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Staining of MUC1 in ovarian cancer tissues with PankoMab-GEX™ detecting the tumour-associated epitope, TA-MUC1, as compared to antibodies HMFG-1 and 115D8

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Summary. PankoMab-GEX™ is a novel humanized and glycooptimized antibody, which recognizes a novel specific tumour epitope of MUC1 (TA-MUC1). The aim of this study was to evaluate PankoMab-GEX™ binding to a variety of ovarian cancer specimens (n=156) and to normal ovarian tissue. In addition, PankoMab-GEX™ staining was compared to that of the well-known anti-MUC1 antibodies HMFG-1 and 115D8. PankoMab-GEX™ showed positive reactivity in serous (100% of cases, mean IRS 8.23), endometrioid (95% of cases, mean IRS 6.40), mucinous (58% of cases, mean IRS 4.17), and clear cell (92% of cases, mean IRS 7.58) carcinomas. In contrast to HMFG-1, healthy ovarian tissue was not recognized by PankoMab-GEX™. Staining with antibody 115D8 was increased with staging. Cytoplasmic PankoMab-GEX™ staining increased with tumour grade, but no correlation was found with staging. Univariate Kaplan-Meier analysis revealed a tendency of reduced survival of patients with high expression of TA-MUC1. The findings are encouraging with respect to a potential use of PankoMab-GEX™ as a new therapeutic antibody for the treatment of ovarian cancer patients.

Key words: Ovarian cancer, MUC1, PankoMab-GEX™ immunohistochemistry, Tumour specificity, Prognosis

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Introduction

PankoMab-GEX™ is a novel humanized IgG1 antibody explicitly tailored to recognize a special tumour-associated epitope on MUC1, TA-MUC1. Its specificity and affinity equals that of its chimaeric and murine version described by Danielczyk et al.(2006). PankoMab-™ has three modes of action against tumor cells (including stem cells), ADCC, phagocytosis and apoptosis induction. PankoMab-GEX™ was glyco-optimized by the GlycoExpress™ platform using human glycoengineered production cell lines in order to enhance ADCC activity. Its epitope consists of a special carbohydrate-induced conformation of the PDTRP motif (Karsten et al., 1998, 2004, 2005) wherein the tumor-specific carbohydrate antigens Tn or TF play a key role (Danielczyk et al., 2006). The Tn or TF glycan is an absolute requirement for the binding of PankoMab-GEX™ to the PDTRP motif of MUC1. PankoMab-GEX™ offers high tumour selectivity, making it very attractive as a therapeutic antibody, which is currently developed in clinical trials.

In a recent study, we demonstrated that the murine antibody corresponding to PankoMab-GEX™ (mPankoMab) may be suitable as a diagnostic antibody in breast cancer. In contrast to the antibodies DF3 and VU-4-H5, mPankoMab reactivity revealed a strong correlation with the expression of the estrogen receptor (ER). No correlation to lymph node involvement was found. However, in this study, the localization of the antigen (membrane versus cytoplasm) was not considered (Dian et al., 2009). mPankoMab has also been shown to be a prognostic marker in lung cancer

(Kuemmel et al., 2009).

For *in vivo* therapeutic applications, the acquisition of data concerning the distribution of TA-MUC1 on different types of cancer is important. In a recent study, the reactivity of PankoMab-GEX™ with a number of different human cancer types such as lung, breast, gastric, colorectal, liver, cervical, thyroid, and others (including several non-epithelial tumors) was investigated (Fan et al., 2010).

In the present study we report on the binding of PankoMab-GEX™ to ovarian cancer and on possible correlations with histological grading, staging, and overall patient survival. Furthermore, PankoMab-GEX™ staining was compared to two other, well-known anti-MUC1 specific mouse monoclonal antibodies, HMFG-1 and 115D8, on both neoplastic and healthy tissues.

Materials and methods

Specimens

Formalin-fixed paraffin-embedded tissues from ovarian cancer patients undergoing surgery at the Department of Obstetrics and Gynaecology - Maistrasse of the Ludwig- Maximilians-University of Munich in the period between the years 1992 and 2002 was investigated. All specimens had histological classification as either serous, mucinous, endometriod or clear cell ovarian cancer by a gynaecological pathologist (D.M.). Grading was also done by a gynaecological pathologist. Out of 156 ovarian cancer cases (Table 1), 93 were graded as low Grade (G1 and G2) and 53 as high grade (G3). Data regarding clinical staging, survival and recurrence-free survival were obtained from the Munich Cancer Registry. Control ovarian tissue came from pre-menopausal (n=10), non pregnant individuals who underwent gynaecological surgery by hysterectomy with salpingo-oophorectomia at the Department of Gynaecology and Obstetrics between 1997 and 2002.

Antibodies

The humanized antibody PankoMab-GEX™ was provided by Glycotope GmbH, Berlin, Germany. For comparison, two commercial murine MUC1 antibodies, HMFG-1 (Burchell et al., 1983) and 115D8 (Zotter et al., 1987), were employed.

Immunohistochemistry

Paraffin sections (3 μm) of ovarian carcinoma tissues were dewaxed for 15 minutes in xylol and incubated for 2 minutes in absolute ethanol. To inhibit endogenous peroxidase activity the tissue slides were treated with 3% hydrogen peroxide in methanol for 20 minutes. Afterwards the tissue slides were rehydrated in a descending series of alcohols. They were washed twice in PBS and incubated with Power Block™ (BioGenex, Fremont, CA, USA) for 3 minutes (PankoMab-GEX™) or horse serum (HMFG1 and 115D8) for 20 minutes. After decanting the blocking solution, the tissue sections were covered with HMFG1 or115D8 for 16 hours overnight, or PankoMab-GEX™ for 60 minutes at room temperature. The slides were washed again with PBS for 2 x 5 minutes. Bound primary antibodies were visualized with 3.3'-diaminobenzidine (Dako, Glostrup, Denmark) for 2 minutes at room temperature. The staining reaction was stopped with distilled water. Counterstaining was done with Mayer's acidic hematoxylin for 2 minutes and tap water for 5 minutes. The tissue slides were dehydrated by an ascending series of ethanol, and coverslipped.

In controls the primary antibody was replaced with normal mouse serum. Positive (human trophoblast tissue for PankoMab-GEX™ and breast cancer tissue for HMFG-1 and 115D8) controls were always included. Two independent observers, including a gynaecological pathologist (D.M.), assessed the specimens using the semi-quantitative immunoreactive score (IRS) according to Remmele and Stegner (1987), which is routinely used for assessing receptor positivity in cancer. This method was employed to evaluate intensity and distribution patterns of staining. The IRS was calculated by multiplication of optical staining intensity (graded as 0=no, 1=weak, 2=moderate and 3=strong staining) and the percentage of positive stained cells (0=no staining, 1≤10% of the cells, 2=11-50% of the cells, 3=51-80% of the cells and 4≥81% of the cells) as described recently (Scholz et al., 2009).

The evaluation of each specimen was performed without knowledge of the pathological diagnosis.

Statistics

The SPSS/PC software package, version 19.0 (IBM, Armonk, New York), was used for collection, processing and statistical analysis of all data. Statistical analysis was performed using the non-parametrical Mann-Whitney U test and in the case of 3 or more groups its extension, the Kruskal-Wallis one-way analysis of variance by ranks.

For the comparison of survival times, Kaplan-Meier curves were drawn. The chi-square statistics of the log-rank test were calculated to test differences between survival curves for significance. All p-values resulting from two-sided statistical tests and ≤0.05 were considered to be significant.

Results

Serous carcinomas

The antibody PankoMab-GEX™ showed positive staining in all (100%) serous carcinomas investigated with a mean IRS of 8.23 (Fig. 1A). We observed a down-regulation of membranous PankoMab-GEX™

staining from G1 (mean IRS 8.94) to G2 (mean IRS 8.11) and G3 carcinomas (mean IRS 7.60) with a p value of 0.080 (Fig. 2 B). In addition, when we compared subgroups G1 and G3, the difference was significant (p=0.017). In contrast, cytoplasmic staining with PankoMab-GEX™ was found to be increased with grading (most applicable for serous carcinomas, with G1 IRS=0.5 vs. G3 IRS=0.86; p=0.094) which was significant within the whole study population (p=0.033; Fig. 2 A). Both HMFG1 (mean IRS=7.64; Fig. 1A') and 115D8 (mean IRS=10.85; Fig. 1A'') also stained 100% of serous carcinoma sections. Although 115D8 revealed an increase of IRS from 10.15 (FIGO I) to 11.25 (FIGO II), the difference was only significant for the whole population (FIGO I vs. III; p=0.005; Fig. 2C). Neither PankoMab-GEX™ nor HMFG1 significantly correlated with tumour staging.

Endometrioid carcinomas

The antibody PankoMab-GEX™ showed positive membrane staining in 95% of endometrioid carcinomas with a mean IRS of 6.40. In this histological type, however, we found no significant differences in PankoMab-GEX™ staining between G1 (mean IRS 6.00), G2 (mean IRS 7.00), and G3 (mean IRS 6.50) carcinomas (Fig. 1B). This was also true for the cytoplasmic expression pattern, although overall IR scores were much lower (mean IRS=0.28). Endometrioid carcinomas examined here were also recognized by HMFG1 (Fig. 1B') or 115D8 (Fig. 1B'') without correlation to grading or FIGO stage.

Mucinous carcinomas

In contrast to the other histological types of ovarian cancer, mucinous carcinomas showed positive

Table 1. Patient data.

	serous	clear cell	endometrioid	mucinous	total
Number of patients	110	12	21	13	156
Grading					
G1+2	65	5	11	12	93
G3	40	5	8	0	53
GX	5	2	2	1	10
Staging					
FIGO 1+2	20	4	10	11	45
FIGO 3+4	90	4	10	2	106
FIGO X	0	4	1	0	5

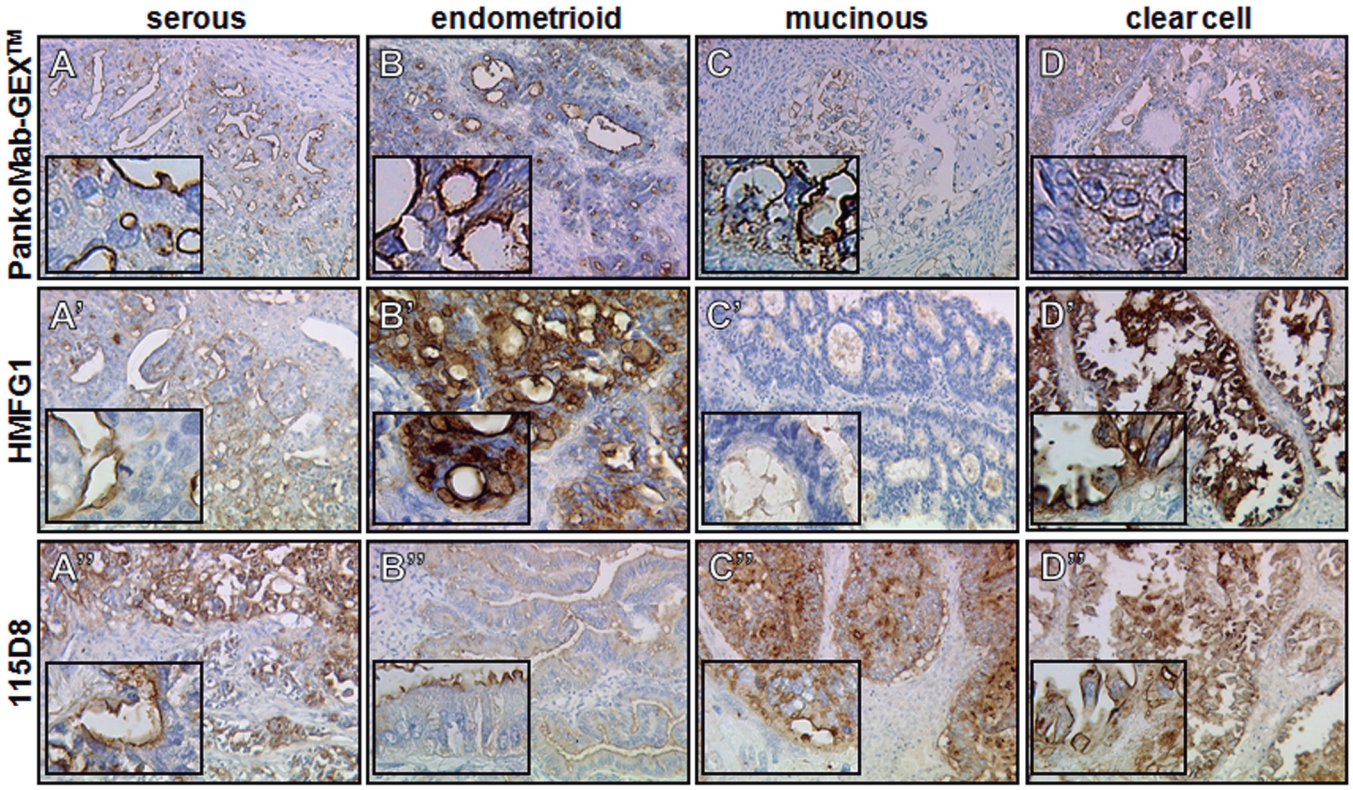

Fig. 1. Expression patterns of PankoMab-GEX™ **(A-D)**, HMFG1 **(A'-D')** and 115D8 **(A''-D'')** are shown. Representative images for serous, endometrioid, clear cell and mucinous carcinomas are shown. Mann-Whitney statistics were calculated for pairwise comparisons and statistical significance was defined for $p<0.05$. A-D, x 10; inserts, x 40.

membrane staining with PankoMab-GEX™ only in 58% of cases with a mean IRS of 4.17. The difference in staining between stage FIGO I (mean IRS 3.75) and II (mean IRS 10.00), although apparent, was not statistically significant. The cytoplasmic staining pattern was less strong than membranous staining, and also not different between stages. Interestingly, in the case of mucinous carcinomas the percentage of positive cases was lower with PankoMab-GEX™ as compared to HMFG1 (92%, Fig. 1C') and 115D8 (92%, Fig. 1C'').

Clear cell carcinomas

In the group of clear cell carcinomas the antibody PankoMab-GEX™ showed positive membranous staining in 92% of the cases investigated with a mean IRS of 7.58. We saw no significant different staining by PankoMab-GEX™ between G1 (mean IRS 10.00), G2 (mean IRS 8.50), and G3 stage carcinomas (mean IRS 7.20) (Fig. 1D). Neither the cytoplasmic PankoMab-GEX™ pattern nor HMFG1 (Fig. 1D') or 115D8 staining (Fig. 1D'') correlated with either grading or staging.

Normal ovarian tissues

Normal ovarian tissue was not stained by PankoMab-GEX™ (Fig. 3A) either at the surface epithelium or in the stroma, whereas HMFG-1 was clearly positive in ovarian surface epithelia (Fig. 3B). Antibody 115D8 was also negative with normal surface epithelium (Fig. 3C).

Overall survival

Statistical analyses were performed to test for a prognostic value of either positive or negative

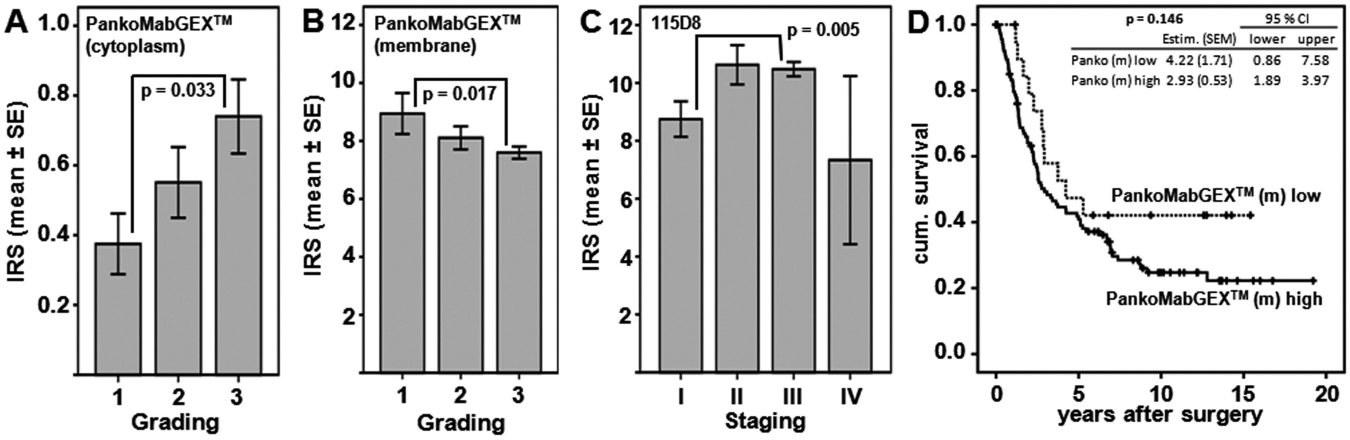

Fig. 2. Statistically significant correlations of cytoplasmic PankoMab-GEX™ whole population **(A)**, membranous PankoMab-GEX™ (serous carcinomas), **(B)** and 115D8 **(C)** staining results with grading or staging are shown as bar charts. Overall survival **(D)** of patients whose tumours were either PankoMab-GEX™ negative or positive was not significantly different.

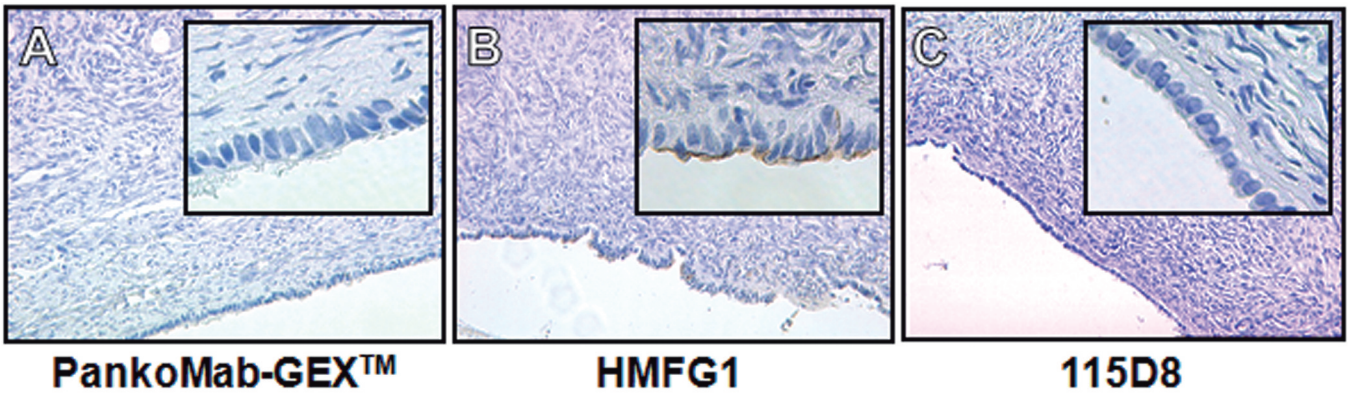

Fig. 3. Representative images of PankoMab-GEX™ **(A)**, 115D8 **(B)** and HMFG-1 **(C)** are depicted. A-C, x 10; inserts, x 40.

PankoMab-GEX™ epitope expression, or of membranous versus cytoplasmatic staining, respectively. The univariate Kaplan-Meier analysis revealed no significantly different overall survival between ovarian cancer patients whose tumor did or did not express the membranous PankoMab-GEX™ epitope (log rank, p=0.146, Fig. 2D). A trend for an improved overall survival was observed for tumours with cytoplasmatic PankoMab-GEX™ staining, though it did not reach statistical significance (p=0.259). HMFG-1 (p=0.777) and 115D8 (p=0.170), as well as the comparison of membrane versus cytoplasmatic staining of PankoMab-GEX™ (p=0.158), was not useful to predict patients' prognosis.

Discussion

Targeted therapies are an important option for cancer treatment (Lee-Hoeflich et al., 2008). Such strategies include treatment with unconjugated antibodies or with antibodies conjugated to cytotoxic drugs or radionuclides, or in combination with immunological effector cells (Hempel et al., 2000; Klement et al., 2002; Buchsbaum et al., 2003; Repp et al., 2003). High specificity and affinity to the cancer cells are essential for the selection of therapeutic antibodies (Bhattacharya-Chatterjee et al., 1994; Collignon et al., 2009; Doalto et al., 1991; Stipsanelli and Valsamaki, 2005; Xia et al., 2005). In the case of ovarian cancer, the epithelial mucin-1 (MUC1) is an obvious target. It is overexpressed in most carcinomas, and its glycosylation and cellular distribution is changed after malignant transformation. Song et al. have shown that radioimmunotherapy with the anti-MUC1 monoclonal antibody C595 can effectively target and kill ovarian cancer cells *in vitro* and *in vivo* (Song et al., 2008). Verheijen et al. (2006), on the other hand, were not able to extend survival or time to relapse with a different anti-MUC1 antibody (HMFG-1) in a multinational, open-label, randomized phase III trial. These discrepancies may be caused by differences in the fine-specificities of the anti-MUC1 antibodies employed. Another approach for a better therapeutic efficacy of antibody-guided therapies would be a multi-antigen targeting strategy (Verheijen et al., 2006). In any case, the choice of the best suited antibody is crucial.

In this study we have investigated the binding characteristics of a novel humanized anti-MUC1 antibody, called PankoMab-GEX™, towards ovarian cancer specimens. PankoMab-GEX™ recognizes a conformational epitope of the PDTRP motif of MUC1 induced by the tumor specific carbohydrate antigens Tn or TF, whereby its binding is fully dependent on the presence of Tn or TF. The main advantage of this antibody is its improved tumour specificity with a lack of binding to blood cells and to accessible normal tissues, as well as a higher affinity and number of binding sites per tumor cell when compared to other MUC1 antibodies such as HMFG-1 (Danielczyk et al., 2006). A further advantage is its manifold more potent anti-tumour modes of action, such as ADCC, phagocytosis and apoptosis, compared to other MUC1 antibodies as shown in *in vitro* and *in vivo* models. Besides its use in naked antibody therapy approaches it is also suitable for a drug targeting approach due to its ability for internalization. The present study confirmed that ovarian cancer is a primary indication for PankoMab-GEX™. With the exception of mucinous ovarian cancers, all other histological types of ovarian cancer were almost complete TA-MUC1-positive, whereas normal ovarian cancer tissue was not stained by PankoMab-GEX™ (Fig. 3). Two well-known murine MUC1 antibodies (HMFG-1 and 115D8) were also employed in this study. Both antibodies stained ovarian cancer tissues. However, HMFG-1 also stained normal ovarian epithelium. Interestingly, 115D8 was negative with normal ovarian epithelium. This was not expected, since its epitope has been described as sialic acid-dependent (Price et al., 1998), and normal MUC1 is known to carry (mostly sialylated) glycans.

We could not find significant correlations between any of the three MUC1 antibody staining results and overall or recurrence-free survival. PankoMab-GEX™ staining, however, tended to correlate with a reduced patients' survival. Cytoplasmic expression of MUC1 has been reported to be associated with worse prognosis in cancer patients (Matsumura et al., 2002). We have found elevated cytoplasmic PankoMab-GEX™ staining levels in high grade carcinomas (Fig. 2A), which is not surprising. High grade cancers predicted worse prognosis in our study population, too (data not shown). Although membrane staining by PankoMab-GEX™ declined slightly in G3 tumours, it still remained a strong and reliable signal (Fig. 2B).

In conclusion, PankoMab-GEX™ reveals excellent tumour specificity in almost all cases of ovarian cancer except the mucinous type, and with a high percentage of tumour cells stained. Normal ovarian epithelia are not stained. This makes PankoMab-GEX™ a promising candidate for a therapeutic antibody in ovarian cancer.

Conflict of Interest Statement. U. Karsten and S. Goletz are employees of Glycotope GmbH. which made the PankoMab-GEX™ antibody. All other authors declare that they have no conflict of interest.

References

Bhattacharya-Chatterjee M., Mrozek E., Mukerjee S., Ceriani R.L., Kohler H. and Foon K.A. (1994). Anti-idiotype antibodies as potential therapeutic agents for human breast cancer. Adv. Exp. Med. Biol. 353, 139-148.

Buchsbaum D.J., Zhou T., Grizzle W.E., Oliver P.G., Hammond C.J., Zhang S., Carpenter M. and LoBuglio A.F. (2003). Antitumor efficacy of TRA-8 anti-DR5 monoclonal antibody alone or in combination with chemotherapy and/or radiation therapy in a human breast cancer model. Clin. Cancer Res. 9, 3731-3741.

Burchell J., Durbin H. and Taylor-Papadimitriou J. (1983). Complexity of

expression of antigenic determinants, recognized by monoclonal antibodies HMFG-1 and HMFG-2, in normal and malignant human mammary epithelial cells. J. Immunol. 131, 508-513.

Collignon J., Gennigens C., Rorive A., Coucke P., Lifrange E., Maweja S., Fillet G. and Jerusalem. G (2009). Monoclonal antibodies and breast cancer. Current therapeutic progress. Rev. Med. Liege 64, 279-283. (In Belgium)

Danielczyk A., Stahn R., Faulstich D., Loffler A., Marten A., Karsten U. and Goletz S. (2006). PankoMab: a potent new generation anti-tumour MUC1 antibody. Cancer Immunol. Immunother. 55, 1337-1347.

Dian D., Janni W., Kuhn C., Mayr D., Karsten U., Mylonas I., Friese K. and Jeschke U. (2009). Evaluation of a novel anti-mucin 1 (MUC1) antibody (PankoMab) as a potential diagnostic tool in human ductal breast cancer; comparison with two established antibodies. Onkologie 32, 238-244.

Doalto L., Paridaens R., Dodion P., Manil L., Rigo P. and Fruhling J. (1991). Value of CEA levels and of immunoscintigraphy (using CEA-marked antibodies) for the diagnosis and therapeutic monitoring of breast cancer: apropos of a case. Acta Clin. Belg. 46, 42-47. (In Belgium)

Fan X.N., Karsten U., Goletz S and Cao Y. (2010). Reactivity of a humanized antibody (hPankoMab) towards a tumor-related MUC1 epitope (TA-MUC1) with various human carcinomas. Pathol. Res. Pract. 206, 585-589.

Hempel P., Muller P., Oruzio D., Behr W., Brockmeyer C., Wochner M., Ehnle S., Riethmuller R. and Schlimok G. (2000). Combination of high-dose chemotherapy and monoclonal antibody in breast-cancer patients: a pilot trial to monitor treatment effects on disseminated tumor cells. Cytotherapy 2, 287-295.

Karsten U., Diotel C., Klich G., Paulsen H., Goletz S., Muller S. and Hanisch F.G. (1998). Enhanced binding of antibodies to the DTR motif of MUC1 tandem repeat peptide is mediated by site-specific glycosylation. Cancer Res. 58, 2541-2549.

Karsten U., Serttas N., Paulsen H., Danielczyk A. and Goletz S. (2004). Binding patterns of DTR-specific antibodies reveal a glycosylation-conditioned tumor-specific epitope of the epithelial mucin (MUC1). Glycobiology 14, 681-692.

Karsten U., von Mensdorff-Pouilly S. and Goletz S. (2005). What makes MUC1 a tumor antigen? Tumor Biol. 26, 217-220.

Klement G., Huang P., Mayer B., Green S.K., Man S., Bohlen P., Hicklin D. and Kerbel R.S. (2002). Differences in therapeutic indexes of combination metronomic chemotherapy and an anti-VEGFR-2 antibody in multidrug-resistant human breast cancer xenografts. Clin. Cancer Res. 8, 221-232.

Lee-Hoeflich S.T., Crocker L., Yao E., Pham T., Munroe X., Hoeflich K.P., Sliwkowski M.X. and Stern H.M. (2008). A central role for HER3 in HER2-amplified breast cancer: implications for targeted therapy. Cancer Res. 68, 5878-5887.

Matsumura N., Yamamoto M., Aruga A., Takasaki K. and Nakano M. (2002). Correlation between expression of MUC1 core protein and outcome after surgery in mass-forming intrahepatic cholangiocarcinoma. Cancer 94, 1770-1776.

Remmele W. and Stegner H.E. (1987). Recommendation for uniform definition of an immunoreactive score (IRS) for immunohisto-chemical estrogen receptor detection (ER-ICA) in breast cancer tissue. Pathologe 8, 138-140.

Repp R., van Ojik H.H., Valerius T., Groenewegen G., Wieland G., Oetzel C., Stockmeyer B., Becker W., Eisenhut M., Steininger H., Deo Y.M., Blijham G.H., Kalden J.R., van de Winkel J.G. and Gramatzki M. (2003). Phase I clinical trial of the bispecific antibody MDX-H210 (anti-FcgammaRI x anti-HER-2/neu) in combination with Filgrastim (G-CSF) for treatment of advanced breast cancer. Br. J. Cancer. 89, 2234-2243.

Scholz C., Toth B., Barthell E., Mylonas I., Weissenbacher T., Friese K. and Jeschke U. (2009). Immunohistochemical expression of glycodelin in breast cancer correlates with estrogen-receptor alpha and progesterone-receptor A positivity. Histol. Histopathol. 24, 467-471.

Song E.Y., Qu C.F., Rizvi S.M., Raja C., Beretov J., Morgenstern A., Apostolidis C., Bruchertseifer F., Perkins A. and Allen B.J. (2008). Bismuth-213 radioimmunotherapy with C595 anti-MUC1 monoclonal antibody in an ovarian cancer ascites model. Cancer Biol. Ther. 7, 76-80.

Stipsanelli E. and Valsamaki P. (2005). Monoclonal antibodies: old and new trends in breast cancer imaging and therapeutic approach. Hell. J. Nucl. Med. 8, 103-108.

Verheijen R.H., Massuger L.F., Benigno B.B., Epenetos A.A., Lopes A., Soper J.T., Markowska J., Vyzula R., Jobling T., Stamp G., Spiegel G., Thurston D., Falke T., Lambert J. and Seiden M.V. (2006). Phase III trial of intraperitoneal therapy with yttrium-90-labeled HMFG1 murine monoclonal antibody in patients with epithelial ovarian cancer after a surgically defined complete remission. J. Clin. Oncol. 24, 571-578.

Xia W., Gerard C.M., Liu L., Baudson N.M., Ory T.L. and Spector N.L. (2005). Combining lapatinib (GW572016), a small molecule inhibitor of ErbB1 and ErbB2 tyrosine kinases, with therapeutic anti-ErbB2 antibodies enhances apoptosis of ErbB2-overexpressing breast cancer cells. Oncogene 24, 6213-6221.

Zotter S., Lossnitzer A., Hageman P.C., Delemarre J.F., Hilkens J. and Hilgers J. (1987). Immunohistochemical localization of the epithelial marker MAM-6 in invasive malignancies and highly dysplastic adenomas of the large intestine. Lab. Invest. 57, 193-199.

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