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Original Article

Autophagy is decreased in triple-negative breast carcinoma involving likely the MUC1-EGFR-NEU1 signalling pathway

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Abstract: Triple-negative breast carcinoma (TN) is a heterogeneous cancer type expressing EGFR in 75% of cases. MUC1 is a large type I sialylated glycoprotein comprising two subunits (α and β chains, also called respectively MUC1-VNTR and MUC1-CT), which was found to regulate EGFR activity through endocytic internalisation. Endocytosis and autophagy use the lysosome pathway involving NEU1. Recently, a molecular EGFR-MUC1-NEU1 complex was suggested to play a role in EGFR pathway. In the aim to understand the relationship between EGFR-MUC1-NEU1 complex and autophagy in breast carcinoma, we compared triple negative (TN) showing a high-EGFR expression with luminal (LUM) presenting low-EGFR level. We studied the expression of MUC1-VNTR, MUC1-CT and NEU1 in comparison with those of two molecular actors of autophagy, PI3K (p110β) and Beclin1. A total of 87 breast cancers were split in two groups following the immunohistochemical classification of breast carcinoma: 48 TN and 39 LUM. Our results showed that TN presented a high expression of EGFR and a low expression of MUC1-VNTR, MUC1-CT, NEU1, Beclin-1 and PI3Kp110β. Moreover, in TN, a positive statistical correlation was observed between Beclin-1 or PI3Kp110β and MUC1-VNTR or NEU1, but not with EGFR. In conclusion, our data suggest that autophagy is reduced in TN leading likely to the deregulation of EGFR-MUC1-NEU1 complex and its associated cellular pathways.

Keywords: Breast, carcinoma, EGFR, MUC1, NEU1, PI3K, beclin-1, autophagy

Introduction

Breast cancers are the most common cause of cancer mortality in women. Most of them are routinely treated following their estrogen receptors (ER), progesterone receptors (PR) and human epidermal growth factor receptor type 2 (HER2) expressions. According to this clinical approach, a biological classification has been recently proposed by Perou *et al* and adopted by the St Galen International Expert Consensus. Briefly, this classification proposes three main molecular subtypes: luminal (ER+PR+HER2-), overexpressed HER2 (ER-PR-HER2+) and triple negative (ER-PR-HER2-) carcinomas [1-3]. However, triple negative breast carcinoma (TN) corresponds to a heterogeneous cancer subtype leading to difficulties to assign an appropriate treatment [4]. Interestingly, about 75% of TN expressed high amount of type 1 epidermal growth factor receptor (EGFR). Unfortunately, the treatments by a monoclonal anti-EGFR alone (Cetuximab) or in combination with carboplatin, were associated with a low rate of clinical response suggesting a complex signalling pathway [5-7].

MUC1, or CD227, is a large trans-membrane O-glycosylated protein affiliated to the insoluble mucin family. Structurally, MUC1 is a heterodimer consisting of a large extracellular α-subunit containing 20 to 125 tandem repeats of 20

amino acids broadly glycosylated (MUC1-VNTR), and a β-subunit containing the transmembrane domain and a cytoplasmic tail (MUC1-CT) [8-10]. Many breast cancers and other epithelial cancers over-express MUC1 presenting severe alterations of their glycosylation pattern leading to the exposure of repetitive peptide core epitopes that may represent potential targets for immunotherapy [11-14]. Kawaguchi *et al* demonstrated that MUC1 glycosylation changes are correlated to the tumoral capacity to develop metastasis [15]. Among the glycosylation processes, sialylation is crucial for a variety of cellular functions such as cell adhesion signal recognition, and biological stability of glycoproteins. Sialylation of glycoproteins is regulated by two opposing enzymatic activities: sialyltransferases and sialidases [16, 17]. It is interesting to mention that NEU1, a well-known lysosome sialidase, has been proposed to regulate EGFR and MUC1 signalling (ref Lillehoj et *al)*. Moreover, NEU1 forms a complex with both EGFR and MUC1 [18]. The β-subunit part of MUC1, MUC-CT, is involved in several cellular signalling pathways that could potentially induce cancerous transformation by either growth/survival pathways induction or apoptosis inhibition [19, 20]. Some authors demonstrated a colocalisation between MUC1-CT and EGFR both at the cell membrane and in the nucleus, involving internalisation of EGFR and activation of the EGFR-PI3K-AKT pathway [20-22].

The phosphoinositide 3-kinases (PI3K) constitute a family of lipid kinases that can be activated by extracellular stimuli. PI3K are involved in tumour cell survival, proliferation and differentiation. They are grouped into three classes of isoforms mainly based on their substrate specificity. The two ubiquitously expressed PI3K isoforms p110α and p110β play different roles in cellular signalling. The p110α isoform promotes the main response of EGFR stimulation, whereas p110-β seems to finely tune this response [23, 24]. Importantly, p110β is also involved in the endocytosis of EGFR and/or to promote autophagy by activation of the Rab5-Vps34-Vps15-Beclin-1 complex [25, 26]. Interestingly, MUC1 expression is associated with increased lysosomal turnover of the autophagic maker LC3-II by stimulation of the AMP-activated protein kinase (AMPK), therefore highlighting the involvement of MUC1 in the regulation of autophagy [21, 27]. Autophagy is a cellular degradation pathway involving double-membrane vesicles and the lysosome machinery, including catabolic enzymes such as NEU1. Autophagy is activated upon cellular stress in order to maintain cell homeostasis. Autophagy plays a role in differentiation, aging, immunity and tumour suppression [28]. Intriguingly, autophagy is also associated with resistance to chemotherapy [29, 30].

To understand the relationship between EGFR-MUC1-NEU1 complex and autophagy in breast carcinoma, we compared TN showing a high-EGFR expression, with LUM presenting low-EGFR level. We studied the expression of MUC1-VNTR, MUC1-CT and NEU1 in comparison with those of PI3K (p110β) and Beclin1.

Materials and methodology

Patient population

Between 2010 to 2013, archival paraffin embedded surgical material and clinical data of 48 triple negative breast carcinomas (TN, age = 61.1 ± 14.9 years) and 39 luminal carcinoma (LUM, age = 60.4 ± 12.4 years, P = ns) using as control group, were available for this study. All cases were classified following the immunohistochemical classification in mean of a preliminary immunohistochemical study confirmed by the tissue microarray (TMA) [1-3]. Among those, 19 patients presented lymph node metastasis (LUM = 15/39 (38.4%) vs. TN = 4/48 (10.4%), *P* = 0.0008) and 15 had haematogenous metastasis (mainly lung, liver and brain; LUM = 1/39 (2.5%) vs. TN = 14/48 (29.1%), *P* = 0.0007). Tumour recurrence was described in 13 patients (LUM = 2/39 (5.1%) vs. TN = 11/48 (22.9%), *P* = 0.02). No neo-adjuvant chemotherapy was performed. The mean of follow-up was 101.6 ± 60.4 weeks.

This study was made according to the approval of the local ethic committee, and all patients were informed and agreed to contribute to this study.

Histological procedures and Tissue Micro Array (TMA) construction

All surgical specimens were initially fixed in 4% buffered formaldehyde solution for 8 to 48

Table 1. Primary antibodies, dilution, antigenic retrieval, incubation times and abbreviations used in this study

Antibodies	Clone	Abbreviation	Manufacture	Dilution	Retrievial	Incubation (minutes)
Beclin-1	H-300	Beclin-1	Santa Cruz	1:50	Citrate, pH 6	60
EGFR wild-type	DAK-H1-WT	EGFR	Dako	1:200	EDTA, pH 9	30
α-Estrogen Receptor	SP1	ER	Dako	RTU	EDTA, pH 9	20
HER2	c-erB-2	HER2	Dako	1:800	Citrate, pH 6	30
MUC1 core glycoprotein	Ma552	MUC1-VNTR	Novocastra	1:50	EDTA, pH 9	10
MUC1-ter C	ARP41446	MUC1-CT	Aviva System Biology	1:400	EDTA, pH 9	60
Neuraminidase1	NEU1	ARP44186_T100	Aviva System Biology	1:1000	EDTA, pH 9	60
Pi3K p110 β	N/A	PI3K	Spring	1:100	Citrate, pH 6	30
Progesterone Receptor	PgR636	PR	Dako	RTU	EDTA, pH 9	20

hours, then embedded in paraffin and cut into 4 µm thick slides. The slides were stained with a classical haematoxylin-eosin stain to perform the initial diagnosis. From these archival formol/paraffin blocs, we built a TMA receive paraffin block that could be used for all immunohistochemical slides. We used an automated TMA device (Minicore2, Mitogen UK) associated with a needle core of 0.6 mm diameter. We chose 3 distant core needle samples of each donor tumour paraffin block. The final TMA receive paraffin block was cut in serial slides. These slides were consecutively used for immunohistochemistry.

Immunohistochemical methods

Immunohistological staining was performed with a Dako Autostainer Link 48® immunostaing system (Dako Glostrub, Denmark). After dewaxing, antigenic retrieval were performed using citrate buffered (pH 6) or EDTA buffered (pH 9) antigenic retrieval solution at 99°C in a warm bath (EnVision Flex Target Retrieval solutions high and low pH, Dako). Endogen peroxydase were inhibiting with a hydrogen peroxide phosphate buffered solution (EnVision Flex Peroxydase Blocking Reagent, Dako). After the incubation of the primary antibodies, the immunological reaction was revealed by a polymer dextran coupled with secondary antibody and peroxydase for 15 min (EnVision Flex HRP, Dako) and diaminobenzidine for 10 minutes (EnVision DAB + chromogen, Dako). Counterstain was made with haematoxylin for 10 min (EnVision Flex haematoxylin, Dako). Negative controls were obtained using mouse IgG1 (Negative Control Mouse, Dako) diluted at 1:100, in place of primary antibodies. Primary antibodies, dilution and antigenic retrieval are described in **Table 1**.

Classification of breast cancers by immunohistochimestry

HER2 immunostaining were considered positive as described in the Guideline of College of American Pathologists and controlled by a FISH technique for all cases (HercepTest® Dako) [31]. ER and PR were subsequently scored using a score consisting to sum the intensity and proportion of the nuclear immunostaining. A result superior to 2 was considered as positive [32]. According the St Galen guideline [3] and the results of these immunostainings, all cases were classified following the immunohistochemical classification [1, 2].

Immunostaining quantification

Staining results were evaluated by CG and CM, based on the intensity and percentage of staining tumour cells, with agreement reached. The parametric results were edited as a score by a multiplication of intensity (0 = none, 1 = weak, 2 = intermediated, 3 = strong) and the percentage of tumour cells (0 = none, 1 = 1%, 2 = between 1% to 10%, 3 = between 10% to 33%, 4 = between 33% to 66% and 5 = between 66 to 100%) [modified from 32].

Statistics

T-test and Spearman's test were performed. A p value < 0.05 was considered significant. The WinSTAT® version 2012 (Fitch Software, Bad Krozinger, Germany) and Excel 2013 (Microsoft Corp., Redmond, Washington U.S.A.) programs were used for statistical analysis. The results were expressed in means and standard error.

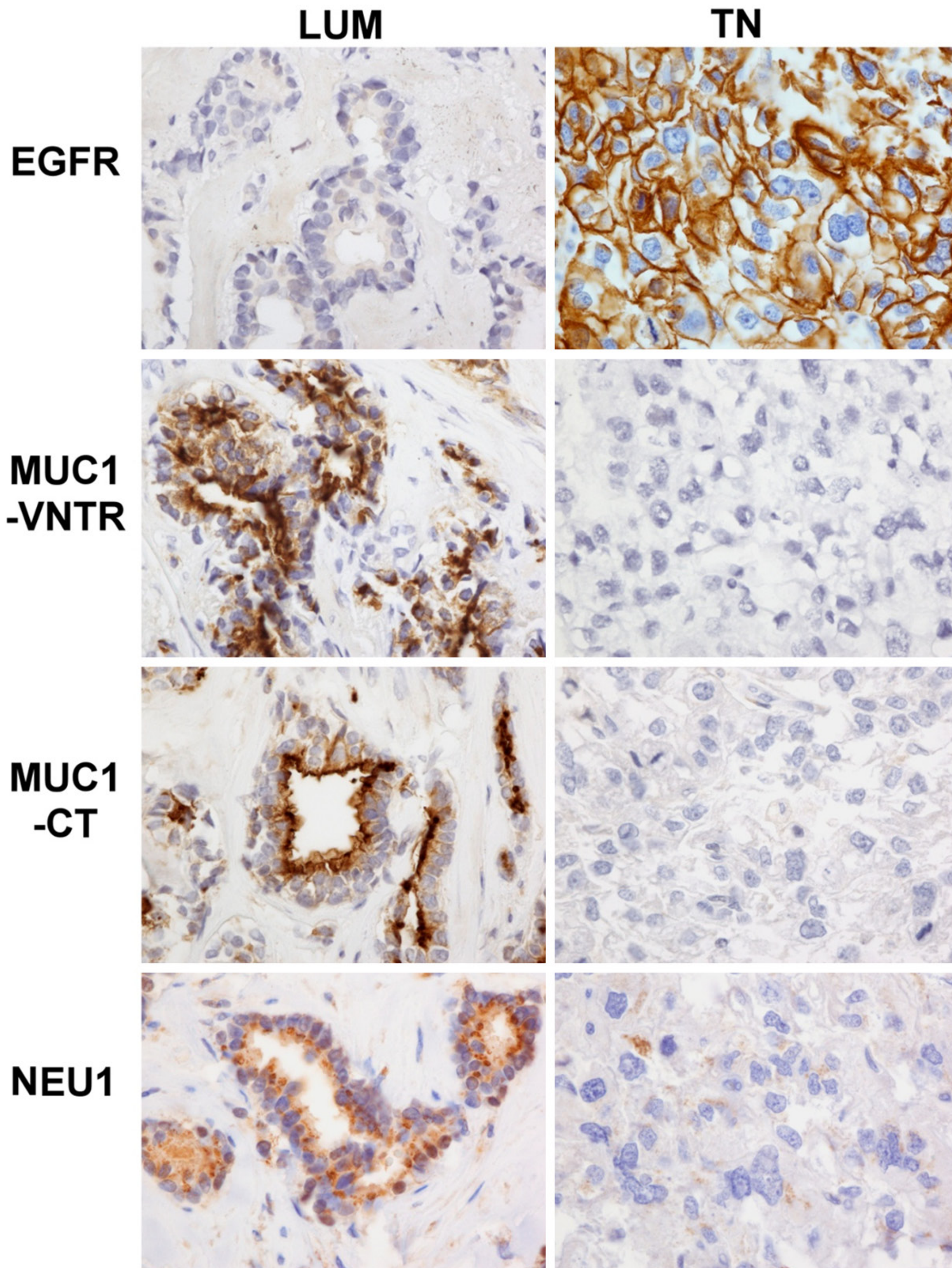

Figure 1. EGFR, MUC1-VNTR, MUC1-CT and NEU1 difference and morphological distribution between luminal (LUM) and triple-negative (TN) breast carcinoma. EGFR is negative in LUM and positive in only membrane in this TN case/ MUC1-VNTR and MUC-CT are positive in cytoplasm of LUM and negative in TN/NEU1 is positive in cytoplasm of LUM and negative in TN. This figure illustrated observations described in **Table 2**: the low-expression of MUC1-VNTR, MUC1-CT, NEU1 and the high-expession of EGFR in TN, suggesting that the EGFR/MUC1/NEU1 molecular complex could be deregulated in breast cancers. Immunohistochemistry on the same LUM and TN cases. Magnification 400×.

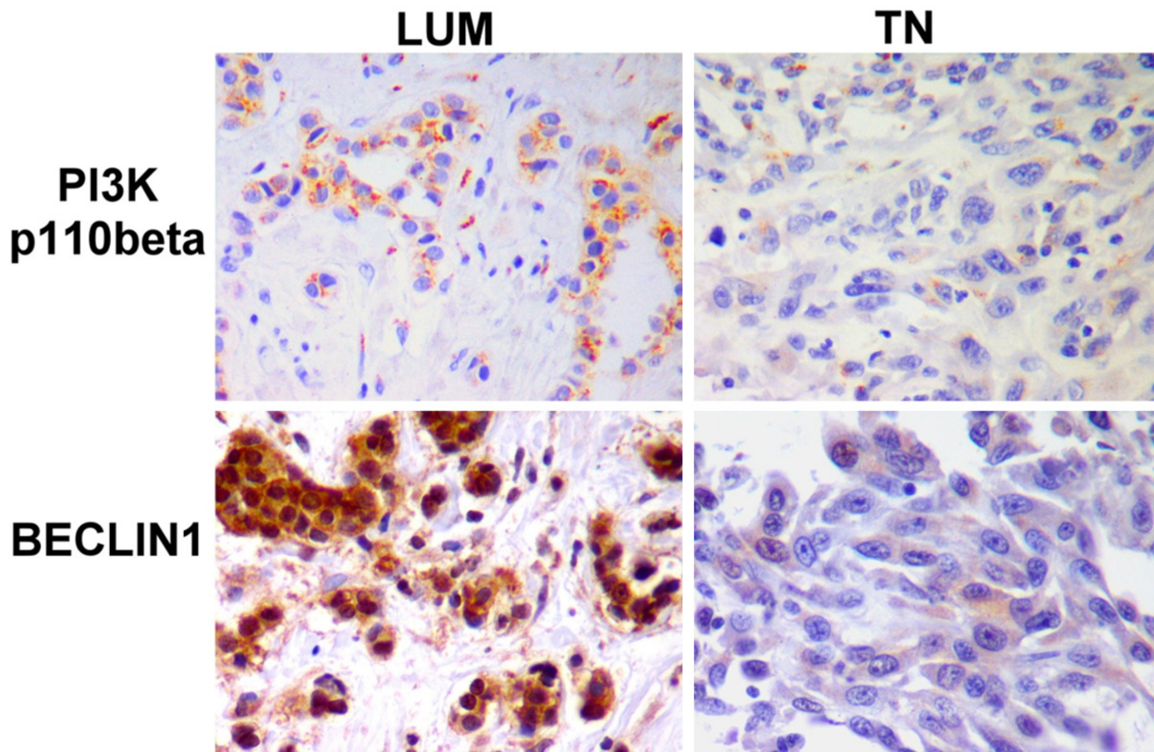

Figure 2. PI3Kp110β and Beclin-1 difference and morphological descriptions between luminal (LUM) and triple-negative (TN) breast carcinoma. PI3Kp110β and Beclin-1 are high-positive in LUM and low-positive in TN. This figure illustrated observations described in **Table 2**: the low-expression of Beclin-1 and PI3Kp110β in TN, suggesting a low action of autophagy. Immunohistochemistry on the same LUM and TN cases. Magnification 400×.

Results

Characteristics of patients with TN and LUM breast carcinomas

In this retrospective study, we analysed data from 87 patients prognosticated either with triple negative breast carcinomas (TN, n = 48, age = 61.1 ± 14.9 years) or with luminal breast carcinoma (LUM, n = 39, age = 60.4 ± 12.4 years, P = ns) [1-3]. Among the whole population of breasts cancer patients, those diagnosed with lymph node metastasis was significantly higher in the LUM group compared to the TN group (15/39 (38.4%) vs. TN = 4/48 (10.4%), respectively, P = 0.0008). Conversely, the number of patients with haematogenous metastasis (mainly lung, liver and brain) was lower within the LUM group than in the TN group (LUM = 1/39 (2.5%) vs. TN = 14/48 (29.1%), P = 0.0007). Tumour recurrence was described in 13 patients (LUM = 2/39 (5.1%) vs. TN = 11/48 (22.9%), P = 0.02). No neo-adjuvant chemotherapy was performed. The mean of follow-up was 101.6 ± 60.4 weeks.

Morphological differences between TN and LUM

First, we studied the morphological cell distribution of EGFR, MUC1-VNTR, MUC1-CT and of NEU1 (**Figure 1**), PI3Kp110β and Beclin-1 (**Figure 2**) in the 48 TN in comparison with the 39 LUM. To that aim, we compared the immunohistological score obtained for each antibody in the TN and LUM groups (**Table 2**). Interestingly, all immunostainings presented a significant statistical difference between LUM and TN, suggesting an important biological difference between these 2 groups of breast tumours. Globally, TN showed a lower expression of MUC1-VNTR (P = 0.002), MUC1-CT (P < 0.0001), NEU1 (P = 0.03), PI3Kp110β (P < 0.0001) and Beclin-1 (P < 0.0001) as compared to LUM. A higher expression of EGFR (P < 0.0001) was observed in TN. Although TN breast cancers are well-known to highly express EGFR, in this study 14 TN were EGFR-negative and 2 LUM were EGFR-positive. However, no change within our data was observed if these cases were discarded EGFR is expressed both in the cytoplasm and the cell membrane.

Table 2. Biological difference between LUM and TN antigenic expressions. Results are expressed in mean and standard deviation of histological score

	LUM	TN	P
N	39	48	
EGFR	0.35 ±1.18	6.60 ± 5.04	< 0.0001
MUC1-VNTR	10.71 ± 4.89	7.14 ± 5.76	0.002
MUC1-CT	9.53 ± 4.62	5.50 ± 4.18	< 0.0001
NEU1	8.05 ± 3.16	6.06 ± 5.01	0.03
PI3Kp110β	9.55 ±4.09	6.10 ± 3.96	< 0.0001
BECLIN1	9.65 ± 4.26	6.23± 3.77	< 0.0001

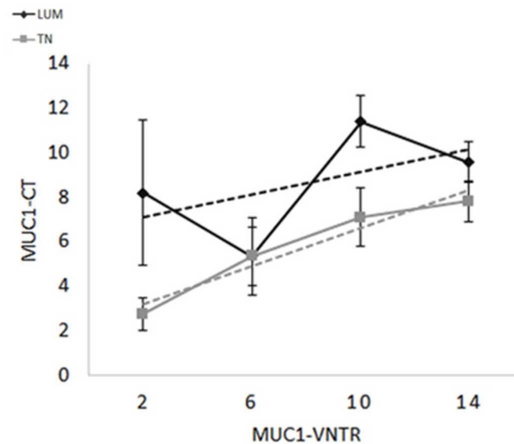

Figure 3. Histological score relationship between MUC1-VNTR and MUC1-CT in LUM (grey, P = ns) or TN (black, $P < 0.0001$). The linear positive correlation between MUC1-VNTR and MUC1-CT observed only in TN, suggests that these both antigens were produced in the same concentration. Consequently, MUC1 seems less modified in TN than in LUM.

Then, we investigated the morphological localisation of these molecules. As we previously described, MUC1-VNTR is expressed both at the cytoplasm membrane and in the cytoplasm [33]. MUC1-CT showed the same expression pattern. Importantly, we noted that MUC1-VNTR and MUC1-CT expression were not always observed at the cell membrane of each patient group, indicating that MUC1 epitopes are not always accessible for a target therapy using monoclonal antibodies. NEU1 and PI3Kp110β were localized mainly in the cytoplasm. Beclin-1 was observed either in the cytoplasm or in the nuclei.

Relative expression of the EGFR/MUC1/NEU1 complex molecules

Although MUC1 expression has been well illustrated in breast carcinoma, to date the comparison of MUC1-VNTR and MUC1-CT in TN and LUM is still not described in the literature. Here, we found a positive correlation between MUC1-VNTR and MUC1-CT within the TN group ($P < 0.0001$, r = 0.64) but not for the LUM group, suggesting that in TN, MUC1-VNTR and MUC1-CT were produced at the same rate (**Figure 3**). Previous studies suggested the possibility of a MUC1-EGFR-NEU1 molecular complex [18, 34]. We then sought a relationship between the expressions of EGFR with each one of these two forms of MUC1 in the two groups of breast cancers. In contrast to the previous documented studies, we did not observe a positive correlation neither between EGFR and MUC1-VNTR, nor with MUC1-CT whatever the breast cancer group analysed (data not shown). Likewise, EGFR expression was not positively correlated to Neu1 expression. Furthermore, in the setting of the above-mentioned results in **Figure 1**, we also questioned the possibility of an inverse correlation between EGFR and MUC1 or NEU1. Though, no correlation was found between these molecules. Conversely, MUC1-VNTR was statistically and positively correlated to NEU1 expression in the TN group (P = 0.04; r = 0.25), but not in the LUM group (**Figure 4**). No correlation was observed with the intracellular MUC1 domain (MUC1-CT) (**Figure 4**). These results suggest that only an interaction between NEU1 and the extracellular domain of MUC1 may occur in the TN group.

Autophagy and TN breast cancer

Autophagy has been involved in breast cancer [29, 30]. We studied two different proteins involved in the autophagy pathway: the subunit PI3Kp110β and Beclin-1 [26-28]. Our results indicate a positive correlation between PI3Kp110β and Beclin-1 either in LUM (P = 0.001, r = 0.41) and TN (P = 0.002, r = 0.40), demonstrating the relationship between the two proteins. TN presented a low-level of both PI3Kp110β and Beclin-1 suggesting a decreasing of autophagy (**Table 2**). Moreover, NEU1 presented a positive correlation for PI3Kp110β and Beclin-1 in TN (respectively P = 0.0003, r =0.48 and P = 0.01, r = 0.31) (**Figure 5**). In the LUM group, NEU1 was positively correlated to PI3Kp110β (P = 0.04, r = 0.29) but not with beclin-1 (**Figure 5**). These observations pointed

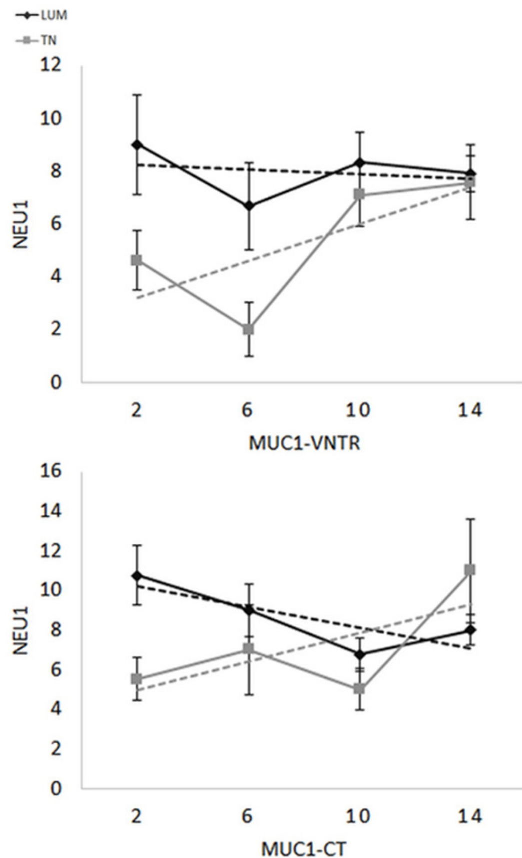

Figure 4. Histological score relationship between NEU1 and MUC1-VNTR in LUM (grey, *p*=ns) or TN (black, *p*=0.04) or MUC1-CT for LUM (grey, *P* = ns) or TN (black, *P* = ns). Correlations between MUC1-VNTR or MUC1-CT and NEU1 were only significant between MUC1-VNTR and NEU1 in TN (*P* = 0.04) suggesting that the extracellular chain of MUC1 is associated with NEU1 only in TN.

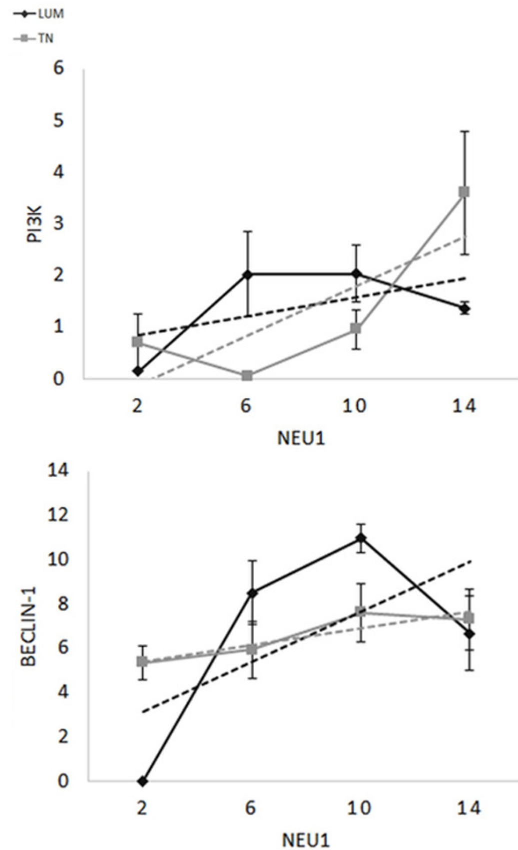

Figure 5. Histological score relationship between NEU1 and PI3Kp110β for LUM (grey, *P* = 0.04) or TN (black, *P* = 0.0003) or Beclin1 for LUM (grey, *P* = ns) or TN (black, *P* = 0.01). NEU1 presented a positive correlation with the two antigens of autophagy: PI3K (p110β) (for TN *P* = 0.01 and for LUM *P* = 0.04) and Beclin-1 (For Tn *P* = 0.0003 and for LUM P = ns). This suggests that the lysosomial enzyme NEU1 is involved in the autophagy pathway.

out the implication of NEU1 in the autophagy through the lysosomal machinery. MUC1-VNTR also showed positive correlations in TN for both PI3Kp110β (*P* = 0.009, r = 0.32) and Beclin 1 (*P* = 0.01, r = 0.32). MUC1-CT was only correlated with PI3Kp110β in the TN group (*P* = 0.04) but not with Beclin-1 (**Figure 6**), suggesting that MUC1-VNTR was the main MUC1 subunit involved in the autophagy process in TN (**Figure 7**). This relationship was less obvious in LUM. We concluded that autophagy is reduced in TN involving likely MUC1-VNTR and NEU1.

Discussion

Recently on routine histological material, we have demonstrated that MUC1 protein was associated with the tumour aggressive biological behaviour of breast carcinoma [33]. Several authors also illustrated the secretion of MUC1 by breast cancer cells [35-37]. Most of them were mainly interested by the extracellular α-subunit of the MUC1 (MUC1-VNTR) because in tumour cells, the antigenic sides of the α-subunit protein core are specifically denuded by an aberrant lack of glycosylation. The core protein is then more exposed and constitutes a potential target for immunotherapy [38]. Deepening the knowledge of breast carcinomas, we here showed an important heterogeneity, both in the quantitative and the qualitative expression of MUC1 in luminal and triple negative breast carcinomas. In agreement with our data, Siroy *et al had already* showed that 67% of early stage basal-like triple negative

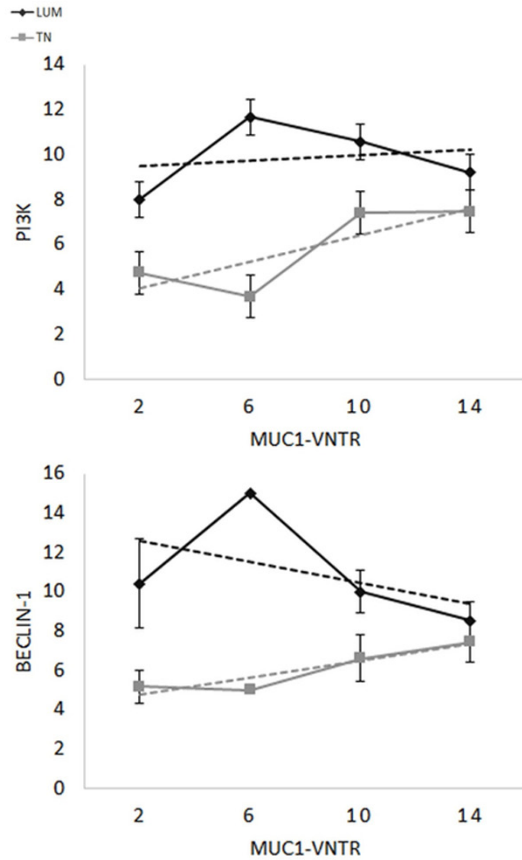

Figure 6. Histological score relationship between MUC1-VNTR and PI3Kp110β for LUM (grey, *P* = 0.04) or TN (black, *P* = 0.009) or Beclin-1 for LUM (grey, *P* = 0.01) or TN (black, *P* = 0.01). Only MUC1-VNTR is correlated with two antigens of autophagy (Beclin-1 and PI3Kp110β) (*P* = ...) in TN and in LUM. This suggests that the extracellular part of MUC1 could be played a role in the autophagy of breast carcinoma.

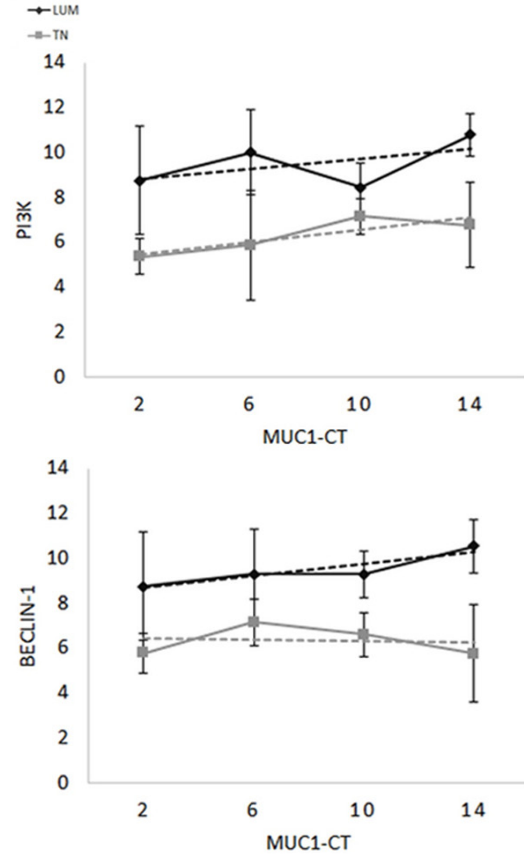

Figure 7. Histological score realtionship between MUC1-CT and PI3Kp110β for LUM (grey, *P* = ns) or TN (black, *P* = 0.04) or Beclin-1 for LUM (grey, P = ns) or TN (black, P = ns). Only MUC1-CT is only correlated PI3K (p110β) in TN (*P* = 0.04). This suggests that the intracellular part of MUC1 is less involved in the autophagy pathway than the extracellular part of MUC1. As illustrated in the **Figure 2**, MUC1 is less modified by the MUC1-VNTR splicing in TN, suggesting that an independent action of the extracellular MUC1-VNTR and the intracellular MUC1-CT.

breast cancers strongly expressed MUC1, 27% showed a weak secretion and 6% were negative [37]. Our study also supports a previous report on MUC1-VNTR expression in a small group of patients (10 cases) [33]. Such variability is of clinical interest. Indeed, we here showed that both MUC1 subunits (MUC1-VNTR and MUC1-CT), and thereof epitopes exposure, are less expressed in TN than in LUM breast cancers.

Lillehoj et al suggested the possibility of a MUC1-EGFR-NEU1 molecular complex [18]. Consequently, we looked at the association between MUC1, EGFR and/or NEU1 in the two groups of breast cancers. However, we did not find any correlation between EGFR and MUC1-VNTR, MUC1-CT or NEU1. We even found that while TN was expressed a high level of EGFR, both MUC1-VNTR and MUC1-CT were down-regulated in TN as compared to LUM breast cancer. These results are discordant with those of Neeraja Dharmanarj *et al* who recently demonstrated a statistical correlation between MUC1 and EGFR and concluded that the activation of EGFR stimulates MUC1 expression in multiple cellular contexts [39], but are in setting with those of others authors who showed that EGFR stimulation promotes the cleavage α-subunit MUC1 [20, 40, 41]. Such discrepancies highlight the importance to define *in situ* the expression of these molecules according to the type of cancer. *In vitro* study on breast cell lines demonstrated that MUC1 and EGFR are associ-

ated in a molecular complex in breast cancers and that MUC1 inhibits EGFR down-regulation and endocytosis [34]. Based on our *in situ* results, we then hypothesize that EGFR overexpression observed in TN is the result of EGFR accumulation in the cytoplasm or cell membrane rather than EGFR-overproduction.

Our results demonstrate that the expression of MUC1-VNTR and MUC1-CT were only correlated in TN, suggesting that the MUC1 is not cleaved yet, and therefore that MUC1 endocytosis is reduced in TN. Accordingly, Wreschner et al demonstrated that full length MUC1 is modified following a limited proteolysis event of its extracellular part (MUC1-VNTR) by the recycling of MUC1 by endocytosis [34, 38]. Subsequently, Crose et al showed that MUC1-CT constitutes a better indicator of MUC1 production than MUC1-VNTR because it does not depend on the MUC1 proteolysis [42]. Therefore, the positive correlation between MUC1-VNTR and MUC1-CT observed in TN, is the indirect reflect of the lack of MUC1 recycling. Reduced recycling of MUC1 in TN therefore bound to a reduced level of MUC1 glycosylation supports the fact that MUC1 epitopes could be better recognized in this type of breast cancer. Furthermore, because MUC1-CT is not or less altered or glycosylated, it constitutes a better indicator of the primary secretion of the MUC1 [42]. This also supports our above mentioned hypothesis that EGFR accumulates in TN.

The high level of EGFR and our hypothesis that MUC1 is not cleaved suggest that EGFR-MUC1 pathway is deregulated in TN. The membrane associated PI3K plays an important role in the EGFR intracytoplasmic signalling. Using conditional gene knockout mice deficient in the class IA PI3K p110α or p110β catalytic subunit, Dou *et al* demonstrated that p110β subunit promotes autophagy by activation of the complex Rab5-Vps34-Vps15-Beclin-1, independently of its kinase activity [26]. This pathway seems to be independent of EGFR stimulating pathway which is associated with the cascade of PI3K p110α/AKT/mTOR, well-known as an inhibitor of autophagy [23, 43]. Our observation of the low expression of both Beclin-1 and PI3Kp110β confirms that the autophagy pathway is reduced in TN breast cancer. In these cancers, the positive correlation between MUC1-VNTR with both PI3K p110β and Beclin-1, strongly advocates for a link between MUC1-VNTR and autophagy [45]. Then, our in situ results support a previous in vitro study showing that MUC1 promotes autophagy in human tumour cells in response to glucose deprivation [27].

Autophagy is an adaptive phenomena widely used by tumour cells using the lysosomial machinery [44]. Debnath *et al* pointed out the important role of autophagy in breast carcinogenesis. Indeed, reduced autophagy can promote tumour development by genomic instability. We found that EGFR was highly expressed in TN. Interestingly, in a series of 107 TN, Tilch *et al* did not identify any mutation of the EGFR gene suggesting that EGFR protein is physiologically normal [45]. In the setting of a reduced level of autophagy in TN breast cancer, it is worth to note that IL17A has been described to attenuate the autophagy process by regulation of PI3K [46]. Recently, in 3 of our patients presenting a TN breast cancer, Cochaud *et al* showed a high production of IL17A, supporting the implication of IL17A as inhibitor of autophagy in TN [47]. Furthermore, EGFR activation in TN surely plays a role in Beclin-1 phosphorylation and, consequently on autophagy suppression. Indeed, Wei *et al* demonstrated that this mechanism could contribute to tumour progression and chemoresistance in lung carcinoma [48]. Another interesting regulator of autophagy is the oncoprotein p53 which is often mutated in TN. Of note, genetically altered p53 was also demonstrated to inhibit autophagy [49-51]. Interestingly, we also observed a high level of p53 in TN (data not show).

Tumour cells are also able to activate autophagy in diverse conditions such as hypoxia, extracellular matrix fragmentation or other metabolic modifications [52]. The recycling of MUC1 through the cytoplasm and the cellular membrane and its cleavage and release in the extracellular matrix are well documented in the literature [34, 38]. Although NEU1 has been recently suggested to be associated with the EGFR-MUC1 membrane complex, we found that NEU1 is less expressed in TN than LUM. Nevertheless, in TN NEU1 was positively correlated with the extracellular MUC1 domain or MUC1-VNTR ($P = 0.04$, $r = 0.25$) but not with the intracellular MUC1 domain (MUC1-CT). Interestingly, NEU1 is a lysosome enzyme that is able to de-sialylate several macromolecules such as MUC1 or EGFR. NEU1 is known as a modulator of cell

receptors, and has been involved in endocytosis and MUC1 regulation [18]. It can activate phagocytosis in macrophages and dendritic cells through de-sialylation of surface receptors [53, 54]. NEU1 is also involved in processing extracellular matrix fragmentation signals such as elastin peptides [55]. Gilmour *et al* showed that matrix metalloproteinase-9 and NEU1 form a complex with EGFR on the cell surface [56]. These observations suggest that the extracellular matrix could play an important role in the engagement of the molecular complex EGFR-NEU1-MUC1 and its associated intracellular signals.

To conclude, we demonstrated that autophagy is reduced in TN breast cancers leading likely to deregulation of the EGFR-MUC1-NEU1 complex and associated cellular pathways. Nevertheless, further studies will be needed to show the colocalizations of EGFR-MUC1-NEU1 and the close regulations of these molecular actors in different type of breast cancers.

Disclosure of conflict of interest

None

Abbreviations

TN, triple-negative breast carcinoma; LUM, luminal type of breast carcinoma; MUC1-VNTR, α-subunit of MUC1; MUC1-CT, β-subunit of MUC1; TMA, tissue micro-array; NEU1, neuraminidase-1; EGFR, human epidermal growth factor receptor type 1; HER2, human epidermal growth factor receptor type 2; ER, Estrogen receptors; PR, progesterone receptors; CK, cytokeratin; PI3K, phosphoinositide 3-kinases.

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