A Study of Glycoconjugates in Nasopharyngeal Carcinoma with Correlation to Clinical Transformation

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Little is known about the glycoconjugate changes in human nasopharyngeal epithelium following neoplastic changes. Glycoconjugate histochemistry (Glycine maximus (SBA), Griffonia simplicifolia II (GSA-II), Ulex europaeus (UEA-I), Arachis hypogaea (PNA) and Canavalia ensiformis (ConA)) were performed on the following nasopharyngeal biopsies: 10 adenoid tissues (benign controls), 10 chronic inflammation, 20 squamous metaplasia, 20 undifferentiated carcinoma and 5 squamous cell carcinoma. These results were correlated with the clinical transformations findings. Strong ConA and PNA staining (after neuraminidase treatment (NA)) characterized a subpopulation of squamous metaplasia subjects who later transformed to nasopharyngeal carcinoma. Strong ConA and PNA (before and after NA) depicted the majority of undifferentiated carcinoma subjects having local recurrence following irradiation therapy. In squamous metaplasia, ConA and PNA (after NA) staining may serve as a warning sign for neoplastic changes. Strong stainings for ConA and PNA (before and after NA) in undifferentiated carcinoma subjects may predict a risk for local recurrence.

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Nasopharyngeal carcinoma is a rare disease among Caucasians. However, alarming frequencies are noted among Chinese, especially the inhabitants south of the Yangtze River and along coastal areas of mainland China and Taiwan. Nasopharyngeal carcinoma is ranked as the fifth most common cancer among men and the seventh most common cancer among women in Taiwan (1). Although the morphological features of undifferentiated carcinoma are well defined in the human nasopharynx, less is known about the histochemical changes occurring within the cells and on their surfaces following malignant transformation (2–4). No data discuss the relevances between glycoconjugate changes and clinical transformations.

Aberrant glycosylation of proteins is a common characteristic of neoplastic changes. This phenomena is considered a major determinant of cancer as is invasive growth or metastasis (5, 6). The terminal carbohydrates located on cellular surfaces determine many of the final structural and functional properties of proteins conferring essential biological attributes (7). Alterations of these cell surface carbohydrates may accompany malignant transformation resulting in the appearance of tumor-associated antigens. This may influence both tumor growth and metastasis. Changes in cell surface glycoconjugate expression may result from the absence of or the activation of new tumor-related glycotransferases (8). These glycoconjugate expression changes may directly be related to the histological differentiation of the tumor cells (9). The carbohydrates may be identified by binding specific glycoconjugates to the oligosaccharides using labels such as biotinylated lectins and avidin-biotin-peroxidase complexes (10).

The lack of clinically relevant glycoconjugate information has prompted our investigation of lectin bindings in nasopharyngeal epithelium of benign and malignant conditions. In order to solve the most urgent issues of nasopharyngeal carcinoma, such as how to make an early diagnosis of fresh case and efficient detection of recurrence to have a proper treatment, additional studies were designed to make lectin histochemistry more clinically relevant. We correlated cell surface glycoconjugate changes to clinical data such as neoplastic transformation and local recurrence following irradiation therapy.

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Table 1

A panel of glycoconjugates including sugar specificities and inhibitory saccharides

Lectin	Abbreviation	Major sugar specificities	Inhibitor sugar
Canavalia ensiformis	ConA	Mannose > glucose	Mannose
Griffonia simplicifoliaII	GSA-II	N-acetylgluosamine	N-acetylgluosamine
Arachis hypogaea	PNA	Gal(1,3)-GalNAc	D-galactose
Glycine max	SBA	a-D-GalNAc	D-GalNAc
Ulex europaeus I	UEA-I	a-L-fucose	L (-)fucose

Gal., Galactose; GalNAc, N-acetylgalatosamine.

MATERIAL AND METHODS

This is a retrospective study enrolling 55 subjects having nasopharyngeal biopsies and consisting of the following groups: chronic inflammation (n = 10); squamous metaplasia (n = 20), undifferentiated carcinoma (n = 20), and well-differentiated squamous cell carcinoma (WSCC) (n = 5). Two of the 10 chronic inflammation patients and 7 of 20 squamous metaplasia patients were eventually diagnosed with undifferentiated carcinoma during their follow-up course. Six of the 20 undifferentiated carcinoma patients had local nasopharyngeal recurrence following irradiation therapy. All pathological diagnoses were classified in accordance with the World Health Organization criteria. Adenoid biopsies were taken from 10 patients following adenoidectomy which served as our benign controls (11).

The tissue was fixed in formalin and embedded in paraffin. Sequential tissue sections were cut using a microtome set at 5μm. These sections were mounted on glass slides pretreated with acid alcohol, dried, deparaffinized using xylene and rinsed in 100% ethanol. Endogenous peroxidase activity was blocked by using 0.3% hydrogen peroxide in 0.01 M phosphate buffer saline (PBS) at pH 7.5. This was placed in a wet chamber for 30 min at 37°C. The sections were then treated with 0.2% bovine serum albumin in 0.05 M tris buffered saline (TBS) for 20 min. After washing with PBS, they were incubated with 50 mg/ml of biotinylated lectins (Sigma) (Table 1) for 2 h and washed in PBS for 10 min. Next, the sections were incubated in freshly prepared avidin-biotin-peroxidase complex (ABC, Vector Lab.) for 30 min. Visualization was achieved by using a developing solution consisting of 0.05% diaminobenzidine (Sigma) in TBS at pH 7.5. Lectin binding specificity was verified by blocking with the various specific sugars (0.2 M) for 20 min prior to incubation (negative controls). The histochemical reactions were scored by microscopical observation of the tissue sections for the presence of oxidized DAB, a brown precipitate. The intensity of these precipitates was arbitrarily assigned the following staining intensity scores: negative (−), weak (+), moderate (++) or strong (+++).

The penultimate sugar residue to sialic acid was detected by pretreatment of the sections in neuraminidase (Sigma) followed by PNA (NA-PNA) and SBA incubations (12). First, the sections were incubated in 0.1 U/ml neuraminidase (NA) prepared in 1% sodium chloride dissolved in 0.05 M sodium acetate buffer, pH 5 for 60 min at 37°C and thereafter washed in PBS for 10 min prior to biotinylated lectin staining. Comparisons were made by using the Chi-square test along with the Yates' correction. The significance level was $p < 0.01$.

RESULTS

The distribution of staining patterns for the various glycoconjugates are illustrated in Table 2. It shows weak ConA staining in normal respiratory and chronic inflammatory epithelia. There were focal ConA staining in the epithelium of squamous metaplasia subjects and an increase in neoplastic cell staining of undifferentiated carcinoma and WSCC subjects. No reactivity to PNA was noted in non-malignant epithelium. Fifty percent of the undifferentiated carcinoma subjects and all the WSCC subjects had positive PNA staining. After NA treatment, adenoid and chronic inflammation subjects showed no staining for both PNA and SBA. Forty percent of the squamous metaplasia subjects had positive PNA stainings following NA treatment. Positive PNA staining after NA treatment was shown in all of the undifferentiated and WSCC carcinoma subjects.

The characteristics of lectin staining intensities for local recurrence after irradiation therapy in undifferentiated carcinoma subjects and malignant transformations in chronic inflammation and squamous metaplasia subjects are illustrated in Table 3. All seven squamous metaplasia subjects, who eventually fell victim to undifferentiated carcinoma, had strong ConA staining patterns (Fig. 1). Eight of the 20 squamous metaplasia subjects had PNA positive staining following NA treatment. During follow-up examination, 6 of these 8 subjects (75%) had malignant transformation to undifferentiated carcinoma, although a total of 7 subjects had malignant transformation. Six (85.7%) of these 7 subjects had positive NA-PNA staining (Fig. 2). The strong ConA and NA-PNA staining in squamous metaplasia are significantly associated with malignant transfor-

Table 2

Glycoconjugate staining patterns in benign and malignant nasopharyngeal epithelium

	ConA	UEA-1	PNA	NA-PNA	SBA	BSL
Adenoid {10}	7/10 [+]	10/10 [+++,++]	0	0	0	0
Ch. Infla. {10}	7/10 [+]	10/10 [+++,++,+]	0	0	0	0
Sq. Meta. {20}	16/20 [+++,++,+]	20/20 [++,+]	0	8/20 [++,+]	0	0
Undif. Ca {20}	20/20 [+++,++,+]	6/20 [++,+]	10/20 [+++,++,+]	20/20 [+++,++,+]	0	0
WSCC {5}	5/5 [++,+]	4/5 [+++,++,+]	5/5 [+++,++]	5/5 [+++,++]	0	0

Staining intensities are given in arbitrary units: [+++, strong; ++, moderate; +, weak; −, negative]. { } indicates the number of cases and ( ) shows the percentage of positively stained tissue. Ch. Infla., chronic inflammation; Sq. Meta., squamous metaplasia; Undif. Ca., undifferentiated carcinoma; WSCC, well-differentiated squamous cell carcinoma.

mation ($p < 0.01$). A total of 6 of 20 patients showed local recurrence of whom 5 were noted as having a strong ConA staining pattern. Strong ConA staining in undifferentiated carcinoma subjects have a significant correlation to local recurrence ($p < 0.01$). Also, strong PNA staining (prior to or after NA treatment) in the undifferentiated carcinoma subjects (Fig. 3) are significantly associated with local recurrence ($p < 0.01$).

DISCUSSION

Cell surface glycoproteins with heterogenous glycosyl residues play an important role in the regulation of cell proliferation and epithelial growth. They are often altered on neoplastic cells and aberrantly proliferating cells (6). Glycoconjugates have been used extensively to study alterations in cell surface carbohydrates associated with malignant transformation (13, 14). They are also ideal molecular probes for studying tumor histogenesis (15, 16), heterogeneity of tumor cell populations (17, 18) and the relationship of tumors to inflammatory and preneoplastic conditions (19, 20). In the present study, the histochemical staining methods were applied to formalin-fixed, paraffin-embedded tissues in order to provide us with the advantage of higher sensitivity (10) and more detailed morphology (21–23).

There is a practical limitation in obtaining the true normal nasopharyngeal tissue as a control. Theoretically, using nasal tissue as a substitution is not appropriate according to histological characteristics. Hence, chronic inflammatory specimen has been used as a control in previous nasopharyngeal studies (24, 25). These tissues are usually from patients with suspicious symptoms, but being diagnosed as 'chronic inflammation'. In the present study we enrolled this patient group to observe their clinical transformation. Thus, we choose the adenoid tissue, which may show the inflammatory cells infiltrations too, as our benign control (11).

In the present study, neither SBA nor A-II lectins stained any of the nasopharyngeal epithelium tissue samples. Absence of SBA and GSA-II staining suggests that the N-acetylgalactosamine and N-acetylglucosamine lectin moieties are not expressed on the cellular surface of either epithelial cells or malignant cells. However, this result may be due to either capping of these sugars with additional sugar moieties or a deficiency in the proper glycotransferase enzymes (26).

PNA staining is predominantly intracellular and probably reflects a change in mucus synthesis within the Golgi apparatus. An increased expression of galactose related sugar has also been found following malignant transformation. This is shown by the increased staining of PNA before NA pretreatment. PNA staining pattern in nasopharyngeal epithelium is similar to previous data showing that laryngeal carcinoma (27), oral carcinoma (28), bladder carcinoma (29), colon carcinoma (30) and breast cancer (29) stain densely whereas non-neoplastic tissue stains weakly or not at all (12). In nasopharyngeal carcinoma tissue, positive PNA staining is indicative of the Thomsen-Friedenreich antigen (T-antigen) being detected. This reflects the loss of sialic acid during neoplastic change. Neuraminidase pretreatment may permit the T-antigen to be detected by removing the terminal sialic acid exposing Gal-b-1.3- or 1.4-GalNAc residues (3, 12). In this study, positive PNA staining with NA pretreatment may characterize squamous metaplasia subjects which transformed into undifferentiated carcinoma. This may indicate that the cells, while not 'neoplastic' with conventional histology, have already undergone some 'neoplastic changes'. Previous data have shown that strong staining by PNA is possibly associated with an early stage of disease for head and neck carcinoma (9). However, this biochemical event is not limited to neoplastic tissue only (19).

Our study found a stronger PNA staining pattern in well-differentiated squamous cell carcinoma tissue com-

Table 3

Glycoconjugate staining in nasopharyngeal epithelium related to clinical transformation

	ConA				PNA				NA-PNA			
	+++	++	+	−	+++	++	+	−	+++	++	+	−
Ch. Infla.	0	0	2*	0	0	0	0	2*	0	0	0	2*
Sq. Meta.	7*	0	0	0	0	0	0	7*	0	4*	2*	1*
Undif. Ca.	5^	1^	0	0	4^	1^	1^	0	5^	1^	0	0

(*), case number of malignant transformations; (^), case number of local recurrences. Staining intensities are given in arbitrary units: +++, strong; ++, moderate; +, weak; −, negative. Ch. Infla., chronic inflammation; Sq. Meta., squamous metaplasia; Undif. Ca., undifferentiated carcinoma.

pared with a weaker staining pattern in undifferentiated carcinoma tissue. Similar results are presented in the study by Lalwani et al. (9). They showed a loss of PNA staining as the tumor further dedifferentiated. Only 4 of the undifferentiated carcinoma subjects showed strong PNA staining. These 4 subjects had local recurrence following irradiation therapy. Five of the 7 undifferentiated carcinoma subjects showing strong PNA staining after NA treatment developed local recurrence after irradiation therapy. Possessing this quality of strong PNA staining, these undifferentiated carcinoma cells may have similar biological characteristics to the cells of well-differentiated squamous cell carcinoma. It is known that WSCC is more resistant to irradiation than undifferentiated carcinoma. This may explain these undifferentiated carcinoma subjects' potential for recurrence. Thus, strong staining of PNA in undifferentiated carcinoma tissue (before and after NA treatment) may serve as a predictor of local recurrence.

UEA-1 showed weaker staining patterns in undifferentiated carcinoma tissue compared with the strong staining patterns associated with WSCC of the nasopharynx. In contrast, some data show no staining in oropharyngeal WSCC tissue (8, 28). In our support, Heng et al. (28) report that WSCC stains positively with UEA-1 while poorly differentiated squamous cell carcinoma is negative. Basal cell carcinoma (31) and nasopharyngeal undifferentiated carcinoma (4) have also shown negative UEA-1 staining. UEA-1 seems to indicate whether a squamous cell carcinoma is well differentiated (positive staining) or undifferentiated (negative staining). UEA-1 binds to L-fucose specific binding sites and may act as marker for terminally differentiated keratinocytes. With further investigation, this marker may become a practical diagnostic tool and assist in the classification of epithelial tumors (8, 32).

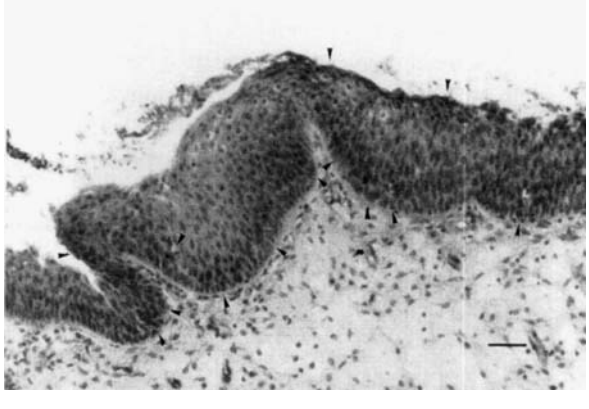

Fig. 1. Focal, strong ConA staining (arrow heads) in the nasopharyngeal epithelium of a squamous metaplasia subject who transformed to undifferentiated carcinoma. (bar = 25μm)

ConA had weak staining in normal and chronic inflammation tissues. It showed an increase in staining for squamous metaplasia, undifferentiated carcinoma and squamous cell carcinoma tissues. In contrast, some have shown a significant decrease of ConA staining in human oropharyngeal squamous mucosa following malignant transformation (8). Our data are supported by others who have shown strong ConA staining in squamous cell carcinoma subjects (9). The discrepancy in the intensity of staining was attributed to an increase in expression of ConA reactive cell surface α-D-mannose units or a change in the distribution of the number of ligand binding sites (33) following neoplastic transformation of the nasopharyngeal epithelial cells. Seven of the 20 squamous metaplasia subjects had strong ConA stainings. All these subjects showed malignant transformation into undifferentiated carcinoma at clinical follow-up. Thus, strong staining patterns for ConA in squamous metaplasia subjects may be a signal of dysplastic change. When biopsies show strong ConA staining for squamous metaplasia subjects, the patients should be closely observed. Eight of the 20 undifferentiated carcinoma subjects had strong ConA staining patterns. Five of these 8 subjects (65%) had local recurrence after irradiation therapy. Hence, a strong ConA staining pattern in the undifferentiated carcinoma subjects may indicate a tendency to local recurrence. Accordingly, strong ConA staining may predict a risk of local recurrence (34) and serve as a prognostic indicator.

Early diagnosis and treatment will achieve a better results in nasopharyngeal carcinoma patients. We may

Fig. 2. After neuraminidase treatment, a moderate PNA staining pattern (arrow heads) was observed in the epithelial cells of a squamous metaplasia subject who later transformed to undifferentiated carcinoma. (bar = 20μm)

ignore the patients with suspected symptoms where biopsy showed squamous metaplasia only. ConA and PNA (after NA) staining may give a warning sign for neoplastic change. This will lead us to a close observation of these patients. How to early detect the recurrence and salvage the recurrent nasopharyngeal carcinoma patients is an urgent issue to improve the survival and life quality of nasopharyngeal carcinoma patients. Strong ConA and PNA staining in undifferentiated carcinoma is a risk factor for local recurrence which will inform our oncologist to follow up these patients carefully.

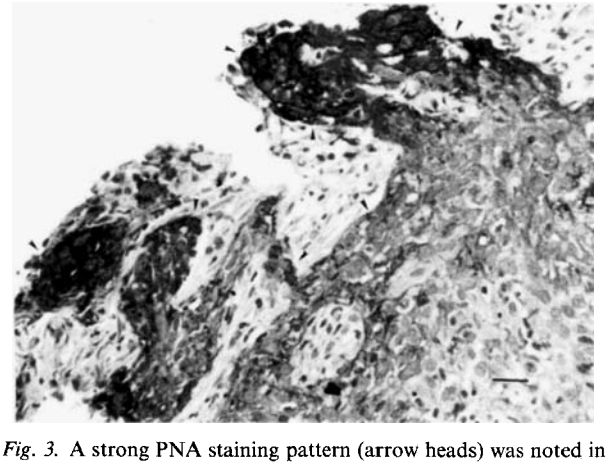

Fig. 3. A strong PNA staining pattern (arrow heads) was noted in the neoplastic cells of an undifferentiated carcinoma subject who had local recurrence following irradiation therapy. (bar = 45μm)

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