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THE LEVEL OF ANTI-(GalNAc BETA) AND ANTI-PARA-FORSSMAN DISACCHARIDE IgG ANTIBODIES IN PATIENTS WITH GASTROINTESTINAL CANCER: RELATION TO SURVIVAL

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Serum anti-(GalNAcβ) and anti-para-Forssman disaccharide (PF_{di}, GalNAcβ1–3GalNAcβ) IgG levels were earlier found to be related to histological grading and progression of gastrointestinal cancer. *Aim*: To study the relation of serum antibodies level to survival in patients with gastrointestinal cancer. *Methods*: The level of anti-GalNAcβ, and PF_{di} IgG was analysed in the serum of patients with gastric (n = 78) and colorectal (n = 48) cancers in the long-term follow-up using ELISA with polyacrylamide glycoconjugates. Survival rate and hazard ratio (HR) were assessed by the Kaplan — Meier method and Cox univariate analysis in different patho-morphological groups. *Results*: Better survival was observed in patients with an increased preoperative level of GalNAcβ antibodies. These were the gastrointestinal group in stages II, III or tumors T2–4 (n = 90–104, *P* = 0.007, HR = 0.48–0.49, 95% CI 0.27–0.83, and the group with gastric cancer in stages I, II (n = 49, *P* = 0.051, HR = 0.39, 95% CI 0.14–1.04). The survival time was significantly longer in the gastrointestinal group in patients whose GalNAcβ antibodies level rose in dynamics (stage III or N1–2: P = 0.031–0.039, HR = 0.29–0.31, 95% CI 0.09–1.00). No significant difference in survival of patients was observed in the evaluation of PF_{di} antibodies. We suggest that the level of antibodies and its change reflect the enteric microbiota colonization, which may influence cancer progression *via* different interrelations between microbiota, the tumor and immune system. *Conclusion*: The preoperative level of GalNAcβ antibodies and its dynamics may be of prognostic significance for clinical outcome assessment.

Key Words: **gastrointestinal cancer, survival, GalNAc beta and para-Forssman disaccharide antibodies, enteric bacteria.**

Enteric microbiota that colonize the alimentary tract stimulate the production of carbohydrate antibodies in humans. The individual pattern and level of carbohydrate antibodies in the serum of patients with gastrointestinal cancer may be changed because of the altered colonization, bacterial translocation and inflammatory response [1, 2]. The antibody level may reflect the adaptive immune response of the host to enteric microorganisms in carcinogenesis that deserves a circumstantial investigation for cancer diagnostics and prognosis. Comparative data about spontaneously occurring carbohydrate antibodies in cancer, their autoreactivity to the glycans of the host including tumor-associated carbohydrate antigens, dynamic changes and relation to survival are scanty. The PAA-glycoconjugates that are homogenous antigens with a single reiterative glycotope [3] were used for the determination of carbohydrate antibodies. Owing to the high reproducibility and low background in ELISA, significant differences in the level of antibodies were demonstrated in comparative investigations [4–8]. We have undertaken a long-term follow-up study to clarify the relation of the serum level of carbohydrate antibodies to survival and its prognostic significance. Patients with elevated levels of IgG antibodies to conjugates of Thomsen-Friedenreich disaccharide (TF, Galβ1–3GalNAc) and Tn (GalNAc*α)* showed a longer survival time while the survival time was shorter in patients with an elevated level of aGal (Galβ1–3Galβ) antibodies. In different patho-morphological groups, the preoperative levels of antibodies and their dynamics were found to be of prognostic value (the manuscript is presented for publication).

We tested the serum of patients by ELISA, using a set of PAA-β-glycoconjugates, and found populations of IgG antibodies to be reactive to GalNAcβ and GalNAcβ1–3GalNAcβ (PF_{di}) ligands [9]. The anti-PF_{di} IgG serum level was significantly higher in patients with gastric and colorectal cancer than in donors. Moreower, the elevated anti-PF_{di} IgG level was associated with the advanced gastric cancer [6].

The aim of the present study was to evaluate whether the level of GalNAcβ and PF_{di} IgG antibodies and the respective changes are related to survival, taking also into consideration the stage of the disease, tumor size status, morphology and the tumor spread in regional lymph nodes.

MATERIALS AND METHODS

Patients. The investigation was carried out in accordance with the ICH GCP Standards and approved by Tallinn Medical Research Ethics Committee, Estonia. The informed consent was obtained from each patient under examination. Altogether sixty-four male and sixty-two female patients, among them seventy-eight with gastric and forty-eight with colorectal cancer in stages I–III, were subjected to a long-term follow-up. Diagnosis was verified by the pTNM system [10]. Patients with distant metastases or those who received chemo- and X-ray therapy, as well as individuals who during dynamic investigations survived less than three months after

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Abbreviations used: GNA – GalNAcß; PAA – polyacrylamide; PF_{di} – the terminal disaccharide of the PF glycolipid; R – responders; TF – Thomsen-Friedenreich disaccharide; Tn – GalNAc*α;* WR – weak responders; $X2_{di}$ – the terminal disaccharide of the X2 glycolipid.

surgery were excluded from the study. The median age of patients was 64 years, mean ± SD: 61.6 ± 9.7. The extended D2 gastrectomy with limphadenectomy and resection of local lesions for colorectal cancer were performed. Regional lymph node metastases were also removed in advanced cancer. Concomitant diseases were documented in some patients. Inflammatory diseases (enterocolitis, pancreatitis and cholecystitis) and postoperative complications were revealed in 17% of patients, anaemia in five cases, and diabetes mellitus in three cases. The other sporadic manifestations were breast cancer, the carcinoma of the uterus, Parkinson's disease, cystitis, pyelonephritis and chronic hepatitis. Venous blood samples were taken before and after a surgical operation, and during the planned visits to the physician for health control. Serum probes were stored at –50 °C before use. The patients were divided into groups by stages, tumor volume T1 and T2–4, tumor grade G1–2 and G3, and regional lymph node metastases N0 and N1–2.

Glycoconjugates. The glycoconjugates of poly[N-(2-hydroxyethyl)acrylamide], namely GalNAcβ-PAA and GalNAcβ1–3GalNAcβ-PAA (PF_{di}-PAA), were received from Lectinity, Russia. The epitope density was 20 mol.%. Tris(hydroxymethyl)aminomethane-PAA (Tris-PAA) was used as a negative control.

The indirect ELISA. The assay was performed as described earlier [11]. The level of serum IgG antibodies reactive to glycoconjugates was estimated as the ratio of $A_{test}/A_{control}$, where A_{test} is the absorbance with glycoconjugate and $A_{control,}$ is the absorbance with Tris-PAA.

Statistical analysis. The level of antibodies and its dynamics were studied in relation to overall survival time starting from the date of the preoperatively taken blood sample. The Kaplan — Meier method and Cox univariate proportional hazards analysis were used (SPSS, version 15.0). The median (M) of the level of GalNAcβ and PF_{di} antibodies (Table 1) was used as a cut-off to discriminate between responders (R) whose antibody level was above or equal to M, and weak responders (WR, the level below M). The difference in survival between patient groups was considered to be significant when $p \leqslant 0.05$. The linear regression analysis was performed using Statgraphics Plus 5.1. The dynamic data were approximated to a quadratic function, using a CurveFinder for CurveExpert (Version 1.34).

Table 1. Median and mean of the preoperative antibody level

Antibodies	Group	n	Median	Mean	SD
GalNAcβ	Gastrointestinal	126	1.34	1.70	1.02
PFdi		118	1.59	2.91	3.27
GalNAcβ	Gastric	78	1.31	1.55	0.72
PFdi		75	1.53	2.90	3.28
GalNAcβ	Colorectal	48	1.39	1.93	1.34
PFdi		43	1.69	2.94	3.30

RESULTS

The relation of the preoperative antibody level to survival. In different patho-morphological groups with gastrointestinal or gastric cancer, GalNAcβ-responders (GNA-R) demonstrated a significantly better survival or a tendency to better survival than GNA-WR while PF_{di}-R versus PF_{di}-WR revealed a weak tendency to worse survival only. In gastrointestinal cancer, the significantly better survival in GNA-R *vs* GNA-WR was observed in stages II, III and tumor volume T2–4. In gastric cancer, a significantly better survival in GNA-R was observed in stages II and II, III, N0 group (Table 2, Figure).

Table 2. The relation of the preoperative GalNAcβ antibody level in the serum of cancer patients to survival

Group	n	p	HR	95% CI	Mean survival time, months*
Gastrointestinal, all	126	0.074	0.62	0.37–1.05	129.0; 108.6
Stages II, III	90	0.007	0.48	0.27–0.82	112.4; 80.3
T2–4	104	0.007	0.49	0.29–0.83	
Gastric, Stages I, II	49	0.051	0.39	0.14–1.04	150.4; 113.9
Stage II	24	0.004	0.24	0.08–0.68	120.3; 54.2
Stages II, III, N0	22	0.010	0.26	0.09–0.77	118.2; 54.7

Note: *Responders *vs* weak responders.

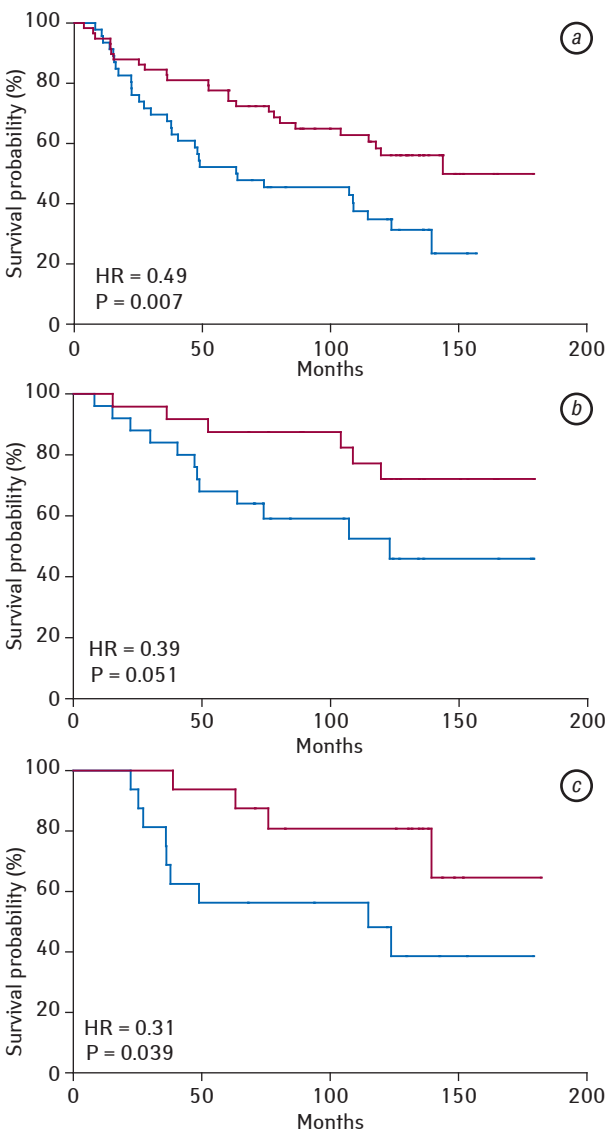

Figure. Probability of survival in GNA-responders (the level of serum GalNAcβ antibodies above or equal to median M, a solid line) *vs* GNA-weak responders (the level below M, a dotted line). *a*, *b* — the relation of the preoperative antibody level to survival; *c* — the relation of the antibody level dynamics to survival evaluated by factor **b**. *a* — patients with T2–4 tumors of gastrointestinal cancer. *b* — stages I–II of gastric cancer. *c* — patients with an N1–2 status of gastrointestinal cancer

No significant differences in survival between GNA-R and GNA-WR, as well as between PF_{di}-R and PF_{di}-WR in coloractal cancer were observed. However, to verify this, more patients need to be investigated. We did not observe the relation of survival to tumor morphology in patients with gastric, colorectal or gastrointestinal cancer (G1–2 *vs* G3, p = 0.24–0.54). Survival was not associated with the age of patients either (p = 0.202). Survival was better in an N0 status (N0 *vs* N1–2, p = 0.046, n = 124, HR = 0.60, 95% CI 1.01–2.76).

The relation of the antibody level dynamics to survival. The level of IgG antibodies to glycoconjugates in the serum of patients changed after surgery [6, 8]. We delineated the change of antibody level as a quadratic function $\boldsymbol{Y = a + bX + cX^2}$, where ***Y*** is the level of antibodies, ***X*** is the time in months, coefficient **a** is the factor characterizing the initial level of antibodies, **b** and **c** are the factors characterizing the change of the antibody level. This function showed a good fit to the data points ($r = 0.82–0.96$) and was applied to the calculation of factors for each patient. We did not examine the dependence of survival on factor **c** because of its insignificant influence on antibody level dynamics. The medians of factors **a** and **b** (not shown) were used as cut-off to discriminate between responders (the value above or equal to M) and weak responders (the value below M).

Similarly to the results of the preoperative assessment, the survival of GNA-R *vs* GNA-WR evaluated by factor **a** was significantly better in patients of the gastrointestinal group having T2–4 tumors. Evaluated by factor **b**, the survival of GNA-R *vs* GNA-WR was found to be significantly better in stage III or N1–2 of gastrointestinal cancer (Table 3). The PF_{di}-R vs WR in G1–2 tumors of gastrointestinal cancer showed a tendency to differ in survival as evaluated by factor **a** or **b**.

Table 3. The relation of the antibody level dynamics to survival

Antibodies	Gastrointestinal group	Factor	n	p	HR	95% CI	Mean survival time, months*
GalNAcβ	all	**a**	79	0.076	0.48	0.21–1.10	161.1; 137.2
	T2–4	**a**	67	0.026	0.39	0.16–0.92	
	Stage III	**b**	28	0.031	0.29	0.09–0.96	150.1; 90.0
	N1–2	**b**	32	0.039	0.31	0.09–1.00	151.6; 104.8
PF	G1–2	**a**	40	0.073	3.86	0.80–18.68	134.0; 170.1
	G1–2	**b**	40	0.079	0.27	0.06–1.29	169.2; 142.2

Note: *Responders *vs* weak responders.

DISCUSSION

This study demonstrates that the survival rate of patients with gastrointestinal cancer in some pathomorphological groups is associated with the serum level of GalNAcβ antibodies. To analyze the relation of antibodies level dynamics to survival, we used factors calculated as coefficients of a quadratic function. A significantly longer survival was observed in patients having T2–4 tumors and whose preoperative or initial level of GalNAcβ antibodies evaluated by factor **a** was increased (Tables 2 and 3, Figure). A tendency close to the significant difference in survival between patients was observed in the whole gastrointestinal group and in stages I, II of gastric cancer. Noteworthy, a significant difference in GNA-R *vs* GNA-WR was observed in stages II, III or II, *i.e.,* in groups apart from stage I (Table 2).

The lower anti-GalNAcβ IgG level has been associated with lower-differentiated carcinomas. Besides, the elevation of GalNAcβ and PF_{di} antibodies level in patients with gastrointestinal cancer was revealed after a surgical removal of G3-tumors [6]. However, this positive dynamics was not associated with survival in G3 group. The elevation of GalNAcβ antibodies assessed by factor **b** was associated with a benefit in the survival rate of patients with advanced cancer (stage III or N1–2).

We demonstrated earlier a significantly higher preoperative level of PF_{di} antibodies in patients with gastric and colorectal cancers than in donors. The elevated antibodies level was associated with the advanced gastric cancer: in stages II, III, IV *vs* stage I and in case of the tumor size T2+T3 *vs* T1. No relation between the antibodies level and regional and distant metastases of gastric or colorectal cancer was found [6]. However, evaluated by the preoperative level of PF_{di} antibodies or that evaluated by factors **a** and **b,** no significant difference in survival rate between patient groups was established in the present study.

In patients with gastrointestinal cancer, the anti-GalNAcβ IgG level has been correlated with the percentage of peripheral blood monocytes [6]. The GalNAcα/β residues are exclusive ligands of the C-type lectin of human macrophages, which is involved in the uptake and presentation of glycosylated antigens [12, 13]. The GalNAcβ-related determinants are present in glycans of the bacterial cell wall. A host-like motif GalNAcβ1–3Gal is present in coaggregation receptor polysaccharides of different streptococcal strains [14]. Enteric bacteria in human are more probable immunogens for antibodies with corresponding specificities. We evaluated the specificity of IgG antibodies in serum probes of patients with gastrointestinal cancer, using PAA-glycoconjugates in the competitive ELISA. IgG demonstrated the moderate cross-reactivity to GalNAcβ and related ligands, namely GalNAcβ1–3Galβ ($X2_{di}$, the terminal disaccharide of the X2 glycolipid, P-like antigen), PF_{di} and GlcNAcβ. From low to moderate cross-reactivity of IgG antibodies to the pair GalNAcβ and Tn (GalNAcα) was observed in serum probes [9]. The IgG antibodies affinity-isolated on GalNAcβ-PAA-Sepharose bound mainly GalNAcβ and $X2_{di}$, but bound other related ligands weakly or not at all. The anti-$X2_{di}$ or anti-PF_{di} IgG antibodies isolated on affinity sorbents were specific to the corresponding ligand used in isolation (manuscript in preparation).

The X2 and sialosyl-X2 glycolipids are present in human erythrocytes as well as normal and tumor tissues as extreme minor components, but are found in blood samples of individuals of a rare blood group p in considerably larger quantities. The PF glycolipid is also present in human erythrocytes in low amounts [15–18]. Whether the antibodies reactive to terminated saccharides can bind self antigens, *i.e.,* linear X2 and PF pentaglycosylceramides, needs investigation.

A significantly higher serum level of anti-GalNAcβ, -GalNAcα and -GlcNAcβ IgG antibodies was found in patients with anti-phospholipid syndrome versus normal controls. Besides, the elevated level of anti-GalNAcβ IgG is associated with fetal loss that may have a predicting importance in pregnancy [19]. These data provide evidence about the possible autoreactivity of GalNAcβ antibodies.

Commensal microbes keep our immune system healthy correcting immune imbalances by different mechanisms [20]. Gastrointestinal tumors germ and develop in the environment of commensal and pathogenic microbiota. The tumor colonization of some genera may cause apoptosis in tumors or, on the contrary, promote the tumor progression [21–23]. We suggest that the level of GalNAcβ antibodies is indirectly associated with survival. The antibody level and its change in patients may reflect the colonization by microorganisms, which beneficially influence survival *via* interrelations within the triad microbiota — tumor — host immune response. Which microorganisms regulate the systemic immune response and increase the antibody level in patients with cancer remains unknown.

We demonstrated lately the prognostic value of determination of TF, Tn and αGal antibodies level in patients with gastrointestinal cancer. The combined determination of TF and αGal antibody level was more informative and could predict survival more accurately than the determination of each level separately (the manuscript is presented for publication). Significant differences in survival, HR and mean survival time values calculated in the present study exhibit a prognostic meaning of the determination of GalNAcβ antibodies level. This study provides additional information to improve the prognostic potential of the combined determination of two or more antibody markers and select patho-morphological groups for clinical outcome assessment.

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