Identification of a Prognostic Signature Based on the Expression of Genes Related to the Insulin Pathway in Early Breast Cancer

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Abstract

Introduction: Insulin and the insulin-like growth factor (IGF) family play a key role in breast cancer (BC). ***Objective:*** In this study, we evaluated on a genomic scale the potential prognostic value of insulin signaling in early BC. ***Methods:*** Candidate genes were selected from the published literature and gene expression profiling experiments. Three publicly available BC datasets, containing gene expression data on 502 cases, were used to test the prognostic ability of the score. The gene signature was developed on GSE1456, containing microarray data from 159 patients, split into a training set (102 breast tumors) and a validation set ($n = 57$). GSE3494 and GSE2990 (350 patients) were used for external validation. Univariate Mann-Whitney test was used to identify genes differentially expressed between relapsed and non-relapsed patients. Expression of genes significantly correlated with relapse was combined in a linear score. Patients were classified as low or high risk with respect to the median value. ***Results:*** On the training set, 15 genes turned out to be differentially expressed: 8-year disease-free survival (DFS) was 51 and 91% in the high- and low-risk group ($p < 0.001$), respectively. In the validation set, DFS was 97 and 54% ($p = 0.009$), respectively. External validation: 8-year DFS was 72 and 61%, respectively, in GSE3494 ($p = 0.03$) and 74 and 55% in GSE2990 ($p = 0.03$). By multivariate analyses, the insulin signature was significantly associated with DFS, independently of age, hormone receptor status, nodal status, and grade. ***Conclusions:*** Our findings indicate that the insulin pathway is involved in BC prognosis at a genomic level and provide a window of selectivity for preventive and treatment strategies targeting the insulin/IGF pathway in BC patients.

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Background

In the past 20 years, a substantial body of evidence has developed regarding the role of insulin and the insulin-like growth factor (IGF) family in breast cancer (BC) [1]. This research, mainly focused on the IGF system, led to the development of antibodies targeting the IGF-1 receptor which have been tested in clinical trials, in many types of cancer, with inconsistent results so far [2].

Along with the metabolic effects of insulin on glucose balance [3], insulin has also been shown to induce cancer cell proliferation. The pathways downstream of the insulin/IGF system are well defined: IGF-I and insulin activate the tyrosine kinase growth receptor pathway, that is, the insulin, IGF-I, and hybrid IGF-I/insulin receptors, all of which are overexpressed in BC cells [4]. Activation of these receptors results in upregulation of the insulin receptor substrate-2 (IRS2), leading to downstream activa-

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tion of the MAPKinase and PI3K-Akt pathways [5]. Moreover, insulin may also modulate circulating levels of IGFs and their binding proteins. Epidemiological observations have provided evidence that higher circulating insulin levels are associated with an adverse outcome in early BC patients [6, 7]. These data suggest that the insulin pathway itself plays a major role in BC prognosis and may represent a therapeutic target, especially in those patients exposed to high plasmatic levels. Hyperinsulinemia generally reflects the presence of insulin resistance, a syndrome characterized by decreased insulin sensitivity in peripheral target tissues, such as muscle and fat, which includes abnormal laboratory findings, such as glucose intolerance, impaired lipid metabolism, and signs of a chronic inflammatory state, and manifests as obesity, hypertension, and diabetes [8, 9]. In nondiabetic women with early BC it has been observed that hyperinsulinemia is associated with the presence of insulin resistance [10].

In an attempt to clarify the role of insulin in cancer outcome, we hypothesized that it might exert its influence on tumor aggressiveness by modulating gene expression of BC cells. With these premises, the aim of this study was to evaluate the prognostic role of genes related to the insulin and the IGF pathways in early BC by using publicly available gene datasets.

Methods

Gene Selection

Candidate genes were selected from the published literature, genomic databases, pathway analysis, and from gene expression profiling experiments performed in peripheral tissues of healthy subjects screened for insulin resistance by the euglycemic insulin clamp technique. In particular, this gene set was identified by microarray profiling (Affymetrix, Santa Clara, CA, USA) on skeletal muscle biopsies of nondiabetic insulin-sensitive and insulin-resistant Pima Indians [11, 12]. These genes can be functionally categorized into various classes including cell growth, signal transduction, ion transport, transcriptional regulation, protein metabolism, structural genes for the cytoskeleton, lipid and carbohydrate metabolism, and chronic inflammation. Data are deposited in NCBI Gene Expression Omnibus (GEO; available at http://www.ncbi.nlm.nih.gov/geo/, GEO Series accession number GSE2508). In addition, due to the well-known interactions between the insulin and the IGF pathways in cancer cells, genes related to the IGF pathway were selected on Gene Ontology. The panel of selected genes and major pathways is shown in Table 1.

Microarray Datasets

We used 3 publicly available BC datasets, GSE1456 [13], GSE3494 [14], and GSE2990 [15], including gene expression data on a total of 590 cases with clinical follow-up. The 3 datasets, containing raw intensity data of Affymetrix HU133A arrays, were downloaded and pre-processed using R/Bioconductor (GCRMA package, quantile normalization, median polish summarization [16]). The 3 datasets were pre-processed together using the supercomputer Michelangelo (www.litbio.org). Since the original datasets were found to contain in part the same patients, 88 samples

Table 1. Genes related to the insulin and the insulin growth factor pathway evaluated in this study

Insulin pathway	Insulin, insulin-induced genes, insulin-degrading enzyme (IDE) Insulin receptor Insulin receptor substrate (IR-S1, IR-S2, IR-S4) Similar to IR-S-like protein Insulin-like factors Insulin promoter factor 1 Homeodomain transcription factor
Glucose metabolism	Solute carrier family 2: GLUT1, GLUT4 Hydroxysteroid (11-β) dehydrogenase 1 CD68 antigen (macrophage marker)
Inflammation	IL-6 (interferon, β2) IL-6 signal transducer IL-6 receptor TNF superfamily, member 2
IGF pathway	IGF 1 (somatomedin C) IGF 2 (somatomedin A) IGF BP (binding proteins) IGF-II mRNA-BP2 IGF-II mRNA-BP 3 IGF 1 receptor IGF 2 receptor IGF 2 antisense IGF II associated protein

present in GSE3494 and in GSE2990 were removed from the latter. This procedure resulted in a total of 502 cases suitable for the present study. Table 2 describes the characteristics of the 3 datasets.

Statistical Methods

The insulin gene signature was developed on GSE1456. The dataset was split by a random procedure into a training and a validation set (ratio 2:1). The signature was developed on the training set. Once the signature had been fully specified, the validation set was accessed once and only for estimating the prediction accuracy of the identified genes. The prognostic value of the signature was tested on the validation set by Kaplan-Meier survival analysis and Cox regression analysis. A multivariate Cox model was run on the whole dataset adjusting for molecular subtype. An external validation was performed on GSE3494, containing 249 early BC patients, and on GSE2990, containing 101 patients, by Kaplan-Meier survival analysis and Cox regression analysis on both datasets (GSE3494 and GSE2990). A stratified multivariate Cox model was run on the merged GSE3494/GSE2990 dataset adjusting for age, tumor size, nodal status, hormone receptor status, and tumor grade. The final model was obtained by means of a background procedure based on the likelihood ratio test. To verify the independence of insulin/IGF signature, we performed a comparison with the 21-Recurrence Score, a clinically validated assay, based on expression of 21 genes in women with ER+, lymph node-negative BC treated with adjuvant tamoxifen [17].

Insulin Gene Signature Development

A univariate Cox regression analysis was run to select genes whose expression levels were significantly correlated with disease-free survival (DFS). When multiple probes were mapped on the

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Table 2. Characteristics of the gene expression datasets included in this study

Dataset	GEO: GSE1456	GEO: GSE3494	GEO: GSE2990
Patients, n	159	249	101
Time of sample collection	1994–1996	1987–1989	1993–1995
Institution	Karolinska Institutet, Stockholm, Sweden	Karolinksa Institutet, Uppsala, Sweden	John Radcliffe Hospital, Oxford, UK
Mean age, years	58	62	58
Mean tumor size, mm	22	22	22
Tumor size <21 mm, %	62	51	58
Grade 3, n (%)	65 (41)	196 (78)	31 (31)
ER positive, n (%)	130 (82)	222 (89)	85 (84)
Positive lymph nodes, n (%)	60 (38)	77 (31)	15 (15)
Adjuvant HT, n (%)	114 (72)	67 (27)	0
Adjuvant chemotherapy, n (%)	30 (19)	60 (24)	0
Mean follow-up, years	6.2	7.1	7.0
Proportion relapsed, %	25	36	40
Platform	Affymetrix	Affymetrix	Affymetrix

HT, hormone therapy.

Table 3. List of the 15 genes of the insulin gene score

Probe set ID	Gene symbol	Low risk		High risk		p value	Score coefficient
		mean	SD	mean	SD		
X201627_s_at	INSIG1	6.9	1.3	7.7	0.8	0.004	0.31
X203328_x_at	IDE	2.9	1.0	3.6	1.5	0.005	0.34
X204686_at	IRS1	8.0	1.4	7.2	1.9	0.01	−0.17
X209184_s_at	IRS2	5.7	1.7	4.8	1.9	0.03	0.22
X204863_s_at	IL6	8.3	1.8	6.8	2.7	0.001	0.02
X209295_at	TNF R10b	7.3	1.0	6.8	1.2	0.04	−0.03
X218368_s_at	TNF R12a	7.4	1.1	7.9	0.8	0.03	0.28
X214581_x_at	TNF R21	3.8	1.0	4.7	1.5	0.002	0.11
X214329_x_at	TNF 10	8.6	1.5	7.8	1.8	0.04	−0.07
X209540_at	IGF1	7.0	2.1	4.8	2.1	<0.001	−0.44
X202410_x_at	IGF2	5.1	2.4	3.8	2.1	0.02	0.09
X203628_at	IGF1R	8.9	2.2	7.8	3.0	0.02	0.05
X201508_at	IGFBP4	10.0	1.2	9.5	1.2	0.05	0.05
X203851_at	IGFBP6	6.3	1.6	4.8	1.9	<0.001	−0.34
X201163_s_at	IGFBP7	12.4	0.5	12.2	0.7	0.04	1.37

same gene ID, the most correlated one by univariate analysis was chosen. Those genes related to DFS with a significance level <0.05 were included in a multivariate Cox regression model. Their expression levels were combined in a weighted linear score:

$$InsulinGeneScore = \sum_i w_i value_i$$

where w_i are weights determined by the coefficients of the multivariate Cox regression model and $value_i$ are the gene expression levels. The median value of the score was chosen as the cutoff to classify patients at low or high risk of disease relapse.

Results

One hundred forty-three probe sets were identified on the Affymetrix chip, corresponding to 85 insulin-related genes. The genes were tested on the GSE1456 dataset. This dataset contains raw data from 159 early BC patients with a median follow-up of 6.1 years. The dataset was split by a random procedure into a training set and a validation set of 102 and 57 primary breast tumors, respectively. On the training set, 15 genes turned out to be differentially expressed between relapsed and nonrelapsed patients with a significance level <0.05. The insulin signature con-
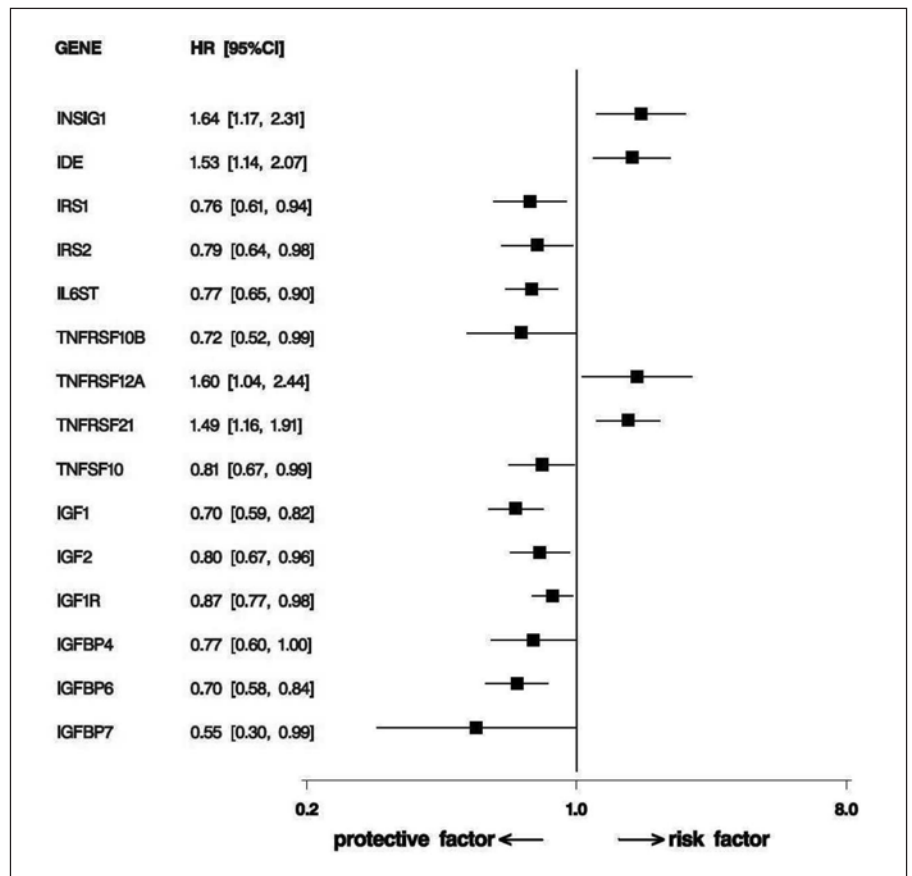
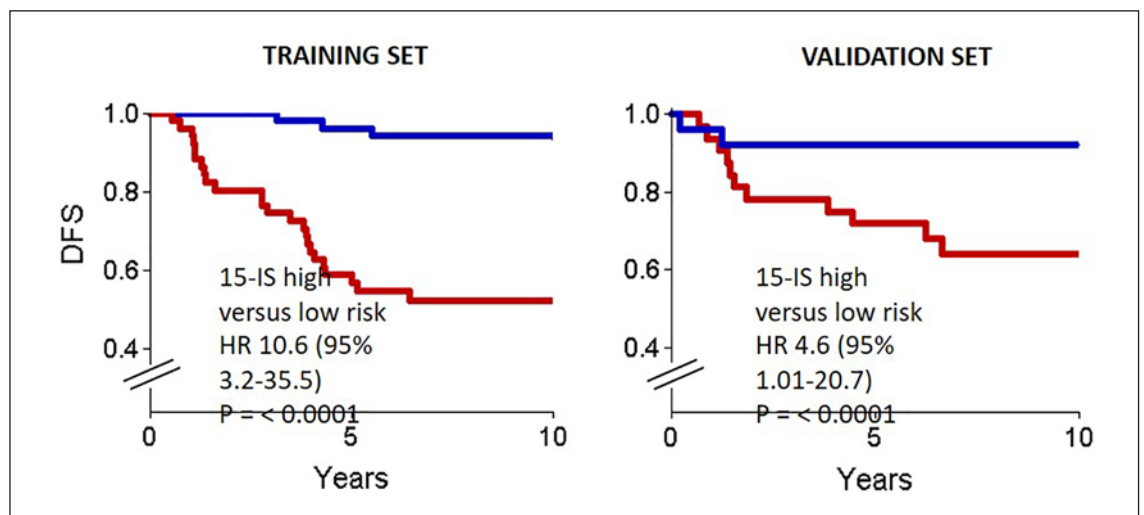**Fig. 1.** Individual HRs related to the over-expression of the 15 genes.

Fig. 2. Kaplan-Meier curves of DFS in GSE1456. DFS, disease-free survival; 15-IS, 15-Insulin Sensitivity.

sists of a score, defined as the linear combination of the 15 genes with the standardized Cox's regression coefficient as the weight (Table 3). The median value of the score was used to classify patients as high and low risk.

The 15 genes can be functionally divided into 3 classes: those belonging to the insulin pathway (4 genes, 27%), the chronic inflammation pathway (5 genes, 30%), and the IGF pathway (6 genes, 40%). Individual hazard ratios (HRs) related to the overexpression of each of the 15 genes are presented in Figure 1. In the training set (102 patients), the 8-year DFS was 91% (standard error [SE] 4%) in the low-risk group and 51% (SE 8%) in the high-risk group
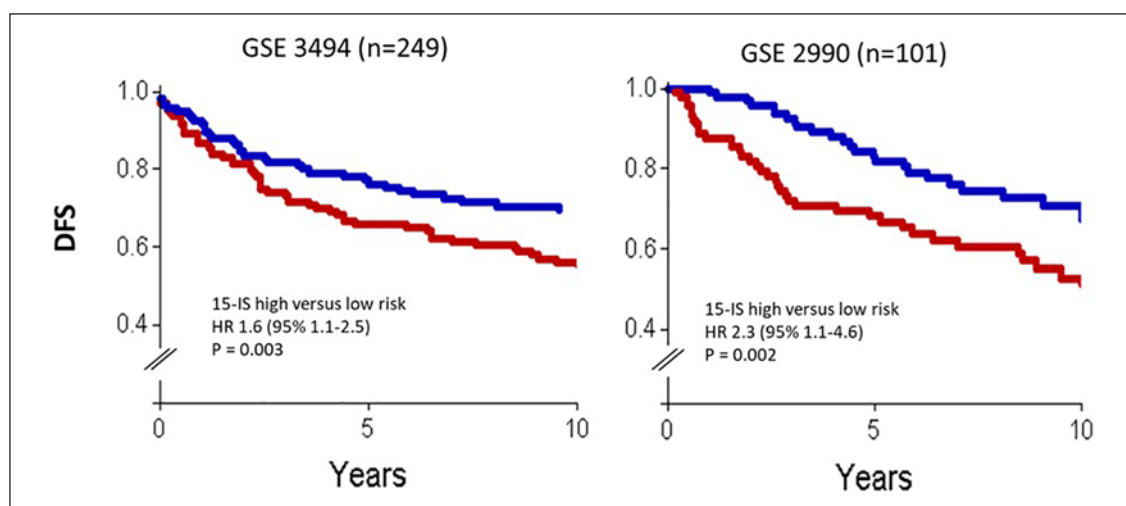
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Fig. 3. Kaplan-Meier curves of DFS in the 2 datasets used for external validation. DFS, disease-free survival; 15-IS, 15-Insulin Sensitivity.

($p < 0.001$). Univariate DFS HR (high vs. low risk) was 10.6 (95% CI 3.2–35.5, $p < 0.0001$). The gene signature was then tested on the 57 patients included in the validation set. The 8-year DFS was 97% (SE 3%) and 54% (SE 10%) in the low-risk and the high-risk group ($p = 0.03$), respectively, supporting the discriminating ability of the identified insulin sensitivity score. Univariate DFS HR was 4.6 (95% CI 1.01–20.7, $p = 0.04$). Figure 2 shows Kaplan-Meier survival curves according to the high- and low-risk group in the training and validation set.

The identified insulin signature was then tested on 2 external independent datasets, GSE3494 including 249 early BC patients and GSE2990 including 101 early BC patients. The score significantly predicted DFS in both datasets: in GSE3494, the 8-year DFS was 72% (SE 5%) in the low-risk group and 61% (SE 4%) in the high-risk group ($p = 0.04$). By univariate analysis, the DFS HR was 1.6 (95% CI 1.1–2.5, $p = 0.03$). In GSE2990, the 8-year DFS was 74% (SE 7%) and 55% (SE 8%) in the low-risk and the high-risk group, respectively ($p = 0.008$). By univariate analysis, the DFS HR was 2.3 (95% CI 1.1–4.6, $p = 0.02$). Kaplan-Meier survival curves obtained in the 2 independent datasets are shown in Figure 3.

By multivariate analysis of the merged datasets (GSE3494 and GSE2990), the insulin signature turned out to be significantly associated with DFS (HR 1.5, 95% CI 1.0–2.3, $p = 0.04$), independently of other established prognostic factors, including age, ER status, nodal status and grade, except for tumor size (Table 4).

Three hundred fifty-seven patients (249 from GSE3494 and 108 from GSE2990) were assessable for type of adjuvant therapy. Of these, 130 (30%) received endocrine therapy and 41 (9%) received chemotherapy according to local clinical practice. The test for interaction between

Table 4. Multivariate Cox analysis of GSE3494 and GSE2990 merged dataset (350 patients)

	HR (95% CI)	p value
Age		
≤50 years	1 (ref.)	
>50 years	0.7 (0.4–1.0)	0.07
Tumor size		
≤2 cm	1 (ref.)	
>2 cm	2.3 (1.5–3.5)	<0.0001
ER status		
Negative	1 (ref.)	
Positive	1.0 (0.6–1.6)	0.9
Nodes		
Negative	1 (ref.)	
Positive	1.4 (0.9–2.1)	0.1
Grade		
1	1 (ref.)	
2	1.2 (0.7–2.0)	
3	1.0 (0.5–1.8)	0.9
Insulin gene score		
Low	1 (ref.)	
High	1.5 (1.0–2.3)	0.04

type of adjuvant systemic therapy (yes or no) and the insulin signature was statistically significant ($p = 0.009$). In patients who received adjuvant systemic therapy, either endocrine therapy or chemotherapy, the insulin signature identified a subset of patients with high risk of relapse despite treatment (HR 2.77, 95% 1.59–4.82, $p < 0.0001$).

Finally, we performed a comparison of the insulin score with the 21-Recurrence Score (21-gene signature) in the validation set (57 patients); a positive correlation between 2 signatures was observed ($r = 0.34$, $p < 0.05$; Fig-
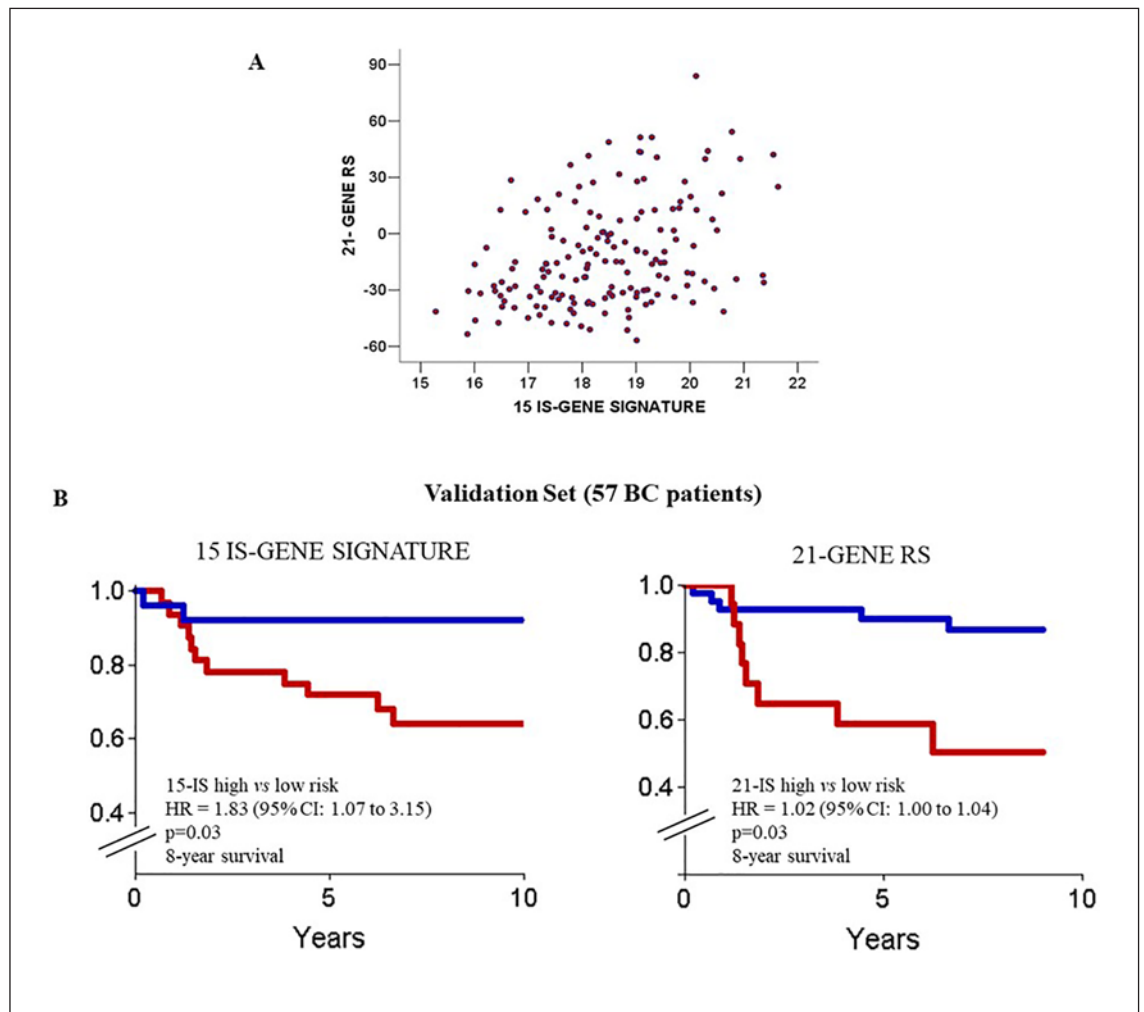
Fig. 4. Scatter plot (**A**) and Kaplan-Meier curves (**B**) of DFS in comparison with 15-IS gene and 21-RS signatures. DFS, disease-free survival; 15-IS, 15-Insulin Sensitivity; 21-RS, 21-Recurrence Score.

ure 4a). When using the median value of the score to classify patients as high and low risk in a multivariate Cox analysis, HR (high vs. low risk) for DFS was 1.83 (95% CI 1.07–3.15, $p = 0.03$) for 15-Insulin Sensitivity signature, while it was 1.02 (95% CI 1.00–1.04, $p = 0.03$) in 21-Recurrence Score. This observation supports the discriminating ability of the identified insulin sensitivity score and its independence from the 21-Recurrence Score (Fig. 4b).

Discussion

In this study, we used a mechanism-driven approach to evaluate the involvement of insulin signaling in BC prognosis on a genomic scale. In particular, we identified a gene signature based on the differential expression of 15 genes related to the insulin pathway that was strongly associated with DFS in early BC. The ability of the insulin signature to predict outcome was maintained after external validation performed on 2 different large independent datasets. In Cox's multivariate analyses, the insulin gene signature was found to be an independent variable in predicting DFS when molecular subtype and other established factors, such as nodal status, histologic grade, hormone receptor status, and age, were taken into account.

The genes included in the insulin signature showed no overlap with other prognostic classifiers, with the exception of the IGF1 gene, shared with the prognostic signature developed by Pawitan et al. [13]. However, this last observation, due to the nature of this analysis, has to be considered as exploratory, since no selection of ER-positive samples was done.

The 15 genes can be functionally divided into 3 classes: those belonging to the insulin pathway (27%), the chronic inflammation pathway (30%), and the IGF pathway (40%). The insulin pathway is present with 4 genes: INSIG1, IDE, IRS1, and IRS2. Preclinical evidence indicates

that INSIG1 (insulin-induced gene 1) is involved in regulation of transcription and is upregulated by exposure to genotoxic agents in cancer models [18]; IDE (insulin-degrading enzyme) is a zinc metalloprotease involved in intracellular degradation of insulin recently detected in malignant tissues, including BC [19]. IRS1 and IRS2 (insulin receptor substrate 1 and 2) are major substrates for the insulin and the IGF receptors and they mediate signals to promote tumor cell survival, growth, and motility in BC cells [20]. Five genes (IL-6, TNFR10b, TNFR12a, TNFR21, and TNF10), pertinent to the chronic inflammation pathway, turned out to be differentially expressed between relapsed and nonrelapsed patients; among these, IL-6 (interleukin-6) and TNF (tumor necrosis factor) are multifunctional cytokines produced by a number of tumor cells, including BC [21, 22], and are correlated with an adverse prognosis in advanced BC stages [23].

In the IGF-related group, high expression of the IGF1 gene was associated with the low risk profile. These data are consistent with available evidence indicating that at a cellular level, high IGF1 expression is correlated with a more favorable outcome in BC patients. Among the other IGF-related genes, IGF-binding protein genes, namely IGFBP4, IGFBP6, and IGFBP7, were also overexpressed in the low-risk profile. IGFBP4 has been shown to be an independent prognostic factor in BC and a predictor of endocrine responsiveness [24]. Finally, both up- and downregulation of IGFBP7 have been reported in BC [25] and are involved in modulating VEGF expression and signaling as well [26].

These data suggest that insulin signaling may be a key regulatory pathway in BC and may represent a therapeutic target. The implementation of therapies that target hyperinsulinemia is one of the most intriguing and emerging fields of research in different types of tumors. In addition to lifestyle interventions that are effective in reducing insulin levels in BC survivors [27], but strongly dependent on patient attitude and compliance, interest has focused on metformin, an anti-diabetic drug widely prescribed for the treatment of hyperglycemia and hyperinsulinemia. Cheap and generally well tolerated [28], meftormin has also been shown to retain antiproliferative properties, through the activation of the AMPK pathway, in preclinical BC models [29]. Epidemiologic evidence also indicated that in diabetic patients, metformin is associated with reduced risk of cancer incidence and mortality [30]. Moreover, in a series of 2,529 BC patients receiving preoperative chemotherapy at MD Anderson Cancer Center, the probability of achieving a pathological complete response was reported to be 30% higher in diabetic patients receiving metformin as compared to nondiabetic patients [31].

In the past few years, these data prompted the exploitation of metformin as an anticancer drug in BC, with inconsistent results. Our group performed 2 randomized clinical trials in early and advanced BC patients. In the first one, 200 nondiabetic patients with operable BC were randomized to receive metformin or placebo in a window-of-opportunity, preoperative study. The primary endpoint was tumor proliferation. Overall, metformin did not significantly impact Ki-67; however, a significant difference was detected according to the level of insulin sensitivity [32].

In the MYME study [33], 122 nondiabetic women with HER2-negative metastatic breast cancer were randomized to receive first-line chemotherapy plus metformin or chemotherapy alone; the final results failed to provide evidence of an additional benefit of metformin in terms of progression-free and overall survival. Noteworthy, a significantly worse outcome was observed in insulin-resistant patients (HOMA ≥2.59, independent of metformin administration). A biological collateral study, Trans-MYME, evaluated in the same patients the prognostic role of IGF-1R expression on circulating tumor cells, indicating that loss of IGF-1R expression was associated with a significantly worse outcome [34]. Yet, a possible limitation of this study was the absence of any information related to individual patient metabolic status, including body mass index, level of insulin sensitivity, and nutritional status.

In conclusion, our study suggests that it is possible to identify a subset of BC patients whose prognosis is strongly modulated by a set of genes related to the insulin pathway. This might help to better individualize lifestyle and therapeutic interventions targeting insulin signaling, by selecting those patients at high risk of relapse according to the insulin signature and to evaluate the prognostic impact of epigenetic modulations induced by these types of interventions.

Statement of Ethics

This study is based on data from publicly available gene datasets; ethical approval was not required.

Conflict of Interest Statement

A.G. received honoraria as expert of testimony and speaker bureau from EISAI, Roche, Lilly, Novartis, Pfizer, TEVA, Astra Zeneca, Celgene and Daichii Sankyo. The remaining authors have no conflicts of interest to declare.

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Author Contributions

A.G., P.B., and U.P. conceived and designed the study. M.S., M.P., V.M., and A.A. downloaded and performed all the analyses on the datasets. A.G., M.S., M.P., V.M., and U.P. wrote the research article. A.G., P.B., M.S., M.P., A.A., V.M., and U.P. interpreted the results and revised the manuscript. All authors contributed to data analysis, drafting, and revising the article and gave final approval of the version to be published.

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