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ORIGINAL ARTICLE

Lewis x Antigen is Associated to Head and Neck Squamous Cell Carcinoma Survival

Martín E. Rabassa[1] · Adrian Pereyra[1] · Liliana Pereyra[1] · Amada Segal-Eiras[1] · Martín C. Abba[1] · Maria V. Croce[1]

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Abstract Head and neck squamous cell carcinoma (HNSCC) is an aggressive disease with poor prognosis without appropriate prognostic markers. Previous research shows that Lewis antigens have been involved in carcinoma dissemination and patients´ survival. Fucosyl and sialyltransferases are the enzymes implicated in the Lewis antigens synthesis. The purpose of this study was to evaluate the prognostic utility of Lewis antigens in HNSCC. We conducted a prospective research including histological samples from 79 patients with primary HNSCC. Lewis x and sialyl Lewis x expression were detected by immunohistochemistry; patient's data, progression free, and overall survival were documented. A statistical correlation study of antigenic expression and patients´ histopathological variables was performed. Cox regression models with internal validation procedures were employed to analyze survival data. By immunohistochemistry, Lewis x was detected in 34/79 (43%) tumor samples, while sialyl Lewis x only in 11/79 (14%). Lewis x expression showed a positive correlation with tumor differentiation and a better overall survival for Lewis x + patients was detected. Moreover, multivariate Cox's regression analysis showed that Lewis x is an independent predictor of better overall survival. The in silico analysis supported the presence of deregulated fucosyl (FUT4) and sialyltransferase (ST3GAL4) in the Lewis synthetic pathway related to patient survival. These results suggest that Lewis x expression is associated with a better outcome in patients with HNSCC.

Keywords Antigenic expression · Head and neck squamous cell carcinoma · Risk and prognostic factors · Survival

Introduction

Head and neck neoplasms represent the sixth cause of cancer worldwide with an age-standardized rate of 9.1 cases per 100,000 inhabitants, not including thyroid tumors. In Latin America incidence is estimated to be lower, with an age standardized rate of 6.3 cases per 100,000 in Argentina [1]. Most head and neck tumors are squamous cell carcinomas (HNSCC) and their carcinogenesis has been associated with cigarette smoking [2], alcoholism [2] and HPV [3]. The reported outcomes of head and neck cancer (HNC) in the literature are either from diverse populations or individual hospital based studies and there are few databases like the Surveillance, Epidemiology, and End Results program of the National Cancer Institute. Historically, 5–10 year survival outcomes have been the choice of researchers to compare the efficacy and potential of ever increasing therapeutic options, and to understand the natural disease course. There is a paucity of scientific literature discussing long term HNC at different primary sites, and cancer cohorts which are representative of a defined population [4]. Most stage I/II patients have a very good prognosis while those with higher stages of the disease show a poor overall survival. Although a better locoregional control of the disease has been achieved, the presence of lymph node metastases is the main factor affecting the survival of HNSCC patients [5].

Glycosylation in oral squamous epithelium follows a sequential elongation of the glycan chain from short precursors

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✉ Maria V. Croce
crocevir@hotmail.com

[1] Centre of Basic and Applied Immunological Research, Faculty of Medical Sciences, National University of La Plata, La Plata, Argentina

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in the basal and para-basal cells to terminal fucosylated and sialylated epitopes in the upper differentiated cells [6]. Alteration of glycosylation is an early event during carcinogenesis [7] and changes in terminal glycan epitopes, both in glycolipids or glycoproteins, are found in oral leukoplakia or in situ carcinoma. Most of these changes are related to deletions of chromosomal regions where glycosyltransferases genes loci are located [8]. The Lewis antigens (Suppl. Fig. 1) are a group of terminal fucosylated epitopes altered during cancer development; one of these carbohydrates, sialyl Lewis x, is thought to increase the metastatic potential due to its interaction with selectin ligands [9]. Lewis-type antigens are present in several glycoproteins and mucins which are considered as their carriers in the glycocalyx in normal as well as transformed cells [10]. This research was developed to study the prognostic utility of Lewis antigens in HNSCC.

Materials and Methods

Patients and Samples

Seventy-nine patients with primary HNSCC who were admitted in the period from July 2003 to May 2005 at the Hospital Municipal de Oncología María Curie, a public hospital in Buenos Aires, Argentina, were included in this prospective study. Thirty-nine patients (49.4%) were initially treated with surgery alone while 3 patients (3.8%) received preoperative radiotherapy. In 27 patients (34.2%) initial therapy consisted of radiotherapy alone or radiotherapy and cisplatin-based chemotherapy. Finally, 10 patients (12.7%) received neoadjuvant chemotherapy followed by surgery. All samples where obtained from diagnostic biopsies.

Patients´ staging was performed according to the American Joint Committee on Cancer Criteria for head and neck carcinoma (AJCC Cancer Staging Manual 6th edition, 2002). Eleven normal samples obtained from oral mucosa belonging to healthy individuals were employed as controls. HNSCC patients´ clinical and histopathological information was obtained from clinical records. Fifty seven out of 79 (72%) were male and 48/79 (61%) were older than 60 years. Thirty-six tumors were in the oral cavity, 27 in the larynx, 11 in the pharynx and 5 in the paranasal sinuses. Twenty five percent of the tumors were well differentiated, 42% moderately differentiated and 33% undifferentiated. Thirty-nine patients (49%) presented lymph node metastasis. Finally, 4% of the patients were found at stage I, while 14% at stage II, 39% at stage III and 43% at stage IV.

Monoclonal Antibodies (MAbs)

The MAbs employed were summarized in Suppl. Table 1.

Methods

Immunohistochemical Analysis

Standard immunohistochemistry was performed following previous reports [11]. All specimens were fixed in formalin and embedded in paraffin; sections were deparaffinized in xylene and hydrated in a graded ethanol series. To block endogenous peroxidase activity sections were placed in methanol with hydrogen peroxide (3%) for 15 min. After three washes with PBS, sections were blocked for nonspecific binding with 50 μl of normal horse serum diluted 1:10 in 1% bovine serum albumin/PBS for 15 min. Following this, sections were incubated with 50 μl MAbs, which were diluted 1:1000 at 4 °C overnight in a moist chamber. After three rinses with PBS, the reaction was developed employing the LSAB2 kit (Dako, USA) together with 3,3′-Diaminobenzidine (Dako, USA), following the manufacturer's instructions. The slides were then counter-stained with hematoxylin, dehydrated in ethanol, washed with xylene and, finally, coverslipped with mounting media. Negative controls were performed by adding PBS instead of the primary antibody.

Evaluation of the Staining

Specimens were examined using a light microscope by two independent observers (MER and MVC), who reached a final result by consensus. The antibody staining patterns were scored in a semi-quantitative manner. Staining intensity was graded as negative (0), low [1], moderate [2] and strong [3]. The number of low power (×10) optical fields (×10) in a specimen with a positive reaction was expressed as a percentage of the total number of optical fields containing tissue. The percentage of positive cells was graded as follows: 0, ≤5% of cells; 1, positive in 6–50%; 2, positive in more than 50% of cells. As for the results, they were scored as either negative (0) or positive (≥1).

The patterns of reaction were classified as linear (membrane), cytoplasmic and mixed pattern (cytoplasmic with plasma membrane staining). The positive reaction of the nucleus and lumen content was also evaluated.

In Silico Analysis

Gene expression data sets from HNSCC samples were obtained from TCGA (The Cancer Genome Atlas) repository data at the cBio Cancer Genomics Portal [12] and raw clinicopathological data was curated to eliminate incomplete records. Other head and neck histological types different from the squamous cell carcinoma (i.e., basaloid tumors) were also excluded from the analysis. Full records available included 522 patients' samples and 20 paired, juxtatumoral, normal epithelial samples.

Statistical Analysis

Correlations between mucins and carbohydrate antigens were assessed employing the Kendall's tau-b coefficient. Differences among groups were evaluated by the Chi squared test, Paired T test or ANOVA test. In all cases statistical significance was set at $p < 0.05$.

The overall survival (OS) was calculated from the date of diagnosis until the last control or date of death of the patients. The progression free survival (PFS) was calculated from the date of diagnosis until the first tumor progression date.

OS and PFS univariate associations with Lewis x and sialyl Lewis x expression were evaluated employing the Kaplan-Meier estimator using the log rank and Breslow's test. Comparisons of stratified groups using the clinical and pathological variables were also considered. Univariate and multivariate Cox regression models addressed OS and PFS associations with the score of antigen expression obtained through immunohistochemistry or gene expression values from the in silico data. Multivariate analysis included the variables gender (female vs. male), age (<60 vs. > = 60), localization (oral cavity vs. other), recurrence (yes vs. no), histological grade (high [3] versus low [1, 2]), pT stage (1–2 vs. 3–4), pN stage (0 vs. 1–2), and M stage (0 vs. 1). A bootstrap procedure was employed to assess internal validation of Cox regression modelling; in all calculations, 1000 random samples from the original population were employed and bias and standard errors for each coefficient (B) were calculated. All variables were normalized and the significance level was set at $p < 0.05$.

The analysis was performed employing the SPSS statistics v19.0 software package.

Results

Immunohistochemical Analysis (Table 1)

The Lewis x antigen was detected in 34/79 (43%) tumor samples with a strong reaction which mainly involved the plasmatic membrane of well-differentiated tumors as well as more differentiated areas within a tumor (Figs. 1c and d). Usually, Lewis x staining was restricted to isolated groups of cells. Analysis of Lewis x expression showed a positive correlation with tumor differentiation (Kendall's t, $p = 0.038$) but no association with the other clinicopathological variables was found. A positive Lewis x reaction involving intermediate and upper layers was detected in 8 out of 11 (73%) normal mucosa samples. The reaction consisted of a strong membrane staining and a lower granular staining in the cytoplasm.

The sialyl Lewis x antigen was detected in small groups of cells in 11/79 (14%) tumor samples; the pattern of reaction was mainly linear while a cytoplasmic reaction was occasionally observed. No association was found with the clinicopathological variables.

Only two out of eleven (18%) normal samples showed sialyl Lewis x staining; the reaction was observed at the upper and medial epithelial layers, involving 10 to 20% of the cells with a mixed pattern (Fig. 1e and f,); in one sample, nuclear staining was also found.

Survival Analysis

Mean survival time of this HNSCC cohort of patients was 44.1 months. When assessing HNSCC patients´ OS, the Kaplan-Meier analysis showed a better performance in positive Lewis x tumors. Breslows' statistical analysis detected a significant difference ($n = 79$; $p = 0.026$), which is evident in the first three years of the survival curves (Fig. 2a).

A multivariate Cox's regression analysis controlling for age, gender, stage and tumor histological grade showed that Lewis x expression is associated with a better OS (Table 2, $p = 0.005$). On the other hand, sialyl Lewis x did not present any association with HNSCC OS or PFS (not shown).

In Silico Analysis

Gene expression analysis was performed in a dataset of 522 HNSCC patients and 20 normal mucosa samples from the TCGA consortium. Taking into account the glycosyltransferases involved in Lewis x, sialyl Lewis x and their precursors during their synthesis, a set of 28 genes was selected which included ABO, B3GALT1, B3GALT2, B3GALT5, B3GNT2, B3GNT3, B3GNT4, B3GNT5, B4GALT1, B4GALT2, B4GALT3, B4GALT4, FUT1, FUT2, FUT3, FUT4, FUT5, FUT6, FUT7, FUT9, GCNT1, GCNT2, GCNT3, GCNT4, ST3GAL3, ST3GAL4, ST3GAL6 and ST8SIA1. Comparison of gene expression between normal and paired tumoral tissues showed that 7/28 (24%) genes were up regulated and 12/28 (41%) were down regulated.

When pathological lymph node infiltration was analyzed, four genes were found to be down regulated (FUT2, FUT3, FUT6 and GCNT4) while a gene was up regulated (B4GALT3) in patients with positive lymph nodes. The analysis of T1/2 tumors versus T3/4 tumors showed an increase in B3GNT4, B3GNT5, B4GALT3, FUT5 and ST3GAL4 expression while B3GALT2, FUT7 and ST3GAL6 were down regulated.

Survival analysis employing univariate and multivariate Cox's regression models showed that ten glycosyltransferases were associated with patient OS, and four (B3GALT1, B3GNT5, FUT4 and ST3GAL4) after controlling for gender, age, histological grade and pathological N and T stage; internal validation was performed by a bootstrap procedure ($n = 1000$). Since FUT4 expression was associated with a better OS while ST3GAL4 was associated with a worse

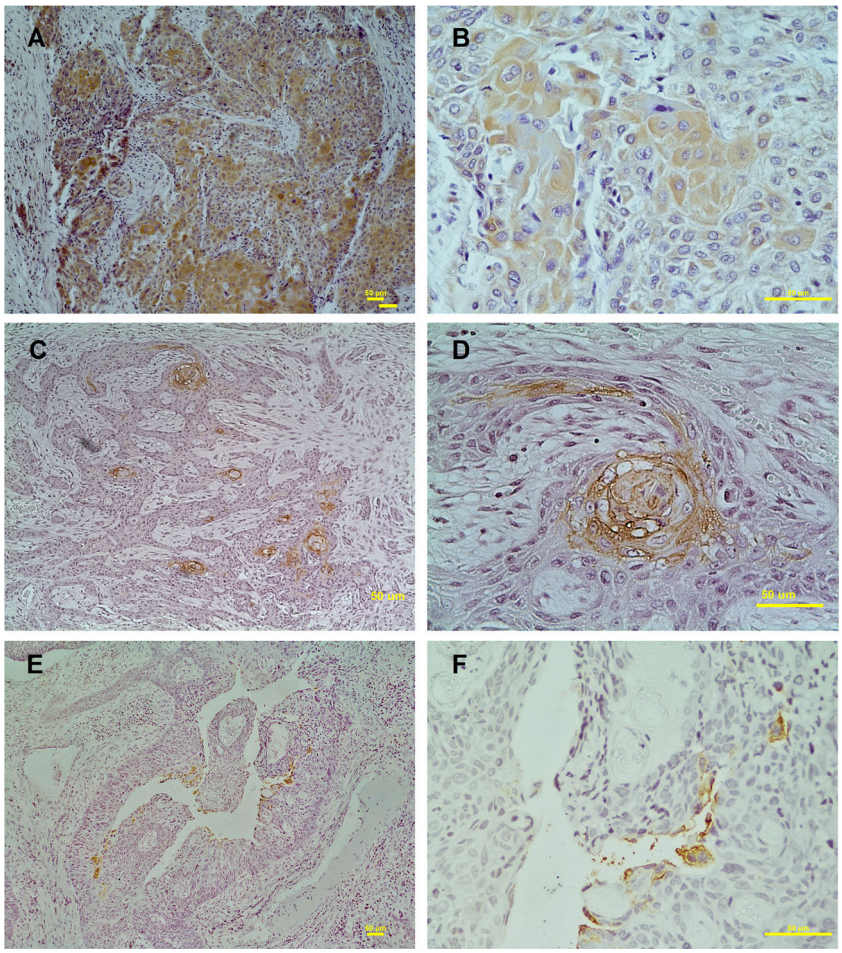

Fig. 1 Microphotographies. Immunohistochemical results of HNSCC staining with anti- Lewis x (**a** - **d**) and anti-sialyl Lewis x (**e** and **f**) MAbs. Microphotographies correspond to an oral carcinoma (**a** - **d**) and a larynx carcinoma samples (**e** – **f**). A higher magnification of a, c and e samples is shown in the right column (**b**, **d** and **f**; ×400)

outcome and a strong negative correlation was found among their regression coefficients ($r = -.347$), a two gene expression index was established (ST/FT Index = ST3GAL4 – FUT4). A high index value was associated with a worse outcome (Supp Fig. 2. Kaplan-Meier analysis, Log Rank test, $p = 0.002$); in a Cox Regression Model, this index showed a similar performance to the pT and pN classifications (data not shown).

Table 1 Summary of immunohistochemical results. Number of positive samples / total number of samples (percentages)

	Lewis x	sialyl Lewis x
Localization		
Normal oral cavity	8/11 (73%)	2/11 (18%)
HNSCC		
Oral Cavity	17/36 (47%)	6/36 (17%)
Nasal Cavity	2/5 (40%)	0/5 (0%)
Larynx	9/ 27 (33%)	3/27 (11%)
Pharynx	6/11 (55%)	2/11 (18%)
Tumor differentiation		
Well	11/20 (55%)	2/20 (10%)
Moderately	16/23 (49%)	9/23 (27%)
Poorly	7/26 (27%)	0/26 (0%)

Discussion

In previous reports, we have exhaustively addressed the expression of Lewis carbohydrate antigens in HNSCC [13–16]. In regard to sialyl Lewis x as a prognostic marker in HNSCC, controversial results were reported. Some authors did not find any association, neither with lymph node metastasis nor with patient prognosis [17, 18], while others did [19, 20]. In the present study, we did not detect any association between sialyl Lewis x and lymph node metastasis.

On the other hand, and considering the Lewis x antigen, we have reported a relationship between Lewis x expression and well-differentiated tumors [13], which is in line with our present findings. Teng YT et al. [21] found expression of this antigen in the stratum spinosum of normal squamous epithelium as well as an association with differentiated cells from invasive carcinomas of the oral cavity.

Lewis x (SSEA-1, CD15, LeuM1) is a carbohydrate structure that functions as an adhesion molecule in glycolipids and

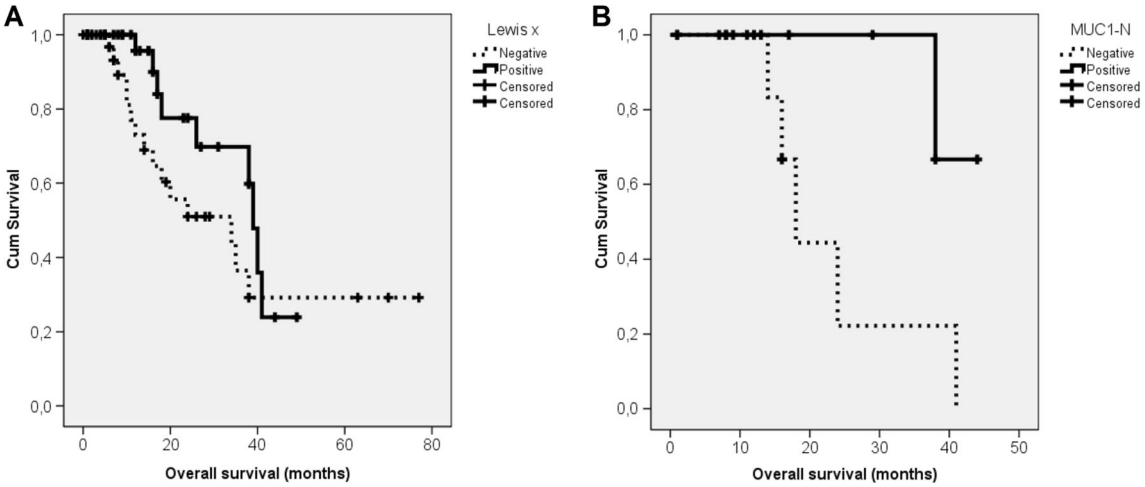

Fig. 2 Kaplan-Meyer plots of overall survival function and Log Rank test analysis. The Kaplan-Meier plots of HNSCC patients´ survival show a better overall survival for positive Lewis x tumors, while applying Breslow's statistical analysis a significant difference is detected ($p = 0.026$) evident in the first three years of the survival curves

glycoproteins. It mediates homophilic and heterophilic adhesion of normal and tumoral cells [22] and is expressed during embryogenesis [23]. In the oral cavity, colon, breast and reproductive tract, Lewis x is expressed both in normal epithelia and derived tumors [24]. It is also expressed in normal myeloid and lymphoid, as well as leukemia cells [25]. It is overexpressed in some tumors and there are some reports relating high Lewis x expression with low patient´ survival. Lewis x has been recognized as a marker of bladder cancer, Hodgkin's lymphoma Reed-Sternberg cells, and a significant prognostic marker in triple negative breast cancer [26]. Interestingly, Derolf AR et al. [27] found an association between CD15 (Lewis x) expression with acute myeloid leukemia favorable prognosis.

The present research consisted in a prospective study and all samples were obtained from diagnostic biopsies, which have been our baseline observation. In a prospective study reporting the performance of a tumor marker related to patients' outcome, baseline observations should be usually performed at the time of definitive surgery or from a biopsy procedure [28] which has been our case. A survival analysis in a cohort of 79 HNSCC patients was performed. The Kaplan-Meier estimator and the Breslow test showed that Lewis x + patients had a better OS than Lewis x- patients, which was statistically significant. These findings are consistent with an early advantage in survival for Lewis x + patients for the first three years of follow up. This association can be explained since Lewis x bearing proteins bind to DC-SIGN and/or MGL lectins which contributes with macrophage clearance [29]. In a recent communication by Chen H et al. [30], expression of Lewis x enhanced an anti- tumoral immune response and prolonged the life span of tumor-bearing mice in a heparanase vaccine model. In consequence, it would be possible that tumors with high expression of Lewis x induce a protective immune response, at least in the early stages of tumor development.

Table 2 Multivariate Cox analysis of survival. The Multivariate Cox's regression analysis of Lewis x and sialyl Lewis x expression in HNSCC controlled for age, sex, stage and tumor histological grade. Lewis x expression is associated with a better overall survival ($p = 0.005$) while sialyl Lewis x shows no association

					95.0% CI	
Variable	B	Wald	Sig.	Exp(B)	Lower	Upper
Age	,372	,371	,543	1450	,438	4799
Sex	-,032	2306	,129	,969	,929	1009
T stage	1960	6608	,010	7101	1,593	31,655
N stage	,951	3699	,054	2589	,982	6828
Grade	-,085	,079	,779	,919	,508	1661
sialyl Lewis x	,615	,745	,388	1850	,458	7474
Lewis x	-1427	6555	,010	,240	,081	,716

Sig Significance, CI Confidence Interval

Our in silico analysis of TCGA HNSCC mRNA expression data revealed that only B3GALT1, B3GNT5, FUT4 and ST3GAL4 remained significantly associated with OS, after controlling for gender, age, histological grade and pathological T and N stages and after internal validation employing a bootstrap procedure. Interestingly, we found a strong negative correlation between FUT4 and ST3GAL4 regression coefficients. Survival analysis employing an index including these two variables (ST/FT index), performed well after controlling for gender, age, histological grade and pathological T and N stages and after internal validation employing a bootstrap procedure. A similar approach was taken by Milde-Langosch K et al. studying C1GALT1 and GALNT1 expression in breast cancer, considering that in both cases (breast cancer and

HNSCC), the enzymes are closely related in the glycan metabolic pathway [31].

FUT4 and ST3GAL4 genes lay on the 11q chromosome flanking a known usually deleted chromosomal region in HNSCC [32] (FUT4 at 11q21 and ST3GAL4 at 11q24.2, Suppl Fig. 3), which might be in accordance with our TCGA data analysis showing that ST3GAL4 was down regulated in tumors respect to normal adjacent tissue while FUT4 at least did not show any significant increment.

ST3GAL4 codifies for an α [2, 3]-sialyltransferase, which has been described as the main responsible for sialyl Lewis x expression and, consequently, for a selectin ligand in lymphoid and myeloid cells [33]. Interestingly, previous research detected a reduction in ST3GAL4 mRNA expression in tumor tissues in comparison to normal counterparts [34] which is in line with our present results, while other authors did not detect any change [35].

On the other hand, FUT4 codifies for an α(1,3/4)-fucosyltransferase which is involved in Lewis x synthesis [36] and it is expressed mainly in embryonic tissues and adult brain and leukocytes [37]. In most gene expression studies, FUT4 is upregulated in tumor tissues compared with its normal counterparts [38, 39]; also, it has been associated with cancer cell proliferation and regulation of apoptosis [40] in association with high Lewis y expression. On the other hand, FUT4 overexpression and α1,3-fucosylation have been linked to suppression of EGFR dimerization and phosphorylation upon EGF treatment [41], which might possibly explain its association with a better patient outcome.

The drawbacks in this study are related to the relative lower incidence of HNSCC in our country, which limited the number of subjects in the study group; also, HNC patients tend to drop-out from long observational studies. Finally, since many glycosyltransferases are involved in the generation of several antigenic determinants and their activities usually overlap, is difficult to address an enzyme-product association [42].

In conclusion, we have found an association of Lewis x expression with a better outcome in patients with HNSCC. Further research is needed in order to verify that FUT4 or ST3GAL4 glycosyltransferases deregulation is involved in the Lewis x synthesis in HNSCC and to assess the behaviour of Lewis x expression in response to chemotherapy or radiation therapy, particularly in resistant tumors.

Compliance with Ethical Standards

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed Consent Informed consent was obtained from all individual participants included in the study.

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