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Quantitative proteomics reveals proteins involved in the progression from non-cancerous lesions to gastric cancer

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ABSTRACT

Gastric cancer is one of the most aggressive malignancies affecting humankind. With almost a million cases globally, it sits in fifth position in terms of incidence, and third in terms of mortality. The progression of this disease is slow, with prolonged and sequential precancerous stages including chronic gastritis, intestinal metaplasia, dysplasia, and finally gastric cancer. Here we used the iTRAQ approach combined with high-resolution mass spectrometry analysis to describe the spectrum of the gastric cancer cascade. Biopsies from three stages: chronic gastritis, intestinal metaplasia, and gastric adenocarcinoma, were selected for analysis by quantitative proteomics. We identified and reported quantitative data for 3914 different proteins quantified with high confidence, uncovering pathways and processes dysregulated between the different stages. Intestinal metaplasia is characterized by the down-regulation of ribosomal proteins, with overexpression of cell survival proteins such as GSTP1 and EPCAM. The transformation to gastric cancer involves overexpression of the DNA replication and the spliceosome pathways. The impairment of mitochondrial pathways was correlated with down-regulation of SIRT3 and SIRT5, and overexpression of enzymes supporting the glycolytic phenotype, such as HK3 and PCK2. Several proteins found dysregulated during the progression of gastric cancer have potential to be used as specific biomarkers and/or therapeutic targets.

Significance

Gastric cancer is among the most aggressive cancers affecting the humankind. The progression of this disease is slow going through prolonged and sequential precancerous stages, lacking of specific symptoms. As a general consensus, gastric cancer evolves from chronic gastritis to premalignant lesions, with the potential to progress into gastric cancer. Quantitative proteomics is a powerful tool to study the progression of this disease. Biopsies from twelve individuals grouped into three different stages were selected for the study; the first group were patients with Moderate chronic follicular gastritis, the second with Incomplete type, intestinal metaplasia and the third with Moderately differentiated, intestinal-type adenocarcinoma. We uncovered several pathways that were dysregulated between the different stages and identified several proteins with potential to be used as biomarkers or even as therapeutic targets. Our results provided highly confident data related to the progression of the disease that can be used by the scientific community interested in studying gastric cancer.

1. Introduction

Gastric cancer (GC) is one of the most aggressive cancers globally. While remaining in fifth position in terms of incidence, with 951,594 cases (6.8% of all cancers), its mortality puts it in third position, with 723,073 deaths (8.8% of all cancer related deaths), according to data reported in the public repository GLOBOCAN 2012 (http://globocan.iarc.fr). This malignancy is more prevalent in the developing countries of Eastern Asia, where its incidence rates are twice as high in men as in

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women. Particularly in Mexico, the current estimated annual number of new GC cases is 7680, making it the sixth most prevalent malignancy, while the estimated number of deaths, including both sexes, is 6281, placing this cancer in third position regarding mortality (8% of cancer related deaths). The main causes of high fatality rates in GC are related to the very poor prognosis derived from the lack of specific symptoms in the early stages of disease progression [1]. Consequently, GC is frequently detected during the advanced stages, which limits the therapeutic options for effective treatment.

GC is a multifactorial disease; however, it is well established that chronic infection with *Helicobacter pylori* (*H. pylori*) is the primary cause, and that this is modulated by host and environmental factors [2]. The risk factors for the progression of GC include, among others, the use of tobacco and a diet high in salt and processed meat [3]. On the other hand, it is the general consensus that a diet rich in vegetables and fresh fruits reduces the risk of progression for GC. Several gene polymorphisms related to the regulation of gastric acid secretion, tumor necrosis factor alpha, and some members of the interleukins family, are involved in the early stages of the progression of the disease [4–6].

Gastric cancer has been classified into two main histomorphological subtypes: Intestinal- and diffuse-type. The progression of GC is slow, with several prolonged and sequential precancerous stages. Particularly, intestinal-type adenocarcinoma is highly correlated with gastric precancerous lesions, starting with *H. pylori* colonization of the gastric mucosa, which in turn leads to a long-standing inflammatory response, which ultimately predisposes development of mucosal atrophy and intestinal metaplasia. These events are widely recognized as the Correa's cascade model of gastric carcinogenesis [7]. In such cases, chronic gastritis is a *sine qua non* condition in cases of atrophy and intestinal metaplasia, as atrophy and intestinal metaplasia are to intestinal-type adenocarcinoma. Intestinal metaplasia is a progressive multifocal process that can progress from small intestinal metaplasia to the colonic-type. Dysplasia occurs in two stages: low-grade and high-grade, which has several cancerous cell morphological and molecular characteristics, including pleomorphism and atypia, accumulation of mutations and hypermethylation [2]. Eventually, dysplasia progresses into early, and then advanced, invasive carcinoma.

High-resolution mass spectrometry-based quantitative proteomics is a powerful tool for evaluating protein expression profiles in most protein samples from organisms, tissues, and fluids from organisms representing all the kingdoms of life. As such, these tools have been extensively used in cancer research for the identification and quantification of proteins from all pathways involved in the progression of cancer. In GC, and in the early stages of its progression, an increasing number of proteomics studies have aimed to find specific biomarkers [8–12]. Among the most useful quantitative proteomics strategies, at least for the analysis of tissue samples where no metabolic labeling can be performed, are the label-free or chemical labeling approaches. The iTRAQ chemistry, for example, can analyze and compare up to eight samples simultaneously in a single experiment [13], and requires only a minimal amount of starting material. Several proteins have been reported as potential biomarkers using these approaches in gastric cancer stem cells or in sera from patients with the disease [12, 14].

Here we performed a quantitative proteomics analysis comparing biopsies from patients with gastric cancer and two early stages during the progression of the disease: chronic gastritis and intestinal metaplasia. For the study, we used the iTRAQ chemistry in combination with high-resolution LC-MS/MS analysis to confidently identify/quantify > 3900 proteins in four replicates. Our results provided important evidence about the transformations required for progression to invasive carcinoma. Our description of specific protein profiles for the premalignant lesions will help in the development of early diagnoses and effective treatments, ultimately resulting in a decrease in gastric cancer mortality.

2. Material and methods

2.1. Patients

This pilot study was conducted in patients attending the Instituto Nacional de Cancerología (INCAN) (National Oncological Reference Center) in Mexico City, from April 2012 to April 2015. Patients older than 18 years who consulted because of gastroduodenal symptoms or with suspected gastric cancer, and who were scheduled for panendoscopy and biopsy for diagnostic purposes, were consecutively enrolled. Patients were excluded if they had received anti-*H. pylori* therapy or proton pump inhibitors (PPI) within the 4 weeks prior to the study, or if they had undergone gastric surgery. Those who had previously received cancer treatment or had other severe chronic diseases were also excluded. All participants included in the study signed a letter of informed consent. The protocol was approved by the Research and Ethics on Research Committees at the Instituto Nacional de Cancerologíaand the Instituto Nacional de Salud Pública.

2.2. Histopathological diagnosis

Diagnosis was based on endoscopic and histopathological findings. From each patient, a total of six biopsies were obtained from gastric fundus (2 samples), corpus (2 samples), and antrum (2 samples), and used in histopathological studies and to detect infection by *H. pylori*. Briefly, gastric biopsies were immediately immersed in a 10% formaldehyde-saline solution and embedded in paraffin. Five-micron thick sections were mounted on glass slides and stained with hematoxylin and eosin (HE), Giemsa, and periodic acid-Schiff (P.A.S.)/alcian blue, when intestinal metaplasia was suspected. The presence of *H. pylori*, non-atrophic gastritis, atrophic gastritis, and intestinal metaplasia was recorded. Intestinal metaplasia was classified as complete or incomplete, according to the modified, updated Sydney classification of gastritis [15]. Adenocarcinomas, either intestinal or diffuse, were classified according to criteria previously established by Lauren [16]. The final diagnosis was determined according to the most severe histological lesion present in any of the biopsies analyzed. *H. pylori* infection was considered to be positive when curved bacteria were observed in any of the biopsies analyzed. All specimens were categorized by an experienced pathologist using blind analysis. Three additional biopsies were obtained from corpus, antrum, and tumor, and were frozen in liquid nitrogen immediately after collection. Another biopsy from the angular incisure was kept at −80 °C in Brucella broth with 5% fetal bovine serum (FBS) containing 16% glycerol, and used for *H. pylori* culture.

2.3. Tissue samples

In order to perform quantitative proteomics, twelve samples were collected and classified in three groups: 1) gastric cancer samples (GC), corresponding to moderately-differentiated intestinal adenocarcinomas (n = 4), 2) premalignant lesions (PL), obtained from patients with intestinal metaplasia (n = 4), and 3) non-cancerous lesions (NC), from patients with moderate chronic follicular gastritis (n = 4). All tissues were immediately frozen in liquid nitrogen and histologically classified.

2.4. Protein extraction

Tissue samples were washed with cold PBS and homogenized in lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.1% SDS, 50 mM DTT, 0.2 mM PMSF, 40 mM Tris; pH 7.4). For each 15 mg of tissue, 100 µL of lysis buffer was added. After homogenization, samples were centrifuged at 12,000 rpm for 15 min at 4 °C. The pellet was discarded, and the protein content of the supernatant was estimated using the 2D Quant kit (GE healthcare). Cysteine residues were alkylated with 100 mM IAA during 30 min at room temperature in the darkness. For this reaction,