

High Farnesoid X Receptor (FXR) expression is a strong and independent prognosticator in invasive breast carcinoma

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Farnesoid X Receptor (FXR), a nuclear receptor superfamily member, is related with bile acids, glucose and lipids metabolism and recently with cancer. In the present study the clinical significance of FXR expression in invasive breast carcinoma was evaluated. FXR protein expression was assessed immunohistochemically on paraffin-embedded breast cancer tissues obtained from 115 breast cancer patients and was statistically analyzed with clinicopathological parameters, estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) expression, as well as with tumor cells' proliferative capacity and overall and disease-free patients' survival. FXR positivity was noted in 91 (79.1%) and high FXR expression in 51 (44.3%) out of 115 invasive breast carcinoma cases. High FXR expression was significantly associated with smaller tumor size ($p=0.0318$) and increased tumor cells' proliferative rate ($p=0.0375$). Invasive breast carcinoma patients presenting high FXR expression showed significantly longer overall and disease-free survival times compared to those with low FXR expression (log-rank test, $p=0.0052$ and $p=0.0058$). In multivariate analysis, FXR expression was identified as independent prognostic factor of overall and disease-free patients' survival (Cox-regression analysis, $p=0.0023$ and $p=0.0049$, respectively). The present data support evidence that FXR may be implicated at the earlier stage of breast malignant disease progression, being a strong and independent prognosticator of favorable overall and disease-free survival in invasive breast carcinoma.

Key words: FXR, breast cancer, immunohistochemistry, clinicopathological parameters, patients' prognosis

Farnesoid X Receptor (FXR), initially cloned at 1995, belongs to a group of metabolic nuclear receptors, including Vitamin D Receptor (VDR), Pregnane X Receptor (PXR), Liver X Receptor (LXR) and Constitutive Androstane Receptor (CAR) [1, 2]. FXR mainly regulates several genes involved in bile acid, lipid and glucose metabolism, by binding to DNA either as a monomer or an heterodimer with common partners for nuclear receptors, Retinoid X Receptors (RXRs) [1, 2]. FXR is highly expressed in the liver, intestine, kidney and adrenals [3]. Two known FXR genes exist, the *Fxrα* and *Fxrβ*. In humans, *Fxrα* gene encodes four FXRα isoforms (FXRα1, FXRα2, FXRα3 and FXRα4) as a result of different promoters and alternative RNA splicing [3], while *Fxrβ* is a pseudogene with uncertain role [3]. Most FXR target genes are independently regulated by all FXRα isoforms, while other target genes, including those encoding intestinal bile acid binding protein (IBABP), syndecan-1, αA-crystallin and fibroblast growth

factor 19 (FGF19), are isoform-specific and mainly response to the FXRα2 and FXRα4 isoforms [1-3].

Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death among females, accounting for 23% of the total cancer cases and 14% of the cancer deaths [4]. Mammary tumors present highly complexity and heterogeneity, while global understanding of the underlined molecular mechanisms governing their origin and progression is still lacking [5]. Molecular imaging has been considered to exert a promising role in complementing and overcoming some of the limitations of traditional biomarkers by providing the ability to perform noninvasive, repeatable whole-body assessments [6]. Estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) define prognosis and identify tumors for targeted therapy, and remain the sole established single-molecule biomarkers defining the minimum breast cancer pathology data set [7].

ER-targeted endocrine therapies are effective for the treatment of patients with ER-positive breast tumors and tamoxifen is currently the most widely used endocrine anti-estrogen treatment [8].

In the last decade, FXR has been implicated in the pathogenesis of cholestatic, non-alcoholic fatty liver and inflammatory bowel disease [9-11]. FXR has also been involved in the development of atherosclerosis, intestinal bacterial growth and liver regeneration [12-14]. Notably, recent accumulative evidence has further suggested that FXR may exert a potential protective role against tumorigenesis by promoting apoptosis and inhibiting cell proliferation, as recently critically reviewed by our group [15, 16]. However, apart from the gradually increasing research conducted on cultured cell lines and animal models, there is no comprehensive clinical data so far concerning the involvement of FXR in human malignant transformation [15]. In fact, certain small pilot cohort studies have currently been conducted on esophageal, breast, hepatocellular, pancreatic and colon carcinoma; however, most of them did not concern potential associations of FXR expression with clinicopathological parameters and patients' prognosis [17-25]. In addition, there are also clinical data suggesting that FXR was highly expressed in breast cancer, being associated with tumor cells' proliferative capacity and ER status [18, 19, 26]; however, its clinical and prognostic value has not been evaluated yet.

In view of the above considerations, the present study is aimed to evaluate the immunohistochemical expression of FXR in invasive breast carcinoma tissue samples in association with multiple clinicopathological characteristics, ER, PR and HER2 expression, as well as with overall and disease-free patients' survival.

Patients and methods

Patients. One hundred fifteen patients aged from 32 to 87 years (mean 57.6 years) who underwent surgical resection due to invasive breast carcinoma were included in this study. None of them had received radiation or chemotherapy pre-operatively. The study was approved by the institutional ethical committee of the Medical School of the University of Athens. Informed consent was signed by all patients under study in order to use for research purposes their biological samples and clinical data [27].

Routine histological examination was performed with haematoxylin and eosin staining. All cases were classified in accordance with World Health Organization criteria [28] and were recorded as invasive ductal or lobular carcinoma. Nuclear grading was based on nuclear pleomorphism. Staging at the time of diagnosis was based on the TNM system [29]. The combined histological grade (1, 2 or 3) of infiltrating ductal and lobular carcinomas was obtained according to a modified Scarff-Bloom-Richardson histological system with guidelines as suggested by Nottingham City Hospital pathologists [30]. The clinicopathological characteristics of the series are shown in Table 1.

Table 1. Associations between FXR expression and clinicopathological parameters in 115 patients with invasive breast carcinoma

Clinicopathological parameters	FXR expression		p-value
	Low (%)	High (%)	
N=115	64 (55.7)	51 (44.3)	
Age (mean±SD;ys)			0.9843
≤ 57.6±12.6 yrs	30 (26.1)	24 (20.9)	
> 57.6±12.6 yrs	34 (29.6)	27 (23.5)	
Menopausal status			0.8453
Premenopausal	19 (16.5)	16 (13.9)	
Postmenopausal	45 (39.1)	35 (30.4)	
Histopathological type			0.9150
Ductal	47 (40.9)	37 (32.2)	
Lobular	17 (14.8)	14 (12.2)	
Histological Grade			0.3141
1	5 (4.4)	5 (4.4)	
2	30 (26.1)	30 (26.1)	
3	29 (22.2)	16 (13.9)	
Nuclear Grade			0.4350
1	26 (22.6)	26 (22.6)	
2	18 (15.7)	14 (12.2)	
3	20 (17.4)	11 (9.6)	
Molecular subtype			0.8580
Luminal-A	28 (24.3)	25 (21.7)	
Luminal-B	9 (7.8)	7 (6.1)	
HER2	8 (7.0)	4 (3.5)	
Triple negative	19 (16.5)	15 (13.0)	
Tumor size			0.0318
pT1	15 (13.0)	16 (13.9)	
pT2	39 (33.9)	34 (29.6)	
pT3	10 (8.7)	1 (0.9)	
Lymph nodes			0.7474
Non infiltrated	27 (23.5)	20 (17.4)	
Infiltrated	37 (32.2)	31 (27.0)	
Histopathological stage			0.5657
I	11 (9.6)	12 (10.4)	
II	42 (36.5)	33 (28.7)	
III	11 (9.6)	6 (5.2)	
ER expression			0.9505
Negative	33 (28.7)	26 (22.6)	
Positive	31 (27.0)	25 (21.7)	
PR expression			0.4161
Negative	35 (30.4)	24 (20.9)	
Positive	29 (25.2)	27 (23.5)	
HER-2 expression			0.4170
Negative	56 (48.7)	47 (40.9)	
Positive	8 (7.0)	4 (3.5)	
Ki-67 protein statement			0.0375
Below median value	37 (32.2)	20 (17.4)	
Over median value	27 (23.5)	31 (27)	

The patients were followed up for a time interval of 8 up to 210 months with a mean survival time of 84.6 ± 45.0 months. Overall survival was defined as the time interval between the date of surgery and the date of death due to breast carcinoma or the last follow-up. Disease-free survival was defined as the time interval between the date of surgery and the date of detection of recurrence or the date of last follow-up without recurrence for breast carcinoma. At the time of the last follow-up, 21 (18.3%) patients had died from disease, 11 (9.6%) were alive with disease and 79 (68.7%) were alive and disease-free. All patients received conventional postoperative treatment depending on the disease extent, including adjuvant chemotherapy, radiation therapy and anti-estrogen therapy, when indicated, according to the consensus recommendations at the time [27].

Immunohistochemistry. Immunohistochemistry was performed on 4 μ m formalin-fixed paraffin embedded breast tissue sections using commercially available rabbit polyclonal anti-FXR (H-130, sc13063, Santa Cruz Biochemicals, Santa Cruz, CA, USA). The immunohistochemistry was performed in automated immunohistochemical stainer (Bond, Leica Biosystems) with Bond Polymer Refine Detection System. The protocol in immunostainer was: Antigen retrieval with ER1 solution (citrate buffer pH 6.0) for 20 minutes, incubation with marker (anti-FXR dilution 1:75) for 30 minutes, incubation with postprimary reagent 15 minutes and with polymer reagent 15 minutes. Appropriate negative controls were performed by omitting the primary antibody and/or substituting it with an irrelevant anti-serum. As positive control, pancreatic adenocarcinoma tissue sections with known increased FXR expression was used [22]. The tumor cells' proliferative capacity was assessed immunohistochemically, using a mouse anti-human Ki-67 antigen; IgG1k antibody (clone MIB-1, Dakopatts, Glostrup, Denmark) as previously described [22]. The expression of ER, PR and HER2 was assessed immunohistochemically, as previously described [27].

Evaluation of immunohistochemistry. Immunohistochemical evaluation was performed by counting at least 1000 tumor cells in each case by two independent observers (S.T. and P.A.) blinded to the clinical data, with complete observer agreement. Specimens were considered "positive" for FXR when more than 5% of tumor cells within the section were positively stained. FXR immunoreactivity was scored according to the percentage of positive tumor cells as 0: negative staining- 0-4% of cells positive; 1: 5-24% of cells positive; 2: 25-49% of cells positive; 3: 50-100% of cells positive, and its intensity as 0: negative staining, 1: mild staining; 2: intermediate staining; 3: intense staining. Finally, the expression of FXR was classified as low; if the total score was 0 or 2 and high; if the total score was ≥ 3 . In this way, we ensure that each group has a sufficient and more homogeneous number of cases in order to be comparable with the other groups [22, 31, 32].

Staining for ER and PR was evaluated according to CAP/ASCO recommendations, i.e. ER and PR assays are considered positive if there are at least 1% positive tumor nuclei in the sample in the presence of the expected reactivity of internal

and external controls [33]. The fraction of HER2 positive stained cells was scored from 0 to 3 according to CAP/ASCO guidelines [34]. Ki-67 immunoreactivity was classified according to the percentage of positively stained tumor cells exceeded the median percentage value into two categories (below and over mean value), as previously reported [22, 31, 32].

Statistical analysis. Chi-square test was used to assess the associations of FXR protein expression with clinicopathological variables and ER, PR, HER2 and Ki-67 protein expression. Survival curves were constructed using the Kaplan-Meier method and the differences between the curves were compared by the log rank test. A Cox proportional-hazard regression model was developed to evaluate the association between the potential prognostic marker and overall survival, at multivariate level. A p-value less than 0.05 was considered the limit of statistical significance. SPSS for Windows Software was used for all analyses (SPSS Inc., 2003, Chicago, USA).

Results

FXR positivity (IHC score > 0) was noted in 91 (79.1%) out of 115 invasive breast carcinoma cases. Fifty-one (44.3%) out of the 115 examined cases presented high FXR expression (IHC score ≥ 3). The subcellular pattern of FXR distribution was cytoplasmic in 35 (38.5%), nuclear in 9 (9.9%) and both cytoplasmic and nuclear in 47 (51.7%) out of the 91 FXR-positive cases. Forty-seven (52.8%) out of 91 FXR-positive invasive breast cancer cases presented mild staining intensity, while 35 (38.5%) and 8 (8.8%) out of 91 FXR-positive invasive breast carcinoma cases presented moderate or intense staining intensity, respectively. Low FXR expression levels were noted in normal breast cells, adjacent to tumor, or in carcinoma in situ cases. Normal ductal cells were either FXR-negative or presented mild to moderate FXR nuclear and/or mild cytoplasmic pattern of immunostaining (Figure 1A). Similar expression pattern was also noted in the case of breast in situ carcinoma (Figure 1B), although increased percentage of positive cells was found when compared with normal breast. Representative FXR immunostainings in breast invasive carcinoma cases presenting mild, moderate and intense staining intensity are depicted in Figure 2A, 2B and 2C, respectively. Fifty-six (58.7%) out of 115 invasive breast carcinoma cases were ER positive. PR positivity was noted in 56 (58.7%) out of 115 cases, while 12 (10.4%) cases were HER2 positive. Fifty-three (46.1%) out of 115 breast carcinoma cases were classified as luminal-A, 16 (13.9%) cases as luminal-B, 34 (29.6%) as triple negative and 12 (10.4%) as HER2-phenotype.

High FXR expression was significantly more frequently observed in invasive breast carcinoma patients presenting smaller tumor size (Table 1, $p=0.0318$). High FXR expression was also significantly associated with increased tumor cells' proliferative rate (Table 1, $p=0.0375$). An increased incidence of high FXR expression was noted in invasive breast carcinoma patients with low histological and nuclear grade, at a no significant level though (Table 1, $p=0.3141$ and $p=0.4350$, respectively).

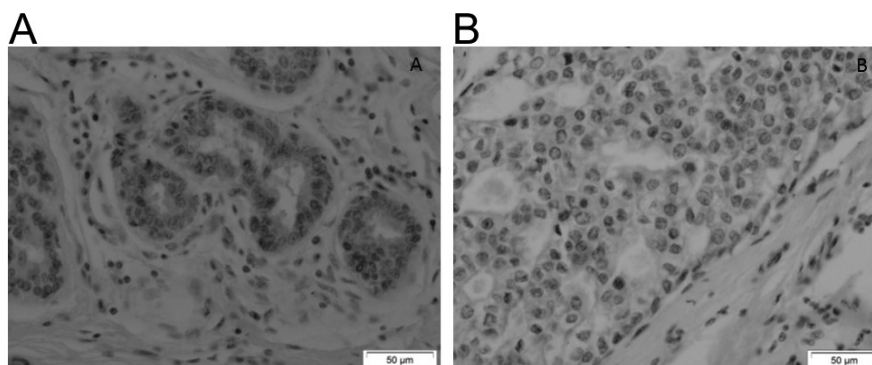


Figure 1. Representative FXR immunostainings in normal breast ductules (A) and in breast carcinoma in situ (B) (original magnification X200, scale bars are present on figures).

High FXR expression was also more frequently observed in breast carcinoma patients presenting earlier disease stage, at a no significant level though ($p=0.5657$). None associations or trends of correlation between FXR expression and the other clinicopathological parameters examined were noted (Table 1). FXR expression was not different between the molecular breast carcinoma subtypes (Table 1).

Kaplan-Meier survival curves indicated that invasive breast carcinoma patients presenting high FXR expression showed significantly longer overall and disease-free survival times compared to those with low FXR expression (Figure 3A and 3B, log-rank test, $p=0.0052$ and $p=0.0058$, respectively). In multivariate analysis, nuclear grade, histopathological stage, Ki-67 protein statement and FXR expression were identified as independent prognostic factors of overall patients' survival (Table 2, Cox-regression analysis, $p=0.0498$, $p=0.0053$, $p=0.0093$ and $p=0.0023$, respectively). Histopathological stage, Ki-67 protein statement and FXR expression were also identified as independent prognostic factors of disease-free patients' survival (Table 2, Cox-regression analysis, $p=0.0018$, $p=0.0437$ and $p=0.0029$, respectively).

Discussion

Undergoing research is currently focused on the role of FXR in crucial biochemical and cellular processes implicated in several pathological disease states, such as cholestatic, non-alcoholic fatty liver and inflammatory bowel disease, atherosclerosis, intestinal bacterial growth and liver regeneration, as recently reviewed by our group [15, 16]. A gradually growing body of in vitro and animal studies has further extended the role of FXR in oncogenic transformation [15, 16]. However, the assessment of the clinical significance of FXR expression in human malignancies remains scarce, being restricted to a small number of pilot cohort studies that mostly did not concern associations with clinicopathological parameters and patients' prognosis [17-25]. Moreover, there is not any clinical data up to now concerning the prognostic significance of FXR in invasive breast carcinoma.

In this aspect, the present study assessed the FXR expression levels in tumoral samples of invasive breast carcinoma patients, investigating for their association with crucial clinicopathological characteristics and patients' survival.

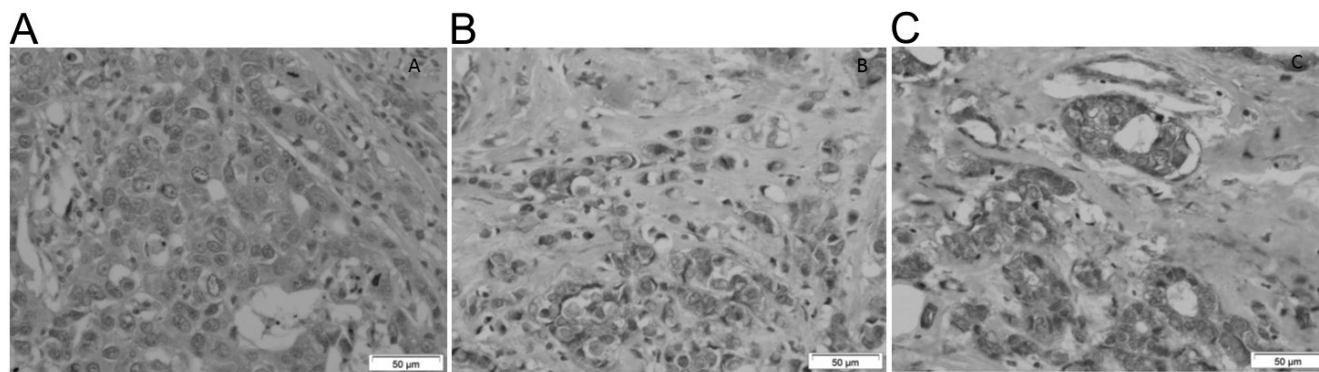


Figure 2. Representative FXR immunostainings in breast carcinomas presenting mild (A), moderate (B) and intense (C) staining intensity (original magnification X200, scale bars are present on figures).

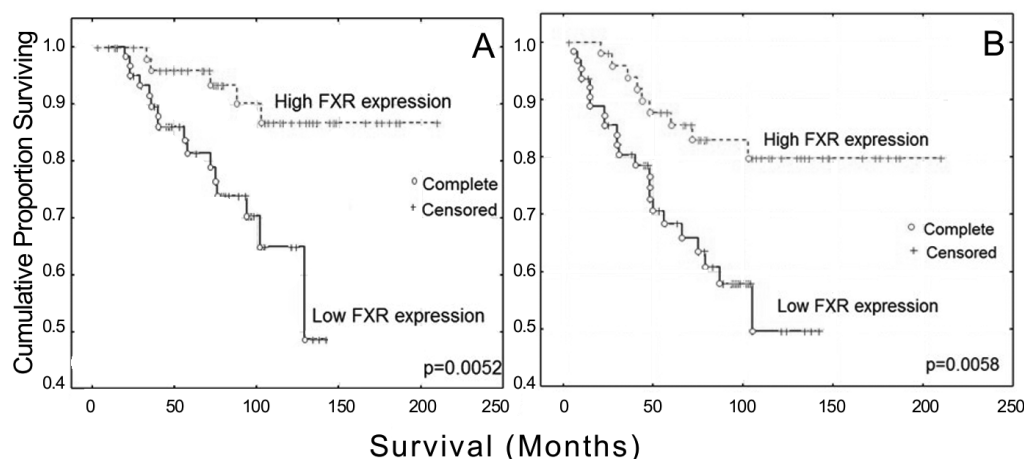


Figure 3. Kaplan-Meier survival analysis stratified according to FXR expression in 115 breast carcinoma patients: (A) Overall patients' survival and (B) Disease-free patients' survival.

According to our results, approximately half of the examined invasive breast carcinoma cases presented high FXR expression levels and all the examined cases presented negative or mild nuclear FXR immunostaining in non-malignant breast tissue. Moreover, it should be noted that FXR subcellular distribution was found predominately cytoplasmic in the vast majority (89.6%) of the examined breast carcinoma cases. The above finding may suggest that either FXR may not be translocated to the nucleus remaining in the cytoplasm or the excess amount produced was retained in the cytoplasm in malignant disease state.

The present study also showed that high FXR expression was associated with smaller tumor size. At the same time, a positive association between FXR and tumor cells' proliferative rate was recorded. Moreover, we found that high FXR expression is associated with longer overall and disease-free patients' survival at both univariate and multivariate level. Thus, it could be speculated that FXR is a strong and independent prognosticator of favorable overall and disease-free survival in invasive breast carcinoma. Such data also reinforces the therapeutic utility of its targeting in breast cancer chemoprevention, since several in vitro and animal studies have documented that FXR may be considered as a potential molecular target in breast neoplasia [15].

As far as concern the existing data so far on breast neoplasia, an immunohistochemical study was initially performed on 10 breast carcinoma tissue samples and identified FXR expression in both the ductal epithelial cells of normal breast and infiltrating ductal adenocarcinoma cells [18]. Moreover, enhanced FXR expression was shown in breast carcinoma cell lines MCF-7 (ER-positive) and MDA-MB-231 (ER-negative) [18]. A more recent study conducted on 204 breast carcinoma patients showed that FXR expression was significantly correlated with the proliferation marker Ki-67 and the nodal status [19]. Moreover, this study documented that FXR expression was positively correlated with ER and PR expression and luminal-phenotype [19]. In contrast, we did not find any association between FXR and ER or PR expression. This controversy may be ascribed to the fact that the above study [19] considered nuclear staining to define FXR expression, whereas in the present study both cytoplasmic and nuclear staining were taken into account to semi-quantify FXR expression. Moreover, the above study [19] reported exclusively nuclear FXR staining, whereas in our study the vast majority of the examined breast carcinoma cases presented cytoplasmic or both cytoplasmic and nuclear staining. This discrepancy may also be ascribed to the different primary antibody used to detect FXR immunoreactivity. In another study by the same

Table 2. Multivariate analysis for nuclear grade, histopathological stage, Ki-67 statement and FXR expression for overall and disease-free patients' survival

Clinicopathological Variables	Overall survival		Disease-free survival	
	HR (95% CI)	p-value	HR (95% CI)	p-value
Nuclear Grade (I / II+III)	2.766 (1.095-3.656)	0.0498	2.015 (0.873-3.976)	0.1636
Histopathological stage (I+II / III)	4.048 (2.875-6.755)	0.0053	3.351 (2.018-6.129)	0.0018
Ki-67 statement (Below/over median value)	3.695 (2.229-6.452)	0.0093	2.124 (0.984-3.685)	0.0437
FXR expression (Low / High)	0.183 (0.022-0.589)	0.0023	0.289 (1.257-2.598)	0.0029

group, immunohistochemical analysis on 65 breast carcinoma samples established significant correlations between FXR expression and ER, Ki-67 and topoisomerase-II alpha expression [26]. Interestingly, activation of FXR by the primary bile acid chenodeoxycholic acid or the synthetic agonist GW4064, inhibited growth of tamoxifen-resistant breast cancer cells, in vitro, through downregulation of HER2 expression, which emphasized the importance of targeting FXR in breast cancer [35].

Concerning the existing clinical evidence so far in other human malignancies, FXR expression was inversely correlated with neoplastic transformation/progression and inflammation severity in ulcerative colitis [24]. Moreover, primary sclerosing cholangitis – ulcerative colitis patients presented diminished FXR expression in the proximal colon compared to ulcerative colitis patients [24]. In human hepatocellular carcinoma, FXR expression was down-regulated, being positively correlated with multiple malignant clinicopathological characteristics [21]. FXR overexpression was also associated with poor histopathological grade, larger tumor size and presence of lymph node metastasis in 59 esophageal adenocarcinoma patients [23]. In another study, FXR was detected in non-dysplastic tissue, but its expression was lost during progression to dysplasia and adenocarcinoma in Barrett's esophagus patients [20]. FXR mRNA levels were reduced in adenomas compared to normal colorectal mucosa, while an even more pronounced decrease in colon carcinomas was recorded [17]. In another study, FXR expression was reduced in colon carcinoma compared to peritumoral nonneoplastic mucosa [25]. Loss of FXR expression was correlated with high tumor grade in the right colon. Moreover, FXR expression in tumor and normal colon tissue showed an inverse correlation with histopathological stage, while FXR expression in tumor was inversely correlated with clinical outcome [25]. At last, in a recent cohort study conducted by our group on 55 pancreatic adenocarcinoma patients, enhanced FXR expression was associated with earlier histopathological stage [22]. Moreover, FXR expression was identified as a strong and independent prognosticator of favorable overall patients' survival [22].

Conclusions. The present study supported clinical evidence that FXR expression is a strong and independent prognosticator of favorable overall and disease-free survival in invasive breast carcinoma. FXR seems to be implicated at the earlier stage of breast malignant disease progression. The present study further suggested that FXR translocation from nucleus to cytoplasm may be a potential event during malignant breast transformation process. In this aspect, additional research conducted on larger cohorts and on each molecular subtype separately could evaluate whether FXR may be considered of diagnostic and prognostic utility in breast neoplasia, exploring also its usefulness as potential therapeutic target in breast neoplasia.

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