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Thioredoxin Interacting Protein (TXNIP) is an Independent Risk Stratifier for Breast Ductal Carcinoma *in situ*

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Abstract:

Current clinicopathological parameters are useful predictors of breast ductal carcinoma *in situ* behaviour, but they are insufficient to define high risk patients for disease progression precisely. Thioredoxin interacting protein (TXNIP) is a key player of oxidative stress. This study aims to evaluate the role of TXNIP as a predictor of ductal carcinoma *in situ* progression. Tissue microarrays from 776 pure ductal carcinoma *in situ* and 239 mixed ductal carcinoma *in situ* and invasive tumors were constructed. All patients were treated at a single institution with a long-term follow-up and TXNIP expression was assessed using immunohistochemistry. TXNIP expression was investigated in terms of associations with clinicopathological and molecular features and patient outcome. Loss/reduced cytoplasmic expression of TXNIP was associated with features of aggressiveness including high nuclear grade (p=1.6x10^{-5}), presence of comedo necrosis (p=0.001) and oestrogen receptor negative (ER-)/HER2- ductal carcinoma *in situ* (p=4.6x10^{-5}). Univariate analysis showed an inverse association between TXNIP expression and outcome in terms of shorter local recurrence free survival (p=0.009). Multivariable analyses showed that independent predictors of ductal carcinoma *in situ* recurrence were low TXNIP expression (p=0.005, HR=0.51 and 95%CI: 0.32-0.81), larger ductal carcinoma *in situ* size and high nuclear grade. TXNIP functions as a tumor suppressor gene with loss of its expression associated with ductal carcinoma *in situ* recurrence. TXNIP can be used as a potentially useful marker in prognostic stratification of ductal carcinoma *in situ* for management decisions.

INTRODUCTION:

The main aim of ductal carcinoma *in situ* management is to prevent local recurrence particularly invasive recurrence (1). Despite a low mortality risk, ductal carcinoma in situ is often treated with mastectomy or breast conservative surgery with frequent re-excisions compared to invasive disease (2). Mastectomy results in very few recurrences but is often considered as over-treatment for screen-detected precursor lesions. Conversely, breast conservative surgery alone increases the risk of recurrence which may be considered as under-treatment in a proportion of high risk ductal carcinoma *in situ* (3). Radiotherapy can reduce the recurrence rate by approximately 50% but has significant side-effects and its application should therefore be rationalized. Accurate risk stratification of ductal carcinoma *in situ* means not only predicting recurrence but also which patients are likely to progress to invasive disease.

Ductal carcinoma *in situ* is observed in more than half of invasive breast cancer (4) and in the majority of these cases, they share morphology, immunoprofile and genetic features suggesting that the invasive component arises from the associated ductal carcinoma *in situ* (5-7). However, there remains a lack of objective predictive markers of ductal carcinoma *in situ* behavior; not only of recurrence but also development of invasive disease that could aid in treatment decisions.

Clinicopathological characteristics are used as prognostic factors in guiding treatment decisions (3) but they are insufficient to reflect the molecular and clinical heterogeneity. Assessment of molecular markers can be used as surrogates of ductal carcinoma *in situ* biology and behavior and have the potential to predict patient outcome (8). It is known that cancer cells continuously experience oxidative stress, resulting from heightened reactive oxygen species generation (9). Enhanced oxidative stress has been implicated in the initiation and progression of cancer, promoting cell survival and drug resistance in some cases (10). However, unresolved high levels of oxidative stress beyond the capacity of the cancer cell to manage causes critical damage to cellular DNA, proteins and lipids ultimately leading to cell death (11). A negative regulator of the major anti-oxidant

thioredoxin (TRX), thioredoxin interacting protein (TXNIP) has potent growth suppressive, metastasis inhibitory and pro-apoptotic functions (12). Loss of TXNIP expression was reported to be associated with the development of some solid tumors (12). In the breast, high expression of TXNIP was reported in normal breast tissues (13) and showed reduced immunointensity in invasive tumors (14). Ectopic expression of TXNIP in the breast cancer cell line MCF-7 drives cells to undergo senescence, accompanied by increased reactive oxygen species release. In a study of 98 locally advanced invasive breast cancer treated with anthracycline-based chemotherapy, Woolston and colleagues (15) have demonstrated that TXNIP is an independent predictor of outcome.

We hypothesized that oxidative stress could play a role in breast cancer progression and that TXNIP might be a potential prognostic marker in ductal carcinoma *in situ*. In this study, TXNIP expression was assessed in a large (n=1015) and well-characterized cohort of ductal carcinoma *in situ* from a single institution with long term follow-up. In addition, the prognostic significance of *TXNIP* gene copy number and gene expression was assessed in a large cohort of invasive breast cancer (n=1980) as a molecular surrogate of ductal carcinoma *in situ*.

PATIENTS AND METHODS

Study Cohort

This retrospective study was conducted on a consecutive series of 1059 primary pure ductal carcinoma *in situ* cases diagnosed, and treated in a uniform manner, between 1990 to 2012 at the Nottingham City Hospital, Nottingham, UK. Exclusion of referral, miscoded and recurrent cases resulted in 776 pure primary ductal carcinoma *in situ* with available formalin-fixed paraffin embedded tumor blocks for tissue microarray construction. A series of 239 cases diagnosed as synchronous ductal carcinoma *in situ* and invasive tumors (mixed ductal carcinoma *in situ* and invasive breast cancer) was also collected as a validation set. Patients' demographic information, histopathological parameters, management, radiotherapy and patient outcome data were collated. No patients were

treated with hormonal therapy. Local recurrence free survival was calculated based on the time (in months) from the date of primary surgical treatment to the time of ipsilateral local recurrence. The median follow-up period, in pure ductal carcinoma *in situ* cohort, was 118 months (range 2 to 240 months), during which 90 patients (14%) developed local ipsilateral recurrence including invasive (60/90; 67%) or ductal carcinoma *in situ* (30/90; 33%). Recurrence following breast conserving surgery and mastectomy occurred in 84 and 6 cases respectively.

Patients who developed contralateral disease following ductal carcinoma *in situ* diagnosis were censored at the time of diagnosis of the contralateral cancer.

Tissue Microarray and Immunohistochemistry

Tissue microarrays were prepared from representative ductal carcinoma *in situ* lesions of the pure cases and from ductal carcinoma *in situ* and invasive tumors from the mixed cases. The tissue microarray was constructed using 3D Histech® Grand Master®, whereby cores of 1 mm and 0.6 mm were taken from ductal carcinoma *in situ* and invasive tumor samples respectively. In addition, a set of whole tissue sections from 10 cases containing ductal carcinoma *in situ* and invasive tumors were assessed to evaluate heterogeneity and the pattern of TXNIP expression in normal and malignant breast lesions.

Validation of TXNIP antibody (Abcam; clone EPR14774) specificity was performed using Western blotting on whole cell lysates of MCF-7, SKBr3 and MDA-MB-231 human breast cancer cell lines (obtained from the American Type Culture Collection; Rockville, MD, USA). TXNIP antibody was used at 1:3000 dilution which showed a single specific band at the predicted size of 44 kDa (**Figure 1A**).

Expression of TXNIP protein in ductal carcinoma *in situ* was assessed by immunohistochemistry using the Novocastra Novolink polymer detection system (Leica, Newcastle, UK). 4 μm tissue microarray and full-face sections were stained with mouse monoclonal TXNIP antibody (1:3000) for 30 min. 3,30-Diaminobenzidine tetrahydrochloride (Novolink DAB substrate buffer) was used as a chromogenic substance.

Sections were counterstained with hematoxylin. Positive staining controls (human tonsil and kidney tissue) were included while a negative control was achieved by omitting the application of the primary antibody. Expression of estrogen receptor (ER) in ductal carcinoma *in situ* was carried out using ER clone SP1, Ventana Benchmark® ULTRA system (Tucson, Arizona, USA), as per the recommended protocol (**Supplementary material 1**). A cut-off of ≥1% for ER staining positivity was used (16). HER2 status was assessed using immunohistochemistry staining (1:400, DAKO, no antigen retrieval) with HercepTest scoring method as previously published (16, 17) (**Supplementary material 1**). In equivocal cases (HER2 immunohistochemistry score 2+), *HER2* gene amplification was determined by chromogenic *in situ* hybridization using ZytoDot 2CSPEC ERBB2/CEN 17 Probe Kit (**Supplementary material 2**) (17, 18)

Cytoplasmic TXNIP staining was assessed using the semi-quantitative H-score taking into consideration the intensity of staining and the percentage of stained tumor cells within each tissue core (19). All cases were scored blinded to clinicopathological and outcome data. Cases with multiple cores (n=210) were scored and the average was used as the final score.

This work obtained ethics approval by the North West – Greater Manchester Central Research Ethics Committee under the title; Nottingham Health Science Biobank (NHSB), reference number 15/NW/0685.

Analysis of TXNIP in invasive breast cancer:

To confirm the prognostic and clinical significance of TXNIP in invasive breast cancer, as a molecular surrogate of ductal carcinoma *in situ* in the breast, *TXNIP* gene copy number and normalized gene expression (mRNA) data were analyzed using the Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) dataset that comprises 1980 tumors with long term follow-up (20).

Statistical analysis

Statistical analyses were performed using SPSS v23 (Chicago, IL, USA) for Windows. X-tile software program (Yale University, version 3.6.1) was utilized to define the optimal cut-off point for TXNIP expression (H-score 120) with corrected *p* value and relative risk against local recurrence free survival (21, 22). Association between TXNIP expression and clinicopathological parameters using categorized data was evaluated using Chi-squared test. Survival rates were determined using the Kaplan–Meier method and compared by the log-rank test. Multivariate analysis using Cox proportional hazard regression model determined the influence of TXNIP expression, when adjusted to other variables, on local recurrence free survival. All tests were 2-tailed and a *p* value of less than 0.05 was considered as statistically significant.

RESULTS

Analysis of *TXNIP* mRNA levels in invasive breast cancer revealed significant association between reduced levels/loss of expression and high histological grade (p=0.004), large tumor size (p=0.003), advanced stage tumors (p=0.01), and with ER-negative and HER2-positive phenotype (p=0.003 and p=0.011 respectively) and luminal B and basal intrinsic subtypes according to the PAM50 classification (p=6.7x10^{-27}) (**Supplementary Table 1)**. Loss of copy number and reduced *TXNIP* mRNA expression were associated with worse outcome in terms of shorter breast cancer specific survival (p=0.003 and p=0.002 respectively) (**Supplementary Figure 1)**. Reduced TXNIP mRNA expression was also associated with loss of copy number (p=0.049). These findings confirmed the tumor suppressor function of TXNP in breast cancer and its potential prognostic value and supported investigating its role in ductal carcinoma *in situ*.

Frequency and localization pattern of TXNIP in ductal carcinoma *in situ*

Assessment of whole tissue sections revealed cytoplasmic expression of TXNIP in a homogenous distribution pattern with occasional nuclear staining in normal luminal and myoepithelial cells. The homogenous pattern of expression confirmed the validity of using tissue microarray technology to assess its expression. Variable staining intensities and localization was detected in the different morphological components, with occasional

strong nuclear staining in normal breast epithelial cells, strong to moderate cytoplasmic staining in ductal carcinoma *in situ* (with occasional membrane accentuation) and mild to negative cytoplasmic staining in invasive tumors (with no nuclear staining). TXNIP positive pure ductal carcinoma *in situ* tumor cells exhibited distinct cytoplasmic staining with occasional peripheral/perimembrane accentuation and infrequent nuclear staining in some low-grade tumors (**Figure 1**). After exclusion of uninformative cores (i.e., loss and/or folding of cores), a total of 636 pure ductal carcinoma *in situ* and 231 tumors with mixed ductal carcinoma *in situ* and invasive components were included in the analysis. TXNIP expression showed a unimodal distribution with a median H-score of 185 (range 0-300) in the pure ductal carcinoma *in situ*.

In mixed ductal carcinoma *in situ* and matched invasive cancer, both components were consistently positive in 27 (12%) cases and negative in 65 (28%) cases. High ductal carcinoma *in situ* /Low invasive breast cancer expression for TXNIP was present in 134 (58%) cases however, there were 5 (2%) cases in which invasive breast cancer was positive/high expressing and ductal carcinoma *in situ* low expressing for TXNIP. When pure ductal carcinoma *in situ* was compared to ductal carcinoma *in situ* mixed with invasive breast cancer, a statistically significant difference was detected, with the TXNIP cytoplasmic mean H-score being higher in pure ductal carcinoma *in situ* tumors ($\bar{X} \pm$SD=185$\pm$ 76, $\bar{X} \pm$SD= 112 $\pm$ 88 respectively; t-test= 15.8 and p<0.0001). In the mixed cases, a significant difference was also observed whereby the TXNIP cytoplasmic mean H-score was higher in the ductal carcinoma *in situ* component when compared with the adjacent invasive component ($\bar{X} \pm$SD= 112 $\pm$ 88, $\bar{X} \pm$SD= 83$\pm$ 78 respectively; Z=16.6 and p<0.0001) (**Supplementary Figure 2**). Similar results were observed when TXNIP was analyzed as a categorical variable (Chi-squared p<0.001 for pure vs mixed ductal carcinoma *in situ* and p<0.001 for ductal carcinoma *in situ* vs adjacent invasive components). No nuclear expression was seen in tumor cells of the cases mixed with invasion.

Association of TXNIP with clinicopathological parameters in pure ductal carcinoma *in situ*

High/positive TXNIP cytoplasmic expression was significantly associated with good prognostic factors in ductal carcinoma *in situ* including low nuclear grade (p=1.6x10^{-5}), absence of comedo type necrosis (p=0.001), positive ER status (p=2x10^{-6}), negative HER2 status (p=0.007) and with the luminal (ER+/HER2-) phenotype (p=4.6x10^{-5}). (**Table 1**).

Association with Patient Outcome of pure ductal carcinoma *in situ*

In univariate analysis, patients with positive TXNIP expression had a significantly longer local recurrence free survival (log rank (LR)=7.99, 95%CI:0.34-0.83 and p=0.009, **Figure 2A**). When the analysis was restricted to patients who underwent breast conservative surgery, to avoid the impact of surgical management on outcome prediction, TXNIP expression retained its significant association with improved outcome (LR=6.67, 95%CI:0.39-0.88 and p=0.04, **Figure 2B**). No significant association was observed between TXNIP expression and the type of local recurrence.

Positive TXNIP expression was associated with improved outcome in the group of patients treated with breast conservative surgery without post-operative radiotherapy (94/220) (p=0.02) but not in those who were offered radiotherapy (21/93) (p=0.5) **(Figure 2C and 2D).**

In ductal carcinoma *in situ* cases treated with breast conservative surgery without post-operative radiotherapy, loss/reduced TXNIP expression was observed in 126 cases and those showed approximately 40% recurrence rate (51/126), such patients could be candidates for radiotherapy to improve local recurrence free survival. low TXNIP expression was also associated with recurrence of 17% (12/72) of cases in the group of patients treated with breast conservative surgeryand postoperative radiotherapy, which points out the importance of TXNIP as a predictor marker. Overall proportion of recurrences after breast conservative surgery that showed low TXNIP expression were 75% (63/84).

When molecular classes were considered, high TXNIP was associated with improved outcome in the luminal (ER+/HER2-) class (p=0.04, **Figure 2E**).

Multivariate Cox regression model including ductal carcinoma *in situ* size, nuclear grade and presence of comedo necrosis showed that high TXNIP expression was an independent predictor of good outcome in pure ductal carcinoma *in situ* (p=0.005, HR 0.51, 95% CI 0.32-0.81) (**Table 2**).

DISCUSSION

The treatment of ductal carcinoma *in situ* remains a challenge as the clinicopathologic features of the disease do not reliably stratify patients into distinct risk groups to guide treatment decision (23). For this reason, some studies have attempted to risk stratify ductal carcinoma *in situ* based on genetic and molecular factors including *Oncotype* Dx® ductal carcinoma *in situ* score (24-28). Although ductal carcinoma *in situ* score minimised the proportion of patients undergoing radiotherapy, the assay seems expensive and not cost-effective (29). In fact, none of the investigated parameters has been translated to clinical practice. Therefore, there is a pressing need to identify cost-effective and robust biomarker(s) to predict outcome for ductal carcinoma *in situ* patients.

The role of TXNIP expression was investigated in different tissues (30-34) including locally advanced invasive breast cancer (15) and we observed that loss of TXNIP expression was associated with features of increasing aggressiveness. It is likely that the effect of TXNIP on cell growth and proliferation are cell-context dependent and might be circumvented via activation of alternate mitogenic pathways (9), nevertheless the data is consistent with TXNIP being a tumor suppressor (30). TXNIP expression in cancer might be downregulated through epigenetic, transcriptional, post-transcriptional, or translational mechanisms (35).

To date, there is no well-established robust publically available database for ductal carcinoma *in situ* mRNA or copy number aberrations. To confirm the prognostic significance of *TXNIP* mRNA in breast cancer and assess the role of copy number alteration

on its expression and function, we have used the METABRIC cohort. This analysis showed that loss of copy number is one of the mechanisms for downregulation of *TXNIP*, which is associated with aggressive behavior and poor outcome. Utilizing a large annotated series of ductal carcinoma *in situ* treated at a single institution, we have demonstrated that TXNIP downregulation is associated with increased aggressiveness and poor outcome in terms of shorter local recurrence free survival. We also found downregulation of TXNIP expression in invasive disease compared to the co-existing ductal carcinoma *in situ*. These results support our hypothesis that TXNIP is a tumor suppressor in breast cancer.

Our results demonstrate that loss of expression of TXNIP in ductal carcinoma *in situ* is not only associated with parameters characteristic of poor prognosis but is also an independent predictor of recurrence. These results are in line with TXNIP functioning as a tumor suppressor that is reported to be commonly silenced by genetic or epigenetic mechanisms in cancer cells (11, 36).

In this study, a significant negative association between the expression of TXNIP and local recurrence in ductal carcinoma *in situ* was identified. A previous study indicated that TXNIP stabilizes cyclin-dependent kinase inhibitor p27, which plays a pivotal role in inhibiting cell proliferation and apoptosis resulting in G_1 arrest and inhibition of cell cycle (37). This observation may explain our finding that high TXNIP expression was associated with low grade, low proliferating ER positive and HER2 negative ductal carcinoma *in situ* and with longer local recurrence free survival. Independent of its interaction with TRX, TXNIP has the ability to inhibit cell cycle progression by indirectly inhibiting mammalian target of rapamycin (mTOR), a regulator of cell growth and metabolism (38). Therefore, in addition to inducing a metabolic shift important to tumor biology, downregulation of TXNIP is likely to promote cell survival, growth, invasion, and metastasis. TXNIP can also reduce tumor invasion and angiogenesis through inhibition of TRX and can directly have an impact on cell survival by promoting a pro-apoptotic environment (39, 40). The exact mechanisms by which TXNIP exerts its tumor suppressive functions in ductal carcinoma *in situ* cells are not yet fully clear. Future studies of the mechanisms by which TXNIP is expressed and

functions in ductal carcinoma *in situ* will improve our understanding of the progression to invasive disease.

In this study, a significant correlation between TXNIP expression and ER+/HER2- status was identified. This finding is consistent with previous reports indicating that TXNIP may interrupt HER2 mediated oncogenic effect (36). To our knowledge, few studies have reported an explicit impact of HER2 signaling on TXNIP expression. It might suggest that there is a direct link between ERBB2 and TXNIP or could be explained by the glucose theory. It is well known that cancer cells experience energetic stress and glucose deprivation especially in HER2 positive cancer cells (37). Glucose induces TXNIP expression, and reactive oxygen species triggers the dissociation of TXNIP from TRX, leading to increased TXNIP availability (41). Therefore, TXNIP may be induced by hyperglycemia regulated TRX- reactive oxygen species activity in the HER2 signaling pathway. Information regarding this mechanism will require further investigation in the future.

The current study demonstrated differential expression of TXNIP between pure ductal carcinoma *in situ* and ductal carcinoma *in situ* associated with invasive disease, which may have potential application for predicting invasive disease in ductal carcinoma *in situ* diagnosed on preoperative biopsy samples, after further validation. In addition, the ability of TXNIP to inhibit the major reactive oxygen species scavenger TRX, further regulate cell cycle progression and metastasis and promote apoptosis, clearly identifies TXNIP as an important therapeutic target.

In conclusion, the difficulty in implementing predictive markers for ductal carcinoma *in situ* remains the availability of large cohorts with consistent treatment for validation, and our study is an exception. We report that TXNIP is an independent prognostic factor and a potential tumor suppressor in breast cancer. Overexpression of TXNIP in ductal carcinoma *in situ* is therefore a good prognostic factor that can potentially improve ductal carcinoma *in situ* risk stratification for management purpose. Further functional studies

are recommended to extensively validate the role of TXNIP as a predictor of progression to invasive disease.

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Authors' disclosures of potential conflicts of interest

The author(s) indicated no potential conflicts of interest.

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Figure 1: Characterization of TXNIP expression in ductal carcinoma *in situ*: (A) Western blot analysis of the anti-TXNIP antibody confirmed a single specific band at 44 kDa in a variety of cell lines, image for MCF7 shown, (B) TXNIP cytoplasmic expression in normal breast tissue showing expression in the epithelium and a few scattered myoepithelial cells. Occasional membrane and/or nuclear staining is also seen. TXNIP immunohistochemistry expression in ductal carcinoma *in situ* showing variable intensities from negative (C), weak (D), moderate (E) and strong (F) staining (x20 magnification). (G) is full face section of a ductal carcinoma *in situ* case mixed with invasive carcinoma and show moderate TXNIP expression in ductal carcinoma *in situ* but reduced/negative expression in the adjacent invasive carcinoma component.

Figure 2: Kaplan Meier curves show that high expression of TXNIP is associated with (A) longer local recurrence free survival in the whole series (p=0.009), (B) improved survival in patients treated with breast conserving surgery (p=0.04), (C) improved outcome in non-radiotherapy treated patients (p=0.02), (D) Local recurrence free survival in radiotherapy treated patients (p=0.50), (E). TXNIP is also associated with Local recurrence free survival when ductal carcinoma *in situ* stratified into luminal ER+/HER2- (p=0.040).

Table 1: Correlation between TXNIP expression and the clinicopathologic variables of pure ductal carcinoma *in situ* cases.

Parameter	n (%)	TXNIP Expression High (n=225) n (%)	TXNIP Expression Low (n=411) n (%)	χ^2 (*p* value)
Age, years*				
Less than 40	23 (3.6)	10 (5)	13 (4)	0.71
Between 40 and 60	371 (58.3)	131 (58)	240 (58)	(0.702)
More than 60	242 (38.1)	84 (37)	158 (38)	
Presentation				
Screening	302 (47.5)	113 (50)	221 (54)	0.73
Symptomatic	334 (52.5)	112 (50)	190 (46)	(0.391)
Ductal carcinoma *in situ* Size (mm)				
Less than 16	218 (34.3)	84 (37)	134 (33)	4.73
Between 16 and 40	246 (38.7)	80 (36)	166 (40)	(0.098)
More than 40	167 (26.3)	57 (25)	110 (27)	
Nuclear Grade				
Low	87 (13.7)	50 (22)	37 (9)	24.88
Intermediate	162 (25.5)	61 (27)	101 (25)	**(1.6×10^{-5})**
High	387 (62.8)	114 (51)	273 (66)	
Ductal carcinoma *in situ* histologic type				
Single	312 (49.1)	113 (50)	199 (48)	0.189
Mixture**	324 (50.9)	112 (50)	212 (52)	(0.664)
Comedo necrosis				
Yes	403 (63.4)	122 (54)	281 (68)	12.45
No	233 (36.6)	103 (46)	130 (32)	**(0.001)**
Coexistent LCIS				
Yes	58 (9.1)	21 (9)	37 (9)	0.02
No	578 (90.9)	204 (91)	374 (91)	(0.890)
Coexistent Paget's				
Yes	31 (9)	7 (6)	24 (11)	2.42
No	304 (51)	102 (94)	202 (89)	(0.463)
Management				
Mastectomy	323 (50.7)	110 (49)	213 (52)	3.56
Breast Conserving Surgery	313 (49.3)	115 (51)	198 (48)	(0.207)
Radiotherapy***				
Yes	96 (15.1)	35 (16)	61 (15)	0.06
No	540 (84.9)	190 (84)	350 (85)	(0.516)
Estrogen Receptor Status				
Positive	412 (75)	150 (87)	262 (69)	22.46
Negative	137 (25)	22 (13)	115 (31)	**(2×10^{-6})**
HER2 Status****				
Negative	471 (80)	178 (85)	293 (76)	12.15
Positive	120 (20)	31 (15)	89 (23)	**(0.007)**
Molecular Classification				
ER+/HER2-	342 (53.8)	139 (79)	203 (60)	24.02
ER-/HER2+	61 (9.6)	12 (7)	49 (14)	**(4.6×10^{-5})**
ER+/HER2+	43 (6.8)	14 (8)	29 (9)	
ER-/HER2-	66 (10.4)	10 (6)	56 (17)	

***p* value in bold: significant**, TXNIP: Thioredoxin Interacting Protein

n: Number, LCIS: Lobular Carcinoma in situ,

*Age: categorized according to the Van Nuys Prognostic Index (VNPI), **Histologic type is a mixture of more than one morphologic type, *** 93 cases who were treated with radiotherapy belong to breast conserving surgery group, the remaining 3 cases were

treated with mastectomy ****HER2 final status is achieved using combination of IHC and chromogenic *in situ* hybridization (CISH).

Table 2: Multivariate Cox proportional hazards for predictors of local recurrence in pure ductal carcinoma *in situ* patients.

Variable	HR	95% CI	*p*-value
Ductal carcinoma *in situ* size	0.60	0.46-0.89	**<0.001**
Ductal carcinoma *in situ* Grade	1.83	1.24-2.70	**<0.001**
Comedo necrosis	0.97	0.54-1.73	0.911
TXNIP expression	0.51	0.32-0.81	**<0.001**

HR: Hazard Ratio, CI: Confidence Interval, TXNIP: Thioredoxin Interacting Protein
Significant *p* values are represented in **bold**