MUC4 is a biomarker of metastasis in TNBC and its downregulation by blocking soluble TNF prevents metastasis in combination with immunotherapy

Mauro, Florencia[1]*; Bruni, Sofia[1]; Dupont, Agustina[2,3]; Inurrigarro, Gloria[2]; Figurelli, Silvina[3]; Barchuk, Sabrina[4], Lopez Della Vecchia, Daniel[4]; Cordo Russo, Rosalia[1]; Gil Deza, Ernesto[5]; Mercogliano, María Florencia[1]; Schillaci, Roxana[1]*

[1]Instituto de Biologia y Medicina Experimental (IBYME - CONICET),[2]Servicio de Patología Sanatorio Mater Dei, [3]Servicio de Patología, Hospital Juan A. Fernández, [4]Servicio de Ginecología, Hospital Juan A. Fernández, [5]Instituto Oncologico Henry Moore.

*flor.luciana@hotmail.com, *roxanaschillaci@gmail.com

#2218

INTRODUCTION

Triple negative breast cancer (TNBC) has the worst survival among breast cancer and systemic chemotherapy has been the treatment option. Recently, immunotherapy based on anti PD-1/PD-L1 antibodies shown to be effective in a subset of TNBC whose tumors have tumor-infiltrating lymphocytes (**TILs**) .

Mucin 4 (MUC4) is a transmembrane glycoprotein expressed in certain types of cancer. We previously demonstrated that the proinflammatory cytokine TNF upregulates MUC4 expression in HER2 positive breast cancer and it is associated with poor disease-free survival in patients treated with trastuzumab (1). In addition, MUC4 has been involved in **metastases dissemination** (2,3).

OBJECTIVE

★ To evaluate MUC4 expression in a TNBC cohort and its clinical impact on overall survival and metastasis-free survival.

★ To study the relationship between MUC4 and PD-L1 expression and TILs in a cohort of TNBC patients.

★ To determine the impact of TNF neutralization on MUC4 expression, epithelial-mesenchymal transition, tumor cell invasion in TNBC cell lines and on the establishment of pulmonary metastases in a preclinical model.

RESULTS

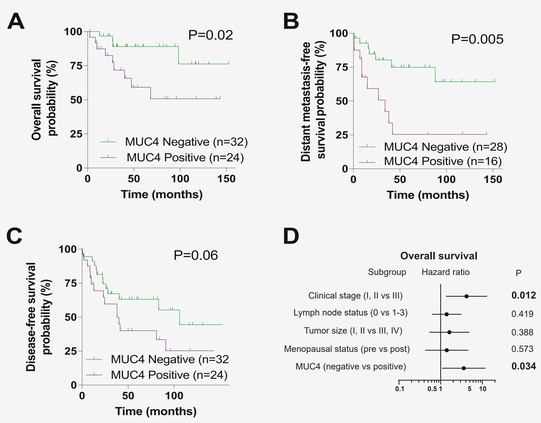

Figure 1. MUC4 expression correlates with poor overall survival and distant metastasis-free survival in TNBC, and is an independent biomarker of poor overall survival. A total of 56 TNBC specimens were analyzed by immunohistochemistry (IHC) at diagnosis. Kaplan–Meier analysis of the overall survival, n=56 **(A)**; probability of distant metastasis free survival, n=44 **(B)**; and disease free survival, n=56 **(C)** of patients based on the expresion of MUC4 are shown. The P value was calculated using the log-rank test. **D.** Data were analyzed using Cox univariate and multivariate proportional hazards regression models, respectively.

Table 1. Patients' baseline characteristics with TNBC

Characteristic	N° patients	%	Median	Range
Total number of patients	55			
Age (years)			54	28-83
Length follow-up (months)			42	4–152
Menopausal status				
Pre	21	38.2		
Post	34	61.8		
Tumor size				
T1	16	29.1		
T2	20	36.4		
T3	14	25.5		
T4	5	9.1		
Lymph node status				
N0	29	52.7		
N1	20	36.6		
N2	5	9.1		
N3	1	1.8		
Clinical stage				
I	10	18.2		
II	28	50.9		
III	17	30.9		
Treatment				
Chemotherapy	11	20.0		
Radiotherapy	5	9.1		
Chemo+Radiotherapy	39	70.9		

Table 2. Correlation between MUC4 expression and clinicopathological characteristics

Clinicopathological characteristics	MUC4 (n= 55) Negative (N, %)	Positive (N, %)	P
Overall population	32 (58.2)	23 (41.8)	
Menopausal status			
Pre	13 (40.6)	8 (34.8)	0.660
Post	19 (59.4)	15 (65.2)	
Tumor size			
T1	8 (25.0)	8 (34.8)	0.871
T2	12 (37.5)	8 (34.8)	
T3	9 (28.1)	5 (27.7)	
T4	3 (9.4)	2 (8.7)	
Lymph node status			
N0	16 (50)	13 (56.5)	0.399
N1	12 (37.5)	8 (34.8)	
N2	4 (12.5)	1 (4.3)	
N3	0 (0)	1 (4.3)	
Clinical stage			
I	5 (15.6)	5 (21.7)	0.746
II	16 (50.0)	12 (52.2)	
III	11 (34.4)	6 (26.1)	
Treatment			
Radiotherapy	2 (40.0)	3 (60.0)	0.331
Chemotherapy			
AC	2 (7.1)	3 (18.8)	
AC+T	21 (75.0)	12 (75.0)	
FAC	5 (17.9)	1 (6.3)	

Data were analized using χ^2 test.

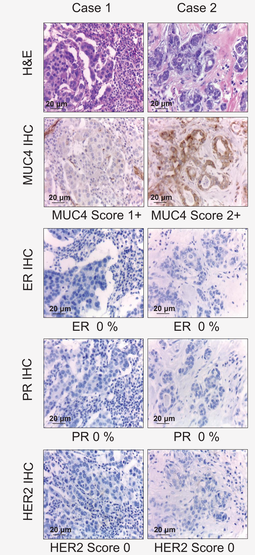

Figure 2. MUC4 expression negatively correlates with TILs in TNBC. Representative H&E and IHC studies of MUC4, ER, PR and HER2 are shown.

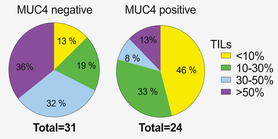

Figure 3. Distributions of TILs according to MUC4 expression in TNBC. Specimens were studied by IHC and H&E. TILs-positive: >30%

Correlation between MUC4 expression and TILs in TNBC

	MUC4 - (%)	MUC4 + (%)	P
TILs (< 30%)	8 (25.8)	18 (78.3)	0.0003
TILs (≥ 30%)	24 (74.2)	6 (21.7)	

Data were analized using χ^2 test.

Figure 4. MUC4 expression negatively correlates with TILs, PD-L1, KI67 and AR. A cohort of 48 TNBC specimens were studied by H&E and IHC. SP142 anti PD-L1 antibody were used. Data were analized using χ^2 test. TILs: tumor infiltrating lymphocytes. PD-L1: programmed death ligand 1. Ki67: proliferation marker. AR: androgen receptor, biomarker of luminal androgen receptor (LAR). CK5: cytokeratin 5, basal marker.

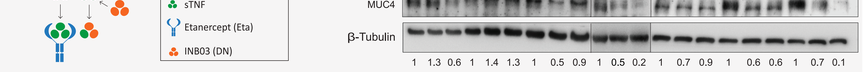

Figure 5. TNFα blockade decreases MUC4 expression in TNBC cell lines. A. We used two **TNF blocking agents**: etanercept (**Eta**, able to neutralize both transmembrane and soluble TNF) and INB03 (**DN**, which only neutralizes soluble TNF). **B.** Cells were treated with IgG (5 µg/ml), Eta (5 µg/ml) or DN (10 µg/ml) for 48 hours. Cellular extracts were obtained and Western blot analysis were performed.

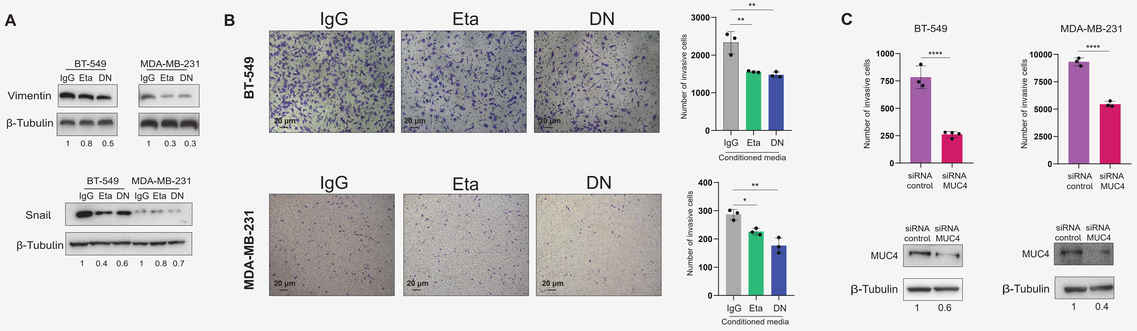

Figure 6. TNF blockade decreases the expresion of mesenchymal markers and reduces the invasive capacity of TNBC cell lines. A. Cells were trated with IgG, Eta or DN for 48 hours. Cellular extraxts were obtained and Western blot was performed. **B.** Invasion assay was performed by placing each cell line on transwells and conditioned media obtained from BT-549 and MDA-MB-231 treated with IgG, Eta or DN were placed on the well. After overnight incubation, the cells present in the lower membrane (invading cells) were stained with crystal violet. **C.** Invasion assay was performed by placing each cell line, previously silenced with siRNA control or siRNA MUC4, on transwells and media were placed on the well. **(B and C)** Cells were quantified by Image J. P values were calculated by one-way ANOVA, coupled with a Tukey post-hoc test. *P<0.05 **P<0.01 ****P<0.0001

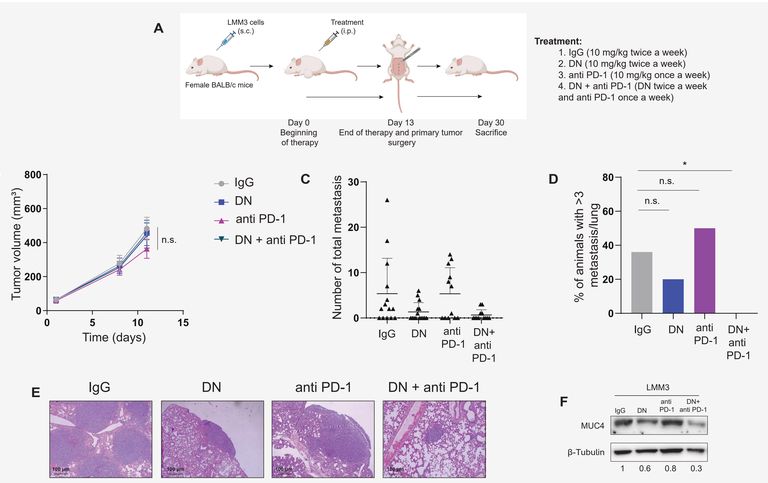

Figure 7. Soluble TNF blockade in combination with anti PD-1 antibody prevents the appearance of lung metastases in the LMM3 tumor. A. Female BALB/c mice were injected with 3x10⁶ LMM3 cells s.c. and, after tumor establishment, mice were randomized and treated (n=10-15 mice/group). **B.** Tumor volume was monitored routinely. P values were calculated by one-way ANOVA. **C,D.** On day 30 the animals were sacrificed and the number of lung metastases were evaluated by a pathologist. Oligometastases were defined as less than or equal to 3 metastases per lung. P values were calculated by Z score statistic. *P<0.05. **E.** Representative H&E studies are shown. **F.** Tumor extracts were obtained and Western blot analysis were performed.

CONCLUSIONS

★MUC4 expression is an independent biomarker of poor overall survival and is associated with an increased risk of metastasis in TNBC patients.

★MUC4 is inversely correlated with TILs, and is associated with tumors with low proliferative rate (Ki67<30%) and negative PD-L1: it would be useful to identify tumors resistant to chemotherapy and immunotherapy.

★TNF blockade decreases MUC4 expression, mesenchymal markers and reduces invasive capacity in TNBC cell lines.

★Soluble TNF blockade in combination with anti PD-1 antibody prevents the establishment of lung metastases in a preclinical model of TNBC.

We propose TNF as a new target for the treatment of TNBC, and MUC4 as a predictive marker to guide a combined treatment of TNF blockers with immunotherapy.

FINANCIAL SUPPORT AND REFERENCES

1. Mercogliano MF, et al. Clin Cancer Res 2017. **2.** Komatsu M, et al. J. Biol. Chem. 1997. **3.** Rowson-Hodel AR, et al. Oncogene 2018.