Expression of the Tumor-associated Mucin 1 Epitope Analyzed with the Humanized PankoMab-GEX™ Antibody in Malignant and Normal Tissues of the Head and Neck

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Abstract. *Background: During neoplasia, glycosylation changes. In this setting, mucins, especially mucin 1 (MUC1), become carriers for oncofetal carbohydrates and relieve invasive growth. The recently described tumor-associated MUC1 epitope TA-MUC1 is primarily restricted to malignancies and is overexpressed in these tissues. The humanized monoclonal antibody PankoMab-GEX specifically recognizes TA-MUC1. Materials and Methods: Laryngeal cancer specimens (n=125) and normal tissue of head and neck (n=7) were used in this study. Paraffin-embedded sections were incubated with PankoMab-GEX. Staining reaction was carried out using peroxidase (POD) labeling and diaminobenzidine (DAB). Breast cancer tissue was used as positive control and negative control used non-specific mouse IgM. Semi-quantitative evaluation by two independent double-blinded investigators, including a pathologist, used the immunoreactive score (IRS) of Remmele and Stegner. Results: A total of 31 out of 125 laryngeal cancer specimens were classified as G1. Of these, 22 (71%) were completely negative for TA-MUC1, the remaining 9 showed very weak staining, with an IRS of 2. A total of 94 cases of cancer specimens were classified as G2 and G3; 34 of them were also negative, but 60 had an IRS of up to 9. All investigated normal tissue of the upper aerodigestive tract was completely negative for TA-MUC1. Conclusion: G1 tumors are completely negative or do not reach an IRS relevant range. The finding that G1 tumors are completely negative for TA-MUC1 or have IRS≤2 can be helpful for histopathological examination, especially concerning tumor grading. Therefore, this antibody holds great potential for use as a therapeutic antibody in laryngeal cancer.*

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Mucins are large membrane-bound glycoproteins. The extracellular part of mucins can be found on epithelial luminal surfaces of a variety of cells, functioning in hydration and protection (1). In addition, mucins are thought to act as cell-signaling molecules (2). Mucins consist of a so-called tandem repeat protein core that varies in the number of repetitions. These tandem repeat units are heavily *O*-glycosylated (3). The degree of glycosylation of mucins is central to their role in tumors (3, 4). During genesis of malignant neoplasms, the glycosylation pattern is altered (3, 5). In this setting, mucins, especially mucin 1 (MUC1), becomes a carrier protein for oncofetal carbohydrates and relieves invasive growth (6, 7).

Some epitopes of MUC1 which are shed to the serum are already used as tumor markers, with a high clinical relevance for diagnosis and follow-up treatment (8, 9). The MUC1 glycan epitope sialyl Lewis a, better known as CA19-9 (10), has been applied in a variety of tumor types (11, 12). MUC1-derived oncofetal glycan epitopes appear mainly in tumors. There are only a few cases where they are expressed in normal adult tissue, such as in the reproductive tissues (13). In contrast, the recently described tumor-associated MUC1 epitope (TA-MUC1) is primarily restricted to malignancies and is overexpressed in those tissues (14). Furthermore and in contrast to the common tumor marker epitopes, TA-MUC1 remains adhered to the cell membrane (15). This is the main basis for the development of specific antibody-based therapies.

The recently established humanized MUC1 antibody PankoMab-GEX™ specifically and exclusively recognizes TA-MUC1 (14). In comparison to all other MUC1 antibodies, it has the highest dependency on glycosylation, the greatest antigen binding capacity and therefore the strongest tumor correlation (14). Besides its potent antibody-dependent cellular cytotoxicity, PankoMab-GEX™ interferes with the cell cycle by blocking or masking MUC1 by binding to it (16-18). It can, therefore, influence different signaling pathways because the intracellular part of MUC1 is an active receptor tyrosine kinase (RTK) (2). During tumor progression, MUC1 loses its apical polarity and is spread all over the cell surface (19). Hence its dimerization with all other RTKs spread over the whole cell surface becomes possible, as has been shown with members of the human epidermal growth factor receptor 2 (ERB-B2) protein family (2, 20).

Tumors of head and neck are the sixth most frequent worldwide (21). Squamous cell carcinoma of the larynx is the most common malignancy of the head and neck (22). Despite improvements in the fields of surgery and radiotherapy, delayed diagnosis is still common worldwide.

TA-MUC1 is a good target for antibody therapy in combination with its perfectly matching humanized antibody PankoMab-GEX™. This already has been confirmed in phase I and II clinical trials for patients suffering from ovarian cancer (Glycotope GmbH, Berlin, Germany 2013) (23, 24). The aim of this study was to determine if this therapy design might also be suitable for other malignancies such as laryngeal cancer. There is neither a proper diagnostic tumor marker nor a really appropriate therapeutic antibody for this type of cancer.

Materials and Methods

Study population. Laryngeal carcinoma specimens from 128 patients were obtained after surgery and histological classification including TNM staging (25). Thereby 30 cases were classified as G1, 58 as G2 and 40 as G3. All follow-up data were well documented and shown in Table I.

Normal tissue, such as tongue, vocal cord, larynx, pharynx and epiglottis, was taken from autopsies from the Department of Legal Medicine of the Friedrich Alexander University Erlangen (n=7). Omission of any kind of cancer was assured. The Ethics Committee of the Friedrich Alexander University of Erlangen Nuremberg approved this study with the title: "Prognostic relevance of the MUC-1 oncoprotein in laryngeal carcinoma" on the 20th of July 2012.

Antibody and immunohistochemistry. Peroxidase-labeled humanized monoclonal antibody PankoMab-GEX™ (Glycotope GmbH, Berlin, Germany) was used at a concentration of 2.7 μg/ml.

Immediately after surgery or autopsy, tissue specimens were formalin fixed and subsequently embedded in paraffin. Paraffin sections of 3 μm were prepared and provided for immunohistochemistry by heating them at 55˚C overnight. Slides were deparaffinized and rehydrated stepwise in ethanol. No antigen retrieval was necessary, but endogenous peroxidase activity was blocked by 3% H_2O_2 in methanol for 20 min. Nonspecific binding sites were of no consequence because of the purity of the antibody. The sections were incubated with PankoMab-GEX™ (2.7 μg/ml) for 90 min at room temperature. Color development was carried out using diaminobenzidine and counterstaining with hematoxylin. Ovarian and breast cancer specimens were used as positive control and omission of the specific antibody as well as incubation with bovine serum as negative controls.

Slides were analyzed blindly by two different investigators who evaluated staining according to the immunoreactive score (IRS) of Remmele and Steger (26). The scores for intensity of staining and the percentage of positively stained cells were multiplied for evaluation. The intensity and distribution pattern of the immunochemical staining reaction were evaluated by two independent blinded observers. In two cases, the evaluation of the two observers differed. These cases were re-evaluated by both observers together. After the re-evaluation, both observers came to the same result.

Statistical analysis. The SPSS/PS software package, version 16.0 (SPSS GmbH, Munich, Germany), was used for collection, processing and statistical analysis of data. Correlation between the IRS of tumor specimens and histological grading was analyzed using the Mann–Whitney *U*-test and the Kruskal–Wallis test. Statistical significance was reached at $p<0.05$.

Table I. *Clinical characteristics of the study population (n=125).*

	No.	%
Gender		
Male	117	94
Female	8	6
HPV-positive		
No	100	80
Yes	25	20
Tumor size (T)		
1	25	20
2	50	40
3	20	16
4	30	24
Tumor grade		
I	31	25
II	54	43
III	40	32
Tumor progression		
No	95	76
Yes	30	24
Outcome		
Alive	90	72
Died	35	28

Results

Positive control staining. Human epithelia cancer tissue was used as positive control tissue. We identified intense staining of TA-MUC1 in breast cancer (Figure 1A) as well as ovarian cancer tissue (data not shown).

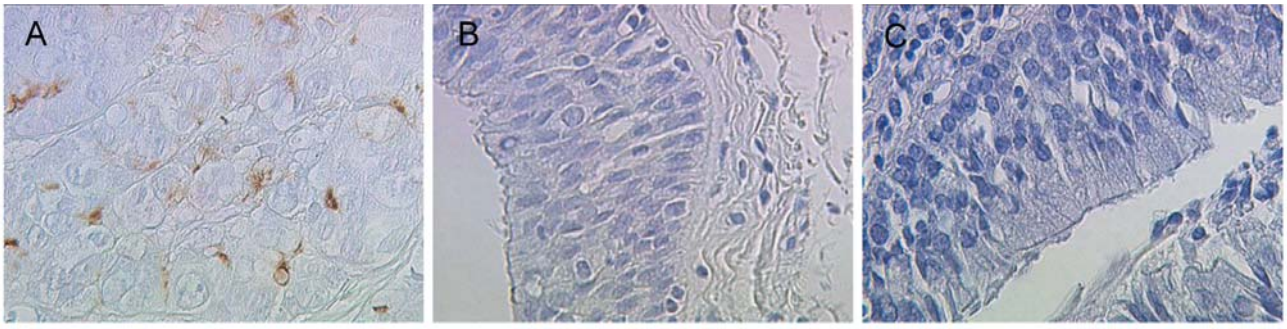

Figure 1. *Breast cancer tissue shows focal but intense expression of tumor-associated mucin-1 (TA-MUC1, A). Laryngeal (B) as well as normal vocal cord (C) tissues did not stain for TA-MUC1 and demonstrated the tumor specificity of the PankoMab-GEX™ antibody. Original magnification, ×40.*

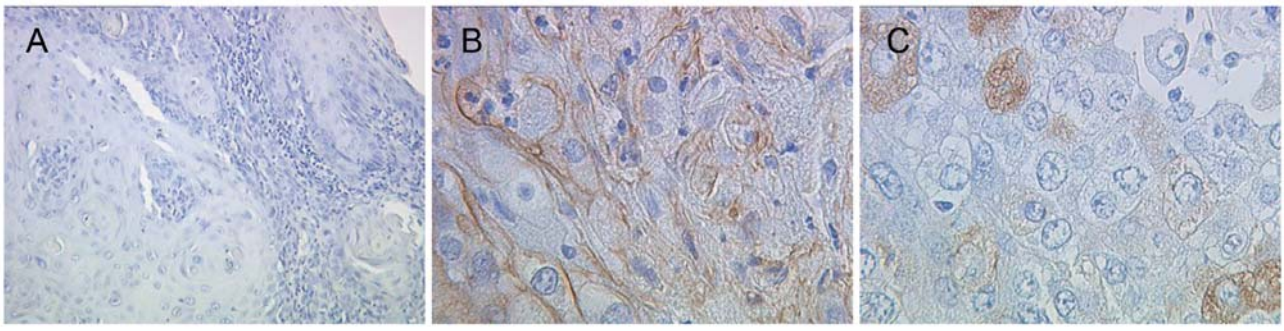

Figure 2. *Staining using PankoMab-GEX™ in relation to tumor grading. G1 tumors did not stain with an immunoreactive score (IRS) higher than 2 (A). In contrast to G1 tumor tissue, we found enhanced staining of G2 (B) and G3 tumor specimens (C). Original magnification, ×40.*

Evaluation of TA-MUC1 in normal tissue of the head and neck. All normal tissues of the upper aerodigestive region, such as the larynx (Figure 1B), vocal cord (Figure 1C), pharynx, tongue and epiglottis (not shown), remained completely negative for staining of TA-MUC1.

Evaluation of TA-MUC1 in laryngeal cancer specimens. A total of 22 cases out of 31 G1 laryngeal tumors (71%) were completely negative (IRS=0) (Figure 2A), while the remaining 11 did not reach an IRS greater than 2. None of the IRS for G1 tumors were clinically relevant. In contrast, all G2 (Figure 2B) and G3 (Figure 2C) tumors (n=94) showed enhanced TA-MUC1 staining. G2/G3 graded tumor specimens did not differ in the range of the IRS. A total of 36% (n=34) of the G2/G3 tumors were negative (IRS=0), but 64% (n=60) were positive (IRS up to 9).

Statistical evaluation of the staining results. The expression of TA-MUC1 significantly positively correlated with tumor grading (p=0.001). G1 tumors exhibited significantly less staining compared to G2 (p=0.001) and G3 (p<0.001) carcinomas (Figure 3).

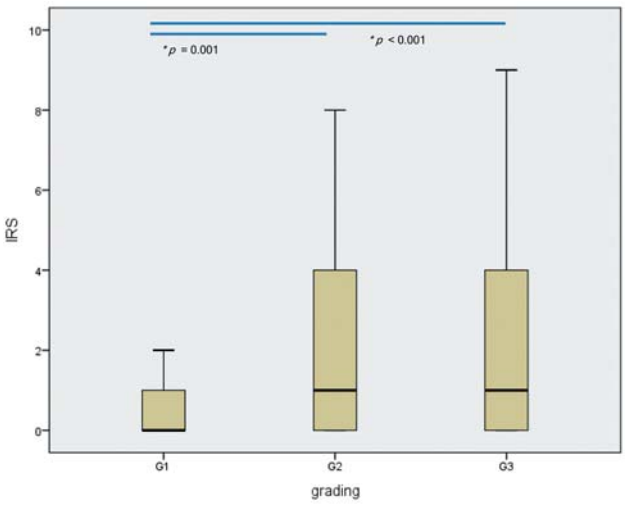

Figure 3. *Statistical evaluation of immunoreactive score (IRS) in relation to tumor grading as shown in box-plot analyses. Significant differences were identified in staining with PankoMab-GEX™ between G1 and G2 tumor tissues (p=0.001) and G1 and G3 tumor tissues (p<0.001). The presentation of staining results is shown as Box-plot. The range between the 25th and 75th percentiles is represented by the boxes with a horizontal line at the median. The bars show the 5th and 95th percentiles.*

Discussion

In this study, we demonstrated the diagnostic potential of the humanized antibody PankoMab-GEX™ for the detection of TA-MUC1 in laryngeal tumors. The results of our study showed a strong correlation of TA-MUC1 with grading of these tumors. Its significant staining according to tumor grading could be used as an additional tool in histopathological decision-making. Histological grading can vary between different pathologists (27). Classifying a tumor is important for treatment and the whole follow-up. A lack of or very weak staining by PankoMab-GEX™ is a good indicator of well-differentiated tumor and therefore of better prognosis.

A very important result of our study is the finding that TA-MUC1 is restricted to malignancies in the head and neck area. In this study, normal tissue was obtained from legal autopsies from differently aged individuals who did not suffer from any tumor. All of the normal material remained completely negative on staining with PankoMab-GEX™. Therefore we assume that PankoMab-GEX™ is able to distinguish between normal and malignant cells. This is a major prerequisite for an antibody in order for it to be used as a future candidate for therapeutic studies of patients with laryngeal tumor.

The number of patients suffering from laryngeal cancer has increased. Especially in economically developed countries, we have seen a major increase probably because of rising tobacco and alcohol consumption (28-30). Unfortunately, laryngeal cancer is diagnosed in an advanced state of the disease in the majority of patients. A short time interval between identification of the tumor and start of treatment and therapy contributes to better outcomes (31, 32). Simultaneous cisplatin-based radiochemotherapy is the most frequently applied non-surgical treatment. In comparison to radiotherapy alone and induction therapy with cisplatin–fluorouracil, combined therapy led to improved locoregional control and laryngeal preservation; unfortunately there was no significant improvement in overall survival (33).

Recently, the therapy of poorly differentiated advanced laryngeal carcinoma has been associated with or followed by use of the epidermal growth factor inhibitor cetuximab, which is a chimeric monoclonal antibody. The addition of cetuximab to standard therapy increased median progression-free and gave an overall survival benefit up to almost three months, but also causes negative side-effects, such as anemia, neutropenia, thrombocytopenia, sepsis and skin reactions (34, 35).

Targeted antibody therapies have become an essential part of treatment of a variety of malignancies (36-38). Most popular is the application of trastuzumab, a humanized monoclonal antibody used in combination with standard chemotherapy that prolongs survival and substantially enhances the quality of life of patients with ERB-B2-positive breast cancer (39, 40). Regrettably an eminent number of patients develop resistance to trastuzumab (41, 42). In the recent past, some groups demonstrated a turnaround to trastuzumab sensitivity *in vitro* by application of MUC1 antagonists (43, 44). MUC1 is a favored binding partner for members of the ERBB family (2, 45). But MUC1 itself, as a self-contained RTK, is an appropriate target for antibody therapies because it affects different pathways (18, 46, 47). In particular, TA-MUC1 is very suitable because it is nearly exclusively expressed in malignancies (14, 23). The matching antibody hPankoMab-GEX™ has already been tested in clinical trials for ovarian cancer and gave good results, but it might also have efficacy against further tumor types (15, 48).

In this study, we demonstrated a universal absence of TA-MUC1 in all normal tissues of the upper aerodigestive tract, but its overexpression in the worst-graded tumors. These data show that PankoMab-GEX™ holds the potential for use in a targeted therapy for laryngeal cancer, even in patients with high-grade tumors.

Conflicts of Interest

The Authors declare no conflict of interest in regard to this study.

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