Author's response to reviews

Title: Proteome analysis of human gastric cardia adenocarcinoma by laser capture microdissection

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Author's response to reviews:

Reviewer: Mitsuhiko Osaki

1. To confirm the validity of proteome analysis in this study, the authors should perform immunohistochemistry or Western blotting for not only HSP27 but also more a few proteins described in Table 2.

We perform immunohistochemistry and western blotting for HSP27, HSP60 and Prx-2.

2. According to Table 1, the 9 clinical samples were composed of 3 well differentiated, 3 moderately differentiated and 3 poorly differentiated types, respectively. Are there any differences or relationship between histological type and protein expression?

In this study, we collect 9 clinical samples, which were composed of 3 differentiated types. To do that, we want to find the common differently expression proteins between tumor and nontumor in these differentiated types. However, it is not the main aim for us to state the differences between histological type and protein expression. So we don't carry out work in this respect. We'll try to find them in the further study.

3. The authors performed proteome analysis using only cardiac adenocarcinomas. It is considered that the data will be important to find out the feature of GCAs. Recently, some papers obtained in gastric non-cardiac adenocarcinoma using proteomics technologies are reported, in particular:
Cardia is the anatomical borderland between the oesophagus and stomach, so there is a constant controversy concerning the relationship about the epidemiology, clinical features, and classification among the adenocarcinoma of distal esophagus, cardiac, and gastric. To compare their protein profiles, we browsed the literature to find these proteins. Thirteen out of 23 proteins have been found in GC previously (Table 2). Among them, HSP27, 60, 70, PDIA3, and CA II have the same expression pattern in GCA and GC. However, in contrast, a low-level expression of HSP27 was found in EA [16-20]. Therefore, the tumorigenesis of GCA is different from EA and HGC, and should thus be a distinct pathological entity.

Reviewer: Ferdinand von Eggeling

1. authors state that "...approx. 25000 - 30000 cells were obtained per cap" and that "tissues from multiple caps were extracted..." (see Materials & Methods; 2.4 LCM). Did the authors use for 2-DE analysis cells derived from only one tissue/patient or were samples of multiple different tissues/patients mixed for one 2-DE?

In order to reduce individual differences, tissue samples were obtained from the same patient, which enabled us to study the differential protein expression under a similar genomic background. Therefore, we perform LCM to rich tissue from every patient containing nontumor and tumor cells. Then, we collect multiple caps of one patient containing nontumor and tumor cells and extract protein from them, respectively. After examine the protein concentration with a Bio-Rad protein assay kit, we carry out 2-DE for one patient. In this study, we carry out work according to this process one patient by another.

2. How many cells were used at all for one 2-DE and how many matched pairs of tumor tissue at all were performed by 2-DE analyses?

Tissues from multiple caps (about 800,000~1,000,000 cells) were extracted into the same lysis buffer until sufficient material had been collected. We used about 300,000~400,000 cells for one 2-DE, and 9 mathed pairs of tumor tissue for 2-DE analyses.
3. The authors state that they found 27 obviously differently expressed protein spots in 2-DE containing proteins classified into different physiological functions. Among these proteins heat shock proteins were identified. In my opinion it would be more appropriate to show the identification by MALDI-MS and characterisation by IHC of one protein and not of different proteins as it was done the MS spectrum of HSP60 and IHC experiments of HSP27.

In this study, we also identified HSP60 by IHC and WB. Therefore, we use the MS spectrum of HSP60 as an example of MALDI-MS TOF.

4. Were the samples used in the Western blot analysis of HSP27 also derived from microdissected tissue?

Yes, the samples used in the Western blot analysis also derived from microdissected tissue

5. Reference 5 I cannot read.

Reference 5 is an article in Chinese. I translate it into english by myself.

Reviewer: Chengchao Shou

1. The authors should confirm the results from 2-D gel with other methods, such IHC in cancer tissue or Western blot.

See 2.8 Immunohistochemical and Western blot analysis

To validate the expression patterns of three proteins in GCA tissues, immunohistochemistry was performed using formalin-fixed and paraffin-embedded tissue specimens that were matched with 2-DE samples. Dewaxed 5um thick sections were treated with a 0.3% hydrogen peroxidase for 3 min and a blocking antibody for 30 min. After heated mediated antigen retrieval, sections were incubated with primary antibody at 4 overnight as follows: HSP27 (Sigma, St. Louis, MO, USA), 1:500; HSP60 (Santa Cruz, America), 1:300; and Prx-2 (Santa Cruz, America), 1:150. Sections were then incubated with a peroxidase-labeled antibody (1:500), developed with diaminobenzine, and counterstained with hematoxylin.

For western blot analysis, the protein samples (30ug) used in 2-DE were run on
12% SDS-PAGE, transferred onto PVDF membranes, and then blocked with PBS/5% skim milk/0.01% Tween for 2-4 hours at room temperature. Primary antibody was diluted in blocking buffer was added as follows: HSP27, 1:200; HSP60, 1:300; and Prx-2, 1:200. Afterward, it was incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody and a HRP-conjugated anti-GAPDH/ beta-acting antibody to confirm equal protein loading in each lane for 1-2 hours at 37 or room temperature. The samples were washed, and detected with enhanced chemiluminescence for 30-60 seconds (Minipore).

2. Meanwhile, some data are needed about the significances of the un-regulated genes in gastric cancer, which were found by authors with LCM pulsing 2-D gel and MOLDI TOP MS.

HSPs are a group of stress proteins that are induced by various types of environmental and pathophysiologic insults. Co-up-regulations of HSP27, 70, and 60 were found in the gastric cardia tumor tissues in this study. As chaperones, HSP27 and HSP70 could inhibit apoptosis through interacting with apoptosome and the caspase of activation complex [24], which underlie their role in tumor progression and resistance to treatment [25]. Numerous studies indicate that HSP27 and HSP70 over-expressions signal a poor prognosis and predict a poor response to chemotherapy. HSP60 appears to be a key endogenous inflammatory mediator and is presumably released by damaged cells. Moreover, HSP60 has a role, together with HSP70 in, antigen presentation in malignant diseases [26]. HSPs are also overexpressed in breast, colorectal, gastric, and prostate carcinomas [11, 25]. Therefore, the over-expression of HSP27, 70, and 60 may be useful biomarkers for carcinogenesis in GCA and may be associated with the degree of differentiation and the prognosis of GCA.

Among the identified proteins, two proteins are involved in the regulation of transcription and expression of tumor-associated genes. PCBP1, up-regulated in GCA, is an RNA chaperone, post-transcriptional regulator. It has been demonstrated that PCBP1, together with PTB-1 and hnRNPK, control some proto-oncogene genes and apoptosis-related genes expression such as Bag-1, c-myc, Apaf-1, XIAP, and DAP5 through stimulating the activity of the internal ribosome entry segment (IRES). PCBP1 is required to open the RNA in the region containing the ribosome entry window, while PTB-1 may be required for ribosome recruitment [27, 28]. Zhi Dai et al. recently described that the glycan parts of PCBP1 might be related to metastatic ability and might play a role in the process of hepatocellular carcinoma metastasis [27]. Then the up-regulation of PCBP1 may regulate the cap-independent mechanism of translation initiation of cardia tumor-associated genes in the development of GCA. As a tumor suppressor, Alpha-enolase can regulate the c-myc promoter activity in the form of a c-myc binding protein (MBP-1). MBP-1 can bind to the P2 element in the c-myc promoter and compete with the TATA-box binding protein (TBP) to suppress the transcription of c-myc [29, 30]. Down-regulation of Alpha-enolase is
in accordance to the work of Chang YS et al. who found that Alpha-enolase predicted aggressive biological behavior and is associated with poor survival in non-small cell lung cancer [29].

What is interesting is that three antioxidant enzymes, including Prx-1, Prx-2, and GSTP, were identified in gastric cardia tumor. All of them are involved in the removal of reactive oxygen species (ROS) which can induce cellular senescence and apoptosis and therefore function as antitumorigenic species [31]. We presume that over-expression of Prx-1 and GSTP may protect gastric cardia tumor cells from apoptosis by scavenging ROS in these cells. Moreover, evidence suggests that enhanced antioxidant mechanisms in tumor cells in vivo contribute to chemoresistance and poor prognosis. Increased expressions of Prx-1 and GSTP have been detected in hepatocellular [32] and pancreatic carcinoma [33]. Downregulation of Prx-2 in GCA may imply its tumor suppressor role which was controversial with some other studies [34]. However, Furuta. J. et al. recently found that Prx-2 was silenced in melanomas due to aberrant methylation of 33 CpG islands [35]. Therefore, disregulations of antioxidant enzymes in GCA may represent tumor cells with a microenvironment which is advantageous for their survival and proliferation.

Other up-regulated proteins involved in physiological processes are ANXA2, ANXA4, and hnRNPA2/B1. Annexins are Ca2+ and phospholipid binding proteins acting as a regulator of membrane fusion and possess the structural properties necessary to form ion channels [36]. While ANXA2 and ANXA4 are associated with several physiological processes (e.g., signal transduction, cellular differentiation, and proliferation), their roles in GCA tumorigenesis have not been described previously. ANXA2 was over-expressed in advanced gastric carcinomas and could contribute to its progression [37]. In relation to this, Zimmermann U et al reported that ANXA4 plays an important role in the morphological diversification and dissemination of the renal cell carcinoma [38]. Therefore, the over-expression of ANXA2 and ANXA4 may be related to the malignant transformation of gastric cardia epithelia. hnRNP A2/B1 is a member of a large family of hnRNP proteins involved in various functions, including the regulation of transcription, mRNA metabolism, and translation [39]. He, Y. et al. presented evidence that hnRNP A2/B1 may play an important role in cell proliferation through the regulation of the expressions of BRCA1 and p21 [40]. Guo, W. et al. found that p21 might have an effect on GCA development [41]. Moreover, hnRNP A2/B1 is a target antigen for MG7, which is an early gastrointestinal cancer-specific monoclonal antibody [42]. Further study is thus needed to discover the pathogenesis of hnRNP A2/B1 in GCA.

CAs are physiologically important enzymes that catalyze a reversible conversion of carbon dioxide to bicarbonate [43]. There are at least 13 active isoenzymes that have been identified in mammals. They are involved in many biological processes such as pH homeostasis and ion transport. CA II is a very efficient enzyme and is expressed in most organs of the alimentary tract, with a high
expression in the gastric and intestinal epithelia [44, 45]. Its main physiological functions are to regulate the acidity of the gastric juice and to assist in forming a HCO3-, covering the epithelium and protecting it from digestion [44]. A significantly low-expression of CA II may make it difficult for CA II to maintain its function for normal gastric cardia cell growth and could therefore lead to the progression of malignant transformation. Recently, both CA and CA II were reported to have down-regulations in colorectal and gastric tumors and were considered to participate in cancer biological aggressiveness and synchronous distant metastasis [46, 16].

ADH1C belongs to the zinc-containing alcohol dehydrogenase family. It takes part in alcohol metabolic process and can catalyze the oxidation of approximately 80% of ethanol to acetaldehyde, a known toxic and carcinogenic compound [47]. Increased acetaldehyde production has been implicated in the pathogenesis of colorectal, hepatocellular, and breast cancers [48-50], thereby indicating that a high intake of alcohol is associated with tumorigenesis. However, our result first found that ADH1C was significantly reduced in GCA. Furthermore, the role of alcohol consumption in GCA is controversial. Some studies considered it as a risk factor for GCA, but a case control study in Sweden revealed that there was no positive correlation between alcohol consumption and esophageal and cardia adenocarcinoma. Therefore, further study is required to elucidate the molecular mechanism of ADH1C in the development of GCA.

Reviewer: Yu-Ju Chen

1. A typical proteomic study aims to identify differentially expressed proteins, which helps delineation of disease mechanism. Although Table 1 shows the list of differentially expressed proteins, yet how much change in terms of fold-change is not clear in the table. The authors should use imaging software to calculate the average fold-changes and include the quantitative information in Table 1.

we used the imaging software to calculated the average fold-chages and showed them in the table 2.

2. There are numerous grammar errors throughout the manuscript. Substantial corrections are required to ensure readability.

I have corrected the grammar errors in the manuscript. I use blue color to show them.

3. The 2DE results revealed 15 commonly over-expressed protein and 8 down-regulated proteins compared to the normal tissue pairs. The clinical data shown in Table 1 indicate that three classes of tissues, moderately differentiated, well differentiated and poorly differentiated, were studied. Is there any inter-group
correlation of disease stage with the fold-change or proteins? Furthermore, a 
heap map of all the differentially expressed proteins for the intra-group 
correlation may be helpful. The information will gain understanding for 
carcinogenesis mechanism.

In this study, we collect 9 clinical samples, which were composed of 3 
differentiated types. To do that, we want to find the common differently 
xpression proteins between tumor and nontumor in these differentiated types. 
However, it is not the main aim for us to state the differences between 
histological type and protein expression. So we don't carry out work in this 
respect. We'll try to find them in the further study.