAGE – sodium dodecyl sulphate, polyacrylamide gel electrophoresis; TMA – tissue microarray

Abstract

Tumor cells often evade killing by the complement system by overexpressing membrane-bound complement inhibitors. However, production of soluble complement inhibitors in cells other than hepatocytes was rarely reported. We screened several breast cancer cell lines for expression of soluble complement inhibitor, complement factor I (FI). We also analyzed local production of FI in tissue microarrays with tumors from 130 breast cancer patients by *in situ* hybridization and immunohistochemistry. We found expression of FI in breast adenocarcinoma cell line MDA-MB-468 and confirmed its functional activity. Expression of FI at mRNA and protein level was also confirmed in tumor cells and tumor stroma, both in fibroblasts and infiltrating immune cells. Multivariate Cox regression analyses revealed that high expression of FI protein in tumor cells was correlated with significantly shorter cancer-specific survival (HR 2.8; 95%CI 1.0-7.5; $p = 0.048$) and recurrence-free survival (HR 3.4; 95%CI 1.5-7.4; $p = 0.002$). High FI expression was positively correlated with tumor size ($p < 0.001$), and Nottingham histological grade ($p = 0.015$) and associated with estrogen and progesterone receptor status ($p = 0.03$ and $p = 0.009$, respectively). Our data show that FI is expressed in breast cancer and is associated with unfavorable clinical outcome.

Keywords: innate immunity, complement system, breast cancer, tissue microarrays, immunohistochemistry

Précis: Breast cancer cell lines express fully functional soluble complement inhibitor factor I. Factor I produced locally in breast cancer tissue may serve as independent prognostic marker.

Introduction

The complement system is a part of innate immunity and aims at elimination of “non-self” from our body. Such elimination is based on identification of molecular patterns that are atypical under physiological conditions, and are recognized by a number of pattern-recognition molecules. Once activated, complement does not distinguish any more between self and non-self constituents and, therefore, additional protection of own cells and tissues from misguided complement attack is supported by endogenous complement inhibitors [1]. There is an increasing amount of evidence that both diminished and excessive activation of complement contributes to pathological processes including cancer. Tumor cells of different origin activate complement [2] and this event was long believed to act only in benefit of the host. This hypothesis was supported by a number of facts: (i) tumor cells developed various strategies to counter-attack complement activation [3], (ii) oncogenic viruses encode their own complement inhibitors [4], (iii) tumor cells with knocked-down complement inhibitors grew slower as compared to control, mock-transfected cells, in mouse xenograft models [5]. However, new reports showed that tumor cells benefit from triggering complement activation due to C5a-dependent recruitment of myeloid-derived suppressor cells or induction of angiogenesis [6,7]. Taken together, the role of complement in the pathogenesis of cancer is apparently more complex than initially believed and will likely differ between different types and stages of cancer.

Although membrane-bound complement inhibitors are abundant on the surface of various cell types, the production of soluble complement inhibitors was considered a rare phenomenon in cells other than hepatocytes [8]. The complement inhibitor factor I (FI) is a soluble serine protease with exceptionally narrow specificity. In the presence of appropriate cofactors, FI cleaves the early, activated complement components C3b and C4b to iC3b and iC4b, respectively. Neither iC3b nor iC4b can fuel the complement cascade any more. However, iC3b is recognized by complement receptors CR2 and CR3, which are present on epithelial cells as well as on a number of immune cells including B cells, follicular dendritic cells, tissue macrophages and mononuclear phagocytes, thymocytes, and a subset of peripheral T cells [9]. Interaction of iC3b with its cellular receptors edits the immune response and influences local inflammation, which in turn may exert both pro- and antitumor effects [10]. While expression of FI was reported in a number of tumor cell lines including those originating from hepatoma [11], B cell leukemia [12], glioma [13], rhabdosarcoma [14] lung cancer [15] and cutaneous squamous cell carcinoma [16], only the latter study describes expression of FI by tumor cells of the skin *in vivo* and shows association of FI production and aggressive tumor phenotype [16]. Herein, we report local

production of FI in breast cancer cells and associations between tumor-derived FI and clinical data obtained from 130 breast cancer patients.

Materials and methods

Cells

MDA-MB-231, MDA-MB-468, MCF-7 (all adenocarcinomas) and T47D (ductal carcinoma) breast cancer cell lines originally derived from metastatic sites, were bought from American Type Culture Collection (ATCC) by prof. Tommy Andersson (Lund University, Sweden), who generously shared these with us. Cells were frozen immediately after re-cultivation of the original aliquot and all the experiments were performed on cultures originating from these secondary aliquots within no more than 5 passages. Cells were *Mycoplasma* negative and monthly tested for contamination with VenorGEM Classic kit (Minerva Biolabs). Clones of HEK293 cells expressing human full length FI or transfected with empty vector were prepared as described in [17].

Purification of antibody against FI

Rabbit polyclonal pk9205 antibody against human FI was raised in-house and successively used before for FI detection [15] [18]. To employ this Ab for immunohistochemical FI staining, we purified it further by FI affinity chromatography. Recombinant FI (2.7 mg) produced as described in [19] was dialyzed and resuspended in 5 ml of 10mM 3-(N-morpholino)propanesulfonic acid (MOPS) pH 7 and mixed with 1 ml of Affi-Gel 10 (Bio-Rad). After 4 h incubation at 4°C, potential protein binding sites were blocked with addition of 100 µl of 1 M ethanolamine pH 8.0. The column was equilibrated with PBS, washed with the same buffer and eluted with 0.1 M glycine pH 2.5. The protein-containing eluate fractions were immediately neutralized with 1M Tris, pH 8.0, pooled, dialysed against PBS and concentrated.

qPCR

MDA-MB-231, MDA-MB-468, MCF-7, and T47D were grown to 80% confluency in DMEM high glucose medium supplemented with 10% fetal calf serum (Gibco). RNA was isolated with the RNAeasy kit (Qiagen) according to the manufacturer's instructions. cDNA was synthesized from 1 µg RNA by using 200 U Superscript III reverse transcriptase and 2.5 µM oligo(dT) primer (Life technologies). For the qPCR, 50 ng cDNA of each sample was used in triplicates. A primer (TaqMan probe set) specific for FI (Hs00989715_m1) and TaqMan gene expression master mix

were bought from Applied Biosystems. Cyclophilin A, TATA box binding protein, and hypoxanthine phosphoribosyltransferase 1 were used as reference genes. The relative expression of FI was calculated according to the Δ Ct method by using the geometrical mean of the three reference genes [20].

Detection of FI by western blotting

Breast cancer cell lines were cultured in T25 flasks (Nunc) in DMEM high glucose medium supplemented with 10% fetal calf serum until 100% confluency. Then the cells were washed twice with PBS and 5 ml serum-free Optimem medium (Invitrogen) was added for 48 h. The medium was harvested and concentrated 50x with a concentrating device (Vivaspin) with molecular weight cut-off value of 10 kDa. The retentate was loaded onto a 10% SDS-PAGE (30 μ l per lane) and separated proteins were then transferred to a PVDF membrane. FI purified from plasma [21] was used as a positive control. The western blot was developed with 3.5 μ g/ml mouse monoclonal anti-FI antibody OX21, generously provided by prof. R Sim (Oxford University) followed by goat-anti mouse F(ab)₂ (Dako) diluted 1:1000. Visualization of the bands was performed with Chemi Doc Visualization System (BioRad) using Immobilon Western reagent (Millipore).

C3b/C4b degradation assay

The functionality of MDA-MB-468 cell-derived FI was analyzed by degradation of ¹²⁵I-labeled C3b and C4b proteins, both purchased from Complement Technologies and iodinated by the chloramine-T method as described [22]. Conditioned or control Optimem medium (8 μ l; concentrated 20x) or PBS buffer (negative control) was mixed with 10 μ l of 0.25 mg/ml plasma purified C4b-binding protein; C4BP [23] and trace amounts of iodinated C3b or C4b. As a positive control, 6 μ g/ml of recombinant FI was added. Two μ l of FI function-blocking mAb #1 (Quidel, stock 1.1 mg/ml) was added to chosen samples to ensure the specificity of cleavage. The mixed ingredients in a total volume of 25 μ l were incubated for 3 h at 37°C, then reducing Laemmli buffer was added and samples were separated using 10% SDS-PAGE. After electrophoresis, gels were dried, subjected to autoradiography (Fuji Film) and visualized in a Typhoon FLA 9500 phosphoimager (GE Healthcare).

Patient material and immunostaining of breast cancer tissue microarrays

We studied tissue microarrays (TMAs) constructed from tumor material obtained from female patients diagnosed with invasive breast cancer at Malmö University Hospital between 2001 and 2002. The cohort consisted originally of 179 patients and was described in detail previously [24]. Furthermore, we prepared sections from normal breast tissue that was removed for cosmetic purposes. Immunohistochemical data regarding hormone receptor status, Ki-67 and human epidermal growth factor receptor 2 (Her2) expression were available from previous studies on the same breast cancer patient cohort [25,26]. Definitions of estrogen receptor (ER) and progesterone receptor (PR) negativity followed current Swedish clinical guidelines (<10% positive nuclei). Similarly, Ki-67 status was assessed based on % of positive staining nuclei and dichotomized into $\leq 25\%$ and $> 25\%$. Her2 status was assessed by semiquantitative analysis according to a standard protocol (HercepTest; DakoCytomation) described in [27]. We grouped the specimens as weakly expressing (scores 0-2) and strongly expressing Her2 (score 3). Two 1.0-mm cores of tumor tissue were obtained from each tumor, and, in general, cores were taken from the peripheral aspect of the tumor, and necrotic tissue was avoided. TMA sections of 4 μm thickness were cut and automatically pretreated using the PT-link system (Dako; Agilent Technologies) and then stained in an Autostainer Plus (Dako) with the affinity-purified pk9205 polyclonal antibody (described above) at a final concentration of 0.25 $\mu\text{g}/\text{ml}$ overnight. Subsequently, EnVision Flex HRP kit (Dako) was applied to the sections for 20 minutes followed by DAB reagent for visualization. Pellets of HEK293 freestyle cells transfected with human FI or vector only, treated in the same way as the TMAs, were used as negative and positive controls. Also, we included another negative control, in which the primary antibody was replaced with buffer. Mayer's hematoxylin (Histolab) was used for counterstaining. After histopathological reevaluation we eliminated detached or damaged specimens as well as those containing very little or no tumor cells. Finally samples from 130 patients were used for scoring and statistical analyses. Ethical permission was obtained from the Lund University Regional Ethics Board, ref. no. 445/2007 whereby written consent was not required and patients were offered the option to opt out.

Scoring, evaluation and statistical analysis.

The immunohistochemical staining was evaluated by an experienced pathologist and three scientists who were all blinded with regard to the clinical information. Since the staining within particular tissue sections was homogenous in the majority of cases, we did not analyze percentage of positive cells. Instead, we assessed the intensity of FI staining in tumor cells and tumor stroma

separately and graded from 0 (negative), 1+ (weak), 2+ (moderate) to 3+ (strong). The final score was appointed to the highest intensity attainable in either of the two samples from the same patient. Original slides were scanned in Aperio ScanScope slide scanner and figures showing the representative stainings were prepared in the Image Scope software (Aperio) followed by Illustrator (Adobe). Kaplan-Meier analyses and Breslow test were used to illustrate and estimate the impact of high FI expression in cells and stroma on overall survival (OS), cancer-specific survival (CSS) and recurrence free survival (RFS). For Kaplan-Meier analyses, FI intensity was grouped into weak expression (score 0, 1, and 2) and strong expression (score 3). Cox regression proportional hazards models were used for estimation of hazard ratios (HR) for death from breast cancer or recurrence-free survival according to cellular and stromal FI expression in both uni- and multi-variate analyses. Covariates, all with a p value < 0.05 in the univariate analysis, included in the multivariate analysis were: tumor size, histological grade, ER, Her2 and Ki-67 status (Ki-67 was only included for CSS since it was not significant in the univariate analysis for RFS). Differences in distributions of pathological and clinical parameters (age, tumor size, histological grade, lymph node status, hormone receptor status, Her2 and Ki-67) between different FI intensity scores in malignant cells and stroma were calculated using Spearman correlations or Mann-Whitney U tests, as indicated in table 1. All statistical tests were two-sided and p < 0.05 was considered statistically significant. The calculations were preformed using SPSS Statistics v. 20 (IBM).

In situ hybridization

Paraffin-embedded slides containing breast cancer tissue or cell pellets were baked at 60 °C for 1 h and deparaffinized in xylene followed by absolute ethanol. Subsequently, detection of FI-specific RNA as well as PPIB housekeeping gene RNA (positive control) and bacterial DapB RNA (negative control) was performed with RNAscope 2.0 BROWN assay (Advanced Cell Diagnostics) [28], according to the manufacturer's instructions. Original slides were scanned with Aperio ScanScope slide scanner. In our experimental conditions hematoxylin counterstaining did not turn entirely blue and intracellular material remained purple/grey. Therefore, to better distinguish between counterstaining (purple) and signal from RNA probes (brown), we used Adobe Photoshop picture enhancement options, which included diminishing vibrance to -100% followed by an increase of saturation to +100%.

Results

Breast cancer cell lines produce functional FI

We analyzed expression of FI in four breast cancer cell lines: MDA-MB-231, MDA-MB-468, MCF-7 and T47D by qPCR (Fig. 1A). Two cell lines, MDA-MB-231 and MDA-MB-468 showed significant expression. In order to verify whether the qPCR results corresponded to FI expression at the protein level, we analyzed samples of concentrated, serum-free media conditioned by breast cancer cells using western blotting. The clear band detected in medium conditioned by MDA-MB-468 showed the same molecular weight as the one in plasma-purified FI sample whereas no band was detected in medium conditioned by T47D and MCF-7 cells. Very faint band was detected in the sample of medium conditioned by MDA-MB-231 cells (Fig. 1B). To determine whether FI produced by MDA-MB-468 cells retained its specific activity, we performed a degradation assay, which tested the ability of cell-derived FI to cleave its natural ligands C3b and C4b in the presence of a cofactor C4BP [29](Fig. 1C). When MDA-MB-468 cells-conditioned, serum-free medium was used as a source of FI, the same pattern of C3b and C4b cleavage was revealed as when recombinant FI was applied. To further ensure that the observed cleavage pattern was solely due to FI activity and not to the activity of other proteases, we used a function blocking anti-FI mAb. When added to MDA-MB-468 cells-conditioned medium, it completely prevented cleavage of C3b and C4b.

FI is produced locally within breast cancer tissue.

We aimed to examine whether FI expression is restricted to certain breast cancer cell lines or whether it also occurs in breast cancer tissue *in vivo*. For that purpose, we performed immunohistochemical staining with an affinity-purified, polyclonal anti-FI antibody. Validation of this method was performed on HEK293 cells transfected with human, full length FI or empty vector. Cell pellets were formalin-fixed, paraffin-embedded and passed through all steps of immunohistochemical staining as routinely applied for tissue specimens. We found staining conditions where mock-transfected cells remained colorless and FI-transfected cells were strongly stained in their cytosolic compartment (Fig. 2, upper left panel), as expected. Using the same conditions, we stained TMAs from 130 breast cancer patients and found cytosolic staining of different intensity not only in breast cancer cells (Fig. 2, bottom panel) but also in tumor stroma including infiltrating immune cells and fibroblasts (Fig. 2, upper right panel). Furthermore, we analyzed TMAs of normal, healthy breast tissue from 37 women and detected a degree of expression of FI in ductal epithelial cells and stroma (supplementary Fig.1). However, the

percentage of the specimens with the high expression of FI in cells was lower compared to tumor tissue (see details in Table 1 and supplementary Fig.1). Given the fact that FI is abundant in blood (the contents of small blood vessel in the tissue were accordingly strongly positive), we wanted to distinguish local production from diffusion and retention from the bloodstream. For this purpose we employed the RNAscope technique, which we first validated on HEK293 cell pellets (Fig. 3A). Further experiments on TMAs revealed expression of FI-specific mRNA in malignant breast cancer cells *in situ* (Fig. 3B).

FI in tumor tissue is associated with clinicopathological variables.

We assessed the intensity of FI staining from score 0 to 3 and performed separate analysis for tumor cells and tumor stroma including infiltrating cells (Table 1). The obtained values were associated with available clinical data: age, tumor size, Nottingham histological grade (NHG), lymph node metastases, ER, PR, Her2, and Ki-67. We also set out for separate assessment of FI staining in inflammatory cells but we have not found any correlations or associations with clinicopathological parameters. There was a statistically significant positive correlation between tumor size and FI staining intensity in tumor cells ($p < 0.001$) but not in stroma ($p = 0.405$). Analyses of tumor diameter medians showed that breast cancer cells expressing the highest levels of FI (score 3) formed the biggest tumors *in vivo*. Tumor-specific ($p = 0.015$), but not stromal ($p = 0.326$), FI expression was also associated with NHG. Since there were virtually no tumors sharing FI score 3 and the lowest possible NHG score meaning good differentiation, tubule formation and low mitotic rate, the trend towards more advanced NHG within tumors expressing more FI was observed. We observed an association of tumour cell-specific and stromal FI expression with ER status ($p = 0.030$ and $p = 0.004$, respectively). Distribution of ER- positive tumors followed the distribution of all tumors within the FI expression grades whereas the majority of ER-negative tumors were associated with high expression of FI (score 2 and 3). Similar statistically significant association was observed for PR status with p values 0.009 and 0.020, respectively. More PR-negative tumors were grouped in high FI grades comparing to PR-positive tumors. Stromal but not tumor-specific FI expression was significantly associated with Her2 status ($p = 0.006$), however the total number of Her2-positive tumors was low thus questioning the confidence of our observation. FI expression was not associated with proliferation as determined by Ki67 expression.

High expression of FI in tumor cells is associated with poor cancer-associated survival and recurrence-free survival.

For survival analyses, a dichotomized variable was created whereby tumors of FI intensity scores 0, 1 and 2 were denoted as having weak expression and tumors of intensity score 3 were considered as having strong FI expression. The rationale for this was a marked difference in FI staining intensity between score 2 and score 3 (see Fig. 2B) and the fact that survival curves for patients with scores 0, 1 and 2 looked very similar (not shown). Kaplan-Meier analyses of cancer-specific survival (CSS) and recurrence-free survival (RFS) revealed statistically significant differences between patients bearing tumors with weak and strong expression of FI (p values 0.004 and < 0.001, respectively) (Fig.4). Also, FI expression in stroma associated with RFS, but the p value was of lower confidence (p = 0.034) comparing to FI expression in tumor cells. Overall survival (OS, not shown) in which all cases of death were taken into account was associated with FI expression in tumor cells (p = 0.02) but not in stroma (p = 0.383). Next, uni- and multivariate Cox regression analyses of CSS and RFS in relation to FI expression were performed using the same dichotomized variables as in the Kaplan-Meier analyses. The result revealed that strong expression of FI in tumor cells but not in stroma was an independent marker for CSS (p = 0.048; HR: 2.8; 95%CI: 1.0 – 7.5) and RFS (p = 0.002; HR: 3.4; 95%CI: 1.5 – 7.4) when the variables described in table 2 were included. However, upon inclusion of nodal stage in the analysis, FI expression in tumor cells remained significant for RFS (p = 0.019; HR: 2.8; 95%CI: 1.2-6.4) but not for CSS (p = 0.123; HR: 2.5; 95%CI: 0.8-8.0). Details of Cox analyses for FI expression in tumor cells are reported in Table 2. Regarding FI expression in stroma, univariate Cox analysis resulted in no or weak statistical associations with CSS (p = 0.207; HR: 2.0; 95%CI: 0.7 – 6.1) and RFS (p = 0.045; HR: 2.5; 95%CI: 1.0 – 6.3).

Discussion

Our study contributes to the ongoing discussion about the exact role of complement in tumorigenesis. Although there are many reports supporting the theory of a dual role of complement in tumor growth (reviewed in [10]), only minor attention was devoted to soluble complement inhibitors. Of the existing studies, the majority relates to the main soluble inhibitor of the alternative complement pathway, factor H (FH). Experimental data show that FH expressed by tumor cells may influence tumor growth, as demonstrated in mouse xenograft experiments [5] and suggested by immunohistochemical staining of tumor tissue obtained from patients [30]. Interestingly for our study, FH is one of the main cofactors required for the activity of FI. Of note, there is only one study showing FI production by tumor cells *in vivo* in cutaneous squamous cell carcinoma [16] where there was association of FI staining intensity with aggressive tumor phenotype [16]. Previously we reported that non-small lung cancer (NSCLC) cell lines secrete several soluble complement inhibitors: FI and its cofactors FH and C4BP [15]. Although NSCLC cells strongly express membrane-bound complement inhibitors, we showed that addition of FI and its cofactors provides additional protection beyond the level attainable for membrane-bound inhibitors only [15]. Another interesting observation was that NSCLC cells seemed to purposely give up their protection from complement under hypoxic condition and this phenomenon remains in sharp contrast to endothelial cells [31]. These results underline the importance of soluble complement inhibitors as diffusible compounds, which may be used by tumor cells to actively control complement activation in their local microenvironment.

The expression of FI may face difficulties in cells other than hepatocytes due to insufficient cellular machinery for posttranslational processing, resulting in secretion of a precursor devoid of proteolytic activity [32]. Herein, we showed for the first time that breast cancer cell lines produce and secrete fully functional FI. This finding stimulated us to analyze whether FI is also expressed in breast cancer tissue *in vivo* and whether its expression associates with clinical outcome. Interestingly, we detected varying degrees of FI staining in ductal epithelial cells as well as in surrounding stroma already in the healthy breast tissue. Importantly we found that this local expression varies in cancer development and that a high expression of FI in breast cancer cells correlates to a more aggressive phenotype including lower differentiation of tumor cells and larger tumor size. We also observed majority of ER and PR receptor-negative tumors to be distributed within high grades of FI expression, as compared to general FI grade distribution. Loss of ER and PR by breast cancer cells is an attribute of the basal molecular subtype, which is associated with poor prognosis [33] and demands harsh adjuvant chemotherapy.

These associations may be reflected in the significantly shorter survival observed for patients with tumors expressing high levels of FI. Notably, our results from FI expression in breast cancer remain in line with the evidence from cell lines. Two out of four breast cancer cell lines identified in our study to express FI, namely MDA-MB-468 and MDA-MB-231 do not express ER and PR whereas MCF-7 cells are ER positive and T47D cells express both ER and PR.

There are several hypotheses why local production of soluble complement inhibitors may be important for tumor cells. In spite of the fact that these proteins are ubiquitous in the bloodstream (FI: 35 $\mu\text{g/ml}$, FH: 500 $\mu\text{g/ml}$, C4BP: 250 $\mu\text{g/ml}$), tumor vasculature is often malformed causing blood flow stagnation [34]. Reduced diffusion would also affect retention of complement components but neighboring cells may produce them instead. For example, adipocytes (usually adjacent to breast tissue) express three complement proteins: C3, factor B and protein D, which are sufficient for activation of the alternative complement pathway. Our current results are in agreement with findings showing that local production of FH favors tumor growth [5,30]. Noteworthy, FI is a more universal inhibitor since it inactivates not only the alternative but also the classical and lectin complement pathways. Another important observation from our study is the FI expression in tumor stroma, implying that not only tumor cells but also tumor-associated fibroblasts and infiltrating immune cells may actively regulate local complement activation within tumor tissue. We revealed that the list of FI-expressing cells is longer than initially believed and a systematic study on FI expression is needed to clarify this issue.

Beside an obvious pro-tumor activity of endogenous complement inhibitors, there are also reports showing tumor-sustaining events driven by complement activation, such as mobilization of immune suppressor cells [6] and angiogenesis [7]. The reason why human immunohistochemical studies has so far only confirmed pro-tumorigenic effects of soluble complement inhibitors may be related to the fact that these studies enable insight only into outgrown tumors but miss the very first stages of tumor formation. It is highly possible that the same mechanisms, which protect outgrown tumors from the complement attack, must be suspended at the earlier stages of tumorigenesis. Keeping that in mind we conclude that FI supports breast cancer at the certain stage when the tumor is palpable. However, one must be careful with extrapolation of our findings to the whole sequence of tumor growth. Conversion of surface-bound C3b into iC3b, an opsonin recognized more readily by CR3 expressing cells including phagocytes, T cells and NK cells, is another consequence of FI activity, yet unfavorable for tumor cells. However, inhibition of complement-mediated cell lysis by FI may be physiologically more relevant than activation of complement dependent cell cytotoxicity (CDCC)

by iC3b due to different accessibility of tumor cells for soluble components and CDCC effector cells. Moreover, iC3b stimulated CDCC may need additional priming, as reviewed in [35].

In conclusion, the results from this study demonstrate, for the first time, that FI is expressed in breast cancer cells *in vivo* and its expression correlates to clinical outcome. We noticed not only correlation of FI expression with tumor size and NHG or association with steroid receptor status but also revealed the prognostic value of FI in tumor cells. When the presence of metastases (nodal stage), an obvious and exceptionally strong prognostic marker for survival and recurrence in breast cancer is excluded, tumor-specific FI expression acts as an independent marker for RFS and CSS. Even if nodal status is included, tumor-derived FI still independently predicted RFS in the current cohort. These findings underline the importance of soluble complement inhibitors in tumor progression.

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Conflict of interests

Authors declare no conflict of interest

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Table 1. Associations between FI expression and clinicopathologic features in primary breast cancer.

Factor	FI staining intensity in tumor cells						FI staining intensity in stroma					
	n	0	1	2	3	p value	n	0	1	2	3	p value
All n (%)	130	7 (5)	42(32)	53(41)	28(22)		129	15(12)	44(34)	55(43)	15(12)	
Age	130					0.143 ^a	129					0.507 ^a
median (range)		59 (47-81)	64.5 (35- 86)	64 (34- 97)	73 (41- 91)			63 (35-91)	64.5 (40- 97)	63 (34-91)	75 (41-89)	
Size (tumor diameter, mm)	130					<0.001 ^a	129					0.405 ^a
median (range)		22 (11- 28)	19 (8- 60)	21 (8- 80)	27.5 (13-145)			26 (11-60)	22 (8- 140)	20 (8-145)	21 (13-73)	
NHG						0.015 ^a						0.326 ^a
I (%)	18	2 (11)	9 (50)	7 (39)	0 (0)		17	2 (12)	6 (35)	8 (47)	1 (6)	
II (%)	58	2 (3)	20 (35)	23 (40)	13 (22)		58	6 (10)	23(40)	24(41)	5 (9)	
III (%)	54	3 (6)	13(24)	23(43)	15(28)		54	7 (13)	15(28)	23(43)	9 (17)	
Nodal status						0.215 ^a						0.824 ^a
0 (%)	65	4 (6)	23(35)	26(40)	12(19)		65	8 (12)	22(34)	26(40)	9 (14)	
1-3 (%)	32	1 (3)	12(38)	14(44)	5 (16)		31	5 (16)	12(39)	11(36)	3 (10)	
4 (%)	21	1 (5)	5 (24)	7 (33)	8 (38)		21	2 (10)	5 (24)	11(52)	3 (14)	
missing	12						12					
ER status						0.030 ^b						0.004 ^b
negative (%)	17	1 (6)	2 (12)	7 (41)	7 (41)		17	1 (6)	1 (6)	11(65)	4 (24)	
positive (%)	113	6 (5)	40(35)	46(41)	21(19)		112	14(13)	43(38)	44(39)	11(10)	
PR status						0.009 ^b						0.020 ^b
negative (%)	40	1 (3)	9 (22)	16(40)	14(35)		39	4 (10)	8 (21)	19(49)	8 (20)	
positive (%)	90	6 (7)	33(37)	37(41)	14(16)		90	11(12)	36(40)	36(40)	7 (8)	
HER2 ^c						0.304 ^b						0.006 ^b
weak (%)	118	6 (5)	39(33)	49(42)	24(20)		117	15(13)	40(34)	52(44)	10 (9)	
strong (%)	9	0 (0)	3 (33)	2 (22)	4 (44)		9	0 (0)	2 (22)	2 (22)	5 (56)	
missing	3						3					
Ki-67						0.516 ^b						0.137 ^b
≤ 25%	54	4 (7)	15(28)	24(44)	11(20)		53	6 (11)	23(43)	19(36)	5 (9)	
>25%	59	2 (3)	18(31)	23(39)	16(27)		59	7 (12)	15(25)	29(49)	8 (14)	
missing	17						17					

^a Spearman correlation, 2-tailed p-value

^b Mann-Whitney U test for comparison of median, 2-tailed exact p-value

^c weak (score 0-2), strong (score 3)

Table 2. Univariate and multivariate Cox regression analyses of the impact on breast cancer specific survival and recurrence free survival for different patient and tumor characteristics. FI score was dichotomized into weak (value 0, 1, 2) and strong (3).

Variable	Univariate				Multivariate			
	<i>n</i>	HR	95% CI	<i>p</i>	<i>n</i>	HR	95% CI	<i>p</i>
Survival (CSS)								
FI (weak vs. strong)	129	3.4	1.4-8.2	0.006	111	2.8	1.0-7.5	0.048
ER (neg vs. pos)	144	0.2	0.1-0.5	0.001	111	0.9	0.3-2.7	0.823
HER2 (weak vs. strong)	139	7.7	3.0-19.8	>0.001	111	5.8	1.7-20.0	0.006
NHG (1, 2, 3)	144	5.1	2.0-13.0	>0.001	111	1.1	0.3-4.0	0.923
Size (\leq 20 mm vs. >20 mm)	144	3.0	1.2-7.8	0.020	111	3.3	0.9-12.8	0.080
Ki-67 (\leq 25% vs. >25%)	123	4.2	1.4-12.5	0.010	111	3.0	0.7-12.6	0.137
Recurrence free survival								
Variable	<i>n</i>	HR	95% CI	<i>p</i>	<i>n</i>	HR	95% CI	<i>p</i>
FI (weak vs. strong)	130	4.4	2.0-9.4	>0.001	127	3.4	1.5-7.4	0.002
ER (neg vs. pos)	144	0.3	0.1-0.6	>0.001	127	0.8	0.3-2.0	0.577
HER2 (weak vs. strong)	139	7.1	3.0-16.9	>0.001	127	5.7	2.3-14.5	>0.001
NHG (1, 2, 3)	144	3.2	1.6-6.2	>0.001	127	1.6	0.7-3.4	0.287
Size (<20 mm vs. >20 mm)	144	3.1	1.4-7.1	0.006	127	2.3	1.0-5.7	0.067

Abbreviations: HR, hazard ratio, CI, confidence interval

Figure legends

Fig. 1 Expression of FI in breast cancer cell lines

A) FI expression in breast cancer cell lines was assessed on mRNA level by qPCR and related to three housekeeping genes. **B)** Production of FI on protein level was confirmed by western blotting performed on serum free medium conditioned by breast cancer cells. **C)** Functional activity of FI produced by MDA-MB-468 cells was shown using C3b/C4b degradation assay in which incubation of C3b or C4b with FI cofactor C4BP and conditioned medium resulted in appearance of degradations products of C3b and C4b. For both C3b and C4b, degradation was abolished when FI blocking antibody was added to the conditioned medium.

Fig. 2 Immunohistochemical staining of FI-expressing cells and breast cancer tissue.

Upper left panels represent pellets of HEK-293 cells transfected with empty vector or full length human FI. The same conditions were used for staining of breast cancer TMAs, which showed FI presence in stroma (infiltrating immune cells and fibroblasts pointed out by red arrow; upper right part) and breast cancer tumor cells (bottom panel). Examples of scoring of FI intensity in tumor cells are given in the bottom panel.

Fig. 3 Detection of FI-specific RNA by RNAscope technique.

A) Validation of RNA probes on HEK-293 cells transfected with empty vector or full length human FI followed by paraffin embedding and treatment identical to breast cancer tissue **B)** representative photographs of consecutive slides of breast cancer tissue stained with negative control RNA probe, positive control RNA probe and FI-specific probe. The same area of tumor tissue stained for FI with immunohistochemistry is given at the left side.

Fig. 4 The effect of weak vs. strong FI expression on survival and recurrence.

Kaplan-Meier estimates of cancer-specific survival (**A, C**) and recurrence-free survival (**B, D**) for weak (scores 0, 1, and 2) vs. strong (score 3) FI expression in tumor cells and stroma. High expression of FI in tumor cells was significantly associated with shorter cancer specific and recurrence free survival. For high FI expression in stroma only association with shorter recurrence free survival was statistical significant.

Fig.1

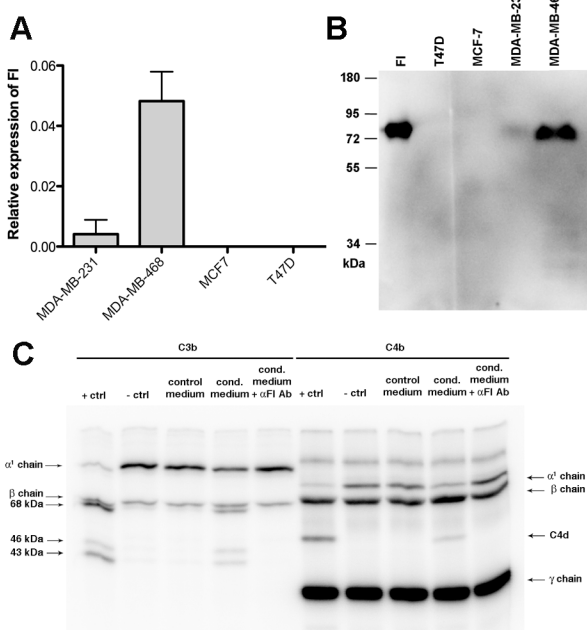
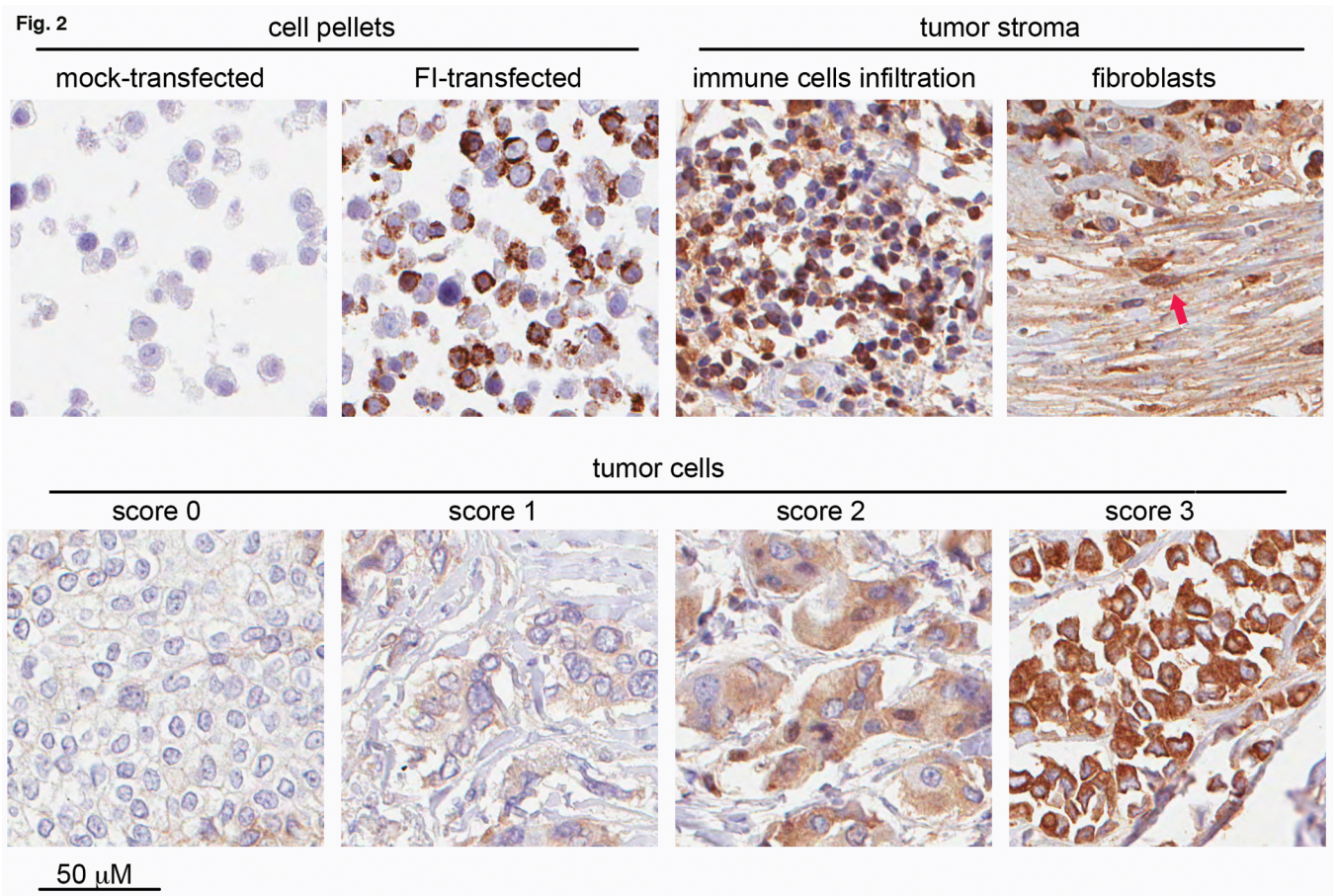


Fig. 2



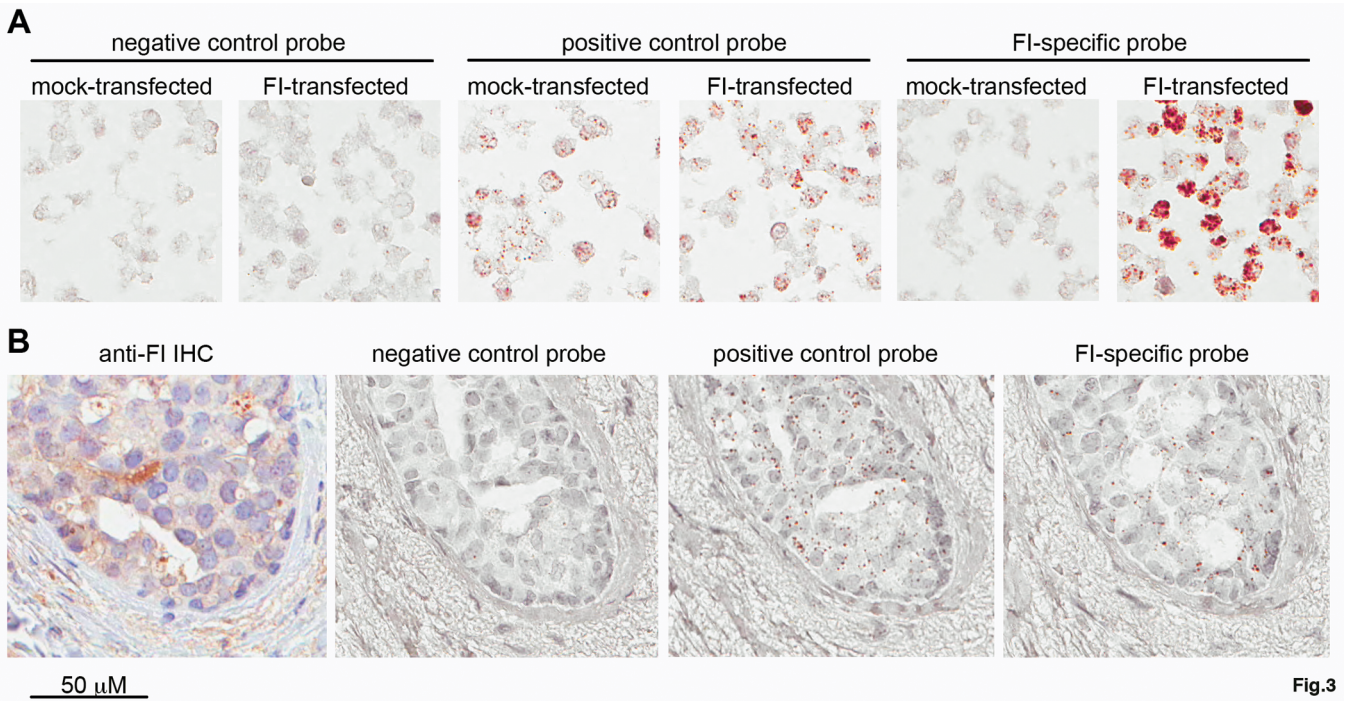
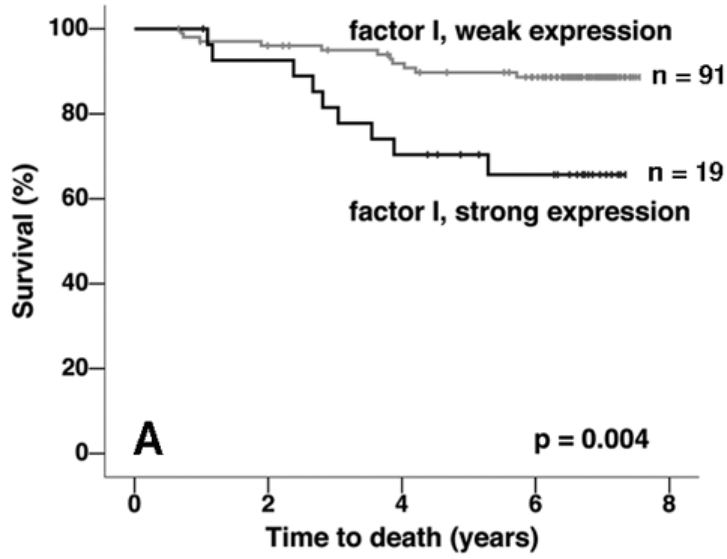
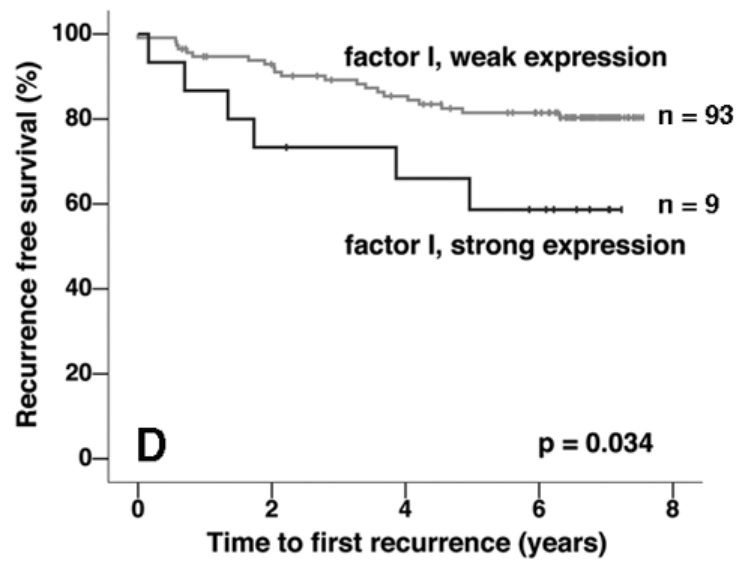
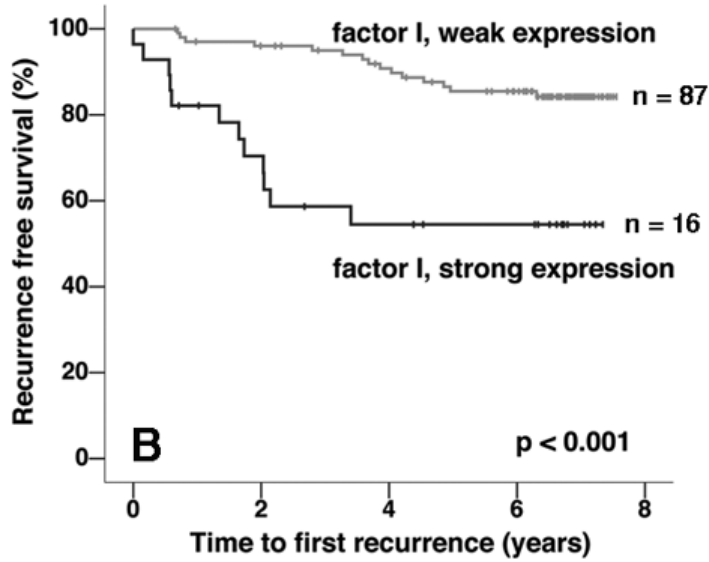
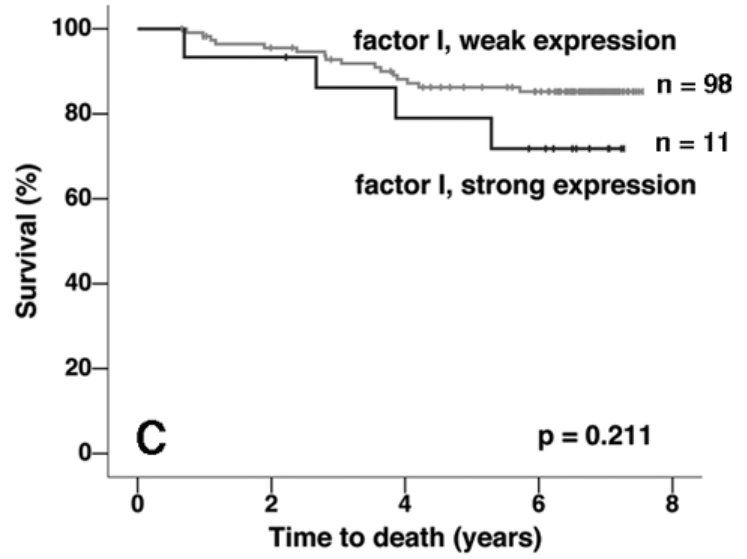


Fig. 4 Tumor cells

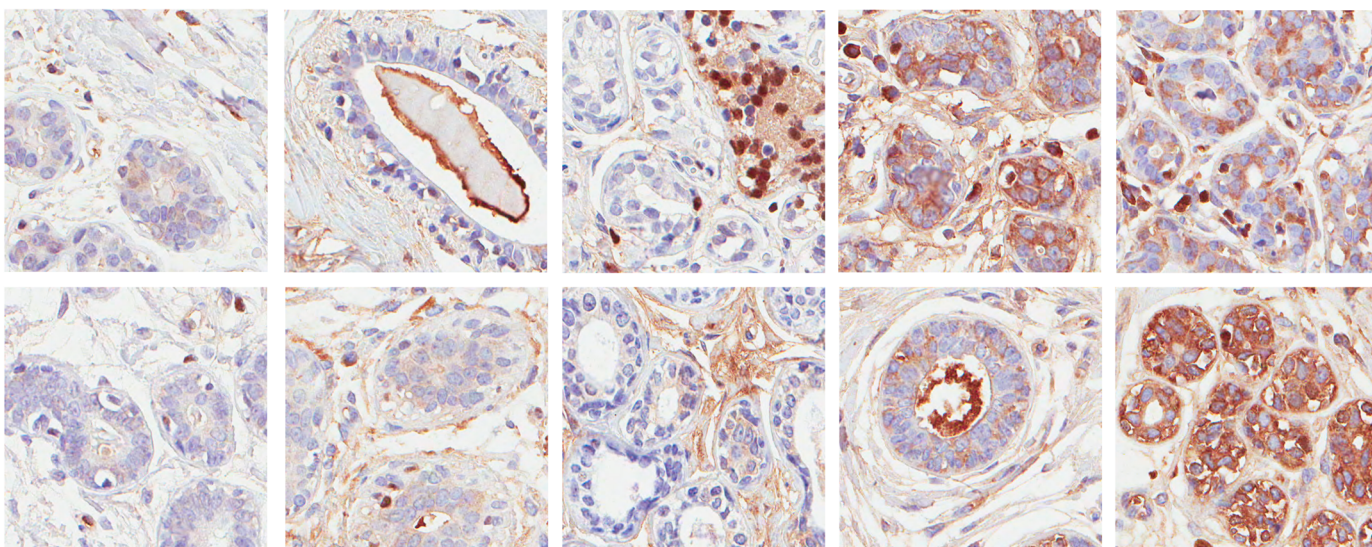


Stroma



Supplementary Fig.1

A



50 μ M

B	Staining intensity in ductal epithelial cells					Staining intensity in stroma				
	all	score 0	score 1	score 2	score 3	all	score 0	score 1	score 2	score 3
<i>n</i>	37	7	18	9	3	35	1	12	19	3
%		19	48	24	8		3	34	54	8

A) Representative sections of normal breast tissue showing different intensity of staining for FI in stroma and ductal epithelial cells. **B)** Table summarizing the total number and percentage of healthy individuals with given FI staining intensity in their breast tissue. Scoring was performed in the same way as described for the patients with breast cancer, summarized in Table 1.