ORIGINAL ARTICLE

Expression of nuclear insulin receptor substrate 1 in breast cancer

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Background: Insulin receptor substrate 1 (IRS-1), a cytoplasmic protein transmitting signals from the insulin and insulin-like growth factor 1 receptors, has been implicated in breast cancer. Previously, it was reported that IRS-1 can be translocated to the nucleus and modulate oestrogen receptor α (ER α) activity in vitro. However, the expression of nuclear IRS-1 in breast cancer biopsy specimens has never been examined.

Aims: To assess whether nuclear IRS-1 is present in breast cancer and non-cancer mammary epithelium, and whether it correlates with other markers, especially ER α . Parallel studies were carried out for the expression of cytoplasmic IRS-1.

Methods: IRS-1 and ER α expression was assessed by immunohistochemical analysis. Data were evaluated using Pearson's correlation, linear regression and receiver operating characteristic analysis.

Results: Median nuclear IRS-1 expression was found to be low in normal mammary epithelial cells (1.6%) and high in benign tumours (20.5%), ductal grade 2 carcinoma (11.0%) and lobular carcinoma (~30%). Median ER α expression in normal epithelium, benign tumours, ductal cancer grade 2 and 3, and lobular cancer grade 2 and 3 were 10.5, 20.5, 65.0, 0.0, 80 and 15%, respectively. Nuclear IRS-1 and ER α positively correlated in ductal cancer ($p < 0.001$) and benign tumours ($p < 0.01$), but were not associated in lobular cancer and normal mammary epithelium. In ductal carcinoma, both nuclear IRS-1 and ER α negatively correlated with tumour grade, size, mitotic index and lymph node involvement. Cytoplasmic IRS-1 was expressed in all specimens and positively correlated with ER α in ductal cancer.

Conclusions: A positive association between nuclear IRS-1 and ER α is a characteristic for ductal breast cancer and marks a more differentiated, non-metastatic phenotype.

Recent experimental and clinical evidence suggests the involvement of the insulin-like growth factor I (IGF-I) receptor (IGF-IR) in breast cancer development and progression.^{1–6} The tumorigenic action of IGF-IR is executed through multiple antiapoptotic, growth promoting and/or prometastatic pathways.^{5–9} Many of these pathways stem from insulin receptor substrate 1 (IRS-1), a major IGF-I signalling molecule that becomes phosphorylated on multiple tyrosine residues upon IGF-IR activation. Tyrosine phosphorylated IRS-1 acts as a scaffolding protein sequestering downstream signalling molecules and propagating IGF-I signal through the PI-3K/Akt, Ras/Raf/extracellular-regulated kinase 1/2, Jak2/Stat3 and other pathways.^{10–13}

Overexpression or downregulation of IRS-1 in breast cancer cell models suggested that the molecule controls several aspects of the neoplastic phenotype, especially anchorage-dependent and anchorage-independent cell growth and survival.^{14–15} In breast cancer cell lines, IRS-1 seems to be expressed at higher levels in oestrogen receptor α (ER α)-positive than in ER α -negative cells, and there is evidence supporting the existence of a crosstalk between IRS-1 and ER α systems.^{1–4, 6, 16–18} Overexpression of IRS-1 in MCF-7 ER α -positive cells has been shown to induce oestrogen independence and mediate anti-oestrogen resistance.^{14–19, 20} High expression of IRS-1 can be partly attributed to ER α activity, as 17 β oestradiol can upregulate IRS-1 expression and function,^{16–21, 22} whereas anti-oestrogens reduce IRS-1 mRNA and protein levels and inhibit IRS-1 signalling.^{19–20, 23} In addition, ER α can directly interact with IRS-1, increasing its stability and potentiating its downstream signalling to Akt.²⁴ Notably, increased activity of IRS-1 is likely to modulate ER α , via extracellular regulated kinase

1/2-mediated and Akt-mediated phosphorylation of ER α on Ser-118 and Ser-167, respectively.^{25–27}

Recent reports suggested that in addition to its cytoplasmic signalling function, IRS-1 is able to regulate nuclear processes in different cell models.^{28–33} For instance, in mouse fibroblasts treated with IGF-I, a fraction of IRS-1 is translocated from the cytoplasm to the nuclear and nucleolar compartments where it modulates the expression of genes controlling cell proliferation (ie, Cyclin D1) and cell growth in size (ie, recombinant DNA) by physically interacting with transcriptional complexes of β catenin and upstream binding factor 1, respectively.^{31–32} Our recent work demonstrated that nuclear IRS-1 is also found in breast cancer cell lines. For instance, in MCF-7 cells treated with 17 β oestradiol, nuclear IRS-1 physically interacted with ER α , modulating its transcriptional activity at oestrogen response element DNA motifs.³³ The exact mechanism of nuclear IRS-1 transport is not clear, but it probably involves other proteins containing nuclear localisation signals (ER α , T antigen, importins).

Despite the evidence that IRS-1 signalling may have a critical role in tumorigenesis, only limited studies examined the clinical significance of IRS-1 expression in human breast cancer specimens.^{18–34–36} In one study, cytoplasmic IRS-1 has been reported to correlate with poorly differentiated breast tumour phenotype (G3) and lymph node involvement.³⁵ Another study correlated IRS-1 with shorter disease-free survival in patients

Abbreviations: Ab, antibody; ER α , oestrogen receptor α ; IGF-I, insulin-like growth factor I; IGF-IR, insulin-like growth factor I receptor; IHC, immunohistochemical; IRS-1, insulin receptor substrate 1; PBS, phosphate-buffered saline; pN, lymph node status; pT, tumour size; ROC, receiver operating characteristic

Table 1 Patient characteristics and clinical parameters of breast tissues and cancers

Sample characteristics				
	Patients with cancers		Controls	
Total specimens	60		34	
Ductal carcinoma	38			
Lobular carcinoma	22			
Benign tumours			19	
Macromasty			14	
Patient age				
	Normal	Benign	Ductal	Lobular
Mean (SE)	53.6 (3.3)	45.4(3.1)	62.9 (2.4)	64.5 (2.7)
Median (range)	56.5 (33–68)	43 (20–68)	61.5 (43–94)	66 (48–78)
Menopause (%)	64	39	87	82
Clinical parameters of breast cancer tissues				
	Ductal (38)		Lobular (22)	
	G2 (19)	G3 (19)	G2 (10)	G3 (12)
pT	1–4	0–4	2–4	0–4
pN	0–2	0–2	0–1	0–2
Ki67	7.7 (0.9), 4–14	14.2 (1.3), 6–21	7.2 (1.5), 4–12	9.0 (1.9), 3–15

pN, lymph node involvement; pT, tumour size.

The age of patients in each group is given as mean value (SE) with median age (range) for each population. The percentage of postmenopausal patients is indicated in each group. The range is reported for pT and pN; median (SE) frequency of expression with range is shown for Ki67.

with smaller tumours.¹⁸ In contrast, Schnarr *et al*³⁴ found that IRS-1 marks a more differentiated phenotype and better prognosis. Furthermore, one study examining cancer and normal specimens reported similar IRS-1 tyrosine phosphorylation in all tissues,³⁶ while other analysis found decreased IRS-1 levels in poorly differentiated cancers relative to normal tissue and benign tumours.³⁴

Regarding nuclear IRS-1, its presence in breast cancer specimens has been noted by Schnarr *et al*³⁴ and Koda *et al*,³⁵ but any association with the disease has never been formally addressed. Consequently, we examined the expression of nuclear IRS-1 in normal mammary tissue, benign breast tumours and breast cancer in relation to ER α and clinicopathological features. Parallel studies were carried out for cytoplasmic IRS-1.

MATERIALS AND METHODS

Patients and tissue specimens

Table 1 summarises information on patient and specimen characteristics. The histopathological examination of sections was based on the World Health Organization and pTN classification of breast tumours. Tumour size (pT) was scored as follows: 0, primary tumour, not detectable; 1, tumour diameter ≤ 2 cm; 2, diameter 2–5 cm; 3, diameter ≥ 5 cm; 4, inflammatory carcinoma of any size. Lymph node status (pN) was scored from 0, no node involved; 1, proximal node involved; 2, distal node involved. The protocol of the present study was reviewed and approved by the local ethics committee.

IMMUNOHISTOCHEMISTRY AND CONFOCAL MICROSCOPY

Samples preparation

Immediately after excision, tissue samples were fixed in 10% buffered formaldehyde solution and embedded in paraffin wax blocks at 56°C. ER α and IRS-1 were analysed by immunohistochemical (IHC) staining using 3 μ m-thick consecutive paraffin sections. The sections were dewaxed in xylene and rehydrated in graded alcohols. Antigen retrieval was achieved by boiling in 0.01 M citrate buffer pH 6.

Immunohistochemistry

Endogenous peroxidase was removed with 3% H₂O₂; non-specific binding was blocked by incubating the slides for 30 min with 1.5% bovine serum albumin in phosphate-buffered saline (PBS). Next, the sections were incubated with the primary antibodies for 1 h at room temperature. ER α was detected using ER α mouse monoclonal antibody (Ab)(DakoCytomation, Glostrup, Denmark) at dilution 1:35. IRS-1 was detected using the C-terminus IRS-1 rabbit polyclonal antibody (Upstate, Lake Placid, New York, USA) at a concentration of 4 μ g/ml. Ab-antigen reactions were revealed using Streptavidin-biotin-peroxidase complex (LSAB kit, DakoCytomation). All slides were counterstained with haematoxylin. Breast specimens previously classified as positive for the expression of the studied markers were used for control and protocol standardisation. In negative controls, primary antibodies were omitted. The expression of ER α and IRS-1 was independently scored by two investigators (CM and CG) by light microscopy in 10 different section fields. For all nuclear markers, mean and median percentage, and the range of epithelial cells displaying positive staining was scored. In some analyses, specimens were grouped into ER α -negative ($<5\%$ of epithelial cells with ER α expression) and ER α -positive ($\geq 5\%$ of cells with ER α). The expression of cytoplasmic IRS-1 was classified using a four-point scale: 0, $<10\%$ positive cells with any staining intensity; 1+, 10–50% positive cells with weak or moderate staining; 2+, $>50\%$ positive cells with weak or moderate staining; and 3+, $>50\%$ positive cells with strong staining. No samples with $<50\%$ of positive cells with strong staining were recorded.

Confocal microscopy

Tissue sections were incubated for 30 min with 3% bovine serum albumin in PBS to avoid non-specific binding, then for 1 h with a mixture of primary antibodies for recognising IRS-1 and ER α .

The anti-IRS-1 polyclonal antibodies (UBI, Lake Placid, New York, USA) at 4 μ g/ml was used for IRS-1 staining; anti-ER F-10 monoclonal Ab (Santa Cruz Biotechnology, Santa Cruz, California, USA) at 2 μ g/ml was used to detect ER α . Following the incubation with primary antibodies, the slides were washed

Figure 1 Oestrogen receptor α (ER α) and insulin receptor substrate 1 (IRS-1) expression in normal mammary epithelium, benign breast tumours and breast cancers. The expression of ER α (ER) and IRS-1 (IRS) were examined by immunohistochemical analysis (IHC), as described in Materials and methods section. (A, B) Normal breast tissue; (C, D) benign breast tumour; (E, F) invasive ductal ER α -positive carcinoma; (G, H) ER α -positive lobular breast cancer. Negative control; IHC of lobular carcinomas with primary antibodies substituted with phosphate-buffered saline. Higher magnification of specific areas is reported as inset in the original images.

three times with PBS, and incubated with a mixture of secondary Abs. A rhodamine-conjugated donkey anti-mouse IgG (Calbiochem, San Diego, California, USA) was used as a secondary Ab for ER α and a fluorescein-conjugated donkey anti-rabbit IgG (Calbiochem) was used for IRS-1. The cellular localisation of IRS-1 and ER α was studied using the Bio-Rad MRC 1024 confocal microscope (Bio-Rad, Hercules, California, USA) connected to a Zeiss Axiovert 135 M inverted microscope (Zeiss, Goettingen, Germany) with $\times 1000$ magnification. The optical sections were taken at the central plane. The fluorophores were imaged separately to ensure no excitation/emission wavelength overlap. In control samples, the staining was performed with the omission of the primary antibodies.

Statistical analysis

Descriptive statistics for nuclear IRS-1 and ER α in normal, benign and tumour samples were reported as mean, median value and range (SE). The relationship between nuclear IRS-1 and ER α was analysed by linear regression and the statistical significance was evaluated by the Pearson's correlation test. The distribution of ER α and nuclear IRS-1 with respect to pT, grade and lymph node involvement are reported in scatter plots. The correlations between nuclear IRS-1, ER α , cytoplasmatic IRS-1

and selected clinicopathological features were examined with the Pearson's correlation test.

The value of nuclear ER α or IRS-1 expression as diagnostic marker of tumour grade, pT, pN and Ki67 was evaluated calculating the areas under the receiver operating characteristic (ROC) curves,³⁷ which assess the performance of a diagnostic test.^{38–40} In the graphical representation of the ROC curve, the X axis is the false positive rate (1-specificity) and the Y axis is the true positive rate (sensitivity). The diagonal line (from 0, 0 to 1, 1) reflects the characteristics of a test with no discriminating power. ROC curve was analysed using MedCalc V.8.1 (MedCalc Software, Mariakerke, Belgium).

RESULTS

Nuclear IRS-1 and ER α expression in normal mammary epithelium and benign breast tumours

In general, the expression of nuclear IRS-1 in normal tissues was very low ($\sim 2\%$ of positive cells; table 2). ER α was expressed in 11 of 14 samples; the median frequency of ER α in all samples was 10.5% (fig 1A, B; table 2). Nuclear IRS-1 was found in 9 of 11 ER α -positive specimens at the median frequency 1.8%. Low expression (3.5%) of nuclear IRS-1 was also recorded in two specimens that did not express ER α (data not shown).

Table 2 Descriptive statistics of nuclear insulin receptor substrate 1 and oestrogen receptor α in all samples

	Normal epithelium		Benign tumours	
	ER α	IRS-1	ER α	IRS-1
Mean (SE)	21.7 (6.1)	2.3 (0.6)	23.4 (4.2)	23 (4.5)
Median (range)	10.5 (0–60)	1.6 (0–7)	20.5 (0–70)	20.5 (0–60)
	G2		G3	
Ductal Carcinoma	ER α	IRS-1	ER α	IRS-1
Mean (SE)	51.8 (10.1)	23.4 (7.1)	6.2 (3.4)	4.1 (1.8)
Median (range)	65 (0–92)	11 (0–72)	0 (0–40)	0 (0–20)
	G2		G3	
Lobular carcinoma	ER α	IRS-1	ER α	IRS-1
Mean (SE)	64.8 (11.3)	32 (9.7)	26.7 (8.9)	30.7 (4.7)
Median (range)	80 (0–90)	35 (0–80)	15 (0–80)	33.5 (0–52)

ER α , oestrogen receptor α ; IRS-1, insulin receptor substrate 1.

The mean (SE) expression with median (range) values for nuclear IRS-1 and ER α in all specimens (ER α -positive and ER α -negative) is given. Samples of cancer of ductal and lobular origin were grouped separately into G2 and G3 populations.

Compared with normal epithelium, benign tumours expressed higher median levels of nuclear IRS-1 (20.5%) and ER α (20.5%; table 2). Nuclear IRS-1 was found in 16 of 19 ER α -positive specimens, but was not present in any of the ER α -negative cases. (fig 1C,D; and data not shown).

Cytoplasmic IRS-1 was expressed in all epithelial cells of normal epithelium and benign tumours at the levels 1+ to 3+ (fig 1B,D and table 3), while no evidence of cytoplasmic ER α staining was revealed in any of the specimens (fig 1A). The co-localisation of nuclear IRS-1 and ER α was determined by confocal microscopy (fig 2).

IRS-1 expression in ER α -positive and ER α -negative breast carcinoma

In invasive ductal carcinoma, nuclear IRS-1 was found in 22 of 38 specimens. The median level of expression in these samples was 13.7%. ER α was detected in 20 of 38 of specimens with a median expression of 29.2% (fig 1 E, F). In all, 22 specimens (15 of 19 in G2, and 7 of 19 in G3) expressed nuclear IRS-1 (fig 1F and table 2). Among nuclear IRS-1-positive samples, 18 specimens also expressed ER α , while four were ER α -negative. Thirteen of G2 ductal carcinomas and five of G3 cancers were positive for both IRS-1 and ER α . In 2 of 38 specimens, ER α was expressed in the absence of nuclear IRS-1.

In lobular cancer, nuclear IRS-1 staining was observed in 16 of 22 samples with the median frequency of 31.2% (fig 1H). Of these 16 samples, 11 were also ER α -positive. Within G2 lobular carcinomas, 6 of 10 specimens displayed nuclear IRS-1 at the median level of 35.0%; all these samples expressed ER α at the median frequency of 80.0% (table 2). In the G3 subgroup, 10 of 12 tumours expressed nuclear IRS-1 (median 33.5%) and 5 of 10 expressed ER α (median 15.0%). In 5 of 16 lobular cancers, nuclear IRS-1 was found in the absence of ER α (table 2).

Cytoplasmic IRS-1 was identified in all ductal and lobular cancer samples displaying a weak to strong staining intensity (table 3). In all specimens, the neoplasm surrounding the tissue appeared normal, and the pattern of ER α and IRS-staining was comparable to that of the normal samples.

Correlation between nuclear IRS-1 and ER α in breast cancer, benign tumours, and normal mammary epithelium

A very strong positive correlation ($p < 0.001$) between nuclear IRS-1 and ER α was found in invasive ductal breast cancer. The markers were also positively associated ($p < 0.01$) in benign tumour cancer samples (fig 3). However, no correlations were found between nuclear IRS-1 and ER α in normal tissues ($p = 0.28$) and lobular breast cancer ($p = 0.24$; fig 3).

Figure 2 Subcellular localisation of insulin receptor substrate 1 (IRS-1) and oestrogen receptor α (ER α) in breast tumours. The localisation of IRS-1 and ER α in ductal cancers was analysed by immunostaining and confocal microscopy as detailed in Materials and methods section. The captured images of IRS-1 (green fluorescence), ER α (red fluorescence), and merged IRS-1 and ER α (Merge, yellow fluorescence) are shown in a representative ductal cancer tissue section.

Table 3 Descriptive statistics of cytoplasmatic insulin receptor substrate 1 in all samples

Cytoplasmic IRS-1 expression (% of cases in class)				
Class	Normal	Benign	Ductal	Lobular
0	0	0	0	0
1+	29	21	16	0
2+	29	21	52	63
3+	42	58	32	37

IRS-1, insulin receptor substrate 1.

Samples are grouped into four classes as described in Materials and methods section. The percentage of specimens with cytoplasmic IRS-1 in each staining category is given.

Nuclear IRS-1 and ER α are correlated with some clinicopathological features in invasive ductal carcinomas

The distribution of nuclear ER α and nuclear IRS-1 was analysed with respect to tumour grade, pT, lymph node involvement and proliferation index (fig 4). The frequency of both ER α and nuclear IRS-1 expression was the highest in node-negative G2 invasive ductal carcinomas of smaller size (fig 4). In the same group, a significant negative correlation between nuclear IRS-1 or ER α and differentiation grade, the pT, lymph node involvement and proliferation rate was found (table 4).

In contrast, in lobular breast carcinomas, the distribution of nuclear IRS-1 or ER α appeared to be independent of, and not correlated with, tumour grade, pT or Ki67 expression (fig 4 and table 4). Interestingly, both nuclear IRS-1 and ER α were more abundant in lymph node-negative samples (fig 4), but no

significant associations were determined between these markers and pN (table 4).

The specificity and sensitivity of nuclear IRS-1 or ER α as a marker of tumour differentiation grade, pT and lymph node involvement was evaluated by the ROC curve analysis. The comparison of the areas under the ROC curves obtained for nuclear IRS-1 and ER α indicated that both nuclear IRS-1 and ER α are good markers for tumour grading in invasive ductal carcinomas, whereas in lobular carcinomas, only ER α could be considered as a marker for grading (table 5; fig 5).

Neither ER α nor nuclear IRS-1 was a useful marker of pT, lymph node involvement or tumour proliferation (data not shown). The distribution of nuclear IRS-1 or ER α was not related to patient's age and menopausal status in cancer, benign and normal samples (data not shown).

Figure 3 Correlations between nuclear (Nuc) insulin receptor substrate 1 (IRS-1) and oestrogen receptor α (ER α) in normal breast tissues, benign breast tumours and breast cancers. Associations between nuclear IRS-1 and ER α in different tissues were analysed with Pearson's correlation test. For each linear regression graph, the linear equation, the correlation coefficient (R) and the statistical significance (p) are reported; *, multiplier of linear correlation.

Figure 4 Distribution of nuclear (Nuc) insulin receptor substrate 1 (IRS-1) and oestrogen receptor α (ER α) in ductal and lobular breast cancers. Distributions of nuclear IRS-1 (%) and ER α (%) relative to tumour grade (Grade), size (pT) and the lymph node involvement (pN) in ductal and lobular breast cancers are shown in scatter plots; *, multiplier of linear correlation.

Relationship between cytoplasmatic IRS-1 and clinicopathological features

In ductal carcinomas, cytoplasmic IRS-1 (each staining intensity group) positively correlated with ER α . Moreover, in ductal cancer low and moderate IRS-1 expression was

positively associated with pT, while high IRS-1 levels negatively correlated with tumour grade (table 6).

In lobular carcinomas, high expression of cytoplasmic IRS-1 directly correlated with Ki67 (table 6). In benign tumours, low expression of cytoplasmatic IRS-1 was negatively associated

Table 4 Correlation between nuclear insulin receptor substrate 1, oestrogen receptor α and selected clinicopathological tumour features					
		Ductal carcinoma		Lobular carcinoma	
		ER α	IRS-1	ER α	IRS-1
G	r	−0.573	−0.511	−0.563	0.029
	p Value	0.001	0.0067	0.065	0.94
pT	r	−0.393	−0.382	−0.326	0.153
	p Value	0.039	0.044	0.310	0.633
pN	r	−0.381	−0.454	−0.082	−0.122
	p Value	0.044	0.015	0.797	0.714
Ki67	r	−0.591	−0.538	−0.329	−0.016
	p Value	0.001	0.003	0.31	0.94

ER α , oestrogen receptor α ; G, tumour grade; IRS-1 insulin receptor substrate 1; pN, lymph node involvement; pT, tumour size; r, correlation coefficient. The association between nuclear IRS-1 or ER α and G, pT, pN, and the expression of the proliferation marker Ki67 was statistically analysed with Pearson's correlation test.

Table 5 Association between nuclear IRS-1, oestrogen receptor α and tumour grade

Diagnostic marker	ROC analysis for tumour grade		
	AUC estimate (95% CI)	Area under the ROC curve	Mann-Whitney test (p value)
Ductal carcinoma			
ER α	71.4 (41.9 to 91.4)	0.809	0.001
IRS-1	78.6 (49.2 to 95.1)	0.778	0.001
Lobular carcinoma			
ER α	80.0 (28.8 to 96.7)	0.817	0.02
IRS-1	60.0 (15.4 to 93.5)	0.533	0.85

AUC, area under the receiver operating characteristic (ROC) curve; ER α , oestrogen receptor α ; IRS-1, insulin receptor substrate 1.

The analysis was performed with ROC curves, as described in Materials and methods section. The AUC describes the value of nuclear IRS-1 or ER α to discriminate between G2 and G3 tumours. AUC estimate reports the CIs considering an error of 5%. The statistical significance was evaluated by Mann-Whitney U test for an area = 0.5.

with ER α , whereas higher IRS-1 levels were not linked to ER α . No correlations between the two markers were found in normal samples (data not shown). Similarly, cytoplasmic IRS-1 expression was not related to age or menopausal status in all analysed material (data not shown).

DISCUSSION

Studies in cellular and animal models established that breast cancer cell growth is controlled by complex crosstalk between ER α and IGF-I systems.^{4-6 14 19 41-44} However, although ER α is an established marker for breast cancer diagnosis and prognosis, and a target for breast cancer treatment and prevention, the value of critical IGF-I system components like IGF-IR and IRS-1 as breast cancer markers needs further examination. Until now, analysis of breast cancer samples could not establish a clear association between IGF-IR and breast cancer progression. Several studies demonstrated higher expression of IGF-IR compared with non-cancer mammary epithelium; however, this feature has been associated with either favourable or unfavourable breast cancer prognosis of breast cancer.^{4 45-53} The value of cytoplasmic IRS-1 as a breast cancer marker is even less clear. Some studies have provided evidence that IRS-1 expression is higher in cancer than in non-cancer breast epithelium, whereas others (including this study) have reported that IRS-1 levels do not increase (but can decrease)

during cancer development and progression.^{18 34 36} Moreover, cytoplasmic IRS-1 has been found either to correlate with ER α and associate with a more differentiated phenotype or be independent from ER α and associated with a more aggressive phenotype.^{16 34 41 52} The significance of nuclear IRS-1 in breast cancer has never been addressed.

In view of the importance of cytoplasmic and nuclear IRS-1 in breast cancer growth evidenced in vitro, and conflicting or lacking data in vivo, we set out to investigate IRS-1 expression in normal mammary epithelium, benign tumours and breast cancer. Using IHC analysis, we assessed cytoplasmic and nuclear IRS-1 abundance, and examined its relationships with some prognostic markers, especially ER α , and clinicopathological features.

Our data on cytoplasmic IRS-1 are consistent with those reported by Schnarr *et al*³⁴ who noted moderate to strong IRS-1 expression in normal and benign tissues, and in well-differentiated carcinomas of both ductal and lobular origin. Similarly, Finlayson *et al*¹⁶ found no difference in IRS-1 phosphorylation in homogenates of normal and breast cancer tissues. In contrast, other groups reported low IRS-1 expression in normal tissue and overexpression in poorly differentiated tumours.^{18 35 48} In agreement with Schnarr *et al*, we found a positive association between cytoplasmic IRS-1 and ER α , and a negative correlation between high expression of IRS-1 and

Figure 5 Value of nuclear (Nuc) insulin receptor substrate 1 (IRS-1) and oestrogen receptor α (ER α) as diagnostic markers of tumour grading. Graphic evaluation of ER α and nuclear IRS-1 in respect to tumour differentiation grade in invasive ductal and lobular carcinomas, showing the true-positive rate (sensitivity) and the false-positive rate (specificity) of the analysis as a function of all possible cut-points for the two markers. ER α , solid line; nuclear IRS-1, dotted line.

Table 6 Correlations between cytoplasmatic insulin receptor substrate 1 and selected clinicopathological features in oestrogen receptor α -positive tumours

		Ductal				Lobular			
		0	1+	2+	3+	0+	1+	2+	3+
ER α	r	—	0.978	0.637	0.987	—	—	0.198	−0.029
	P Value		0.025	0.019	0.013			0.671	0.970
G	r	—	0.375	−0.082	−0.962	—	—	0.204	−0.376
	P Value		0.625	0.790	0.037			0.661	0.624
pT	r	—	0.973	0.553	−0.577	—	—	0.009	−0.225
	P Value		0.026	0.050	0.423			0.984	0.775
pN	r	—	0.00	0.301	—	—	—	−0.069	—
	P Value		1.00	0.318				0.883	
Ki67	r	—	0.724	−0.241	−0.905	—	—	−0.223	0.978
	P Value		0.276	0.428	0.095			0.631	0.022

ER α , oestrogen receptor α ; G, tumour grade; IRS-1, insulin receptor substrate 1; pN, lymph node involvement; pT, tumour size.

The associations between cytoplasmatic IRS-1 and ER α positivity, G, pT, pN and the expression of the proliferation marker Ki67 were statistically analysed with the Pearson's correlation test. The statistically significant correlations given in bold. The absence of value is due to either the absence of samples in the group or to the homogeneity of samples (variance = 0); —, no samples analysed.

tumour grade in ductal carcinomas. This observation is also consistent with coexpression of IRS-1 and ER α noted in less invasive breast cancer cell lines.⁶ In other studies, ER α and IRS-1 were not positively correlated in primary tumours.^{18–35} The reasons for these different results are not clear, but could be related to different IHC protocols, including different Abs used.

We did not find any correlation between cytoplasmic IRS-1 and lymph node involvement in ductal and lobular cancers. This partially confirms data of Koda *et al*³⁵, who did not observe such a correlation in the whole group of primary tumours, but only in the subgroup of better differentiated (G2) cancers. Our results also suggested a positive correlation between cytoplasmatic IRS-1 (weak to moderate) and pT in ER α -positive ductal cancers. This association has not been noted by others. Regarding cell proliferation, we found a positive correlation between IRS-1 and Ki-67 only in ER α -positive lobular cancers expressing high levels of IRS-1 and no associations in all other samples. Similarly, no link between cell proliferation and cytoplasmatic IRS-1 levels was reported by Rocha *et al*.¹⁸ In contrast, a negative correlation was reported by Schnarr *et al*³⁴, whereas Koda *et al*³⁵ noted a positive IRS-1/Ki-67 correlation in ER α -positive primary tumours. Taken together, these data are still too few and inconsistent to suggest cytoplasmic IRS-1 as a marker for breast cancer prognosis and diagnosis.

Instead, our results suggest that nuclear IRS-1 is tightly linked to ER α expression and might serve as an additional clinical breast cancer marker. As expected, ER α levels were low in normal mammary epithelium, higher in benign tumours and

strongly increased in moderately differentiated (G2) cancers. ER α expression was downregulated in poorly differentiated (G3) ductal cancers but not in G3 lobular cancers, confirming the value of ER α as a marker of differentiation in ductal carcinoma.^{54–56} Notably, the levels of nuclear IRS-1 were very low in normal tissue, increased in benign tumours and G2 ductal cancer, and decreased in G3 ductal cancer, displaying an expression trend similar to that of ER α .

In lobular cancer, the levels of nuclear IRS-1 were relatively high in both G2 and G3 tumours (~30%) and were not related to the abundance of ER α . Indeed, statistical analysis of data confirmed a very strong correlation between nuclear IRS-1 and ER α in ductal, but not lobular, cancers. Importantly, in ductal, but again not in lobular cancers, both nuclear IRS-1 and ER α negatively correlated with tumour grade, pT, lymph node involvement and proliferation rate, suggesting their association with a less aggressive phenotype. The ROC analysis confirmed that nuclear IRS-1 as for ER α , is highly reliable as a diagnostic marker of differentiation grade. The observation that nuclear IRS-1 expression increases in benign as well as in highly and moderately differentiated tumours, compared with normal tissues, strongly supports this assumption.

Taken together, our data indicate that nuclear IRS-1 could serve as a novel predictive marker of good prognosis in ductal cancer. The lack of association between nuclear IRS-1 and ER α in lobular cancer and benign tumours, might suggest that, in this setting, IGF-I and ER α systems are not tightly linked.

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AUTHORS' CONTRIBUTIONS

DS and CM participated in the design of the study, performed the statistical analysis and drafted the manuscript. CG carried out the immunostaining and participated in the statistical analysis. FR participated in the design of the study. LM and SC participated in the statistical analysis. FC prepared the histological samples. EM carried out the immunostaining. SA and ES participated in the design of the study and drafted the manuscript.

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Take-home messages

- This is the first report examining the expression of nuclear insulin receptor substrate 1 (IRS-1) in normal mammary tissue, benign breast tumours and breast cancer in relation to oestrogen receptor α (ER α) and clinicopathological features.
- Nuclear IRS-1 is more prevalent in cancer specimens than in normal mammary tissues.
- Nuclear IRS-1 and ER α negatively correlated with tumour grade, size, mitotic index and lymph node involvement.
- A positive association between nuclear IRS-1 and ER α is a characteristic for ductal breast cancer and marks a more differentiated, non-metastatic phenotype.

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