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Article

Independent Negative Prognostic Role of TCF1 Expression within Wnt/β-Catenin Signaling Pathway in Primary Breast Cancer Patients

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Abstract: Wnt pathway is involved in breast cancer (BC) progression. Our aim was to evaluate the expression of some components of the Wnt pathway (β-catenin, FZD4, LRP5, LRP6 and TCF1) in order to detect potential associations with NHERF1 protein. In addition, we assessed their impact on patients'clinical outcome. We evaluated 220 primary BC samples by immunohistochemistry (IHC) and protein localization by immunofluorescence. We found a significant correlation between NHERF1 and FZD4, LRP5, LRP6 and TCF1. Univariate analysis showed that β-catenin ($p<0.0001$), FZD4 ($p=0.0001$), LRP5, LRP6 and TCF1 over-expression ($p<0.0001$ respectively) was related to poor disease free survival (DFS). A Kaplan-Meier analysis confirmed univariate data and showed a poor DFS for cNHERF1+/FZD4+ ($p=0.0007$), cNHERF1+/LRP5+ ($p=0.0002$), cNHERF1+/LRP6+ ($p<0.0001$) and cNHERF1+/TCF1+ phenotypes ($p=0.0034$). In multivariate analysis, TCF1 and β-catenin expression were independent prognostic variable of worse DFS ($p=0.009$ and $p=0.027$, respectively). In conclusion, we found that β-catenin, FZD4, LRP5, LRP6 and TCF1 overexpression was associated to poor prognosis. Furthermore, we first identified TCF1 as independent prognostic factor of poor outcome, indicating it as a new potential biomarker for BC patients management. In addition, Wnt pathway proteins expression, both alone and in association with NHERF1, suggests original associations of biological significance for new studies.

Keywords: breast cancer; Wnt pathway; TCF1; NHERF1

1. Introduction

Breast Cancer (BC) is one of the most common malignant cancers in women [1]; this makes it important to better understand the molecular mechanisms that underly evolution and the possible interaction between the different molecules which play a role in its development and progression.

Na+/H+ exchanger regulatory factor 1 (NHERF1) is a scaffold protein, formed by two tandem PDZ domains and a carboxyl-terminal ezrin-binding (EB) region [2]. This structure makes NHERF1 a sticky protein, able to interact with a lot of signal molecules and implicated in different diseases [3]. In the last decade, a lot of studies highlighted its involvement during carcinogenesis and tumor progression [4-9], and in particular in BC [10-12]. A previous study of ours demonstrated the prognostic significance of nuclear NHERF1 expression (nNHERF1) in a cohort of patients with primary BC [13]. It is worth mentioning that NHERF1 plays a significant role during regulation of several oncogenic signaling pathways, including Wnt/β-catenin [14,15]. Wnt/β-catenin pathway is an evolutionarily conserved pathway that controls cell proliferation, differentiation and the maintenance of stem cells [16]. The β-catenin activity is deregulated in numerous cancer diseases and it is a promising therapeutical target [17]. Its role is already well-established in colorectal cancer, even though no effective therapies have been identified.

Wnt/β-catenin signaling activation needs both Frizzled receptors (FZDs) and low density lipoprotein receptor-related protein (LRP) 5 and 6 co-receptors. Ten human isoforms of FZD receptors are known with 7 transmembrane domains and the majority terminates with a PDZ binding region [18]. The LRP5/6 also belong to a family of 10 LRP isoforms, with a pivotal role in endocytosis, cellular communication, embryonic development, lipid homeostasis, and disease [19]. The molecular interaction between NHERF1 and FZD receptors and their modulation activity has been studied [14], but little is known about NHERF1- LRP co-receptors interaction in physiological and pathological conditions.

One of the downstream transcription factors of Wnt signaling is T-cell factor (TCF) and lymphoid enhancer factor (LEF), TCF1 [16]. Recent evidences showed an interaction between TCF1 and nherf1 promoter, interfering with WNT/β-catenin signaling [4].

In this research, we analyzed the expression and the relationship among NHERF1 and some of the major important players of WNT pathway, such as β-catenin, FZD4, LRP5, LRP6 and TCF1 in a cohort of primary BCs. For this study regarding biomarkers, our primary objective was to correlate, for the first time, NHERF1 with β-catenin, FZD4, LRP5, LRP6 and TCF1 expression and to verify if an interaction exists between NHERF1 and these proteins in BC samples. A secondary goal was to verify their prognostic potential and their impact on patients' clinical outcome.

A full understanding of the action and interaction of these biomarkers could improve current prognostic and therapeutic approaches and contribute in finding new ways, ensuring more precise BC management.

2. Results

2.1 *Profiling of expression and relationship between tumor markers and clinicopathological features*

The 48% (106/220) of tumors presented an higher cNHERF1 respect to nuclear staining 10% (23/220). Cytoplasmic β-catenin expression was evaluated in whole cohort with a high cytoplasmic staining in the 50% of samples (110/220). FZD4 was over-expressed in the 50.4% of patients (111/220). We found a predominant cytoplasmic expression both of LRP5 and LRP6, which result over-expressed in

48.6% (107/220) and 44.5% (98/220) of tumor samples, respectively. Finally, nuclear TCF1 over-expression was present in 51% of cases (112/220). Figure 1 (panel A) shows an example of immunohistochemical staining pattern of the analyzed proteins. The relationship between NHERF1, β-catenin, FZD4, LRP5, LRP6, TCF1 expression and the clinicopathological characteristics are listed in Table S2. We found an increase of cNHERF1expression in tumors >2 cm (p=0.0020), and a low nNHERF1 expression in tumors with a higher histological grade (G3), (p=0.0386). Cytoplasmic β-catenin expression was observed in IDCs (p=0.0138), and it was related to ER negative (p<0.0001), PgR negative (p=0.0050), HER2/neu negative (p=0.0002) and high proliferative activity (Ki67 index) (p=0.0082). FZD4 was over-expressed in IDCs (p=0.0199), in tumors >2 cm (p=0.0010), and in tumors with a high histological grade (p=0.0004). LRP5 was also over-expressed in tumors >2 cm (p=0.0009), in high histological grade (p=0.0002) and high Ki67 index (p=0.0049). LRP6 was over-expressed in older patients (p=0.0175), in tumors >2 cm (p=0.0089), in G3 tumors (p=0.0010) and high Ki67 index (p=0.0235). LRP6 was inversely related to ER (p=0.0012) and PgR status (p=0.0043). TCF1 expression was only directly related to ER positive (p=0.0432).

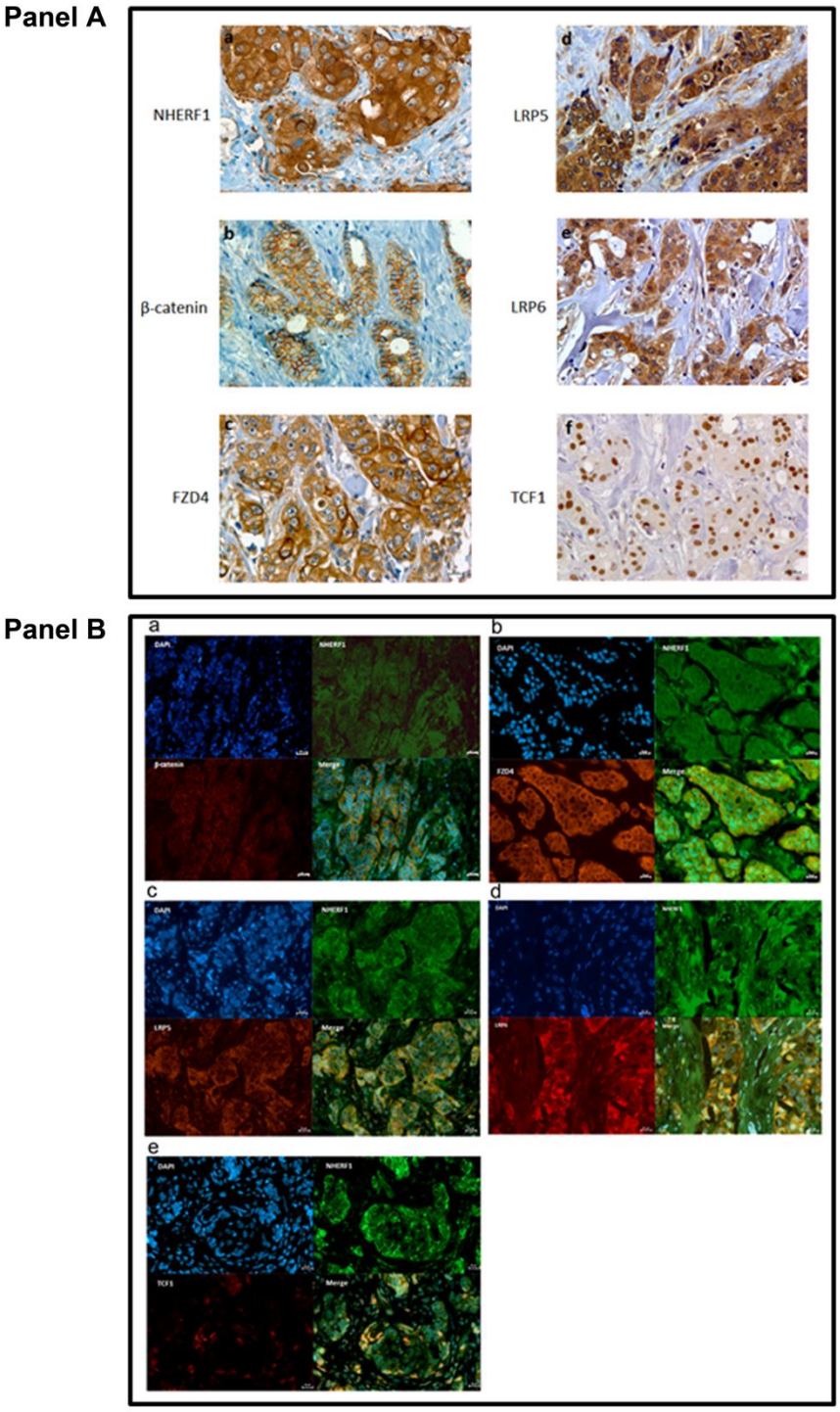

Figure 1. Panel A. Representative images of immunohistochemical staining in BC tissues. The panel displays the representative expression of molecular biomarkers in tumor zone: **a)** positive high cytoplasmic NHERF1 expression; **b)** membranous and cytoplasmic β-catenin expression; **c)** cytoplasmic FZD4 over-expression; **d)** and **e)** cytoplasmic LRP5 and LRP6 over-expression; **f)** nuclear TCF1 expression (original magnification, ×400). **Panel B.** Immunofluorescence assay of NHERF1 and Wnt/pathway proteins expression. Representative tumor tissue samples stained with: **a)** NHERF1 and β-catenin; **b)** NHERF1 and FZD4; **c)** NHERF1 and LRP5; **d)** NHERF1 and LRP6 and **e)** NHERF1 and TCF1 primary antibodies and detected with Alexa Fluor 488 (green) and Alexa Fluor 568 (red) secondary antibodies. The nuclei were stained with

DAPI (blue) (original magnification, ×400). Images were obtained on a Axion Image 2 upright microscope (Zeiss, Germany) with a Axiocam 512 color camera.

2.2 *Association between protein expressions analyzed*

We evaluated the relationship between cNHERF1 and nNHERF1 and the other biomarkers on continuous and dichotomized variables. The Spearman correlation test on continuous variables revealed a direct relation between cNHERF1 and FZD4 (rs: 0.34; p<0.0001), LRP5 (rs: 0.24; p=0.0022) and LRP6 (rs: 0.26; p=0.0010). In addition, a direct correlation between cNHERF1and TCF1 was also observed (rs: 0.18; p=0.0169). Further, nNHERF1 was inversely related to LRP5 (rs: -0.21; p=0.0079), LRP6 (rs: -0.25; p=0.0017) and TCF1 (rs: -0.21; p=0.0048) (Table 1). No significant association between NHERF1 and β-catenin has been found. These data were confirmed for dichotomized variables using the $\chi 2$ test (data not shown).

Table 1. Spearman for rank-based correlations between protein expression in BC patients on continuous variables.

	β-catenin		**FZD4**		**LRP5**		**LRP6**		**TCF1**	
	r	*p-value*	*r*	*p-value*	*r*	*p-value*	*r*	*p-value*	*r*	*p-value*
cNHERF1	0.01	0.883	0.34	**<0.0001**	0.24	**0.0022**	0.26	**0.0010**	0.18	**0.0169**
nNHERF1	0.047	0.5394	-0.12	0.1110	-0.21	**0.0079**	-0.25	**0.0017**	-0.21	**0.0048**

Spearman correlation coefficient r (Rho) and p-value. Bold values indicate significance

2.3 *Immunofluorescence Co-localization*

We identified protein localization by immunofluorescence assay. Firstly, we detected the intracellular distribution of NHERF1 and β-catenin in invasive cellular cluster of BCs. No co-localization was observed for these proteins (Figure 1, panel B-a).

Cytoplasmic NHERF1 and FZD4 proteins co-localized when they were found over-expressed within the cytoplasmic compartment (Figure 1, panel B-b). Immunofluorescence provided also the co-localization of cNHERF1 both with LRP5 and LRP6 (Figure 1, panel B-c and panel B-d, respectively). No co-localization was observed for NHERF1 and TCF1 (Figure 1, panel B-e).

2.4 *Expression of proteins and patient clinical outcome*

Univariate and multivariate analyses were carried out including the expression of cNHERF1, nNHERF1, β-catenin, FZD4, LRP5, LRP6 and TCF1 proteins and all clinicopathological characteristics, as dichotomized variables.

The subgroup of patients with low β-catenin expression had a better 5-year % DFS compared to patients with high β-catenin expression 92% vs 67% (p<0.0001). Patients with high FZD4 expression had a worse 5-years % DFS compared with patients with low FZD4 expression (71% vs 91% p=0.0001). A worse 5-years % DFS for patients with high respect to low LRP5 expression (70% vs 93%; p<0.0001) and for patients with high respect to low LRP6 expression (68% vs 95%; p<0.0001) was also observed. Further, a worse 5-years % DFS was found for patients with high respect to low TCF1 expression (68% vs 92%; p<0.0001). No significant results emerged from cNHERF1, nNHERF1

and OS analyses. In addition, univariate analysis indicated a worse 5-years % DFS for tumor size > 2cm respect to ≤ 2cm (68% vs 88%; p=0.002), positive respect to negative nodal status (72% vs 87%; p=0.001), high (G3) respect to low (G1-2) histological grade (63%vs 94%; p<0.0001), negative respect to positive ER (58% vs 92%; p<0.0001) and PgR (68% vs 90%; p<0.0001) expression, high respect to low Ki67 (68% vs 90; p<0.0001). Then, a poor OS was observed for high (G3) respect to low (G1-2) histological grade (90%vs 99%; p=0.026), negative respect to positive ER expression (88% vs 99%; p=0.024) and high respect to low Ki67 (90% vs 99; p=0.004) (Table 2).

Multivariate analysis, according to the Cox proportional hazard regression model, showed that β-catenin and TCF1 expression were independent prognostic factors, associated to a shorter DFS (Hazard Ratio (HR)=3.26, 95% Confidence Interval (CI) 1.14-9.33, p=0.027; HR=4.86, 95% CI 1.47-16.05, p=0.009, respectively). Further, high grade (G3) was also an independent prognostic marker for DFS (HR=5.28, 95% CI 1.60-17.4, p=0.006) (Table 3).

Kaplan-Meier analysis confirmed the univariate data and it didn't show significant differences in the DFS between patients with high and low cNHERF1 protein expression (p=0.9427) (Figure 2a); a poor DFS in the subgroup of patients with high β-catenin expression, high FZD4 expression, high LRP5 and LRP6 expression and in the subgroup of patients with high TCF1 expression (p<0.0001 for each group) (Figure 2b-f) was observed.

Moreover, a poor DFS was observed for the following phenotypes: cNHERF1-/FZD4+ and cNHERF1+/FZD4+ (p=0.0007); cNHERF1-/LRP5+ and cNHERF1+/LRP5+ (p=0.0002), cNHERF1-/LRP6+ and cNHERF1+/LRP6+ (p<0.0001) and cNHERF1-/TCF1+ and cNHERF1+/TCF1+ (p=0.0034) (Figure 3a-d).

Table 2. *Univariate analysis of DFS and OS*

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			DFS				OS				
Characteristc	**N. pts**	**N. events**	**5-yrs % DFS**	**p**	**HR (95% CI)**	**p**	**N. events**	**5-yrs % OS**	**p**	**HR (95% CI)**	**p**
Overall	200	51	80	-	-	-	10	95	-	-	-
β-catenin											
<5	75	9	92		1.00		2	98		1.00	
≥5	101	41	67	**<0.0001**	4.70 (2.20-10.04)	**<0.0001**	8	92	0.139	3.03 (0.64-14.28)	0.161
FZD4											
<77	76	9	91		1.00		3	97		1.00	
≥77	101	39	71	**0.0001**	3.76 (1.81-7.82)	**0.0004**	7	93	0.399	1.77 (0.46-6.87)	0.406
LRP5											
<83	66	6	93		1.00		1	98		1.00	
≥83	99	39	70	**<0.0001**	5.44 (2.29-12.96)	**0.0001**	8	91	0.068	5.56 (0.70-44.51)	0.106
LRP6											
<74	65	5	95		1.00		2	98		1.00	
≥74	92	38	68	**<0.0001**	6.14 (2.39-15.75)	**0.0002**	7	91	0.290	2.28 (0.47-10.98)	0.304
TCF1											
<16	78	9	92		1.00		2	100		1.00	
≥16	98	40	68	**<0.0001**	4.15 (2.00-8.61)	**0.0001**	7	91	0.165	2.89 (0.60-13.96)	0.185
cNHERF1											
<80	85	23	80		1.00		7	92		1.00	
≥80	96	25	77	0.981	1.01 (0.57-1.79)	0.981	3	97	0.160	0.39 (0.10-1.52)	0.177
nNHERF1											
0	159	43	80		1.00		7	96		1.00	
>0	22	5	84	0.621	0.79 (0.31-2.01)	0.623	3	89	0.124	2.77 (0.71-10.74)	0.141

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Age (years)											
≤54	104	33	78		1.00		6	95		1.00	
>54	96	18	81	0.339	0.75 (0.42-1.36)	0.342	4	95	0.707	0.78 (0.22-2.78)	0.708
Histotype											
Ductal	172	45	79		1.00		9	95		1.00	
Lobular	18	4	84		0.59 (0.18-1.94)	0.673	0	100		0	0.727
Other	10	2	87	0.666	0.85 (0.20-3.54)		1	83	0.446	2.32 (0.29-18.43)	
Tumor size (cm)											
≤2.0	117	19	88		1.00		4	97		1.00	
>2.0	75	29	68	**0.002**	2.44 (1.36-4.37)	**0.003**	6	92	0.165	2.38 (0.67-8.45)	0.179
Node											
Negative	121	21	87		1.00		5	96		1.00	
Positive	75	26	72	**0.001**	2.58 (1.42-4.67)	**0.002**	5	93	0.359	1.77 (0.51-6.12)	0.366
Grade											
1-2	108	7	94		1.00		2	99		1.00	
3	87	42	63	**<0.0001**	7.50 (3.35-16.78)	**<0.0001**	8	90	0.026	4.87 (1.03-22.93)	**0.045**

ER (%)											
≤10	63	36	58		1.00		7	88		1.00	
>10	135	13	92	**<0.0001**	0.19 (0.10-0.37)	**<0.0001**	3	99	0.024	0.24 (0.06-0.93)	**0.039**
PgR (%)											
≤10	92	38	68		1.00		8	91		1.00	
>10	106	11	90	**<0.0001**	0.28 (0.14-0.55)	**0.0002**	2	99	0.055	0.25 (0.05-1.16)	**0.077**
Ki67 (%)											
≤20	113	12	90		1.00		1	99		1.00	
>20	84	36	68	**<0.0001**	3.68 (1.90-7.13)	**0.0001**	9	90	0.004	11.25 (1.42-88.83)	**0.022**
HER2											
Negative	173	48	78		1.00		10	94		1.00	
Positive	26	2	88	0.137	0.36 (0.09-1.48)	0.155	0	100	0.275	0	0.993

178 Bold values indicate significance

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182 ***Table 3.*** *Multivariate analysis of DFS and OS*

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	DFS		OS	
	HR (95% CI)	**P**	**HR (95% CI)**	**p**
Grade (3 vs 1-2)	5.28 (1.60-17.41)	**0.006**	-	-
β-catenin	3.26 (1.14-9.33)	**0.027**	-	-
TCF1	4.86 (1.47-16.05)	**0.009**	-	-

184 Bold values indicate significance

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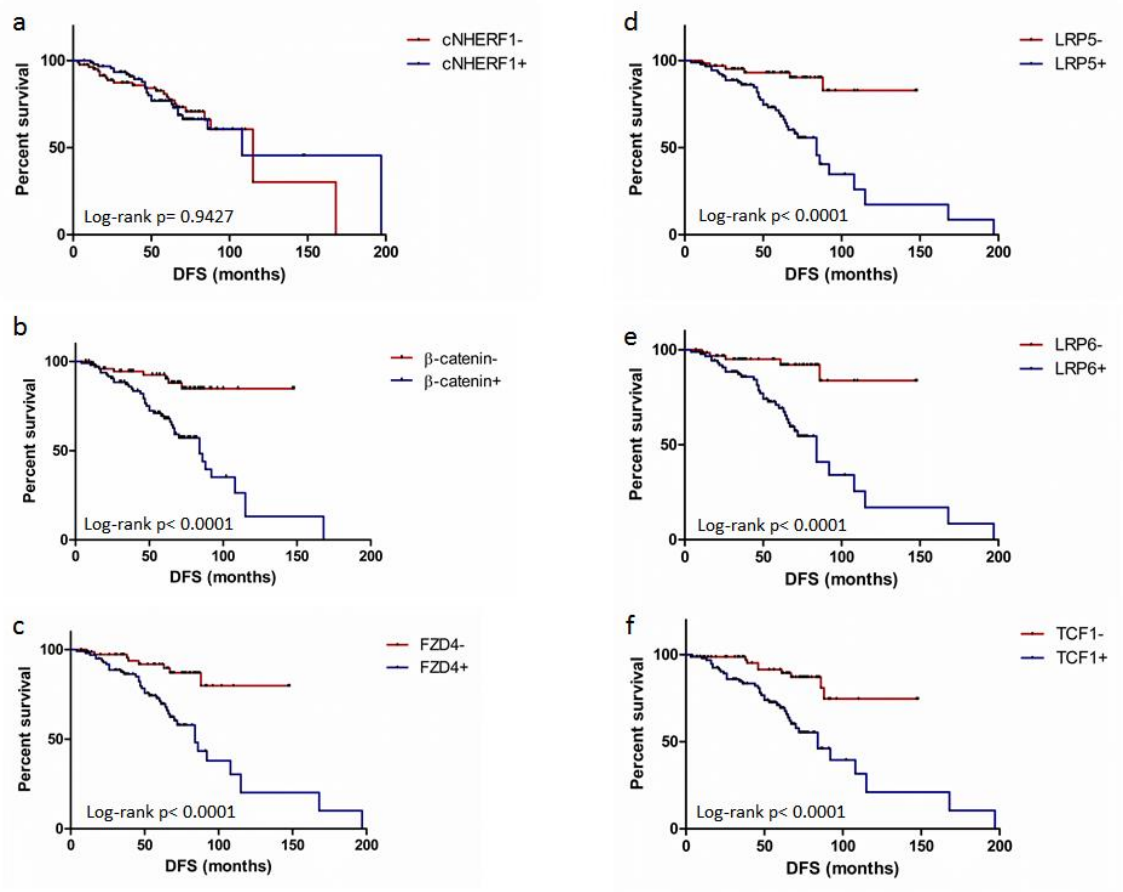

Figure 2. Survival analyses. DFS curves for patients with **a)** cNHERF1- versus cNHERF1+ expression (p=0.9427); **b)** β-catenin- versus β-catenin+ expression (p<0.0001); **c)** FZD4- versus FZD4+ expression (p<0.0001) ; **d)** LRP5- versus LRP5+ (p<0.0001); **e)** LRP6- versus LRP6+ (p<0.0001); **f)** TCF1- versus TCF1+ (p<0.0001).

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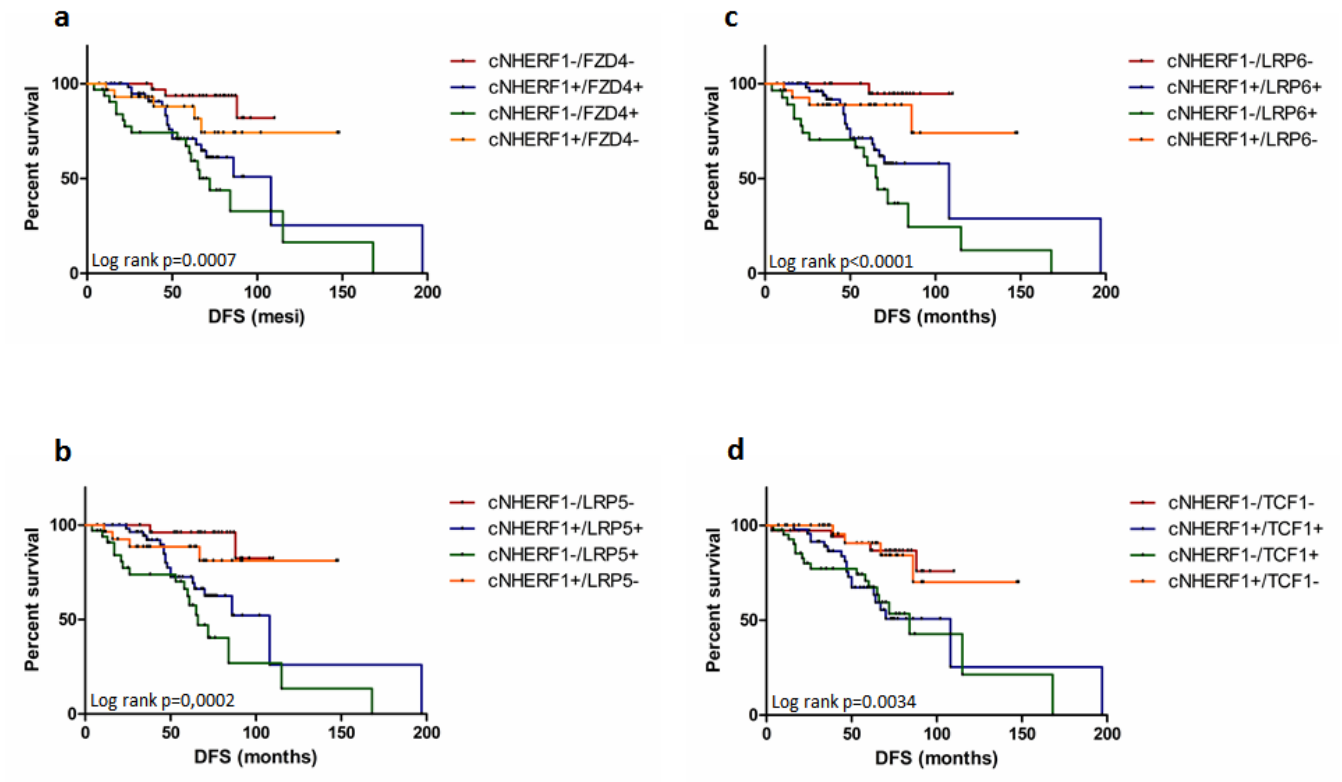

Figure 3. Survival analyses according to both NHERF1 and Wnt/pathway proteins co-expression. DFS curves for patients with **a)** cNHERF1/FZD4 co-expression, four subgroups were compared: cNHERF1-/FZD4-, cNHERF1+/FZD4+, cNHERF1-/FZD4+ and cNHERF1+/FZD4- with a p=0.0007; **b)** cNHERF1/LRP5 co-expression, four subgroups were compared: cNHERF1-/LRP5-, cNHERF1+/LRP5+, cNHERF1-/LRP5+ and cNHERF1+/LRP5- with a p=0.0002; **c)** cNHERF1/LRP6 co-expression, four subgroups were compared: cNHERF1-/LRP6-, cNHERF1+/LRP6+, cNHERF1-/LRP6+ and cNHERF1+/LRP6- with a p<0.0001; **d)** cNHERF1/TCF1 co-expression, four subgroups were compared: cNHERF1-/ TCF1-, cNHERF1+/ TCF1+, cNHERF1-/ TCF1+ and cNHERF1+/ TCF1- with a p=0.0034.

3. Discussion

Understanding the expression of proteins involved in cancer development could improve knowledge of the pathways that contribute to BC onset and progression. Among the various signaling pathways related to cancer, an important role is played by Wnt/β-catenin pathway. This pathway is a central actor of embryo development and maintenance of cellular homeostasis. Its deregulation has been associated to different human disease, comprising cancer [20]. In BC, β-catenin expression in cytoplasm/nucleus has proven to be a significant prognostic factor [21], and the interaction β-catenin-NHERF1 reported by different authors [14,22].

In this study, we analyzed the expression of some of the major constituents of Wnt pathway (β-catenin, FZD4, LRP5, LRP6, TCF1) and their relationships with the scaffold protein NHERF1. Furthermore, we evaluated the expression of these markers and the relation with clinical-pathological characteristics and their impact on patients survival.

Little is known about the cancer tissue expression of FZD4, LRP5, LRP6 proteins and we assessed their behavior for the first time in a clinical BC cohort. Interestingly, these proteins showed a higher expression in our series of BC samples mislocalized in the cytoplasm. Both LRP5 and LRP6 transcriptomic analysis showed high RNA levels in a subgroup of TNBCs [20]. Moreover LRP6

overexpression had been associated to a more aggressive BC phenotype in a smaller patients cohort, and its down-regulation was sufficient to inhibit tumorigenesis, suggesting it as a possible, new, promising therapeutic target [24].

The relation between these markers and some of the more aggressive clinicopathological characteristics (tumor size, histological grade, Ki67 index) highlighted their involvement in BC progression, by contributing to define the role in cancer growth.

The association analyses between NHERF1 and the other markers revealed a positive direct relation between cNHERF1 and FZD4, LRP5, LRP6 and TCF1 expression, but no with β-catenin, suggesting a possible novel tumor progression mechanism, that involves Wnt signaling components. Previous studies have reported a high cNHERF1 expression linked to tumor aggressive features, such as metastases, poor grade and lymphovascular invasion [9,25]. The inverse relation among nNHERF1 and LRP5, LRP6 and TCF1 supported the results of our previous study [13]. Furthermore, we also observed, by immunofluorescence, the co-localization between NHERF1 and FZD4, LRP5, LRP6 expression in the cytoplasmic compartment. NHERF1-FZD4 direct interaction has been previously reported by co-immunoprecipitation in an in vitro model of ovary cells [14]. We observed evidence of a similar distribution of NHERF1 and LRP5/LRP6 in the cytoplasm. The lack of co-localization of TCF1 and NHERF1 is not surprising, because they act in different cellular compartments during the tumor progression [13,26,27]. Furthermore, high TCF1 expression was identified in over half of cases and it was related to ER expression, probably implicated in the modulation of potential target genes, as previously reported by El- Tanani and colleagues. In fact, they demonstrated that some of the different family members of the two sets of downstream transcription factors ERα/ERβ and Tcf-1/Tcf-4 interacted directly, modulating the promoter activity of target genes [28]. TCF1 has been also reported associated to Nherf1 promoter in an "in vitro" model of colorectal cancer by β-catenin knockdown, increasing Nherf1 mRNA levels [4].

Multivariate analysis revealed the prognostic power of TCF1 and β-catenin, indicating them as independent biomarkers of prognosis for DFS in our cohort. Other authors reported β-catenin expression associated with poor prognosis in invasive ductal carcinoma [29], and with unfavorable outcomes for BC patients [30]. We found a prognostic role for the transcription factor TCF1 and this probably could be related to its close relationship with β-catenin, with which contributes to the recruitment of factors that create transcription "hot-spots" [31]. To our knowledge, this is the first study in which TCF1 constitutes a new and independent prognostic factor in BC and it could be used to reveal new research possibilities in the panorama of specific pharmacological inhibition as part of targeted therapy.

The univariate analysis identified TCF1 and also β-catenin, FZD4, LRP5 and LRP6 poor prognosis biomarkers, associating them to a shorter 5-years % DFS.

When we analyzed the outcome of patients with co-expression of cNHERF1 and other biomarkers, the cNHERF1+/FZD4+, cNHERF1+/LRP5+, cNHERF1+/LRP6+, and cNHERF1+/TCF1+ phenotypes showed a worse DFS. These results confirmed the discriminatory capability of FZD4, LRP5, LRP6 and TCF1 as prognostic markers and highlighted the weak discriminatory power of cNHERF1. It was not able to identify the actual worst-case scenario, supporting the evidence of the previous behavior observed by our group [12,32]. TCF1 has proven itself once again a prognostic markers for DFS in BC also when co-expressed with cNHERF1. Our results were mainly in relation to DFS, and not to OS, due to the low number of deaths in our cohort.

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4. Conclusions

The heterogeneous nature of breast cancer renders the identification of new prognostic biomarkers increasingly necessary. In this study, we analyzed the expression of biomarkers involved in Wnt pathway to identify patients at risk of poor prognosis. We found that TCF1 was an independent prognostic factor of poor outcome, and we showed that the over-expression of the Wnt pathway proteins was associated to a worse disease free survival. These results suggest the possible capability of these proteins to stratify breast cancer patients.

5. Materials and Methods

5.1 Patients and Clinicopathological Characteristics

This study was carried out on a retrospective series of 220 primary BCs, diagnosed between 1994 to 2012 at the IRCCS Institute, Istituto Tumori "Giovanni Paolo II" of Bari, Italy. The patients were selected retrospectively according to the availability of the biological material and the clinical follow up. Our patient series was not consecutive. All patients provided an informed consent form to use their removed biological tissue for research purposes, according to ethical standards. Patients were eligible if they had histological diagnosis of invasive breast carcinomas of any size and no evidence of metastatic disease. Patients were excluded if they had a previous history of invasive breast cancer, or other previous or concomitant malignancies or concomitant diseases. The study was approved by the Ethics Committee of the Istituto Tumori "Giovanni Paolo II" with the reference 657/CE 13–12-2018.

Supporting Information Table S1 summarizes the clinicopathological characteristics of the whole cohort. The median age of patients was 54 years (range 28-80). The median follow-up was of 64 months (range 6-235). Forty-nine patients (22.3%) developed a relapse. TNM classification, tumor size, histological grade, estrogen receptor (ER), progesterone receptor (PgR), proliferative activity (Ki67) and human epidermal growth factor receptor 2 (HER2) status were provided by the Pathology Department of our Institute. ER, PgR, Ki67 and HER2 classification has been previously reported8. HER2 status was classified as negative (score 0,1+ and 2+ not amplified) or positive (when scored 3+ by IHC or HER2 amplified by FISH) according to guideline for BC ASCO/CAP-2007 [33].

5.2 *TMA and Immunohistochemistry*.

Immunohistochemistry (IHC) was performed on tissue microarrays (TMAs) sections of 220 BC patients. TMAs were constructed using the Galileo Tissue MicroArrayer CK 4500 (Transgenomic) [12]. The TMA slides were processed and stained by the indirect immunoperoxidase method, using the BenchMark XT automated staining instrument (Ventana Medical Systems, Tucson, AZ,USA), as previously reported [12] or alternatively with a standard manual procedure [11].

Slides were probed with 1:350 anti-EBP50 rabbit polyclonal antibody (ThermoFisher Scientific, Rockford, IL, USA), 1:100 rabbit monoclonal antibody anti- β-catenin (clone: E247, Abcam, Cambridge, UK), 1:30 anti-Frizzled4 rabbit polyclonal antibody, 1:100 anti-LRP5 goat polyclonal antibody, 1:100 anti-LRP6 rabbit polyclonal antibody (all Abcam, Cambridge, UK), 1:50 anti-TCF1 rabbit monoclonal (Cell Signaling Technology). The dilution of the primary antibodies was based on preliminary dilution experiments.

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For automated staining method the UltraView DAB IHC Detection Kit (Ventana Medical Systems) was used to detect NHERF1 and β-catenin protein expression. Tissues were counterstained with Haematoxylin II and Bluing Reagent for 12 min and 4 min, respectively. Samples were dehydrated by sequential washes, cleared in xylene and then mounted.

For manual staining method a polymer-based-IHC detection system was used as the amplification system (EnVision + System-HRP Labelled Polymer Anti-Rabbit or Anti-Mouse secondary antibody, Dako, Carpinteria, CA, USA) according to the manufacture's instruction. For LRP5 antibody, a donkey anti-goat HRP conjugated was applied. The signaling was revealed by incubating the sections in 3,3'-diaminobenzidine (Liquid DAB + Substrate Chromogen System, Dako, Carpinteria, CA, USA) for 8–10 min. Cell nuclei were counterstained with Mayer's Haematoxylin (Bio-Optica, MI, Italy). Known positive controls were included in each staining run. All antibodies used in this study have been validated and the procedures standardized in a pre-analytic phase. Omission of the primary antibody was used as negative controls.

5.3 Immunohistochemical assessment

Immunoreactivity was assessed independently by 2 observers, who were blinded to clinicopathological data. The results from two observers were identical in most cases, and discrepancies were resolved by re-examination and consensus. For all markers a three field average percentage was assessed and the median values of protein expression were considered as cut-off.

NHERF1 and β-catenin immunostaining was assessed as previously described [12,15]. NHERF1 immunostaining was predominantly cytoplasmic (cNHERF1), and in some cases an intense nuclear (nNHERF1) staining was also observed. This was evaluated and scored separately. The cases were classified as positive when cNHERF1 immunoreactivity was present in ≥80% of tumor cells, and when nNHERF1 immunoreactivity was present in >0% of tumor cells observed.

Only cytoplasmic β-catenin immunostaining was considered and the cases were classified as positive when β-catenin immunoreactivity was present in ≥ 5% of tumor cells, nuclear staining was completely absent. FZD4 immunostaining was largely cytoplasmic. FZD4 was assessed by counting the number of immunoreactive cancer cells over total cancer cells (%) for fields (more of 500 tumor cells) at x400 magnification [34]. LRP5 and LRP6 immunoreactivity was observable as a brown cytoplasmic coloration, and a percentage of positive cells for field was reported. TCF1 was positive when strong nuclear staining was present (percentage of positive nuclei for field). For FZD4, LRP5, LRP6 and TCF1 proteins, the cases were classified positive when the immunoreactivity was present in ≥ 77%, ≥ 83% and ≥ 74% and ≥16% of tumor cells, respectively.

4.4 Immunofluorescence

The immunofluorescence method was described previously [8]. In brief, formalin-fixed and paraffin embedded tissue serial sections of 3 μm were deparaffinized and rehydrated in an ethanol series. Saline citrated buffer (pH 6.0) at 0.01 M at 95°C for 30 min was used for antigen retrieval, then 0.1% Triton X100-Phosphate Buffered Saline was applied for 15 minutes. The blocking was performed with 1% Bovine Serum Albumin-Phosphate Buffered Saline for 30 min and then the slides were incubated overnight at 4°C with all the primary antibodies used for the immunohistochemistry assay together with a mouse anti-EBP50 (BD Transduction Laboratories, diluition 1:150). The Alexa Fluor 488 and Alexa Fluor 568 immunoglobulin G secondary conjugated antibodies (1:2000 dilution; Molecular Probes Inc., Eugene, OR, USA) were incubated at room temperature for 1 h and then the

slides were mounted with DAPI (ProLong® Gold antifade reagent; Molecular Probes Inc.). Positive control slides were run simultaneously to assess the quality of immunoreactivity. For negative controls, slide sections were treated with 1% Bovine Serum Albumin instead of the primary antibody, and no reactivity was observed in any of these controls. Images were obtained on a Axion Image 2 upright microscope (Zeiss, Germany) with a Axiocam 512 color camera.

4.5 Follow up and statistical analysis

The Chi-squared test was used for the analysis of the association between marker expression and clinicopathological characteristics. Spearman correlation from ranks was used to analyze the interaction between two continuous variables. These statistical evaluations were performed with the Prism version 5.00 software package (GraphPad Software, San Diego, CA, USA), with the statistical significance set at $p < 0.05$.

Analysis was carried out in relation to disease-free survival (DFS) and overall survival (OS). DFS (in months) was calculated as the time-frame between the date of surgery and the date of loco-regional/distant relapse (second invasive BC, second primary cancer and/or death without evidence of BC) to the date of last contact. OS (in months) was calculated as the time-frame between the date of surgery and date of last contact or the date of death from any cause. DFS and OS survival curves were computed by Kaplan-Meier method and compared by the log rank test. Cox proportional hazard regression model was performed to assess prognostic factors, including the variables that were statistically significant in univariate analysis. The model was optimized using a backward stepwise regression. Statistical significance level was p-values <0.05. Statistical analyses were made using the SAS statistical software, version 9.4 (SAS Institute Inc, Cary, NC, USA).

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, **Table S1**: Tumor characteristics of 220 invasive breast cancer patients; **TableS2**: Relationship between tumor markers and clinicopathological features.

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