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Expression of the carbohydrate tumour marker Sialyl Lewis A, Sialyl Lewis X, Lewis Y and Thomsen-Friedenreich Antigen in normal squamous epithelium of the uterine cervix, cervical dysplasia and cervical cancer

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Summary. The carbohydrate molecules Sialyl Lewis X (SLeX), Sialyl Lewis A (SLeA), Lewis Y (LeY) and Thomsen-Friedenreich antigen (TF) are known to mediate the adhesion between tumor cells and endothelium. They are used as serum markers in diagnosis and treatment in a broad spectrum of human carcinomas, but their expression profile and role in the development of cervical cancer remains unclear. The aim of this study was to investigate the expression of SLeX, SLeA, LeY and TF in normal cervical squamous epithelium, cervical dysplasia and cervical cancer. Slides of paraffin-embedded tissue were fixed and incubated with monoclonal antibodies against SLeX, SLeA, LeY and TF. Immunohistochemical staining was evaluated by using a semi-quantitative score (IRS Score).

We found a significant difference of SLeA expression in invasive cervical cancer compared to normal epithelium (p=0.006) and all grades of dysplasia (p=0.002). The expression of SLeX in normal epithelium was less intense than in carcinoma in situ (p=0.036). Staining for LeY showed the weakest results of the investigated markers. Significant differences were found when normal epithelium was compared to CIN I (p=0.011), to CIN II (p=0.013) and to invasive cervical cancer (p=0.005). For TF, significant differences were found in normal epithelium compared to CIN I (p=0.011), CIN II (p=0.013) and compared to invasive cervical cancer (p=0.005).

This is the first study on the expression of SLeA, SLeX, LeY and TF in normal cervical endothelium, cervical dysplasia, carcinoma in situ and invasive cervical cancer. Further studies and higher numbers are desirable.

Key words: Tumor markers, Uterine cervical neoplasms, Cervical intraepithelial neoplasia, Carbohydrate antigens

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Introduction

Cervical cancer is found in about 15% of female cancers in developed countries (Lynge et al., 2009). About 80% of these arise from squamous cell dysplasia, whereas 15% are adenocarcinomas and 5% are clear cell adenocarcinomas (Bergauer et al., 2009). Knowledge of the molecular mechanisms underlying the development and metastasis of cervical cancer, except human papilloma virus infection, is limited (Liao et al., 2011). Therefore, the search for new marker molecules involved in the development and/or metastasis of cervical cancer is useful. Sialyl Lewis X (SLeX), Sialyl Lewis A (SLeA), Lewis Y (LeY) and Thomsen-Friedenreich antigen (TF) are carbohydrate molecules that mediate adhesion between tumour cells and endothelium. They are usually not expressed in non-malignant tissue. Malignant transformation is associated with abnormal glycosylation, resulting in synthesis and expression of altered carbohydrate determinants, including SLeX and SLeA, which are associated with hematogenous metastasis of cancer (Kannagi et al., 2004; Kannagi, 2004, 2007). The increased expression of carbohydrate determinants might be associated with an increase of UDP-galactose transporter mRNA as shown in colorectal cancer (Kumamoto et al., 2001). In

Japan, these determinants are used for serum diagnostics of cancer, but the literature review also shows several examples of immunohistochemical analyses of the expression of carbohydrate molecules in normal epithelial or tumour tissue: In squamous cell carcinoma of the larynx, SLeA shows a highly increased expression compared to normal or phlogistic laryngeal tissue (Wiest et al., 2010). The same results can be shown for breast cancer in regard to the expression of SLeA and SLeX (Jeschke et al., 2005). On the normal healthy epithelium of the penis and vagina an expression of SLeA, SLeX, Ley and TF can also be observed (Wiest et al., 2007).

However, the expression of carbohydrate molecules in cervical epithelium and cervical cancer as well as their role in tumour development remains unclear.

The aim of this study was to investigate the expression of SLeX, SleA, LeY and TF in normal cervical squamous epithelium, cervical dysplasia grade 1-3 (CIN I-III), carcinoma in situ and invasive cervical squamous cancer.

Materials and methods

Specimens

Tissue samples of normal cervical epithelium, mild, moderate and severe dysplasia, carcinoma in situ and invasive cervical carcinoma were obtained from the department of pathology, Ludwigs-Maximillians-University.

Immunohistochemistry

Immunohistochemistry on paraffin sections of the different tissue specimens was performed as described previously. The sections were incubated in methanol/H_2O_2 for 30 minutes to inhibit endogenous peroxidase activity, washed in PBS for 5 minutes and treated with goat serum for 20 minutes at 22°C to reduce non-specific background staining. Incubation with the primary antibody (Table 1) was done overnight at 4°C. The sections were then thoroughly incubated with the biotinylated secondary anti-mouse antibody for one hour at 22°C and with avidin-biotinylated peroxidase for 45 minutes at room temperature. Between each step, sections were washed with phosphate-buffered saline (PBS, pH 7.4). Peroxidase staining reaction was performed with diaminobenzidine/H_2O_2 (1 mg/ml) for five minutes and stopped by dipping the sections in tap water for 10 minutes. This was followed by counterstaining of the nuclei in sections with haemalaun solution for one minute and the sections were cover-slipped afterwards.

For negative control the primary antibody was replaced by pre-immune horse serum (see table 1 for used antibodies).

The intensity and distribution pattern of the immunochemical staining reaction was evaluated blindly by a pathologist, an attending physician with long experience in cytology and immunohistochemistry and two physicians with experience in immunohisto-chemistry and a strong research background. One slide per case and marker was evaluated by a magnification of 250x. In 15 cases (n=7,2%), the evaluation of the four observers differed. This case was the jointly re-evaluated by the observers. After the re-evaluation all observers came to the same result. The concordance before the re-evaluation was 193 (92,8%).

The specific immunohistochemical staining was evaluated by using a semi-quantitative method by Remmele and Stegner (Immunoreactive-Score IRS, Remmele and Stegner, 1987). Therefore, the staining intensity (SI) is graded as no staining=0, week staining=1, moderate staining=2 or strong staining=3 and the percentage of positively stained cells (PP) is defined as no staining=0, <10% of stained cells=1, 10-50% of stained cells=2, 51-80% stained cells=3 and >80% of stained cells=4. For calculating the IRS score, SI and PP are multiplied.

Slides were evaluated and digitalized with a Zeiss photomicroscope (Axiophot, Axiocam, Zeiss, Jena, Germany).

Statistics

Statistical analysis was performed using SPSS 18.0 (PASW Statistic, SPSS Inc., IBM, Chicago, IL). Analysis of the tumour marker expression in normal epithelium, all degrees of dysplasia and invasive cancer was performed using the non-parametric Kruskal-Wallis rank-sum test and analysis of two independent variables was performed using the Mann-Whitney test.P values below 0.05 were considered statistically significant.

Results

We aimed to investigate 10 samples each of normal squamous epithelium, mild dysplasia, moderate dysplasia, severe dysplasia, carcinoma in situ and invasive cervical cancer. 32 out of 240 samples (13%)

Table 1. Antibodies used for immunohistochemistry.

Antigen	Antibody	Isotype	Source
Sialyl Lewis A	KM231	Mouse IgG1 2 µg/ml	Calbiochem
Sialyl Lewis X	KM93	Mouse IgM 2 µg/ml	Calbiochem
Lewis Y	A70-C/C8	Mouse IgM 2 µg/ml	Glycotope GmbH
Thomsen-Friedenreich	NemodTF1/2	Mouse IgM 2 µg/ml	Glycotope GmbH

were excluded as not being valid for evaluation (e.g., because no intact epithelium was shown on the slide, insufficient staining reaction). For SLeA 49 of 60 samples (82%) were eligible for evaluation, for SLeX 54 of 60 (90%), for LeY 50 of 60 (83%) and for TF 55 of 60 samples (92%).

In the following we refer to "mild staining/expression" for a Remmele score of 0-4, to "moderate staining/expression" for a Remmele score of 5-8 and to "strong staining/expression" for a Remmele score of 9-12. Table 2 shows the number of patients for each antibody regarding normal epithelium, different degrees of dysplasia and invasive cervical cancer as well as median scores.

Expression of SLeA (median scores shown in Fig. 1A)

Of all the investigated markers SLeA expression showed the highest median scores and the strongest variability. The difference of SLeA expression in the different degrees of dysplasia was significant (p=0.019). Normal epithelium (Fig. 1B), CIN II, and carcinoma in situ showed a moderate median expression, CIN I and CIN III (Fig. 1C) showed a strong expression, and invasive cervical cancer (Fig. 1D) expressed SLeA only mildly. We found a significant difference of expression in normal epithelium compared to invasive cervical cancer (p=0.006) and of the expression in all grades of dysplasia compared to invasive cervical cancer (p=0.002).

Expression of SLeX (median scores shown in Fig. 2A)

The median expression of SLeX was moderate in all the different tissue types. Normal epithelium (Fig. 2B) and CIN II showed the least expression with a median score of 5, CIN III and carcinoma in situ (Fig. 2C) showed the highest expression with a median score of 7.

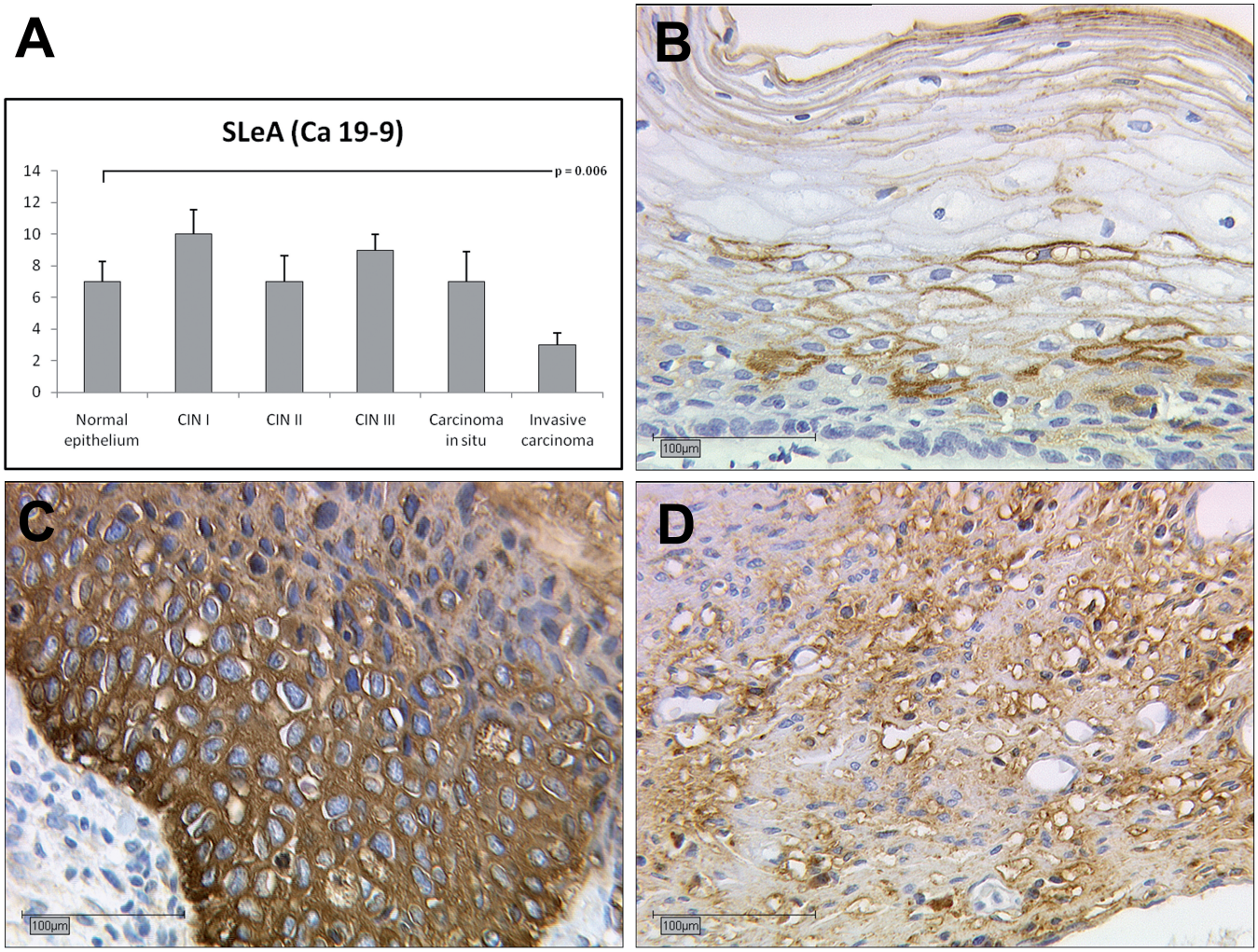

Fig. 1. Median scores of SLeA (Ca 19-9) expression ranged from 3-10 evaluated by IRS. The difference of SLeA expression in the different degrees of dysplasia was significant **(A)**. Normal epithelium showed a median score of 7 **(B)** and was lower compared to staining in CIN III (median score 9, **C**). In invasive cervical cancer the median score decreased to 3 **(D)**. Error bar in 1A denotes standard error of mean. x 250

Invasive cervical cancer showed a median expression of 6 (Fig. 2D). The difference of expression in normal epithelium compared to carcinoma in situ was significant (p=0.036).

Expression of LeY (median scores shown in Fig. 3A)

Staining for LeY showed the weakest results of the investigated markers, but the difference of LeY expression in the different samples was significant (p=0.042). All samples from normal epithelium to invasive carcinoma only showed a mild expression. Normal epithelium (Fig. 3B) and CIN II showed the least expression with a median score of 2; CIN III (Fig. 3C), Carcinoma in situ and invasive cervical cancer (Fig. 3D) showed the highest expression with a median score of 4. Significant differences in marker expression were found when normal epithelium was compared to CIN III (p=0.048), normal epithelium was compared to carcinoma in situ (p=0.040) and compared to invasive cervical cancer (p=0.021). Normal epithelium compared to all grades of dysplasia also showed a significant difference (p=0.047).

Expression of TF (median scores shown in Fig. 4A)

The difference of TF expression in the different subtypes was significant (p=0.041). Normal epithelium (Fig. 4B) showed mild staining, whereas CIN III (Fig. 4C), carcinoma in situ and invasive carcinoma (Fig. 4D) showed moderate staining for TF. CIN I and CIN II showed a strong expression. The least expression showed normal epithelium with a median score of 4 and the highest median score of 9 showed CIN I and CIN II. Significant differences were found when normal epithelium was compared to CIN I (p=0.011) and

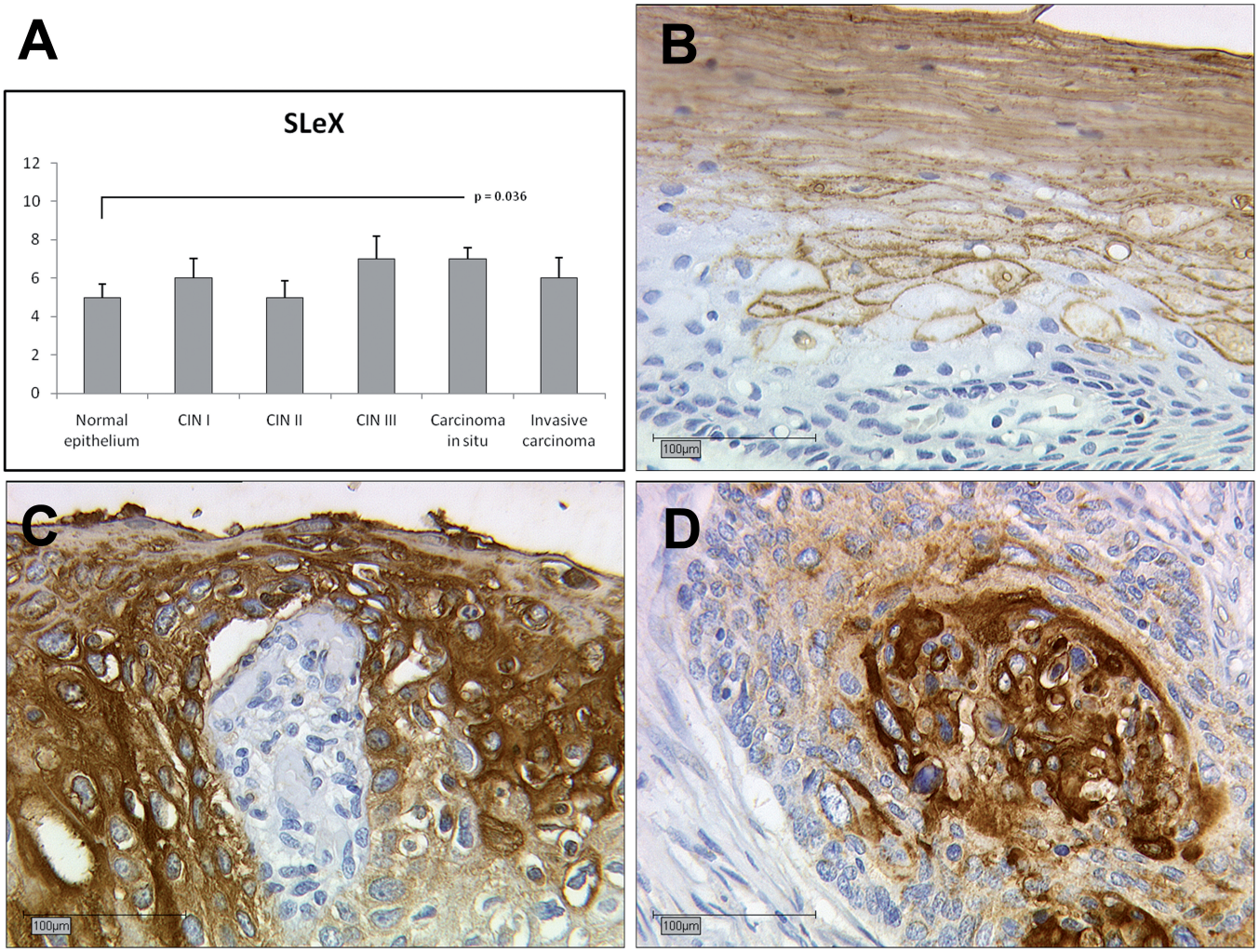

Fig. 2. Median scores of SLeX expression ranged from 5-7 evaluated by IRS **(A)**. Only the difference of expression in normal epithelium **(B)** compared to carcinoma in situ **(C)** showed significant results. SLeX expression in invasive cervical cancer is shown in **(D).** Error bar in 2A denotes standard error of mean. x 250

compared to CIN II (p=0.013), and when normal epithelium was compared to invasive cervical cancer (p=0.005). The comparison of normal epithelium and CIN III was borderline significant (p=0.052). All grades of dysplasia compared to normal epithelium also showed significant differences (p=0.006).

Discussion

To identify new markers for the development of cervical dysplasia and carcinogenesis, we investigated the combined expression of SLeA, SLeX, LeY and TF. Normal cervical endothelium, tissue of cervical dysplasia, carcinoma in situ and invasive cervical cancer tissue blocks were analysed.

As the tissue samples for this study were obtained retrospectively, there were samples diagnosed as CIN 3 and others diagnosed as Carcinoma in situ; the WHO international Classification of Tumours separated these entities as "dysplasia extending into the upper third of the epithelium but not involving the full thickness" versus "involving the full thickness" (Scully and Bonfiglio, 1978). As these histologic differences are often quite subtle, intra- and interobserver variability is high and both diagnoses are equally monoclonal proliferations of abnormal squamous epithelium with aneuploid DNA content, the current nomenclature makes no difference (Kurman, 2002).

SLeA and SLeX are natural ligands for selectins (Munro et al., 1992; Paavonen and Renkonen, 1992; Kannagi, 2007) and selectins are glycoproteins that are in turn specifically recognized by the tetrasaccharides SLeX and SLeA. There are different types of selectins which are expressed on leukocytes (L-selectin) (Green et al., 1992), platelets (P-selectin) (Foxall et al., 1992) and endothelial cells (E-selectin) (Erbe et al., 1992). These selectins are often involved in the development of hematogenous metastases (Wiest et al., 2007). SLeA and

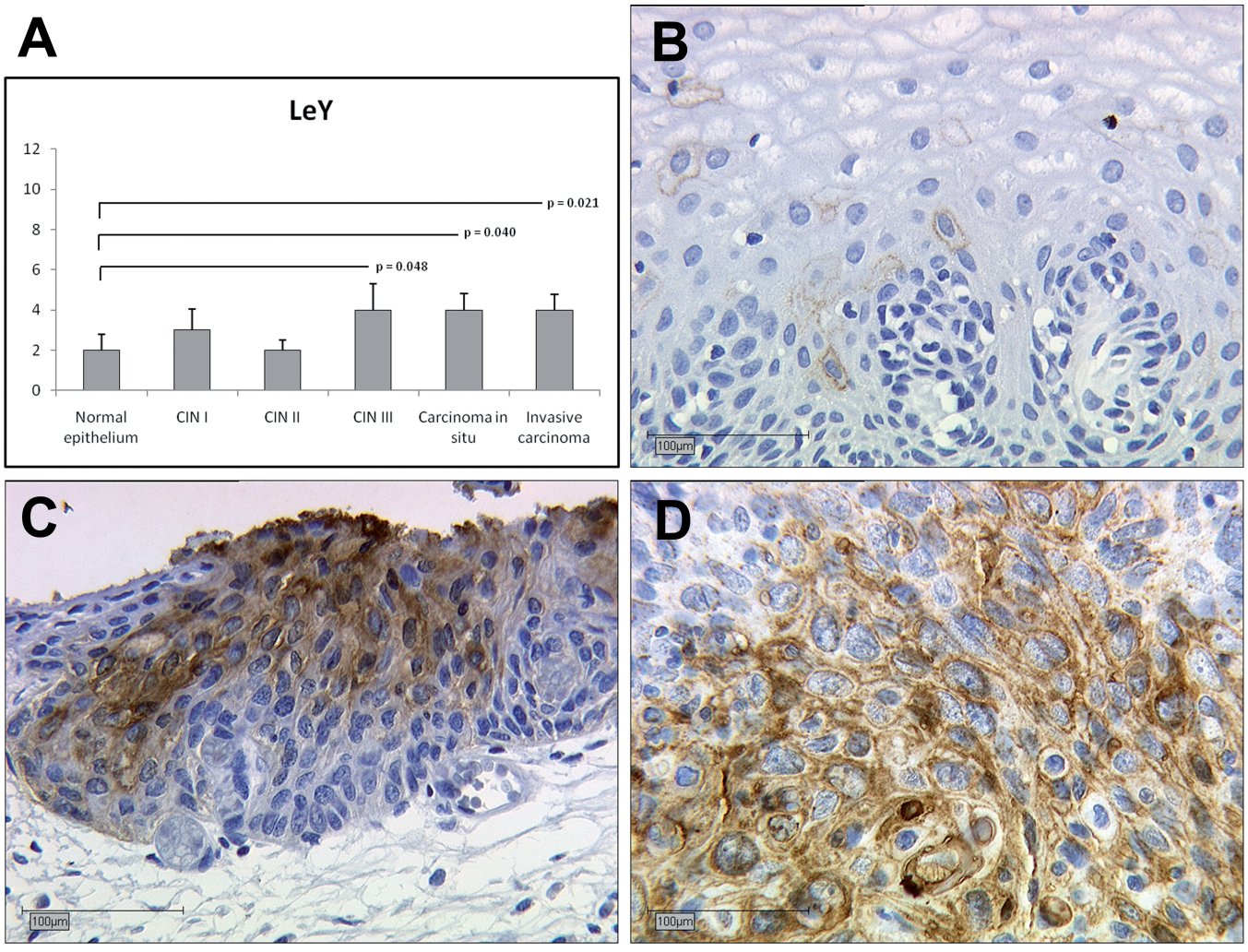

Fig. 3. Staining for LeY showed the weakest results of all markers. Median IRS scores of LeY expression ranged from 2-4 (**A**). Significant differences in marker expression were found when normal epithelium **(B)** was compared to CIN III **(C).** The latter, carcinoma in situ and invasive cervical cancer **(D)** showed the same expression with a median score of 4. Error bar in 3A denotes standard error of mean. x 250

SLeX were found not to be expressed on normal tissue until Wiest et al. showed that they are also expressed by vaginal epithelium and in penis glans and shaft squamous epithelium. This was surprising and cannot easily be explained. It seems possible that SLeA and SLeX mediate the adhesion of fertility related antigens. In our study we found a mild to strong expression of SLeA and SLeX of all samples from normal epithelium to invasive cervical cancer. The expression in normal epithelium is thus not surprising since the cervical epithelium is also involved in the process of human reproduction. There was no clear tendency of both markers towards up or down regulation on the way to tumour progression. Interestingly, the expression of SLeA was less in invasive carcinomas compared to the other tissue types. This might represent the loss of physiological function during tumour development. When observing with the main focus not on quantitative but qualitative staining patterns it was conspicuous that in invasive carcinoma samples the tissue surrounding vessels or areas of tumour necrosis were intensively stained which might stress a role in the process of hematogenous metastasis (possible mediation of adhesion to vessel endothelium which enables tumour cells to invade the vessel lumen or conversely phagocytes to evade and clear away necrotic debris). Compared to SLeA, SLeX showed a less intensive expression in all samples, but the different expression of invasive carcinomas and the other samples was not as potent as with SLeA.

LeY is thought to behave as an onco-developmental cancer-associated antigen and its oligosaccharide structure may play a key role in lymphocyte traffic and inflammatory response (Shur, 1983; Kuijpers, 1993; Wakabayashi et al., 1995; Wiest et al., 2007). It was shown that the LeY antigen correlates to the cancer-associated antigen in colorectal lesions and in uterine cervical cancer (Abe et al., 1986; Umezaki et al., 1997);

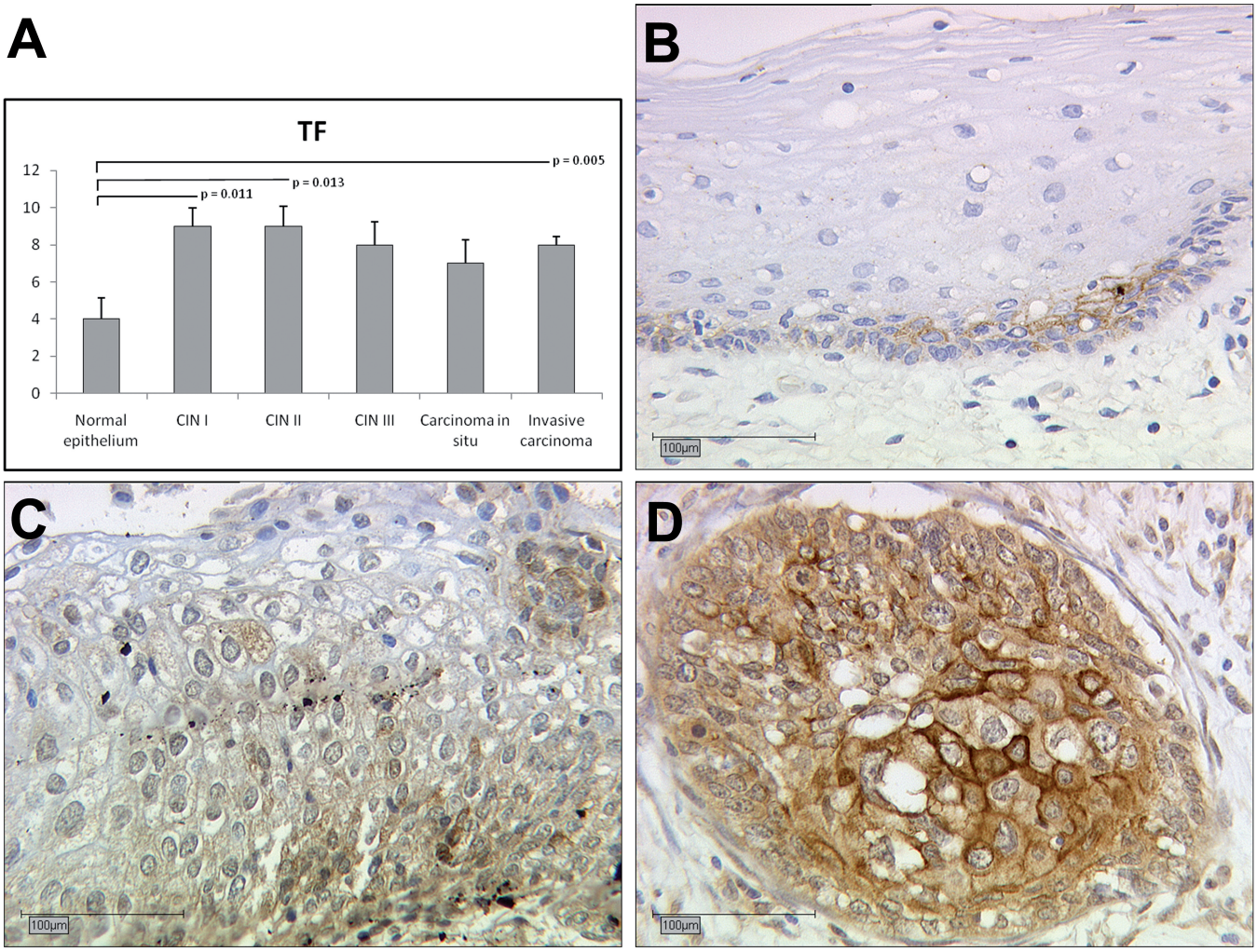

Fig. 4. TF expression was significant in the different degrees of dysplasia, median IRS scores of TF expression ranged from 4-9 **(A).** Normal epithelium **(B)** did not express TF significantly different compared to CIN III **(C)**, but did when compared to invasive cervical cancer **(D)**. Error bar in 4A denotes standard error of mean. x 250

its presence is associated with oncogenesis in adenocarcinoma of the cervix (Umezaki et al., 1995). Circulating Lewis antigens can be acquired by red cells, lymphocytes and platelets (Henry et al., 1995). In our study we found a rather weak staining in all different tissue types and a tendency towards a higher expression in dysplastic or tumour tissue compared to normal epithelium except for CIN II.

Like SLeA and SLeX, TF was thought to be only expressed in tumour tissue until it was found to be expressed by normal penis epithelium, by foetal epithelia, mesothelia, amniotic fluid and trophoblast.

TF has long been recognized as a tumour-associated antigen in primary and metastatic carcinomas that are by definition of epithelial origin. In tumours that originate from other tissues, TF is masked and not accessible to the immune system. However, in carcinomas TF is uncovered and immunoreactive (Springer, 1984; Barr et al., 1989; Hanisch and Baldus, 1997; Van Rooijen et al., 1998; Streu et al., 2000; Yu, 2007). It seems that all humans possess anti-TF antibodies, but patients with breast cancer for instance showed a statistically significant alteration of titer levels. TF is known to be expressed in a variety of cancers such as breast, lung, liver and colorectal cancer, but previous studies about the expression of TF in cervical cancer could not demonstrate a positive expression (Carrilho et al., 2000; Lin et al., 2011; Schindlbeck et al., 2005, 2007). Wiest et al. (2007) concluded that the presence of immunoreactive TF during an early phase of fetal development and its known absence in noncarcinomatous postfetal tissues suggests that TF is a stage-specific oncofetal antigen in pretolerogenic differentiation phase. In contrast to Carrilho et al, we found a mild expression of TF in normal cervical epithelium, but a strong expression in cervical dysplasia grade I and II and a moderate expression in cervical dysplasia grade III, carcinoma in situ and invasive carcinomas. Even though we only investigated small numbers, the results demonstrate that TF is not as highly expressed in healthy tissue compared to invasive cervical carcinomas and their precursor lesions.

This is the first study on the expression of SLeA, SLeX, LeY and TF in normal cervical endothelium, cervical dysplasia, carcinoma in situ and invasive cervical cancer. Within this rather small study cohort SLeA, LeY and TF could differentiate between normal epithelium and invasive cervical cancer. SLeX did not show much variation of expression, but showed significant differences between normal epithelium and Carcinoma in situ. LeY in general showed a rather weak expression with a tendency to higher expression in dysplastic or tumour tissue and TF was expressed higher in all tissues from CIN I to invasive carcinomas. These two antigens might be able to differentiate between normal and dysplastic tissue. Due to small numbers, further studies are needed to describe in more detail possible functions in the course of physiologic or pathologic events or the application as a diagnostic tool.

Table 2. Number of patients and median scores for each antigen.

	Sialyl Lewis A	Sialyl Lewis X	Lewis Y	Thomsen-Friedenreich
Normal epithelium				
Number of Patients	9	10	10	10
Median Score	7	5	2	4
Range	4-12	0-6	0-8	0-12
Mild dysplasia (CIN I)				
Number of patients	7	8	8	8
Median Score	10	6	3	9
Range	2-12	2-12	0-9	4-12
Moderate dysplasia (CIN II)				
Number of patients	8	9	8	9
Median Score	7	5	2	9
Range	1-12	1-9	0-4	3-12
Severe dysplasia (CIN III)				
Number of patients	9	9	8	9
Median Score	9	7	4	8
Range	4-12	2-12	1-12	3-12
Carcinoma in situ				
Number of patients	7	9	9	10
Median Score	7	7	4	7
Range	2-12	4-9	0-6	1-12
Invasive carcinoma				
Number of patients	9	9	7	9
Median Score	3	6	4	8
Range	0-6	2-12	1-6	8-12

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