Lectin Histochemistry of Murine WAP-T Mammary Cancer Reveals Similar Glycoconjugate Changes to Those in Human Breast Cancer

STEFFEN SCHREIBER[1], ANDREAS GOCHT[1,2], FLORIAN WEGWITZ[3], WOLFGANG DEPPERT[3] and UDO SCHUMACHER[1]

Institutes of [1]*Anatomy and Experimental Morphology, and* [3]*Tumor Biology, University Cancer Center, University Medical Center Hamburg-Eppendorf, Hamburg, Germany;* [2]*Joint Practice of Pathology, Lübeck, Germany*

Abstract. *Background: The WAP-T mouse model is an established clinically relevant model of breast cancer. Lectins have been used to study malignant progression in clinical studies. We investigated lectin binding sites to test for the clinical relevance of this model. Materials and Methods: Samples of the WAP-T mouse mammary tissues, from normal tissues to undifferentiated higher tumor grades were stained using an indirect technique with nine different lectins for intensity of lectin binding. Results: HPA bound to the luminal epithelium in higher tumor grades in a similar pattern to that in human breast cancer. BSA-IB4 bound to luminal epithelium in hyperplasia and increased towards higher grades, comparable to previous clinical studies. PHA-L-binding to myoepithelium and luminal epithelium increased from hyperplasia to higher grades, comparable to findings in human breast cancer. Conclusion: The results of our study support the hypothesis that lectin binding sites change similarly in WAP-T and human breast cancer, stressing the similarity of this model with the clinical setting.*

Breast cancer is the leading cause of cancer-associated death in women in the Western world. However no real progress has been made in reducing the breast cancer burden over the past decades (1). One of the major problems in breast cancer research has been the lack of suitable experimental models equivalent to the human situation to study malignant progression. For closely mimicking the pathophysiology of human breast cancer, xenograft models have served as a useful tool in breast cancer research. However, they have a major disadvantage over syngenic models of breast cancer as these xenograft mouse models by nature lack a specific immune response. As naturally-occuring mammary cancer in mice is often induced by the mammary tumor virus, this model also cannot serve as a good clinical model of breast cancer in humans as no viral agent is implied in the pathogenesis of human breast cancer (2). To overcome these two problems in generating clinically relevant models of mammary cancer in mice, in previous experimental designs, a viral oncogene was introduced as a transgene, driven by a tissue-specific promoter into the germline of mice (3, 4). Several transgenic mouse models have been established using this approach (5-8). Schulze-Garg *et al.* established a transgenic mouse model which introduced the early genome region of the simian virus 40 (SV40), driven by whey acidic protein promoters (WAP) into different mouse lines (9). The WAP promoter is regulated by lactotrophic hormones (10, 11) and can be induced by mating. The transgene expression is detectable in the epithelial cells (TDLU) (12) of the lactating gland of mice (13). The neoplastic potential of SV40 is based on the large tumor antigen (T-Ag), which inactivates the two tumor-suppressor proteins phosphorylated retinoblastoma protein (pRB) and protein 53 (p53), and on small T-antigen (ST), which inactivates protein phosphatase 2 (PP2A, catalytic subunit, alpha isoform), thereby activating the Wingless related integration site pathway (Wnt-pathway). This often reflects the molecular changes observed in human tumors, in which the pRb checkpoint is usually inactivated (14). This transgenic mouse model has been shown to be useful for the study of oncogene-induced carcinogenesis in the mammary gland of adult mice (15-17), as it closely reflects tumors commonly present in humans (9, 18, 19).

In addition to classical prognostic markers used in clinical medicine, including histological subtype, grade of differentiation of the primary tumor (20), hormone and

Correspondence to: Steffen Schreiber, Institute of Anatomy and Experimental Morphology, University Cancer Center, University Medical Center Hamburg-Eppendorf, Martinistraße 52, D-20246 Hamburg, Germany. Tel: +49 40741052586, Fax: +49 40741055427, e-mail: steffen_schreiber72@yahoo.com

growth factor receptor status (21, 22), lectins (23), which are carbohydrate-binding proteins, have been employed as a prognostic tool in breast cancer (24-27). In particular, the lectin *Helix pomatia* agglutinin (HPA) has served in clinical (24, 25, 28) as well as in xenograft models of spontaneous mammary cancer metastasis as a useful prognostic marker (29-33).

The aim of the present study was to analyze the lectin binding pattern in the well-established WAP-T mouse mammary model and to compare these results with previous studies of lectin binding in human breast cancer.

Materials and Methods

Mice. All mice were housed under specific pathogen-free conditions in accordance with offical regulations for the care and use of laboratory animals (UKCCCR Guidelines for the welfare of Animals in Experimental Neoplasia) and approved by Hamburg's Authority for Health (no. 88/06) (34). The animals had been bred in in the Heinrich Pette Institute of Experimental Virology, Hamburg, Germany (9).

Mammary fat pads were excised from transgenic mice of the strains WAP-T-T1 and WAP-T-NP8 at necropsy (size: average 28,96g, standard deviation 3,37 age: average 255 days, standard deviation 56,8). The tissues were formalin fixed and paraffin embedded and were drawn from the archives of the Heinrich Pette Institute.

The temporal development of mammary neoplastic lesions were classified beginning with gland hyperplasia (HP), followed by dysplasia and ductal carcinoma *in situ* (G0), ending with manifested invasive carcinoma (15, 18) (G1-G4) (Table I). Notably, all ductal carcinoma *in situ* in the WAP-T mice were high-grade lesions, assessed by their nuclear anaplasia. Invasive carcinoma exhibited different grades of histological differentiation, comparable to the findings in human breast cancer, in which a three-tiered system for histological grading of invasive carcinomas is applied, *i.e.* well-, moderately, and poorly-differentiated, depending on tubule formation, nuclear pleomophism and mitotic counts. In invasive carcinomas of the WAP-T transgenic mice, only the degree of glandular formation (tubular and papillary growth patterns) defines the histological grades, as described previously (9), since all these carcinomas present with high nuclear pleomorphism and high mitotic activity.

Histology and histochemistry. Sections 5-μm-thick were applied to Adhesion Micro Slides (Histo Bond®; Marienfeld GmbH, Lauda-Königshofen Germany). Slides were then de-paraffinized in xylene and rehydrated through a series of graded ethanols to distilled water. One section was stained with hematoxylin and eosin according to standard procedures.

Lectin histochemistry. Lectin histochemistry was performed using an indirect technique with biotinylated lectins (see Table II). All lectins were obainted either from Sigma-Aldrich-Chemie (Steinheim, Germany) or Vector Laboratories Inc. (Burlingame, CA, USA).

Staining for each lectin was performed in one batch to avoid interbatch variability. Deparaffinized sections were briefly incubated in lectin buffer (LB) consisting of tris-buffered saline (TBS contained Trizma Base, 50 mM Tris; Sigma, Steinheim, Germany), sodium chloride (150 mM; J.T. Baker, Deventer, the Netherlands) and hydrochloric acid (Merck, Darmstadt, Germany) in distilled water (adjusted to pH 7.6) with addition of 1% $MgCl_2$ and 1% $CaCl_2$ (Merck, Darmstadt Germany).

Sections were then treated with 0.1% trypsin (Biochrom KG, Berlin, Germany) dissolved in LB and incubated for 10 min at 37°C. To stop the digestion of the sections with trypsin, the sections were washed in running tap water for 5 min. The sections were then washed three times (5 min each) in LB and incubated with the biotinylated lectins (10 μg/ml) for 60 min in a humid chamber at room temperature. This step was followed by a further three washes (5 min each) in TBS. Afterwards the sections were incubated with alkaline phosphatase streptavidin complex (Vectastain® ABC-Kit; Vector Laboratories) in a humid chamber for 30 min.

After final three rinsings (5 min each) in TBS, sections were transferred into the visualizing mixture containing naphthol-AS-biphosphate, hexatozised new fuchsin, dimethylformamide and tween 20 (all from Sigma-Aldrich Chemie, Darmstadt, Germany) applied in the dark for 20 min to visualize enzyme reactivity. To stop the enzyme reaction, the sections were washed in running tap water (7 min) and were then transferred into distilled water (2 min).

Counterstaining was performed using Mayer's hemalum (Merck). The sections were dehydrated and subsequently covered using a resinous permanent mounting medium (Eukitt, Kindler GmbH, Freiburg, Germany).

A section of normal human tonsil or colon, respectively, was included in each batch of staining as a positive control. Control sections (negative as well as positive) were incubated in the same way, omitting the lectins as the negative control.

To confirm specificity of binding, lectins were preincubated in the presence of 0.1 M monosaccharide: BSA-IB4 and RCA-I with galactose; AAA and UEA-I with fucose; GNA with mannose; *N*-acetylgalactosamine with HPA; and *N*-acetyl-glucosamine with WGA; PHA-L was preincubated in the presence of 0.1 M of a complex-type carbohydrate such as bovine thyreoglobulin and SNA-I in inhibition with 0.8% periodic acid.

The sections were examined using a light microscope (Zeiss Axioplan; Zeiss, Jena, Germany). The histological grading (and thereby sections per grading) was determined according to the degree of differentiation (see Table I).

The intensity of staining was scored as follows: −: no staining, (+): very weak, +: weak, ++: moderate, +++: intensive.

The staining pattern of lectins was determined for each section in the histological grading. If the staining pattern differed in the sections of one histological grading, the predominant staining pattern (maximum count) was recorded for the total histological grading.

The percentage of stained epithelial cells in each section was visually estimated and the approximate percentages of all section of the particular stages were used to calculate an average value.

According to previous studies, the epithelium of the mammary gland is subdivided into an internal layer of epithelial cells lining the lumen of the ductal structure and an external layer of myoepithelial cells, which rest on the basement membrane, the ducts being surrounded by dense stroma (35-37). The luminal layer of the epithelium exhibits cuboidal or pyramidal, low columnar to flattened shape, single or multilayered. The thickness of epithelial layer and the degree of differentiation vary as a function of specific structure in which they are located (37).

Accordingly, we refer to stained cells of the epithelium as luminal epithelium for the internal layer and myoepithelium for the external layer. Additionally, the staining intensity of inflammatory mononuclear cells which invaded the tumor tissue was determined.

Results

AAA. In HP, AAA labelled myoepithelium intensively and the luminal epithelium weakly, in stage G0, the myoepithelium stained moderately and the luminal epithelium weakly, with a similar staining pattern in G1, in stage G2 and G3 the myoepithelium and luminal epithelium stained weakly; in stage G4 both the myoepithelium and luminal epithelium stained moderately. The basement membrane was not decorated in any stage (Figure 1).

AAA labelled the connective tissue in the HP intensively, and in stages G0-G4 moderately. AAA labelled the tumor tissue-invading mononuclear cells in stages G1-G3 weakly and in stage G4 moderately.

The luminal epithelium showed an increase from being weakly labelled in HP, G0, G1, G2 and G3 to moderately in G4, as well as the approximate percentage staining increasing from 10% in almost all previous stages to 35% in G4. The mononuclear cells showed a progression from weak to moderate labelling accompanying malignant progression.

BSA-IB4. In HP, BSA-IB4 labelled the luminal epithelium weakly, in stages G0 to G3 weakly, and in stage G4 weakly to moderately (Figure 2). The basement membrane was not decorated in any stage.

BSA-IB4 labelled the tumor tissue-invading mononuclear cells in stage G2 and G3 weakly and in stage G4 weakly to moderately.

The percentage of the labelled luminal epithelium increased from hyperplasia to G4 (except G3), and the intensity increased from weak in hyperplasia-G3 to weak to moderate in G4 (Figure 1). Likewise an increase was detected for mononuclear cells from weak in G2 and G3 and finally weak to moderate in G4.

GNA. GNA labelled between 5 and 15% of the myoepithelium in HP, and in stage G0, G1, G2, G3 and G4 weakly (Figure 1). The basement membrane was not decorated in any stage.

GNA labelled the tumor tissue-invading mononuclear cells in stage G1-G4 weakly.

HPA. HP and stage G0 were not labelled by HPA, additionally in stages G1 and G2, HPA labelled no epithelium. HPA labelled stage G3 luminal epithelium very weakly and stage G4 luminal epithelium weakly to moderately (Figure 3). The basement membrane was not decorated in any stage.

HPA labelled the connective tissue in stages G1 and G4 weakly. HPA labelled the tumor tissue-invading mononuclear cells in stages G1-G3 weakly and in stage G4 weakly to moderately.

The luminal epithelium was labelled very weakly in G3 and increased to weak to moderate staining in G4, the percentage of labelled luminal epithelium increasing from G3 to G4 (Figure 1). Likewise an increase of staining of mononuclear cells from weak in G1-G3 to weak to moderate in G4 was detectable.

PHA-L. PHA-L generally labelled myoepithelium very weakly and luminal epithelium weakly to moderately, except in stage G4, in which myoepithelium stained weakly and luminal epithelium moderately to intensively (Figures 1 and 4). The basement membrane was not decorated in any stage. PHA-L labelled the tumor-invading mononuclear cells in stages G1, G2 and G3 weakly and in stage G4 moderately.

RCA-I. In HP-G0, RCA-I labelled myoepithelium and luminal epithelium intensively, with staining intensity declining with increasing grade to moderate to moderate/ intensive in G4. The basement membrane was not decorated in any stage.

RCA-I labelled the connective tissue in all stages intensively. Tumor-invading mononuclear cells in stages G1 and G2 were stained intensively, in stage G3 moderately and in stage G4 moderately to intensively (Figure 1).

SNA-I. SNA-I labelled myoepithelium moderately and luminal epithelium weakly, with a tendency for this pattern to change to increasing luminal intensity with increasing grade (Figure 1). The basement membrane was not decorated in any stage. SNA-I labelled the connective tissue in all stages moderately. SNA-I labelled tumor-invading mononuclear cells in stages G1-G4 moderately. The luminal epithelium showed a slight increase in staining from weak in hyperplasia, G0 and G1 to moderate in stages G2 and G4.

UEA-I. In 50% of the sections of HP, UEA-I labelled 5% of the myoepithelium very weakly, in 60% of the sections in stage G1 1% of the myoepithelium very weakly and in 50% of the sections in stage G4 5% of the myoepithelium very weakly. Stages G0, G2 and G3 were not labelled by UEA-I (Figure 1). The basement membrane was not decorated in any stage. UEA-I labelled the connective tissue in HP, G1 and G4 very weakly.

WGA. WGA generally labelled myoepithelium weakly and luminal epithelium intensively, except in stage HP where WGA labelled moderately to intensively. The basement membrane was not decorated in any stage.

WGA labelled the connective tissue in HP and stage G2 moderately, in stages G0, G1 and G4 intensively and in stage

Table I. *Histological grading of the tissue specimen and sections (n) per grading.*

Histological stages of neoplastic transformation and grading of invasive carcinomas; number of sections (n)		
Hyperplasia (HP)	Hyperplasia	n=10
Dysplasia/ DCIS (G0)	Noninvasive, intraepitheliale neoplasia, forms of dysplasia and carcinoma *in situ*	n=12
Grade 1 (G1)	Well-differentiated invasive adenocarcinoma (low grade)	n=10
Grade 2 (G2)	Moderately (to poorly) differentiated invasive adenocarcinomas (low grade)	n=11
Grade 3 (G3)	Poorly differentiated (to undifferentiated) invasive adenocarcinomas (high grade)	n=12
Grade 4 (G4)	Undifferentiated invasive adenocarcinomas with anaplastic changes (high grade)	n=10

Table II. *Abbreviation, common name and primary major sugar specificity of lectins used in this study.*

Lectin		
Origin (Common name)	Abbreviation	Major sugar specifity
Aleuria aurantia	AAA	Fucose: α-1-3fuc
Bandeiraea simplicifolia, (griffonia)	BSA-IB4, GSA-IB4	Galactose: D-gal, Gal α-1-3gal , linked
Galanthus nivalis, (snowdrop)	GNA	Mannose: α-1-3, α-1-6 linked
Helix pomatia, (edible snail)	HPA	N-Acetylgalactosamine,, N-acetylglucosamine: GalNAc $>$ GlcNAc
Phaseolus vulgaris	PHA-L	β1-6 Branched oligosaccarides, (complex-type carbohydrates)
Ricinus communis	RCA-I	Galactose, N-acetylgalactosamine: Galβ1-4glcNAcβ1-R
Sambucus nigra, (elderberry)	SNA-I	N-Acetylneuraminic acid, α2,6 sialic acid: Neu5Acα2-6gal(NAc)-R
Ulex europeus, (gorse)	UEA-I	Fucose: α-L-fucose
Triticum vulgaris, (wheat germ)	WGA	N-Acetylglucosamine: GlcNAcβ1-4glcNAcβ1-4glcNAc, Neu5Ac (sialic acid)

G3 moderately to intensively. WGA labelled tumor-invading mononuclear cells in stages G1-G4 intensively.

Discussion

The aim of the present study was to describe the lectin binding pattern in a well-established mouse mammary cancer model (9, 15-17) and to compare these with results of studies of lectin binding in human breast cancer.

A number of studies showed that the lectin HPA labelled cells in different human adenocarcinoma (38-43), including breast carcinoma (24, 25, 44-46), as well in xenograft animal models of mammary cancer (29, 47, 48). HPA recognizes changes in glycosylation (25), particular *N*-acetylgalactosamine and *N*-acetylglucosamine glycans (49, 50) which have been associated with the development of metastases in clinical studies (24, 28, 51). In breast carcinoma, HPA binding is strongly associated with the presence of local lymph node metastases (24, 32, 38, 52, 53), 80% of the metastases of primary tumor being HPA-positive (38). HPA binding in breast cancer indicates a poor prognostic outcome, with shorter disease-free and overall survival times (24, 25, 32).

In our study, no HPA labelling was found in HP or G0 epithelia, while a slight increase in quantity and intensity of HPA staining of the luminal epithelium was observed during malignant progression towards undifferentiated higher grades. These results support the findings of previous studies with human cancer cell lines and animal xenograft models which either detected no or only very low or negligible HPA binding in benign breast disease and primary tumors (50, 54, 55), while positive HPA binding preferentially occurred in primary tumor cancer cells, which are capable of invading local tissue, and in malignant progression (24, 25, 52, 55-57). However, metastasis formation is a relatively rare event in our mouse model (9), which may be due to the short life-span of the mice, as metastasis formation in human breast cancer is often a relatively late event.

Furthermore, there is one study which showed that the distribution of binding sites for HPA was almost identical to that of the lectin BSA-IB4 (58), which recognizes α-D-galactose residues (58). The presence of these residues are also associated with the formation of metastases in murine and human breast carcinoma (59-62).

In our study, we found an increase in quantity and intensity of BSA-IB4 binding for luminal epithelium, from weak in all stages towards weak to moderate labelling in G4. Labeling by BSA-IB4 of tumor-invading mononuclear cells increased from weak in G2 and G3 to weak to moderate in G4. The binding pattern of BSA-IB4 showed some similarity to HPA binding of luminal epithelium and mononuclear cells in our study and can support the findings in the previously described study. Our findings also support studies which
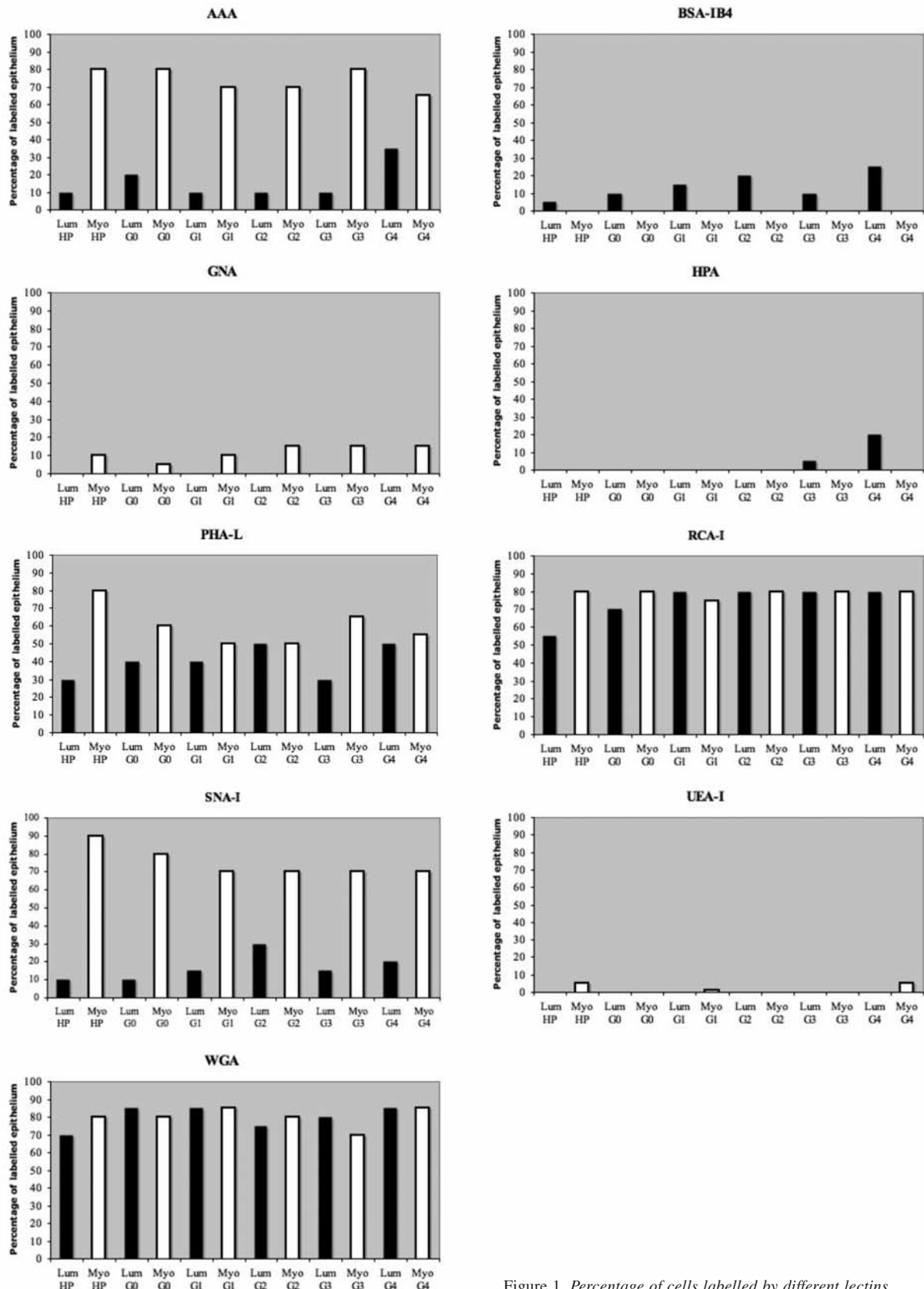
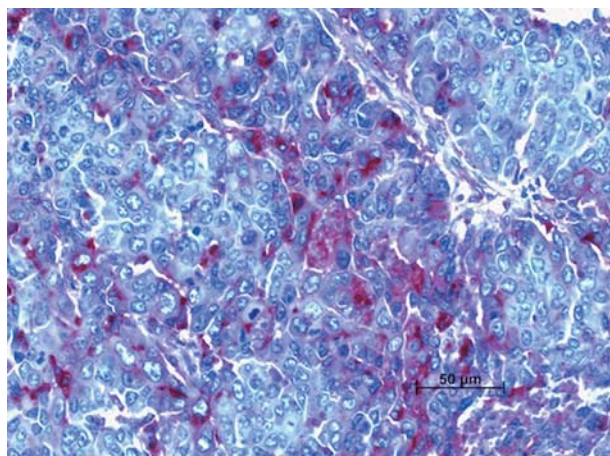Figure 1. *Percentage of cells labelled by different lectins*.

Figure 2. *BSA-IB4 labelling in sections of mammary carcinoma grade 4 in WAP-T transgenic mice.*
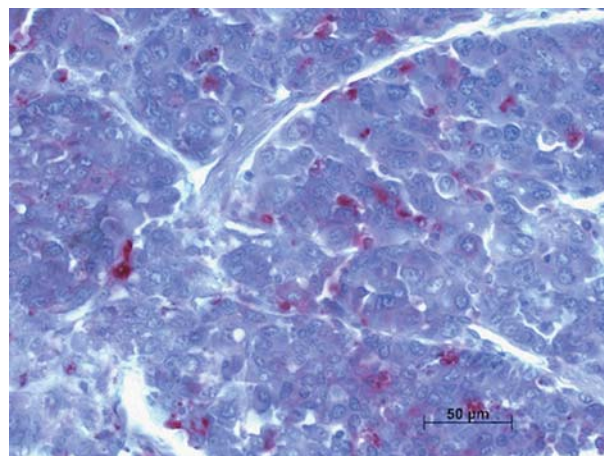

Figure 3. *HPA labelling in sections of mammary carcinoma grade 4 in WAP-T transgenic mice.*

described that BSA-IB4 binding exists in normal human and murine mammary epithelium and increases during malignant progression (53, 62-64).

PHA-L binds to complex-type carbohydrates, namely β-1-6 branched oligosaccharides (26, 55). PHA-L binding is seen in a tissue-specific and developmentally-regulated manner in epithelial transformation, particularly in cells with migratory capacity (26, 65). In previous clinical studies (26, 66) and xenograft animal models (55), PHA-L binding was found to be strongest in primary human breast and colonic cancer compared to benign breast (67) and colonic tissue (26). Additionally, PHA-L binding is seen in human breast cancer cells which are capable of invading locally and in those which formed distant metastases (26, 55, 65, 66) Therefore PHA-L seems to be a possible prognostic marker for tumor progression in breast and colonic cancer (26,55). However, there are studies which showed no correlation between histochemical PHA-L staining reaction and patient outcome (68).

In our study, the number of PHA-L-binding cells increased for myoepithelium and luminal epithelium towards undifferentiated higher grades and therefore towards malignant progression. However, in general, the percentage of PHA-L binding was more variable towards undifferentiated higher grades. The number of PHA-L-binding mononuclear cells also increased towards higher stages of malignant progression. Our findings, thus, corroborate previous studies which showed no PHA-L reactivtity in normal human and murine mammary gland epithelium and fibroadenomas except for a slight staining at the apical/luminal surface of ductal epithelium (26, 66, 67). In human breast cancer cells, these studies showed an increase in intensity and in quantity of the stained malignant cells towards undifferentiated higher stages of breast cancer.
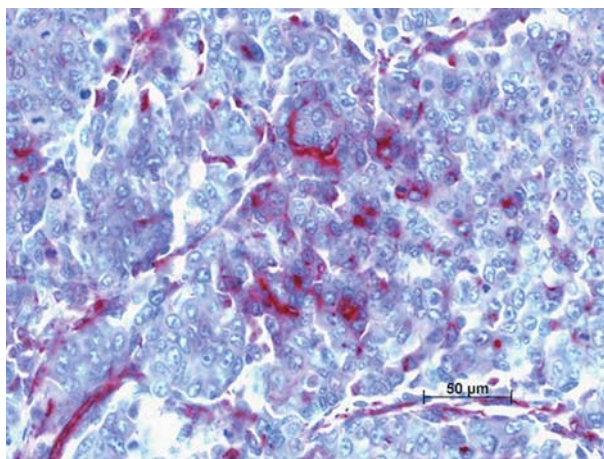

Figure 4. *PHA-L labelling in sections of mammary carcinoma grade 4 in WAP-T transgenic mice.*

Nevertheless, all noted more variable, sometimes diffuse staining reaction in the histological manifestation within human breast cancer and benign breast tissue, as well in xenograft animal models (55), probably due to biological variations in cell transformation. Additionally, this variation could also explain failure to detect correlation between PHA-L binding and patient outcome in some studies.

UEA-I, which has specifity for L-fucose residues (68), only bound to myoepithelial cells in stages of hyperplasia, G1 and G4 and labelled only 1% to 5% of the myoepithelium very weakly. Furthermore, only carcinomas in about half of the animals in these stages were labelled. There was no labelling of tumor cells in the other stages by UEA-I. These results match those of previous studies, which detected variable binding of

UEA-I to normal and malignant breast epithelium (58, 70) in humans and no relationship to any prognostic factors such as stage, grade of differentiation, size of tumor or steroid hormone receptor status (44). In contrast, there was a significant relationship with disease-free interval and overall survival (44) and for the labelled cell proportion and earlier local recurrence (44). However, a previous study showed that a deficiency of fucosylation in murine tumors is related to a metastatic capacity (71), which could explain the findings in our study from a transgenic mouse model with local aggressive and destructive growth.

For AAA, we detected an increase in quantity and intensity of the labelled luminal epithelium from weak in all stages to moderate in stage G4. The mononuclear cells showed a similar pattern. These results could support some clinical studies which also detected labelling of AAA in human breast cancer (72, 73).

For SNA-I, we observed an increase in intensity of labelled luminal epithelium from weak in HP, G0 and G1 to moderate in G2 and G4. These findings could support previous studies which showed that cell-surface sialic acid (74) detected by SNA-I plays a role in cell–cell and cell–matrix adhesion of human breast carcinoma cell lines (75, 76), that SNA expression is significantly higher in human breast cancer cell lines (49, 73), and might confer a poor prognosis (77) or could at least be a marker for different tumor subtypes (73).

Conclusion

In the present study, we showed that the transgenic WAP-T mouse model of mammary cancer and the binding of several lectins at least partially mirrors findings in previous studies on human breast cancer and murine mammary cancer. Thereby, the transgenic WAP-T mouse model seems to be a reliable animal model for studying the role of carbohydrate expression during malignant progression in breast cancer research.

In particular, HPA, PHA-L and BSA-IB4 showed a similar distribution pattern of labelled cells comparable to previous clinical studies (26,50,54,66) and animal models (29,47,48,55).

Competing Interests

The Authors declare that they have no financial or non-financial competing interests.

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