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Hsp60 Quantification in Human Gastric Mucosa Shows Differences between Pathologies with Various Degrees of Proliferation and Malignancy Grade

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Abstract: Background: Stomach diseases are an important sector of gastroenterology, including proliferative benign; premalignant; and malignant pathologies of the gastric mucosa, such as gastritis, hyperplastic polyps, metaplasia, dysplasia, and adenocarcinoma. There are data showing quantitative changes in chaperone system (CS) components in inflammatory pathologies and tumorigenesis, but their roles are poorly understood, and information pertaining to the stomach is scarce. Here, we report our findings on one CS component, the chaperone Hsp60, which we studied first considering its essential functions inside and outside mitochondria. **Methods:** We performed immunohistochemical experiments for Hsp60 in different samples of gastric mucosa. **Results:** The data obtained by quantitative analysis showed that the average percentages of Hsp60 were of 32.8 in normal mucosa; 33.5 in mild-to-moderate gastritis; 51.8 in severe gastritis; 58.5 in hyperplastic polyps; 67.0 in intestinal metaplasia; 89.4 in gastric dysplasia; and 92.5 in adenocarcinomas. Noteworthy were: (i) the difference between dysplasia and adenocarcinoma with the other pathologies; (ii) the progressive increase in Hsp60 from gastritis to hyperplastic polyp, gastric dysplasia, and gastric carcinoma; and (iii) the correlation of Hsp60 levels with histological patterns of cell proliferation and, especially, with tissue malignancy grades. **Conclusions:** This trend likely reflects the mounting need for cells for Hsp60 as they progress toward malignancy and is a useful indicator in differential diagnosis, as well as the call for research on the mechanisms underpinning the increase in Hsp60 and its possible roles in carcinogenesis.

Keywords: chaperone system; Hsp60; gastritis; gastric dysplasia; gastric carcinogenesis; intestinal metaplasia

1. Introduction

Quantitative variations of molecular chaperones in cells and tissues during carcinogenesis have been recognized for a long time. However, it is still unclear what these variations signify for the carcinogenic process. There is abundant information suggesting that tumors

require one or more of the components of the chaperone system (CS), including Hsp60 discussed in this work, for growth and proliferation, epithelial-to-mesenchymal transition, dissemination, and anti-drug resistance [1–15]. Thus, it appears likely that the increase in the levels of chaperones observed in the cells of various types of cancers reflects the response of the cell to the needs of the carcinogenic process. However, due to the close correlation often observed between a progressive increase in the level of certain chaperones with advancing carcinogenesis, with metastasization, and with resistance to anti-cancer drugs, it appears possible that chaperones play a distinct etiologic–pathogenic role in the initiation of malignancies, or at least in their maintenance and progression.

Hsp60 is among the components of the CS studied during carcinogenesis in various tumors by several groups of investigators. For example, a steady increase in Hsp60 levels in tumor tissue paralleling tumor progression in uterine and colon cancers is among the earliest reported observations of the quantitative changes in a chaperone in relation to carcinogenesis [16]. Along the same lines, other reports pertain to other tumors, such as prostate [17–20], thyroid [21], and salivary gland [22] cancers. Here, we present data from a recent study of Hsp60 levels in gastric mucosa obtained with immunohistochemistry, comparing cancer with other gastric pathologies. These pathologies cause tissue disorganization and remodeling as exemplified by benign inflammatory, benign proliferation (e.g., hyperplastic polyps), metaplastic and dysplastic conditions, and malignant proliferations. Metaplasia is a para-physiological condition in which gastric glandular epithelium is replaced by another type of cell. Metaplasia occurs in response to chronic inflammation and is benign and typically reversible, but it can sometimes degenerate and evolve to dysplasia, a pre-cancerous condition [23]. Gastric adenocarcinoma is the most frequent gastric epithelial malignancy [24], and is aggressive and invasive. Gastric carcinogenesis is an orderly process that requires time for the tumor to become established and start its expansion. However, tumor progression can be triggered and accelerated by pre-cancerous conditions, such as gastric dysplasia, which facilitates cell proliferation, escape from pro-apoptotic stimuli, and genetic mutations [25–27]. In the study reported here, we performed an immunomorphological evaluation of the tissue levels of Hsp60 in specimens of gastric mucosa with inflammation (mild or moderate/severe gastritis), hyperplastic polyps, metaplasia, dysplasia, or cancer. The objective was to present an immunomorphological standard of Hsp60 quantitative variations that would be useful for the microscopic analysis of gastric pathologies aiming at differential diagnosis. Elucidation of the underlying molecular mechanisms is beyond the scope of the present work, but the data reported provide the basis for initiating a mechanistic–molecular dissection of the Hsp60 roles in various pathologies that are different from one another at the immunomorphological level.

2. Materials and Methods

2.1. Samples

Biopsy specimens of normal gastric tissue and samples with mild-to-moderate gastritis and hyperplastic polyp were obtained from the archives of the Biotechnology Laboratory, Euro-Mediterranean Institute of Sciences and Technologies (IEMEST). Gastric samples with severe gastritis, intestinal metaplasia, dysplasia, and carcinoma were collected from the archive of the Surgical Pathology laboratory, Department of Sciences for the Promotion of Health and Mother and Child Care, University of Palermo. Formalin fixed and paraffin embedded blocks of human gastric tissue were collected (10 cases for each group). The group of normal mucosa (NM) consisted of 5 males and 5 females with an average age of 51 $\pm$ 4 years. The group of specimens with mild-to-moderate gastritis (MMG) consisted of 3 males and 7 females, with an average age of 54 $\pm$ 4 years, with no signs of activity and a negative history of cancer or polyps. The group of hyperplastic polyp samples comprised 4 males and 6 females, with an average age of 56 $\pm$ 5 years, and a negative history of tumors and/or familial hereditary polyposis syndromes. Endoscopically, these polypoid formations had a diameter below 1 cm. The group of severe gastritis comprised 8 males and 2 females, with an average age of 52 $\pm$ 2 years. The group of gastric dysplasia

comprised 7 males and 3 females, with an average age of 51 ± 2 years. All patients had a negative history of *Helicobacter pylori*. The grade of dysplasia was moderate to severe. The group of gastric carcinoma samples comprised 5 males and 5 females, with an average age of 58 ± 5 years. These samples were obtained from total surgical gastrectomy and presented the histopathological diagnosis of intestinal type adenocarcinoma (type I according to Lauren), with a moderate degree of differentiation (G2). Together with infiltrating neoplasm, all samples also showed areas of normal mucosa and areas of intestinal metaplasia. The pathological staging (pTNM) of the gastric carcinoma group was $T_2N_0M_X$ and $T_3N_0M_X$ for 6 and 4 cases, respectively.

2.2. Immunohistochemistry

Immunohistochemistry (IHC) reactions for Hsp60 were carried out on 5 µm thick tissue sections obtained from paraffin blocks with a cutting microtome. The IHC reactions were performed using the automated IHC system of the Biotechnology Laboratory of the Euro-Mediterranean Institute of Sciences and Technologies (IEMEST) (IntelliPath Flx, Biocare Medical, distributed by Bio-Optica, Milan, Italy). The primary antibody used was a rabbit polyclonal anti-human Hsp60 (Clone H300, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA, catalog no. Sc-13966, dilution 1:300). After the end of the immunostaining cycle, the slides were prepared for observation with coverslips, using a permanent mounting medium (Vecta Mount, Vector, H-5000). The observation of the sections was performed with an optical microscope (Microscope Axioscope 5/7 KMAT, Carl Zeiss, Oberkochen, Germany) connected to a digital camera (Microscopy Camera Axiocam 208 color, Carl Zeiss, Oberkochen, Germany). Two independent pathologists (F.C. and F.R.) examined the specimens on two separate occasions, blinded, i.e., the slides were unidentifiable by the pathologist performing the examination (the k values for each group are: NM k = 0.88; MMG k = 0.88; SG k = 1; HP k = 0.88; IM k = 1; GD k = 0.88 and GC k = 0.75) and performed a quantitative analysis to determine the percentage of cells positive for Hsp60. The evaluation of the percentage of immunopositivity was calculated in a high-power field (HPF) at 400× of magnification and repeated for 10 HPF. The average of the percentages of all immuno-quantifications performed in each case for each group described was considered as a conclusive result, and this value was used for the statistical investigation.

2.3. Statistical Analysis

The one-way analysis of variance (one-way ANOVA with Bonferroni post hoc multiple comparison) was applied to comparatively evaluate the results, using the GraphPad Prism 4.0 software (GraphPad Inc., San Diego, CA, USA). The data are presented as the arithmetic mean (AM) ± the standard deviation (SD), and the statistical significance limit was set at mboxemphp ≤ 0.05.

3. Results

The immunomorphological analysis was performed on epithelial cells and revealed that the immunolocalization of Hsp60 was uniformly widespread in the cytoplasm, but with some granular appearance at times. The average percentages of Hsp60-positive epithelial cells were 32.8 ± 6.9 in normal mucosa; 33.5 ± 15.28 in mild-to-moderate gastritis; 51.8 ± 14.3 in severe gastritis; 58.5 ± 17.4 in hyperplastic polyps; 67 ± 11.8 in intestinal metaplasia; 89.4 ± 3.4 in gastric dysplasia; and 92.5 ± 4.4 in gastric adenocarcinomas.

The statistical analysis revealed significant differences between some of the groups analyzed, showing a gradual increase in Hsp60-positive cell numbers from gastritis to hyperplastic polyp, gastric dysplasia, and gastric carcinoma (Figure 1).

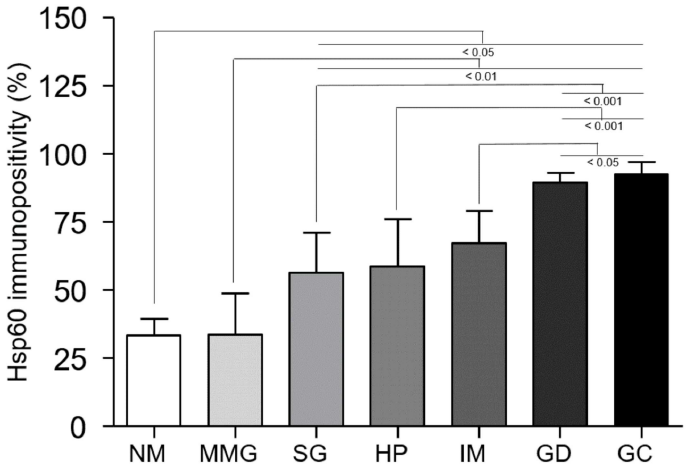

Figure 1. Histogram showing the percentage of Hsp60-positive epithelial cells in normal mucosa (NM), mild-to-moderate gastritis (MMG), severe gastritis (SG), hyperplastic polyp (HP), intestinal metaplasia (IM), gastric dysplasia (GD), and gastric adenocarcinoma (GC). Each group consisted of 10 cases. Data are presented as the arithmetic mean (AM) ± SD.

Gastric dysplasia was no different from gastric carcinoma. Representative immunohistochemical images are displayed in Figure 2.

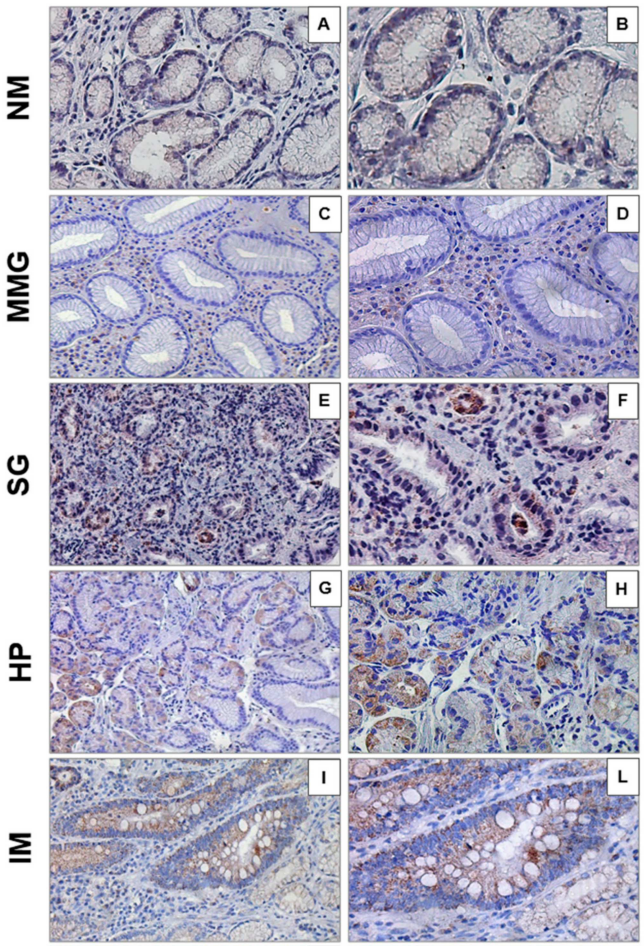

Figure 2. *Cont.*

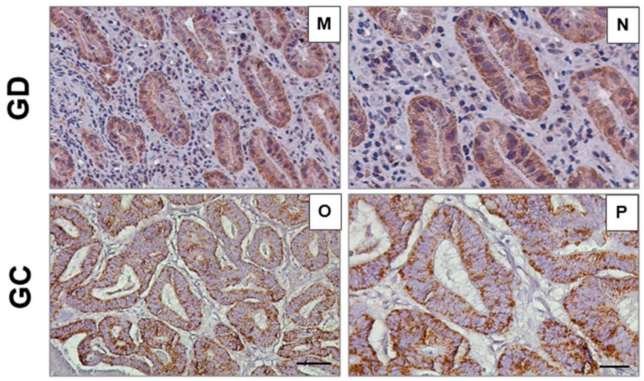

Figure 2. Representative images of immunohistochemical detection of Hsp60 in biopsies of human gastric tissue: (**A**,**B**) normal mucosa (NM), (**C**,**D**) mild-to-moderate gastritis (MMG), (**E**,**F**) severe gastritis (SG), (**G**,**H**) hyperplastic polyp (HP), (**I**,**L**) intestinal metaplasia (IM), (**M**,**N**) gastric dysplasia (GD), (**O**,**P**) gastric adenocarcinoma (GC). (**A**,**C**,**E**,**G**,**I**,**M**,**O**) Magnification 200×, scale bar 50 μm. (**B**,**D**,**F**,**H**,**L**,**N**,**P**) Magnification 400×, scale bar 20 μm.

Details of the normal mucosa, intestinal metaplasia, and gastric carcinoma with increasing numbers of Hsp60-positive cells (see percentages above) are shown in Figure 3.

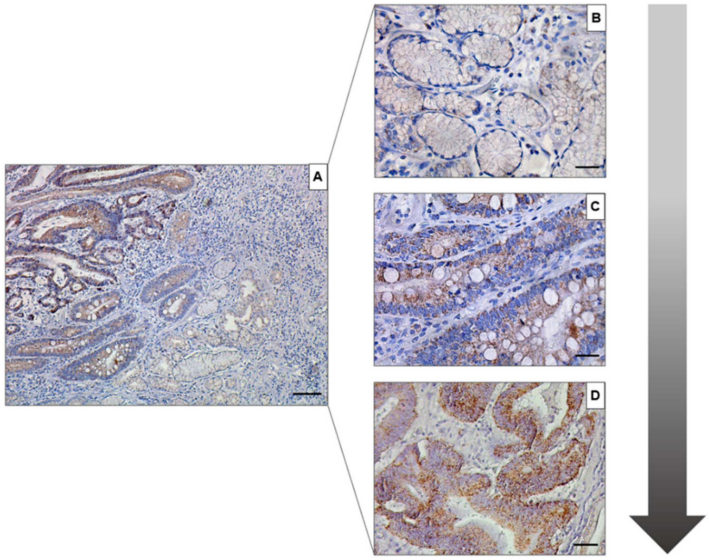

Figure 3. (**A**) Representative image of immunohistochemical detection of Hsp60 in gastric adenocarcinoma with associated areas of normal mucosa and intestinal metaplasia (magnification 100×, scale bar 100 μm). (**B**) Normal gastric mucosa (magnification 400×, scale bar 20 μm). (**C**) Intestinal metaplasia (magnification 400×, scale bar 20 μm). (**D**) Gastric adenocarcinoma (magnification 400×, scale bar 20 μm).

In some biopsies, there were areas of severe gastritis associated with areas of gastric dysplasia. The latter areas showed changes in nuclear and gland morphology typical of dysplasia with increased numbers of Hsp60-positive cells (Figure 4).

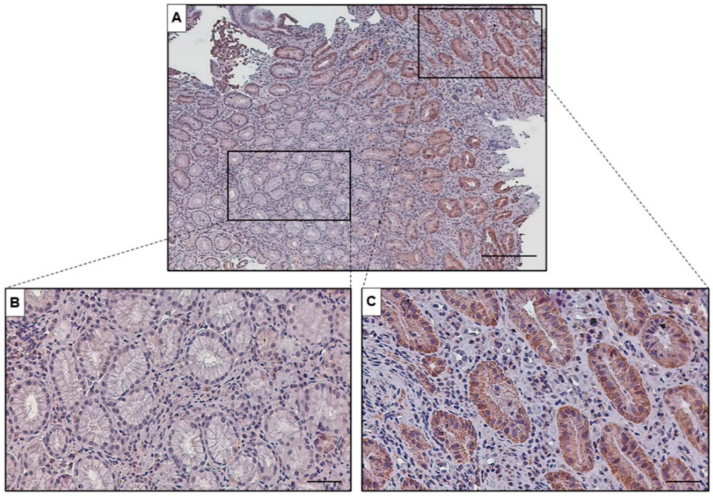

Figure 4. (**A**) Representative image of immunohistochemical detection of Hsp60 in gastric mucosa with areas of severe gastritis and areas with glandular dysplasia (magnification 50×, scale bar 200 μm). (**B**) Higher magnification of severe gastritis (magnification 200×, scale bar 50 μm). (**C**) Higher magnification of gastric dysplasia (magnification 200×, scale bar 50 μm).

4. Discussion

We found that the number of cells positive for Hsp60 and the positivity of the individual cells in gastric adenocarcinoma (GC) are high compared to the normal mucosa (NM). We also compared GC with other gastric pathologies, as follows: (1) mild-to-moderate gastritis, an inflammatory process without damage or destruction of the glandular structure; (2) severe gastritis, also an inflammatory process but associated with the injury and destruction of the glandular structure; (3) hyperplastic polyp, a paradigm of benign proliferation; (4) intestinal metaplasia, a benign reversible condition for the most part, characterized by morphological variations in the mucosa that probably reflect adaptive changes to a chronic inflammation; and (5) gastric dysplasia, a precancerous condition.

The highest levels of Hsp60 positivity were found in GC, but they were also high in gastric dysplasia (GD) in comparison with all the other pathologies studied, which reaffirms the close relationship between GC and GD, with the latter being a prelude to malignant transformation with the atypical alteration of epithelial cells. Additionally, it is of interest that (i) the Hsp60 positivity in hyperplastic polyps (HP) was higher than in normal mucosa (NM) but lower than in GC, even though HP and GC are both characterized by increased cell proliferation; (ii) the Hsp60 positivity was higher in the malignant version of these two examples of increased cell proliferation, HP and GC, but with the difference that GC is undifferentiated and disrupts the tissue structure and infiltrates neighboring spaces, properties not shown by polyps. This indicates that Hsp60 quantitative changes are strongly associated with malignancy (Figure 5).

Epithelial cell proliferation in polyps is characterized by an increase in mitotic replication cycles and a reduction in pro-apoptotic stimuli [28,29] also seen in carcinogenic proliferation, in which genetic mutations may occur [28,30]. Therefore, both pathologies share tissue remodeling events and factors, including an increase in Hsp60. Previous studies have found that Hsp60 increases in cases of alteration of the transmission of the apoptotic signal [31,32], and this may occur in the gastric polyps such as those included in this study, in which an altered cell turnover leads to an increase in proliferation. The involvement of Hsp60 in the carcinogenic process also includes its functional participation in many metabolic and biomolecular mechanisms of the cancer cells, encompassing interactions with various other molecules involved in programmed cell death, cell proliferation, and other pathways leading to malignant transformation [31].

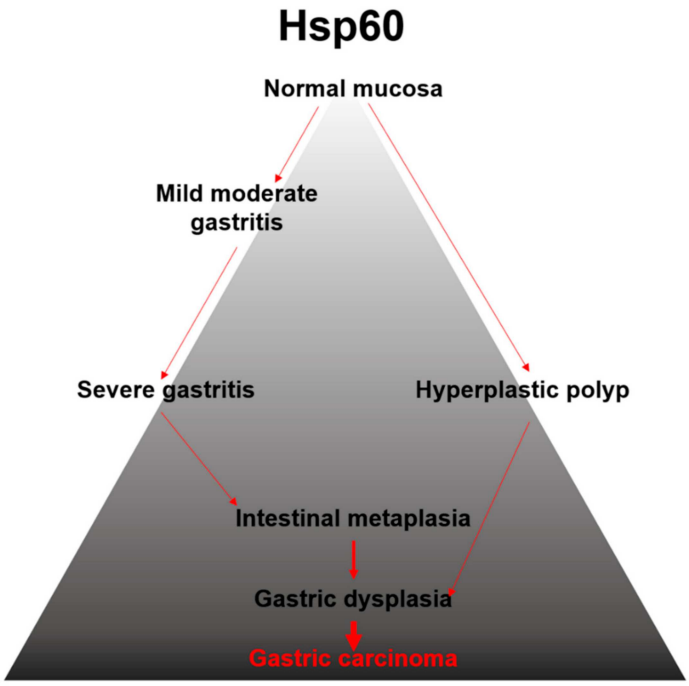

Figure 5. Schematic image visualizing the progressive increase in the number of Hsp60-positive epithelial cells in gastric mucosa as measured in this work. The increase is represented by the increment in darkness from top (lower proliferation) to bottom (higher proliferation), during the progression from normal mucosa (**top**) toward gastric carcinoma (**bottom**), passing through intermediate stages, such severe gastritis, hyperplastic polyp, intestinal metaplasia, and gastric dysplasia.

Other authors have also reported an increase in Hsp60 in the process of gastric carcinogenesis [11,33], ascribing to the chaperonin a fundamental role in the replication mechanisms and in cell survival [33]. Thus, our data are consistent with those from other investigators, showing that the Hsp60 increase is not only associated with the onset of GC but also correlates with the progression of the tumor mass and worsening of the clinical prognosis [11]. Regardless of the mechanisms by which Hsp60 increases in tumor cells, it is likely that the chaperonin aids them and should, therefore, be the target of anti-cancer treatments. The use of compounds to block Hsp60 activity, i.e., negative chaperonotherapy, has been proposed and is a promising field for investigation to develop means to defeat not only GC but also various other malignancies [34,35].

The Hsp60 positivity in mild-to-moderate gastritis is the same as that in normal mucosa, but in severe gastritis, it reaches higher levels, suggesting that in inflammatory processes the chaperonin is also involved to some extent. Here, the non-canonical functions of Hsp60, for example, stimulation of the production of pro-inflammatory cytokines [36–40], may be at play, an issue that deserves further investigation. Along the same lines, it has to be mentioned that infection with *H. pylori* can be associated with an increased risk of gastric neoplastic transformation [41]. Likewise, it has been observed that the *H. pylori* cytotoxin CagA assists in suppressing the heat shock response in gastric cancer cells, including Hsp60 expression [42]. In our study, it is relatively unlikely that *H. pylori* infection contributes to the results in any way, because the specimens we examined were derived from patients with a negative history of this infection.

5. Conclusions and Challenges for the Future

The increase in Hsp60 levels in the gastric mucosa during the progression from hyperplasia to carcinoma, passing through dysplasia, reflects the mounting need for cells for the CS, including Hsp60, to deal with their escalating metabolism and proliferation as they become malignant. It is possible that Hsp60 plays a distinct etiologic role in these

processes, different from its canonical functions pertaining to the maintenance of protein homeostasis inside mitochondria. It is likely that the chaperonin helps the tumor to grow and metastasize and, therefore, a Hsp60 chaperonopathy by mistake or collaborationism is in operation: a normal chaperone (as far as it can be determined with current technology) functions to favor cancer rather than protect the organism against it. This is a key concept to consider while developing treatment strategies because it puts the chaperone at the center of the carcinogenic mechanism. Consequently, Hsp60 becomes a preferential target against which one must develop anti-cancer drugs. This concept also paves the way to investigating the mechanism underpinning the quantitative increase in Hsp60 in tumor cells. Is it the increased expression of the *hsp60* gene? Is it an increase in the life span (low degradation rate) and/or rate of translation of the Hsp60 mRNA? Is it the low degradation rate of the protein Hsp60? What is the role of Hsp60-related miRNAs in the regulation of Hsp60 levels in cancer cells? Finding answers to at least one of these questions will greatly help in choosing approaches to develop anti-cancer drugs targeting Hsp60 as an inside collaborator with the "enemy." Another key issue to keep in mind is that Hsp60 could play other roles unrelated to the maintenance of protein homeostasis inside mitochondria that could favor tumor growth and dissemination, as suggested by the redistribution of the chaperonin outside the mitochondria in tumor cells, and even outside these cells. These extramitochondrial Hsp60 molecules could play non-canonical roles, some of which could favor the tumor. Whether these Hsp60 extramitochondrial and extracellular Hsp60 molecules are normal or abnormal is unknown, but they could bear post-translational modifications, enabling them to play roles that favor tumor growth and dissemination, and resistance to stressors and anti-cancer compounds. The above considerations indicate that research focusing on Hsp60 in gastric tumors could provide information suitable not just for the possible uses of the chaperonin and its quantitative variations as a biomarker in diagnosis and patient monitoring but also for developing therapeutic drugs targeting the chaperonin.

Author Contributions: Conceptualization: F.C. and F.R.; Methodology: A.P. and P.L.P.; Validation: D.C.; Investigation: F.R. and P.L.P.; Resources: A.P., S.I., A.F. and S.D.; Data Curation: F.C. and F.R.; Writing—Original Draft Preparation: F.R. and S.B.; Writing—Review and Editing, F.B., A.J.L.M. and E.C.d.M.; Supervision: A.J.L.M. and E.C.d.M. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: This study was ethically approved by the institutional review board of the Euro-Mediterranean Institute of Science and Technology of Palermo, Palermo, Italy (PIC 01/2018).

Informed Consent Statement: This study is an observational study in which all patients involved gave informed consent before performing the gastroscopy / biopsy or surgery. The same biological material (tissue) used for histopathological diagnosis was used for this work.

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Conflicts of Interest: The authors declare that they have no conflicts of interest.

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