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SHORT COMMUNICATION

The Uncontrolled Sialylation is Related to Chemoresistant Metastatic Breast Cancer

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Abstract Among the scientific communities, there is a convergence of results supporting a direct relationship between dysregulated sialylation and poor prognosis in many human cancers. For this reason, we have retrospectively investigated 169 cases of invasive ductal carcinoma of the breast, coming from female patients aged between 31 and 76 years old. The whole series was subdivided into two prognostic groups: the first group consisted of 138 patients, who showed a post-treatment survival time more than 5 years, while the second group was made up by 31 patients, died within 5 years despite of chemotherapy. All the surgical specimens were fixed in 10 % neutral buffered formalin, paraffin embedded and, then, submitted to routinely haematoxylin/eosin staining and to a further histochemical (Alcian Blue, DDD-Fast Blue B, Mercury Orange), immunohistochemical (ST3GAL5 sialyltransferase, Ki67, c-erbB2, ER, PR) and chemico-elemental characterization. In the 31 cases of breast cancer belonging to the second group, an overexpression of sialomucins and sialyltransferases has been detected. Our results lead us to support that in aggressive chemoresistant breast cancers, the altered expression of sialic acid, due to an uncontrolled sialylation, creates an excessive negative charge on cell membranes, which stimulates repulsion between neoplastic cells and their subsequent access into the blood stream. This event implies an early metastatization and a rapid disease progression with fatal outcome. The early application of Alcian Blue stain on diagnostic biopsies of breast cancer is able to cheaply reveal the sialomucin accumulations, providing for the disease course.

Keywords Breast cancer · Invasive ductal carcinoma · Sialic acid · Sialomucins · ST3GAL5 sialyltransferase · Sialidase (neuraminidase) · Alcian Blue · DDD-Fast Blue B · Mercury Orange · Elemental microanalysis · Sulphur (S)

Introduction

The term ‘sialic acid’ derives from the Greek word *sialon* (*σίαλου*), which means saliva. It is a generic term for the N- or O-substituted derivatives of neuraminic acid, a monosaccharide with a nine-carbon structure [1]. It is also the name for the most common member of this group, that is N-acetylneuraminic acid (Neu5Ac or NANA). Sialylation is the chemical reaction that introduces a sialyl group into a molecule. Sialic acid-rich glycoconjugates (sialoglycoproteins, sialoglycolipids) are widely distributed in animals and, to a lesser extent, in plants and fungi [1]. The sialic acid-rich regions contribute to create a negative charge on the cells’ surfaces [2]. Since water is a polar molecule with partial positive charges on both hydrogen atoms, it is attracted to cell membranes, favoring the fluid uptake. Among the scientific communities, there is a convergence of results supporting a direct proportionality between dysregulated sialylation and poor prognosis in many human cancers [3, 4]. Our interest has been focused on breast cancer, the most common invasive neoplasia in women.

Dedication In memory of Manuela Duzzi, our esteemed laboratory technician, recently passed away due to a chemoresistant metastatic triple-negative breast cancer at only forty-one years old.

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Materials and Methods

Our research group has retrospectively investigated 169 cases of invasive ductal carcinoma of the breast, coming from female patients, aged between 31 and 76 years at the time of diagnosis. The whole series was subdivided into two prognostic groups: the first group consisted of 138 patients, who showed a post-treatment survival time more than 5 years, while the second group was made up by 31 patients, died within 5 years despite of chemotherapy. More in detail, all the surgical specimens were fixed in 10 % neutral buffered formalin, paraffin embedded and, then, submitted to routinely haematoxylin/eosin staining and to a further histochemical (Alcian Blue, DDD-Fast Blue B, Mercury Orange) and immunohistochemical characterization. After deparaffinization, hydration, endogenous peroxidase blocking and heat-induced antigen retrieval, the tissue sections were incubated for 30 min at room temperature with anti-sialyltransferase (ST3GAL5 antibody, prediluted; Novus Biologicals, Littleton, CO, USA), anti-Ki67 (clone MIB-1, 1:75; Dako, Glostrup, Denmark), anti-cerbB2 (HercepTest; Dako), anti-estrogen receptor (ER, clone SP1, prediluted; Ventana, Tucson, AZ, USA) and anti-progesterone receptor (PR, clone 1E2, prediluted; Ventana). Biotinylated secondary antibody was applied and the staining product detected with avidin-biotin complex (ABC) against a hematoxylin counterstain. Detection of the staining reaction was achieved by an enzyme conjugated polymer complex adapted for automatic stainers from Roche Ventana Medical Systems, with 3–3′ diaminobenzidine tetrahydrochloride (DAB) as chromogen. The validation of the histochemical stains and immunohistochemical reactions was obtained by using normal breast tissue, coming from reduction mammoplasty. Finally, 20-μm-thick sections were prepared from representative formalin-fixed and paraffin-embedded blocks and used for elemental microanalysis according to Gatti and Montanari [5]. These paraffin sections were deposited on an acetate sheet, deparaffined with xylol and mounted on an aluminium stub. Thereafter, they were inserted in the chamber of a field emission gun - environmental scanning electron microscope (FEG-ESEM Quanta 250, FEI Company, Eindhoven, the Netherlands) equipped with an energy dispersive system (EDS, EDAX, Mahwah, NJ, USA), in order to obtain a chemico-elemental characterization of the neoplastic tissue.

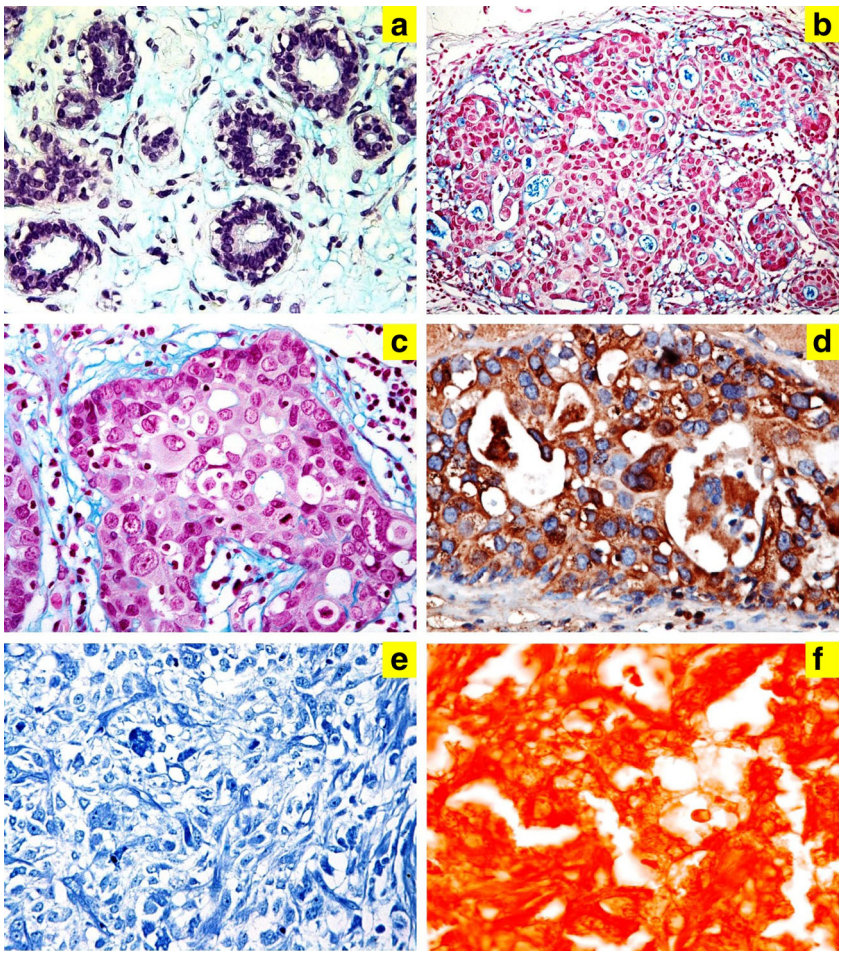

Fig. 1 In a normal breast, neutral mucins are present, while acid mucins (sialomucins) are almost absent (**a** Alcian Blue staining, pH 2.5; ×40). In chemoresistant metastatic breast cancers, conspicuous collections of blue-stained sialomucins are observable inside the neoplastic ducts (**b** Alcian Blue staining, pH 2.5; ×20). These collections disappear after a pre-treatment with sialidase (**c** Alcian Blue staining, pH 2.5; ×40). The immunohistochemistry for sialyltransferase shows its overexpression only in the aforementioned aggressive cases (**d** ST3GAL5 antibody, Novus Biologicals, Littleton, CO, USA; ×40). The accumulation of sialoglycoconjugates is confirmed by both DDD-Fast Blue B (**e**; ×40) and Mercury Orange (**f**; ×40) staining methods

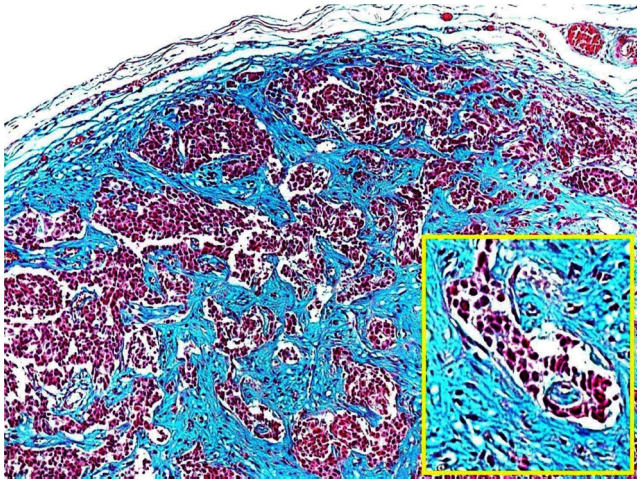

Fig. 2 In a metastatic axillary lymph node from aggressive chemoresistant breast cancer, large amounts of blue-stained sialomucins are well noticeable inside (insert) and around the ductal structures (Alcian Blue staining, pH 2.5; ×4)

Results

In a normal breast, neutral mucins are present, while acid mucins (sialomucins) are absent or present as traces of stain inside the ductal spaces (Fig. 1a). In the 31 cases of breast cancer belonging to the second group, an overexpression of sialomucins and sialyltransferases has been detected. More in detail, in chemoresistant metastatic breast cancers, extensive accumulations of blue-stained sialomucins are well noticeable inside (Fig. 1b) and around (Fig. 2) the ductal spaces. This finding can be referred as malignant alcianophil hypersecretion. The ductal sialomucins disappear after a pre-treatment with sialidase (Fig. 1c), which selectively cleaves the glycosidic linkages of sialic acids. The immunohistochemistry for sialyltransferase reveals its overexpression only in the above mentioned aggressive cases (Fig. 1d). The accumulation of glycoconjugates rich in sialyl groups is further confirmed by both DDD-Fast Blue B (Fig. 1e) and Mercury Orange (Fig. 1f) staining methods for thiols and by elemental microanalysis, which reveals a Sulphur (S) peak (Fig. 3). For the 31 cases belonging to the second prognostic group, tumour subtype with histological grade, estrogen and progesterone receptor expression, Ki-67 labelling index (LI), HercepTest score, pTNM status with specification for metastatic site and chemotherapy regimen have been provided (Table 1). All the above mentioned 31 aggressive cases are no special type (NST) ductal carcinoma, in clear prevalence (80 %) with a high histologic grade (G3), according to the Elston and Ellis' criteria [6]; 4 cases of the series are triple negative breast cancers (anti-ER negativity; anti-PR negativity; anti-cerbB2 negativity). The most frequent pT class is pT2 (48 %), followed by pT1c (39 %). In the 71 % of cases, lymph node metastasization has been detected (N1: 45 %; N2: 10 %); moreover, in the 16 % of cases a massive lymphatic involvement has been ascertained, with more than 10 metastatic axillary lymph nodes (N3) at the onset. The mean Ki-67 LI amounts to 41 %, while the preferential metastatic sites are, in order of frequency, liver, lung, bone and brain.

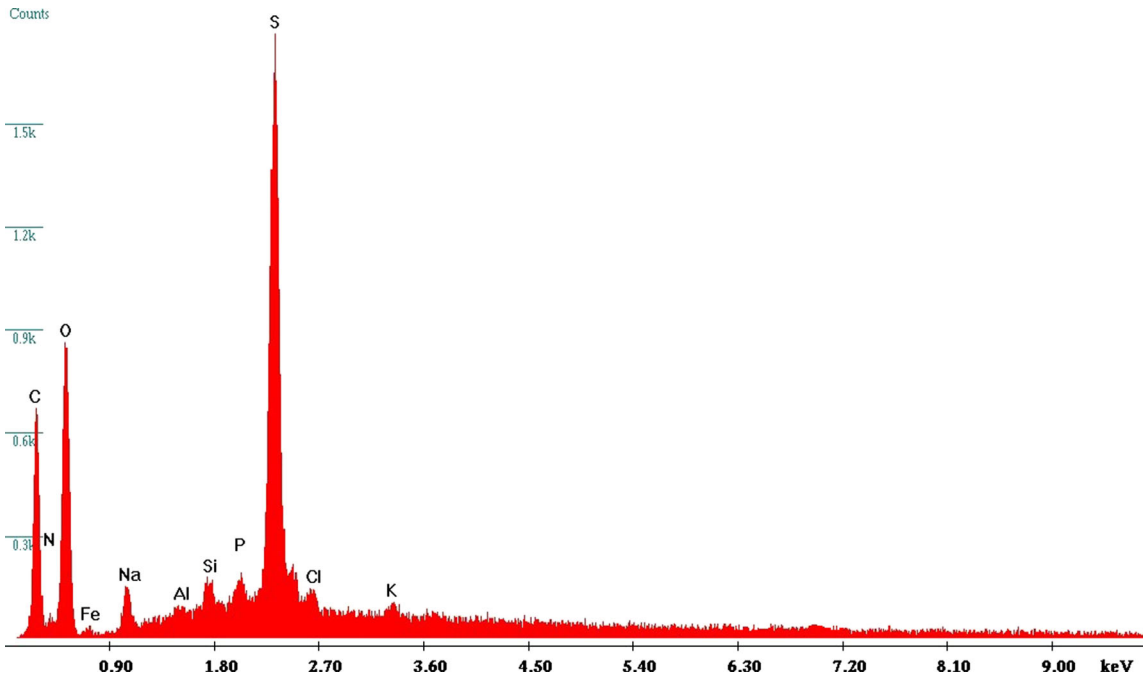

Fig. 3 An EDS spectrum, captured from a neoplastic area of sialomucin collection, shows a well-noticeable Sulphur (S) peak in a background of various elemental composition (X axis = keV; Y axis = counts)

Table 1 Patient age at the time of diagnosis, tumor subtype with histological grade, estrogen and progesterone receptor expression, Ki-67 labelling index, HercepTest score, pTN status with specification for metastatic site and chemotherapy regimen of the 31 aggressive cases, belonging to the second prognostic group (survival time ≤ 5 years)

Case number	Age (years)	Tumor subtype (g)	Er and pr expression (%)	Ki67 li (%)	Herceptest score	Ptn status	Metastatic site	Chemotherapy regimen
1	55	ductal NST (G2)	100 and 10	20	1+	T1cN0	liver	C + F + M, L
2	40	ductal NST (G3)	90 and 90	16	0	T2 N3	liver	Cp + P, B, D, Ta, Fu
3	57	ductal NST (G3)	99 and 20	40	2+	T2 N3	lung	Cp + P, B, D, L, Tr
4	50	ductal NST (G3)	95 and 80	35	0	T2 N3	liver	Cp + P, B, D, Ta, Fu
5	49	ductal NST (G2)	70 and 50	40	0	T1bN0	lung	C + E + F, L
6	56	ductal NST (G3)	0 and 0	70	0	T2 N0	lung	C + F + M, C + E + F, Cp + P, V
7	52	ductal NST (G2)	100 and 70	10	1+	T1cN1	bone, liver, brain	C + F + M, Ta, Fu, Z
8	66	ductal NST (G3)	0 and 0	30	0	T1cN1	bone, liver	C + F + M, C + E + F, Cp + P, V, Z
9	59	ductal NST (G3)	95 and 0	30	3+	T1cN2	liver	C + E + F, L, Tr
10	57	ductal NST (G2)	80 and 0	40	0	T1bN0	liver	C + F + M, L
11	50	ductal NST (G3)	90 and 0	80	1+	T2 N3	liver	Cp + P, B, D, L
12	54	ductal NST (G3)	80 and 0	15	1+	T1bN0	liver	C + E + F, L
13	52	ductal NST (G3)	0 and 0	30	0	T1cN0	bone	C + F + M, C + E + F, Cp + P, V, Z
14	31	ductal NST (G2)	80 and 0	70	0	T2 N1	liver	C + F + M, L
15	32	ductal NST (G3)	90 and 0	50	0	T2 N3	liver	Cp + P, B, D, L
16	47	ductal NST (G3)	0 and 0	60	3+	T2 N1	liver	Cp + P, B, La
17	46	ductal NST (G3)	60 and 25	70	3+	T2 N1	lung	C + E + F, L, Tr
18	58	ductal NST (G3)	100 and 100	20	3+	T1cN1	liver	C + F + M, Ta, La, Fu
19	64	ductal NST (G3)	100 and 25	20	2+	T1bN1	liver	C + F + M, Ta, Tr, Fu
20	48	ductal NST (G2)	95 and 20	25	3+	T2 N1	liver	C + E + F, L, La
21	66	ductal NST (G3)	0 and 0	60	1+	T2 N1	lung	C + F + M, C + E + F, Cp + P, V
22	50	ductal NST (G3)	95 and 95	30	1+	T2 N0	liver	C + E + F, Ta, Fu
23	75	ductal NST (G3)	95 and 90	30	2+	T1cN0	liver	C + F + M, Ta, Tr, Fu
24	76	ductal NST (G3)	100 and 100	40	0	T2 N1	bone	C + E + F, Ta, Z
25	45	ductal NST (G3)	99 and 99	80	2+	T1cN1	liver	C + F + M, Ta, La, Fu
26	68	ductal NST (G3)	0 and 0	60	1+	T2 N1	lung	C + F + M, C + E + F, Cp + P, V
27	76	ductal NST (G3)	100 and 100	40	0	T2 N1	bone	C + E + F, Ta, Fu, Z
28	60	ductal NST (G3)	95 and 2	25	3+	T1cN2	brain, liver	C + F + M, L, Tr
29	75	ductal NST (G3)	95 and 90	30	2+	T1cN0	liver	C + E + F, Ta, La, Fu
30	56	ductal NST (G3)	90 and 5	35	0	T1cN1	lung, brain	C + F + M, L
31	39	ductal NST (G3)	0 and 0	80	0	T1cN2	bone	C + F + M, C + E + F, Cp + P, V, Z

B: bevacizumab; Cp: carboplatin; C: cyclophosphamide; E: epirubicin; D: docetaxel; ER: estrogen receptor; F: fluorouracil; Fu: fulvestrant; G: histologic grade; L: letrozole; La: lapatinib; LI: labeling index; M: methotrexate; NST: no special type; P: paclitaxel; PR: progesterone receptor; pTN: pathological tumor-node staging; Ta: tamoxifen; Tr: trastuzumab; V: vinorelbine; Z: zoledronate

Discussion

The prognostic variability recorded within homogeneous groups of women for disease stages has lately led to a more detailed characterization of breast cancer. The concepts of tumor histotype, histological grade, vascular invasion and tumor-infiltrating lymphocytes have been introduced and new prognostic indicators have been revealed, such as hormone receptor status (ER, PR), proliferative activity (Ki67), gene expression (ERBB2), with important repercussions in the choice of the therapeutic strategy (e.g. hormone blocking therapy, targeted therapy). Our findings led us to support that in aggressive chemoresistant breast cancers, the altered expression of sialic acid [7–9], due to an uncontrolled sialylation, creates an excessive negative charge on cell membranes, which stimulates repulsion between neoplastic cells and their subsequent access into the blood stream. This event implies an early metastatization and a rapid disease progression with fatal outcome, requiring a new targeted therapeutic approach.

Conclusion

The application of Alcian Blue stain on diagnostic biopsies of breast cancer is able to cheaply reveal the sialomucin accumulations, providing for the disease course. In the near future, the implementation of a laboratory technique for the serum detection and quantification of sialyltransferase, as tumor marker for diagnosis and follow-up, is advocated.

Compliance with Ethical Standards

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Conflict of Interest The authors declare no conflict of interest.

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