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DOUBLE STAINING GLYCOHISTOCHEMICAL ANALYSIS OF TUMOUR ASSOCIATED GLYCOCONJUGATES IN HUMAN MAMMARY CARCINOMA

PRATHAP.P*, JAGANNATHAN.N[1] AND SUKUMARAN.N[2]

*[1] *VELS University, Department of Biotechnology, School of Life Sciences, Chennai, Tamilnadu, INDIA, Pincode:600117.*
[2] *Joint Director, ABC Technolabs, Anna Nagar West, Chennai, Tamil Nadu, INDIA. Pincode: 600 040*

ABSTRACT

One of the most common cancer death in the world id due to Cancer of the Breast. It should be noted breast cancer can occur irrespective of sex, but predominantly in women. In order to demonstrate the expression of cancer associated carbohydrate antigens representing the first steps in glycosylation, different glyco histochemical methods viz. Carbohydrate staining were employed in addition to the conventional Haematoxylin and Eosin staining, to recognize the relation of the glycocalyx in infiltrating mammary adenocarcinoma tissue. Glycoconjugates are macromolecules generally carbohydrates with a protein/lipid component. Double staining glycohistochemical analysis of tumour associated glycoconjugates in human mammary carcinoma was performed based on the above methods to highlight the expression of the glycans or glycoconjugates, different staining techniques were employed on infiltrating ductal adenocarcinoma section of the breast. A total nine cases of infiltration ductal carcinoma cases were studied. This study reveals the localization of various tumor glycoconjugates the aberrant structures in most of the different mammary cancer tissues using PAS staining and Alcan Blue- PAS staining.

KEYWORDS: **Cancer, Breast, Staining, Histochemical, Immunohistochemical, Glycoconjugates, Mammary, Carcinoma.**

PRATHAP.P
VELS University, Department of Biotechnology, School of Life Sciences, Chennai, Tamilnadu, INDIA, Pincode:600117.

***Corresponding author**

INTRODUCTION

Breast cancer is the most common malignancy in women and the second leading cause of cancer death[1]. The incidence of breast cancer has been increasing steadily from an incidence of 1:20 in early 1970's to 1:5 in 1990's. Further the incidence is very low in the twenties gradually increases and plateaus at the age of forty-five and increases dramatically after fifty. Both women and men can develop breast cancer, but it is very rare in men.[2]. Breast cancer is most often spread to the bones, lungs, brain, and liver, but any tissue can be affected.[3] Infiltrating ductal carcinoma is the commonest form and is seen in middle-aged or elderly women. Owing to an abundance of fibrous tissue the lump feels very hard, while its contour tends to be irregular.[4] Whilst most histology is performed on tissue that is obtained by surgical biopsy, in certain circumstances it is possible to achieve excellent classification from cytology alone.[5] Both frozen and routine microscopic sections are traditionally stained with a combination of the dyes eosin and haematoxylin which color the cell nuclei blue and the cytoplasm pink. Most cells have a reproducible staining pattern, regardless of the tissue. For example, cell nuclei usually are deeply blue or basophilic, preferentially stained with hematoxylin. Squamous cells, such as those cells comprised in the skin, are usually red or eosinophilic.[6] Additional staining methods, focused on specific cellular elements, are used when indicated. "Immunohistochemical" stains utilize specific antibodies to cellular constituents. "Special" stains utilize chemical reactions to impart different colors to molecules of interest.[7] Special stains are staining methods that use dyes or chemical reactions to demonstrate tissue elements such as carbohydrates, proteins, connective tissue, muscle, microorganisms, pigments, minerals, and cytoplasmic granules. Selective demonstration is achieved based on the chemical properties of the target.[8] Alcian blue technique facilitates recognition of complete and incomplete variants of metaplasia.[9] Periodic acid Schiff with Haematoxylin is appropriate for representation of mucoploysaccharides and fungi in tissue. Glycogen, mucin and some basement films are stained red/purple and the background blue.[10] Glycoconjugates are macromolecules generally carbohydrates with a protein/lipid component. Their appearance and changes in blood plasma concentration or other body fluids and related to and correlated to the presence or progress of neoplasms.[11] The carbohydrate structures change dramatically during development and specific sets of carbohydrate structures and characteristic of different stages of differentiation and very often these carbohydrates are recognized by specific antibodies, thus providing differentiation antigens. In the nature organism, expression of distinct carbohydrates is eventually restricted to specific cell types. The aim of the present study is to analyze the tumour glycoconjugate expression using various histochemical staining procedures in combination with carbohydrate stains in different grades of cancers including various stages of mammary cancers. This work has been divided into three phases. Phase I - depicts the preparation of the routine haematoxylin and eosin stain and testing the various cancer tissue samples, diagnosed for human infiltrating ductal adenocarcinoma of the breast cancer. Phase II- depicts the preparation of the special glycohistochemical stain Periodic acid Schiff's in combination with Haematoxylin stain and testing the various cancer tissue samples, diagnosed for human infiltrating ductal adenocarcinoma of the breast cancer. Phase III - depicts the preparation of the special glycohistochemical stain Alcian Blue pH 2.5 which reveals the acidic mucosubstances. This Alcian blue pH 2.5 stain combination Periodic acid Schiff's is tested in various cancer tissue samples, diagnosed for human infiltrating ductal adenocarcinoma of the breast cancer.

MATERIALS AND METHODS

General laboratory and aseptic techniques were followed as described by Dodds and Roberts.[12] Aseptic techniques were carried out in a laminar airflow chamber equipped with UV germicidal lamps. Autoclaving of glassware was performed at 120^0C and 15 lbs pressure. Clean glassware of borosil brand was used. They were washed with detergents in running tap water then rinsed thoroughly with distilled water and autoclaved. Pre-sterilized plastic wares purchased from Tarsons (India) were used. Grade Biochemical's were used for the entire experiments. Laboratory grade distilled water was purchased and redistilled in glass distillation unit checked for neutral pH was used for the preparation of reagents.

Tissue Source

A total nine cases of infiltration ductal carcinoma tissues were retrieved from the Histopathology department of Dr.Kamakshi Memorial Hospital, Pallikaranai after obtaining ethical clearance from institutional ethics committee. They were routinely fixed in 10% Phosphate buffered Formalin, processed in various percentages of alcohols in their increasing strengths', cleared in xylene and finally embedded in paraffin wax. The pathological details of mammary carcinomas were provided by the above pathology department chief after classification with respect to topographic origin and histologic differentiation. Carcinomas with marked ductal infiltration were classified as infiltrating ductal carcinoma, according to the classification criteria of the World Health Organization. Paraffin blocks of the fixed tissues were cut into 4-5 um thick sections using a rotary microtome and analyzed with various general and carbohydrate stains.

Phase: 1.Haematoxylin and Eosin staining

The breast tissue sections were initially warmed to melt the raw wax and then washed in xylene to remove them from adhering to the slide. Then the sections were deparaffinized in xylene and hydrated in decreasing strengths of alcohol and brought to Phosphate buffered saline. The sections were stained using haematoxylin for 2.5 minutes and washed with phosphate buffered saline to remove excess stain adhering to it. Such excess stained sections can be easily differentiated by a dip in 1% acid alcohol and then washed in phosphate buffered saline. If the washing in distilled water doesn't turn the section blue, then the sections can be treated in sodium carbonate solution for 30 seconds and rinsed in phosphate buffered saline. Then the tissue sections or

the slide are immersed in eosin stain for 30 seconds or less & washed in phosphate buffered saline. The tissue slides were finally dehydrated in increasing strengths of alcohols and cleared in xylene and mounted using DPX or any resinous medium.

Phase: 2 Haematoxylin and Periodic acid schif's staining

The breast tissue sections were initially warmed to melt the raw wax and then washed in xylene to remove them from adhering to the slide. Then the sections were deparaffinized in xylene and hydrated in decreasing strengths of alcohol and brought to Phosphate buffered saline. The sections were stained Meta Periodate for 10 minutes and washed with phosphate buffered saline to remove excess stain adhering to it. Then the sections were placed in Schiff's reagent for 15 minutes where the sections turn light pink in color. Wash in lukewarm water for 5 minutes (Immediately sections turn dark pink color). Then the sections were treated with Meta bi sulphite for 10 minutes and washed with phosphate buffered saline. Sections were counter stained in Mayer's haematoxylin solution for a minute and washed in phosphate buffered saline for 5 minutes. The slides were finally dehydrated and covered with coverslip synthetic mounting medium such as DPX.[13]

Phase: 3 Haematoxylin and Alician blue staining

The tissue sections were initially warmed to melt the raw wax and then washed in xylene to remove them from adhering to the slide. Then the sections were deparaffinized in xylene and hydrated in decreasing strengths of alcohol and brought to Phosphate buffered saline. The sections were stained in Alcian blue solution for 15 minutes and washed with phosphate buffered saline for 2 minute. The sections were treated with Sodium Meta Periodate for 10 minutes and washed with phosphate buffered saline to remove excess periodate adhering to it. Then the sections were placed in Schiff's reagent for 15 minutes, where the sections turn light pink in color. Wash in lukewarm water for 5 minutes (Immediately sections turn dark pink color). Then the sections were treated with 0.55 Meta bi sulphite for 10 minutes and washed with phosphate buffered saline. Section were counter stained in Mayer's haematoxylin solution for a minute and washed in phosphate buffered saline for 5 minutes. The slides were finally dehydrated and covered with coverslip using a synthetic mounting medium such as DPX.[9]

RESULTS AND DISCUSSION

A total nine cases of infiltration ductal carcinoma cases were studied and the microscopic descriptions are as follows. These microscopy reports were reported by the concerned Pathologists. Case # 1, Multiple sections from the lumpectomy site shows granulation tissue, Lobules of adiposities and presence of small clusters of neoplastic cells with Pleomorphic vesicular nucleus. Such neoplastic cells are also seen to infiltrate into the surrounding fatty tissue. Sections from two of the eight lymph nodes, show evidence of secondary metastatic deposits of similar tumour cells. Sections from remaining six lymph nodes show features of reactive hyperplasic. Case # 2, Multiple sections studied shows a malignant neoplasm disposed off in small sheets clusters and glandular pattern. The neoplastic cells are round to polygonal with plemorphic vesicular nucleus. Also seen are neoplastic cells arranged in small clusters and trabeculae pattern. Breast ducts show features of ductal carcinoma insitu. Case # 3, Cellular smear showing many decisive clusters of ductal epithelial cells exhibiting marked cellular pleomorphism with nuclear hyperchromatism. Similar neoplastic cells are also seen in singles. Back ground show haemorrhage and inflammatory cells. Case # 4, Multiple sections studied show a malignant neoplasm disposed off in small sheets clusters and glandular pattern. Sections from one of the three lymph node shows presence of small clusters of malignant ductal epithelial cells in the subcapsular space. Case # 5, Multiple sections studies shows a malignant neoplasm disposed off in small sheets clusters, separated by fibrocollagenous septa. The neoplastic cells are round to polygonal with pleomorphic vesicular nucleus. Increased mitosis seen. Sections shows features of ductal carcinoma insitu (Comedo, solid and cribriform pattern).Case # 6, sections studied show a malignant neoplasm disposed off in small sheets clusters, also infiltrating in adjacent fatty tissue. The neoplastic cells are round to polygonal with pleomorphic hyper chromatic nucleus and moderate cytoplasm. Increased mitosis was seen. Case # 7, reveals many decisive clusters of ductal epithelial cells exhibiting marked cellular pleomorphism and nuclear hyperchromatism. Such neoplastic cells are also seen in singles. Background shows haemorrage and inflammatory cells. Case # 8, multiple sections show granulation tissue, Lobules of adiposities and presence of small clusters of neoplastic cells with Pleomorphic vesicular nucleus. The neoplastic cells are round to polygonal with pleomorphic hyper chromatic nucleus and moderate cytoplasm. Case # 9, reveals many decisive clusters of ductal epithelial cells exhibiting marked cellular pleomorphism and nuclear hyperchromatism. The neoplastic cells are round to polygonal with pleomorphic vesicular nucleus. Increased mitosis seen. Section shows features of ductal carcinoma insitu. To delineate the expression of the tumor associated carbohydrates, different glycohistochemical techniques, were employed on a chosen malignant specimen of breast adenocarcinoma to identify the disease progression and understand the total picture of the glycocalyx in mammary tissues as summarized in table-1. The histochemistry of carbohydrates has undergone considerable changes in the last two decades, particularly with regard to the mucins in tumour diagnosis. In order to demonstrate the expression of cancer associated carbohydrate antigens representing the first steps in glycosylation, different glyco histochemical methods viz. Carbohydrate staining were employed in addition to the conventional Haematoxylin and Eosin staining, to recognize the relation of the glycocalyx in a infiltrating mammary adenocarcinoma tissue.

Table 1
Summary of histochemical staining observations on human breast adenocarcinomas

		Routine Staining	Glyco histochemical staining	
S.NO	Slide Details*	H&E	PAS	AB-PAS
1	2016/1728	+++	+++	++
2	2016/1776	+++	++	++
3	2016/1825	+++	+++	+++
4	2016/1845	+++	++	++
5	2016/1855	++	++	-
6	2016/1896	++	+++	++
7	2016/1256	+++	+	++
8	2016/1274	++	++	++
9	2016/1357	+++	+++	+

**General Reference for research purpose*
#-Scoring of histochemical staining was done by two individual evaluators as follows: - none, +weak, ++moderate, +++ good staining.

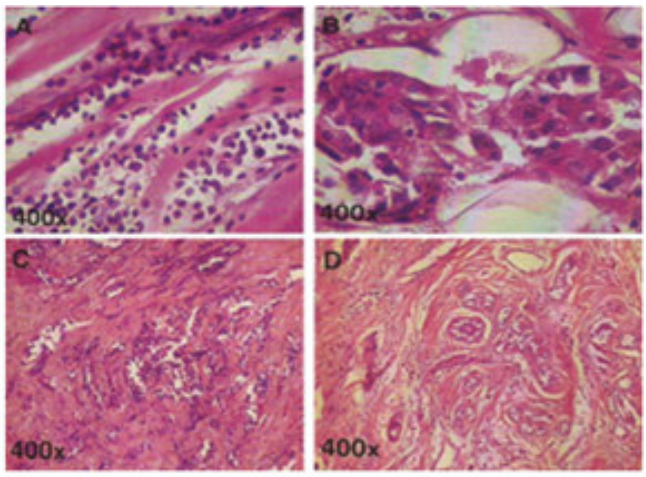

Figure 1
Haematoxylin and Eosin Staining of Human mammary Adenocarcinoma
Slides A-D represents cases from infiltrating ductal carcinomas; H & E staining reveals intense nuclei staining, with a moderate to strong staining in the cytoplasmic areas. All Photographs were photographed at 400X magnification

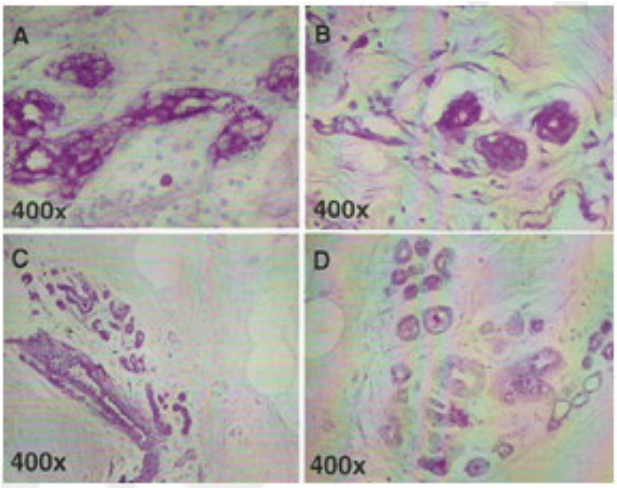

Figure 2
Glycoprotein Staining of Visualised By PAS Technique
Slides A-D represents cases from infiltrating ductal carcinomas; Periodic acid's Schiff staining reveals very strong staining of cell membranes localizing neutral carbohydrate expression. All Photographs were photographed at 400X magnification

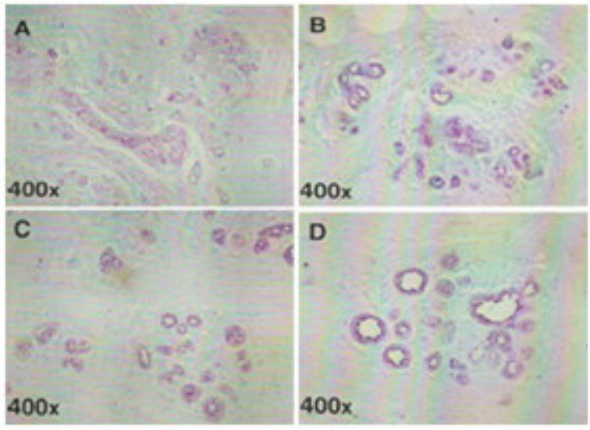

Figure 3
Glycoprotein Staining of Visualised By Alcian Blue-PAS Technique
Slides A-D represents cases from infiltrating ductal carcinomas; Alcian Periodic acid's Schiff staining reveals weak to moderate staining of cell membranes localizing acidic and neutral carbohydrate respectively. All Photographs were photographed at 400X magnification

Double staining glycohistochemical analysis of tumour associated glycoconjugates in human mammary carcinoma was performed based on the above methods to highlight the expression of the glycans or glycoconugates, different staining techniques were employed on infiltrating ductal adenocarcinoma section of the breast. The common stain haematoxyline and eosin was used in combination to differentiate the nucleus and cytoplasm in the different types of cells of the tissues. Mucins and other glycosylated proteins in the extracellular matrix pick up the eosin very weakly in the haematoxylin and eosin staining. Images from A-E in figure:1 represents cases from infiltrating ductal adenocarcinoma of the breast. H&E staining reveals intense nuclear staining, with a moderate to strong staining in the cytoplasmic areas. All the photographs were captured at 400X magnification. In the case of the most severe form of infiltrating carcinoma both the H&E and Schiff' stain shows deep staining in the multi layered gland cells with hyperchromatic and pleomorphic nuclei and luminal tufting of the cellular outlines. Mitosis seem to be a frequent event. Figure:2 represents periodic acid Schiff's staining of infiltrating ductal adenocarcinoma of the human breast reveals very strong staining of the cell membranes localizing neutral carbohydrate expression.

Glycohistochemical staining for the detection of mucins-PAS staining and other glycoproteins were homogenous in localization with respect to the glycoproteins and other mucins bearing tumor marker structures as seen in almost all cases of the infiltrating ductal adenocarcinoma. Therefore this study reveals the localization of various tumor glycoconjugates the aberrant structures's in the most of the different mammary cancer tissues using PAS staining and Alcian Blue- PAS staining (Fig-3). Cellular glycoconjugates are known to be modified with the development, differentiation and maturation of cells. [14,15,16]. Glycoconjugates have been used extensively to study alterations in cell surface carbohydrates associated with malignant transformation In parallel with the studies of Richard[17], Aguirre[18], Dall Pai[19], and Gorgees & Rashan[20],we also clearly noticed that various staining reveals at least three different types of glycoconjugates intermingled in the same cell. As reported by Herlant-Meewis[21] this can be consistent with the fact that the neutral mucous material converts into acidic mucous material. The study of Fukotomi, 1991[22] and colleagues showed that there is a good association between gene amplification and carbohydrate structure in breast cancer cells. Dall Pai[19], showed the presence of small amounts of acidic mucopolysaccharides and neutral polysaccharides. Accordingly, we observed variable degrees of reactions in histochemical staining patterns of acidic mucopolysaccharides and neutral polysaccharides present in mammary cancer tissues.

CONCLUSION

The histochemistry of carbohydrates has undergone considerable changes in the last two decades, particularly with regard to the mucins in tumour diagnosis. In order to demonstrate the expression of cancer associated carbohydrate antigens representing the first steps in glycosylation, different glyco histochemical methods viz. Carbohydrate staining were employed in addition to the conventional Haematoxylin and Eosin staining, to recognize the relation of the glycocalyx in a infiltrating mammary adenocarcinoma tissue.

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CONFLICT OF INTEREST

Conflict of Interest declared none.

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