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RESEARCH ARTICLE

CA72-4 may contribute to real-time reconnaissance of gastric cancer [version 1; peer review: 1 approved, 1 approved with reservations]

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Abstract

Objective: Data from prospective studies indicate a positive impact of gastric cancer screening programs on mortality associated with the disease. Unfortunately, endoscopic procedures, widely regarded as uncomfortable, face low patient compliance, thus underscoring the need for reliable biological markers capable of detection of tumor growth in bodily fluids. Furthermore, in light of the emerging patient-friendly, still devoid of histopathological capabilities, capsule endoscopy, gastric fluid may prove valuable for biomarker-assisted cancer diagnosis. We set out to determine whether CA72-4 measurement in gastric fluid may be of benefit for detection of gastric cancer.

Design: Open prospective study.

Setting: Sample collection was performed at a tertiary referral center for patients with gastroenterological diseases; immunological analysis was performed at the R&D facility of a commercial biotechnology company. Studies were part of an EU-FP6 project (NEMO).

Patients: 176 patients referred for endoscopy due to gastrointestinal complaints.

Interventions: Gastric juice was aspirated endoscopically according to standard operating procedures, volume and pH were measured immediately and samples stored at -80°C.

Outcome measures: Concentration of CA72-4 tumor marker was evaluated by enzyme-linked immunosorbent assay (ELISA).

Results: Median CA72-4 levels were about 4-fold higher in cancer patients compared with patients with normal gastric findings, gastric inflammation, intestinal metaplasia or other diseases ($p=0.001$). Multivariate linear regression analysis revealed that elevated CA72-4 was significantly predicted by gastric carcinoma adjusted for *H. pylori* status, age, smoking status, PPI dose, and pH of aspirate ($R^2=0.27$, $p<0.0001$). In this model, diagnosis of gastric carcinoma had by far the

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Approval Status ? ✓

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greatest influence. At a cut-off level of 100 U/ml, CA72-4 had 75% sensitivity and 89% specificity for detection of gastric cancer.

Conclusions: Based on our findings, CA72-4 level assessment in gastric fluid, featuring yet unmatched accuracy of malignant neoplasia detection may prove beneficial for gastric cancer screening.

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Editorial note:

Please note that the refereeing status of this article was changed from "indexed" to "[v1; ref status: approved 1, approved with reservations 1]".

When this article was first published, *F1000Research* was still in its beta phase; during this period articles that received any two of "Approved" or "Approved with Reservations" statuses from the reviewers were labelled as "indexed". When the journal was formally launched in January 2013, the requirements for indexing were tightened, and only articles that are given either two "Approved" or one "Approved" plus two "Approved with Reservations" statuses by the reviewers are labelled "indexed". The new criteria for "indexing" can still be met in the future if a new revised version receives the necessary approval status from the reviewers.

Background and aims

Despite continuous decline in incidence rates in both sexes, gastric cancer is still the fourth most common cancer worldwide, with 934,000 newly diagnosed cases per year¹ and a vast annual death toll of more than 800,000, according to WHO 2004 statistics. With only little improvement over the past decades, the long-term survival from gastric cancer is poor, since patients are often diagnosed with advanced disease. In the USA, for example, five-year survival is 24%². In a hope for an overall improvement in the forlorn statistics, worldwide efforts are invested to shorten the time-to-diagnosis. Stomach cancer screening has been first introduced in Japan in 1963, followed by The Republic of Korea in 1996, and has recently commenced in less developed countries, with Venezuela, Chile, and Costa Rica adapting the Japanese model to implement pilot screening routines. Notably, the fact that to date no randomized trial of stomach cancer screening has ever been conducted sets hurdles for reliable assessment of efficacy of such policy. Nevertheless, data from recent prospective studies shows reductions in mortality from gastric cancer among participants in screening programs in Japan and Costa Rica³⁻⁵. Importantly, in this regard, endoscopic procedures, although of undisputable great diagnostic value, face a lack of patient compliance, for being widely regarded as uncomfortable. Henceforth, to circumvent this obstacle, reliable biological markers, suitable for detection and monitoring of tumor growth in bodily fluids, have long been searched after, in both blood and gastric fluid. Understandably, being easily accessible, blood has long been the substance of choice for marker evaluation. However, as appears from the peer-reviewed sources, the vast majority of the proposed gastric cancer marker candidates share the lack of diagnostic potential. This flaw, expressed in low sensitivity and specificity parameters, obviously negates the idea of utilization of such markers in gastric cancer screening practice.

As new potential screening methodologies, such as capsule endoscopy⁶, arise, gastric fluid is becoming an attractive milieu for cancer diagnosis as it contains both secreted soluble and exfoliated cellular proteins from the entire gastric mucosa. Unfortunately, early reports on the diagnostic and prognostic utility of the preponderant indicators of neoplasia, such as carcinoembryonic antigen (CEA) and CA 19-9, yielded unsatisfactory results⁷⁻⁹, thus diverting researchers' attention to other biomarkers, including CA72-4. Historically, following its identification [a sialosyl-2-6- α -N-acetylgalactosaminyl epitope of TAG-72 mucin;¹⁰⁻¹⁵], and characterization, the applicability

of the marker to cancer detection was examined in several neoplastic conditions. As second generation high-affinity monoclonal antibody (recognizing a different epitope;¹⁶) has enabled establishment of a double-determinant immunoradiometric assay^{17,18} capable of detection of CA72-4 in bodily fluids of carcinoma patients, CA72-4 presence was assessed in the serum of patients diagnosed with gastrointestinal malignancies¹⁹⁻²¹. These studies disclosed a complementarity between CA72-4 and CEA, as the former was often elevated in samples from cancer patients in which the levels of the latter remained low. Moreover, serum CA72-4 in patients during post surgical follow-up was predictive of recurrent disease²⁰. Several recently demonstrated lines of evidence also described CA72-4 as a potential serum and peritoneal wash-fluid marker of gastric malignant neoplasia, supreme to CEA²²; and references therein). In the present report we show that CA72-4, whose levels had to date been mainly assessed in serum samples²³⁻²⁶, has the potential to become a major biomarker in the gastric fluid-based gastric cancer diagnosis.

Methods**Settings**

Collection of gastric juice samples and clinical data was performed at Israelitic Hospital (IH) in Hamburg, Germany, an academic hospital and tertiary referral center for patients with gastroenterological diseases. Immunological analysis was performed at the R&D laboratory of Novamed Ltd., an ISO 9001-compliant facility, in Jerusalem, Israel.

Patients and experimental protocol

All patients older than 18 years of age undergoing an esophago-gastro-duodenoscopy (EGD) for clinical reasons were generally eligible for the study. Study participants gave written informed consent before any study related procedures were performed.

In all volunteers the EGD was performed as indicated clinically and according to routine procedures, including biopsies and interventional therapeutic measures, e.g. dilation therapy, if indicated. However, juice samples were only collected and analyzed if investigators could intubate the stomach and thoroughly aspirate gastric contents under visual control into separate vials before taking biopsies or performing other measures that might have altered gastric contents (e.g. rinsing of the mucosa with saline in order to improve visibility of potential mucosal alterations).

Volume and pH of each gastric juice sample were measured and recorded immediately, (pH was reassessed prior to immunological analysis). Subsequently, the pseudonymized samples were stored at -20°C until the end of the day and then transferred to -80°C for further storage until evaluation of biomarker concentration.

Collection of clinical data

Clinical data may be of pivotal importance for correct identification of biomarkers and was collected prospectively from all study participants. Data were pseudonymized and included the following information: results of endoscopic and histologic investigations, age, sex, height, weight, symptoms, time of last food and fluid intake, diet (e.g. vegetarian), concomitant medication (in particular intake of proton pump inhibitors (PPI)), alcohol consumption,

smoking habits and relevant previous and concomitant diseases including abdominal surgery.

Definition of patient groups

Patient groups were defined according to the results of the endoscopic and histologic investigations:

A) Normal stomach: Normal EGD and normal histology according to representative biopsies from the antrum and the corpus.

B) Gastric inflammation: Endoscopic diagnosis of gastric ulcer(s), erosions or gastritis and/or histologic findings of more than mild gastritis (endoscopic diagnosis of gastritis based on reddening and swelling of the mucosa was only accepted if confirmed histologically).

C) Intestinal metaplasia: Histologic evidence of intestinal metaplasia, irrespective of other endoscopic and/or histologic findings (except gastric cancer).

D) Gastric cancer: Endoscopic and histologic evidence of gastric cancer, no previous therapy.

E) Miscellaneous: Other diseases of the stomach diagnosed endoscopically and/or by histology, or diseases of the esophagus or duodenum.

ELISA

Assays were performed by a laboratory technician blinded to patient clinical data, including diagnosis. Samples were analyzed, immediately following thawing, with CA72-4 ELISA assay (DRG instruments GmbH, Marburg, Germany), according to the manufacturer's protocol, using 10 µl of sample. At least duplicate absorbance readings were obtained for each sample at 450nm wavelength using FL600 microplate reader with KC4™ data analysis software, (Bio-Tek® Instruments Inc., Winooski, VT, USA). Samples demonstrating high level of CA72-4 on the initial reading were assessed, in duplicates, up to 5 times on different days using a different assay plate and reagent set, depending on sample availability. Data collection, processing, and initial statistical analysis were performed on-site by a senior scientist, also blinded to patient clinical data. Data was then transferred to the IH site for detailed statistical analysis.

Statistical methods

Statistical analyses including ANOVA, Wilcoxon or Kruskal-Wallis test and univariate and multivariate linear regression analysis were performed using JMP software (version 6.0.3; SAS Institute Inc., Cary, NC, USA). Data are expressed as mean±SD or median with interquartile ranges depending on whether data were normally distributed or not. Multivariate linear regression analyses were used to investigate the influence of patient grouping and clinical parameters on CA72-4 concentrations in gastric juice. For the multivariate analyses, manual stepwise model building was performed and the following parameters were tested as predictors: age, gender, BMI, *Helicobacter pylori* (*H. pylori*) status (according to histology), smoking habits (never vs. active or ex-smoker), alcohol intake (never vs. current or ex-alcohol intake), PPI dose (in multiples of standard dose), endoscopic evidence of gastric bleeding and histologic diagnosis of gastric carcinoma, intestinal metaplasia, gastric

inflammation and nonmalignant histologic alterations of the gastric mucosa.

Results

Study participants

Overall 380 patients consented to participate in the study. Collection of gastric juice and clinical data was performed in 262 patients, in most of the others no juice samples could be obtained because the stomach contained insufficient amounts of fluid to be aspirated endoscopically or solids that could not be aspirated. In a minority of patients clinical data were insufficient for study purposes.

CA72-4 concentrations were measured in 176 gastric juice samples, based on sample volume sufficiency, from subjects with normal stomach (N=28), gastric inflammation (N=58), intestinal metaplasia (N=26), gastric carcinoma and no previous therapy (N=8) and patients with miscellaneous diseases (N=56). 108 out of 176 patients were female. Mean age of the patients was 60.8±16.8 years, mean BMI was 25.1±4.9 kg/m².

CA72-4 concentrations in gastric juice

CA72-4 concentrations in gastric juice ranged from 0.3 to 287 U/ml (Figure 1). Median CA72-4 concentration was 35.5 [19–68.5] U/ml. There was a marked and highly significant difference between the CA72-4 concentrations observed for the various diagnostic groups

Figure 1. Distribution of CA72-4 concentrations in the gastric juice of 176 patients undergoing routine esophago-gastro-duodenoscopy. Individual values ranged from 0.3 to 287 U/ml. The box plot shows median CA72-4 concentration (35.5 U/ml) and interquartile ranges (19–68.5 U/ml).

($p=0.0005$). While CA72-4 concentrations in gastric juice were similar in patients with an endoscopically and histologically normal stomach, gastric inflammation, intestinal metaplasia of the gastric mucosa or miscellaneous diseases, patients with gastric cancer had markedly elevated CA72-4 levels (Figure 2). Compared with all other participants, median CA72-4 concentrations in gastric juice of cancer patients were increased about fourfold (144.5 [54.0–220.3] U/ml vs. 34.5 [18.1–64.0] U/ml, $p=0.001$) (Figure 2). Six out of eight patients with gastric cancer and 18 out of 168 patients with other diagnoses had CA72-4 concentrations above 100 U/ml. Thus, at this cut-off level elevated CA72-4 concentrations had 75% sensitivity and 89% specificity for detection of gastric cancer.

Univariate linear regression analysis

In the univariate linear regression analysis, the diagnosis of gastric cancer significantly predicted increased CA72-4 concentrations in gastric juice ($p<0.0001$). Age or smoking habits were predictors of borderline significance for increased CA72-4 levels in older subjects and smokers ($p=0.051$ and $p=0.090$, respectively).

CA-72-4 levels were not significantly predicted by diagnosis of intestinal metaplasia ($p=0.964$), gastric inflammation ($p=0.656$), *H. pylori* status ($p=0.874$), gastric bleeding ($p=0.491$), sex ($p=0.206$), BMI ($p=0.218$), alcohol intake ($p=0.857$), PPI dose ($p=0.252$) or pH of the aspirate ($p=0.426$). If patients with gastric inflammation, intestinal metaplasia and miscellaneous diseases of the gastrointestinal tract were combined to one group of patients with pathologies other than gastric carcinoma, prevalence of this diagnosis did also not predict CA-72-4 concentrations in gastric fluid in the univariate analysis ($p=0.226$).

Figure 2. Concentration of CA72-4 in gastric juice of various groups of patients undergoing routine esophago-gastro-duodenoscopy (EGD; 176 in total). Patients with an endoscopically and histologically normal stomach, gastric inflammation, intestinal metaplasia of the gastric mucosa or miscellaneous diseases had similar CA72-4 concentrations. By contrast, in patients with gastric cancer CA72-4 concentrations were significantly elevated compared with all other groups (** $p<0.001$). The figure shows individual values and box-plot diagrams for the various groups. At a cut-off level of 100 U/ml (dotted line), elevated CA72-4 concentrations had 75% sensitivity and 89% specificity for detection of gastric cancer.

Multivariate linear regression analysis

Multivariate linear regression analysis confirmed that CA72-4 concentration in gastric juice was significantly predicted by diagnosis of gastric carcinoma adjusted for age, smoking habits, *H. pylori* status, PPI dose, and pH of the aspirate ($R^2=0.27$, $p<0.0001$). In this model, diagnosis of gastric carcinoma had by far the greatest influence ($p<0.0001$). Age ($p=0.033$) and smoking status ($p=0.002$) were additional independent significant predictors of CA72-4 concentrations. The other parameters achieved only borderline significance ($p=0.079$ to $p=0.138$), but the overall significance of the model was reduced when these parameters were not taken into account.

Multivariate linear regression analysis further revealed that CA72-4 concentrations were significantly predicted by pathologies other than gastric carcinoma adjusted for *H. pylori* and smoking status, age, PPI dose, and pH of the aspirate. However, gastrointestinal diseases other than gastric carcinoma had opposite (decreased levels in patients with such diseases) and much weaker effects ($R^2=0.07$, $p=0.028$) on CA72-4 concentrations compared with diagnosis of gastric cancer. In this model, diagnosis of *H. pylori* and smoking status were independent significant predictors of CA72-4 concentrations ($p=0.0384$ and $p=0.009$, respectively), while the other parameters achieved borderline significance ($p\geq 0.073$).

Dataset 1: Anonymized full data set CA72-4

<http://dx.doi.org/10.6084/m9.figshare.96382>

Discussion

The combination of a relatively high incidence, especially in north-east Asia, and a frequently late stage diagnosis of gastric cancer, due to the patient- or physician-side misinterpretation of symptoms, has proven deadly over the years, making the disease the second most frequent cause of cancer death worldwide^{27,28}. Generally, the cancer progression is aggressive upon late stage diagnosis with 5-year survival rates usually less than 30%. The existing means of screening are very accurate in providing a diagnosis, but face low patient-side compliance due to known discomfort associated with endoscopic procedures, thus imposing indirect constraints on the diagnostic value of fiberoptic endoscopy. The emerging capsule endoscopy, although holding a promise to circumvent the issue of compliance, still lacks diagnostic capabilities beyond visual identification of lesions. Thus, it will inevitably, require a reliable substitute for histological analysis, readily available through biopsy collection using a “conventional” endoscope, to outcompete the latter in screening efficiency, simplicity, and time-to-diagnosis. One such alternative is “lab-on-capsule” cancer marker-based molecular recognition of gastric malignancies. This option can be made possible through utilization of one or more marker(s) featuring high sensitivity and specificity in detection of gastric malignant tumors. In this work, we embarked upon identification of such a marker through assessment of the above mentioned parameters for a selected group of cancer markers, levels of which were measured in the gastric juice collected from patients with various disorders of the upper GIT, as well as in the gastric juice of patients in whom no disease was detected by either or both EGD and histopathology.

During this study we have established several ELISA assays for markers previously implicated in relation with gastric cancer, such as: CEA^{7-9,23}, pepsinogen II (PGII;²⁹⁻³³), regenerating islet-derived family, member 4 (RegIV;^{34,35}), and cytokeratin 8 (CK8;³⁶⁻³⁸), and have also made use of commercially available assays for CA 19-9^{7-9,23}, Gastrin^{17,30}, and pepsinogen I (PGI;³¹⁻³³). However, in our preliminary large-scale sampling analysis we failed to find any difference between the levels of these markers (including PGI/PGII ratio) in the gastric juice of cancer and non-cancer patients (data not shown). Quite the reverse, data generated in the course of this study points to a great diagnostic potential of CA72-4 direct measurements in gastric fluid. Notably in this regard, the CA72-4 concentrations in the gastric fluid of the majority of cancer patients were prominently elevated, compared to those measured in the gastric fluid obtained from patients with a completely normal stomach, patients with gastric inflammation, intestinal metaplasia or miscellaneous diseases of the upper gastrointestinal tract, including other malignant tumors.

One of the most obdurate hurdles to detection of cancer markers in gastric juice is the hostility of the gastric milieu imposed, among other factors, by high proteolytic activity and high acidity. Univariate linear regression analysis did not reveal a significant association between pH of gastric juice and CA72-4 concentrations. In multivariate linear regression analysis the “protective” effect of higher pH on CA72-4 concentrations was small, and failed to show statistical significance. In this regard, the apparent superiority of CA72-4 over other potential biomarkers in gastric juice may be speculated to stem from its relative stability in a wide range of pH, possibly due to the nature of the detected epitopes.

At a cut-off level of 100 U/ml CA72-4 had a sensitivity of 75% and a specificity of 89% for detection of gastric cancer. These data are also encouraging, although, with respect to the sensitivity parameter, the rather low number of cancer patients included in the study limits reliability of these findings and, generally, represents the most important drawback of our study. Gastric cancer was newly diagnosed in 8 out of 176 patients (5%), a proportion somewhat higher than that of cancer diagnoses expected in unselected patients undergoing EGD because of dyspeptic symptoms [1–2%;³⁹). Notably, the IH is a tertiary referral center for patients with gastrointestinal diseases where gastric cancer patients are observed much more frequently. However, in most patients satisfactory endoscopic and histologic investigations have been performed prior to referral to IH. The experimental design did not allow performance of an EGD for reasons other than clinical, hence, certain patients could not be included in the study. Moreover, for final analysis of data only patients with newly diagnosed disease were selected in order to limit potential confounders. Interestingly, an ostensible decrease in CA72-4, measured in a single patient concomitantly with cancer detection (150 U/ml), was observed following chemotherapy (87 U/ml). Thus, in the future, effort should be invested in further exploration of this phenomenon and the possibility that CA72-4 may also be used to monitor treatment efficiency.

Contrary to sensitivity, the specificity data are based on analysis of a large number of gastric juice samples from patients with completely normal gastric findings or various gastric or other diseases, advocating for data reliability. We observed a trend towards lower intragastric CA72-4 concentrations in patients with pathologies other than gastric cancer and an accordingly high specificity of nearly 90% for CA72-4-based detection of gastric cancer at the cut-off level chosen. This is particularly encouraging for a putative screening marker, as low specificity would be associated with a large number of futile invasive diagnostic tests in patients that are, in fact, free from gastric cancer.

In conclusion, work will be needed to accurately establish the precise sensitivity and the cut-off level for cancer detection. However, more than a fourfold, and thus a highly biologically significant, increase in the mean value of CA72-4 in the cancer group patients in this study, compared to all other participants, along with the apparent high specificity of the marker-based cancer detection, underscore the validity of our findings, and suggest that CA72-4 may contribute to real-time detection of gastric cancer in the future.

Consent

Study participants gave written informed consent before any study related procedures were performed.

Author contributions

Jutta Keller – acquisition of data, study concept and design, analysis and interpretation of data, drafting of the manuscript, statistical analysis.

Ella Reiss-Sklan – acquisition of data, analysis and interpretation of data, statistical analysis.

Miri Refael – acquisition of data.

Viola Andresen – statistical analysis.

Yael Herman-Levy – acquisition of data.

Igor Ruvinsky – acquisition of data, study concept and design, analysis and interpretation of data, drafting of the manuscript, statistical analysis, study supervision.

Jutta Keller and Ella Sklan have contributed equally to this work.

Competing interests

No competing interests were disclosed.

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Janusz Jankowski

Department of Clinical Pharmacology, Oxford University, Oxford, UK

This is essentially a good paper, however there are three areas where it needs to be improved.

1. There needs to be a succinct but informative overview of the marker. In addition, could the authors present a Kaplan-Meier curve of the marker in pathological samples?
2. The sensitivity and specificity ratios aren't good enough. Despite the authors mentioning this in the discussion, both ratios ought to be well above 90% perhaps even 95% as both won't be good enough for a screening test. The risk of missing a cancer is so huge otherwise.
3. The authors need to follow the NIH sequence of validation of biomarkers and explain how they plan to proceed with further validation.

Competing Interests: Janusz Jankowski is the chief investigator of the AspECT trial and receives funding from Astrazeneca. Janusz discloses no other competing interests.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

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Alex Boussioutas

Department of Medicine, The University of Melbourne, Victoria, Australia

I have read this article with interest and I think it is a good paper, however I do have some reservations.

The main one being there is no independent validation cohort and this is based on n=8 gastric cancers. I think if the authors tested their method on an independent cohort of samples (blinded) it would provide a very good validation.

The other point is more pragmatic; do the authors still require an endoscopy to collect the gastric juice? If they will do this by less invasive means (e.g. nasogastric tube suction) they should evaluate the technique using that method. Otherwise if a patient is having an endoscopy you will be able to visualize a gastric cancer.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 30 Oct 2012

Igor Ruvinsky, Novamed Ltd and Nano-based capsule-Endoscopy with Molecular Imaging and Optical biopsy (NEMO) project, Israel

Dear Dr. Boussioutas,

I absolutely agree with you in regard with the number of gastric cancer samples assessed in this study, and we expressed our reservations, accordingly, in the article.

However, in our defense, I should stress the fact that this study was conducted as a part of a greater, FP6-sponsored, project (Nano based capsule-Endoscopy with molecular imaging and optical biopsy – NEMO) and, consequently, influenced by the time and budget constraints of the latter.

Procedure-wise: one of the aims of the NEMO project was to combine capsule endoscopy with nano-based molecular recognition of gastric malignancies (as well as with several other diagnostic tools and abilities), which required identification of high-value biomarker(s) alongside establishing a suitable detection platform. Ultimately, sampling and sample analysis would and, hopefully, will be performed on board of the capsule (a job for NEMO 2.0), with performance parameters of the detection platform attuned separately to each biomarker selected.

Competing Interests: No competing interests were disclosed.

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