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Epidermal growth factor receptor type 2 (HER-2) expression in gastric cancer – a correlation between clinical and histological features in patients at the Oncology Centre in Bydgoszcz

Ekspresja naskórkowego czynnika wzrostu typu 2 (HER-2) w raku żołądka - korelacja cech klinicznych i histologicznych u pacjentów Centrum Onkologii w Bydgoszczy

Summary
Gastric cancer is one of the most common tumours in Poland and it is often diagnosed at the advanced stage. Progression of the disease is the most important prognostic factor. It is assessed according to the TNM classification; however the prognosis in patients at the same clinical stage is often different. Therefore, in these cases there is a need to evaluate additional factors in order to determine the biological course of the disease. HER-2 (epidermal growth factor receptor type 2) expression is one of the prognostic predictors recently evaluated in the clinical studies. The first reports on HER-2 protein overexpression in gastric cancers were published in the late twentieth century, but its importance as a prognostic factor is still inconclusive and further research is needed. The aim of our study was to evaluate a relationship between the expression of HER-2 and microscopic features (histological type according to Lauren and WHO classification, grading, Ki-67 expression) in gastric cancers in 194 patients treated at the Oncology Centre in Bydgoszcz. HER-2-positive gastric cancers were more common in women than in men (11 (14.5%) versus 7 (5.9%) patients). The highest incidence of cancers with HER-2 overexpression was found in the group of tubular cancers (28.6%) and papillary cancers (14.3%). Among mucinous cancers and mucocellulare carcinoma, HER-2 receptor overexpression was found in 2.6% and 5.9%, respectively. However, there was the highest percentage of HER-2 receptor overexpression (20.3%) in tumours classified by Lauren as intestinal. There was higher statistically significant ($p = 0.0021$) level of Ki-67 expression in the group of HER-2-positive cancers.
Summarize, evaluation of HER-2 expression in gastric cancers facilitates qualification of patients for the treatment with the anti-HER-2 antibody. The introduction of trastuzumab into gastric cancer therapy significantly improves survival in patients with HER-2 receptor overexpression. Furthermore, HER-2 overexpression in intesti-

nal and highly differentiated cancers, which are generally regarded as having a better prognosis, may be an independent risk factor for worse prognosis. This is comparable to diffuse or poorly differentiated cancers. Further research conducted on larger cohorts is needed to explain the reasons for such divergent results.

Key words: gastric cancer, epidermal growth factor receptor type 2, HER-2, predictive markers

Streszczenie

Rak żołądka jest jednym z najczęstszych nowotworów złośliwych w Polsce. Nowotwór ten rozpoznawany jest często w stadium zaawansowanym. Najważniejszym czynnikiem rokowniczym jest zaawansowanie, oceniane według klasyfikacji TNM, jednak rokowanie u chorych o tym samym stopniu zaawansowania klinicznego często jest różne. Z tego powodu dla określenia przebiegu biologicznego choroby w tych przypadkach potrzebna jest ocena dodatkowych czynników. Jednym z ostatnio ocenianych w badaniach klinicznych czynników prognostycznych i jednocześnie predykcyjnych jest ekspresja naskórkowego czynnika wzrostu typu 2, HER-2 (ang. Human Epidermal Growth Factor Receptor 2). Pierwsze doniesienia dotyczące nadekspresji białka HER-2 w rakach żołądka opublikowano pod koniec XX wieku, jednak jego znaczenie jako czynnika rokowniczego jest wciąż niejednoznaczne i konieczne są dalsze badania. Celem naszych badań była ocena zależności pomiędzy ekspresją HER-2 i cechami obrazu mikroskopowego guza (typ histologiczny według Laurena, według klasyfikacji WHO, gradingu, ekspresją Ki-67) w rakach żołądka u 194 pacjentów Centrum Onkologii w Bydgoszczy. HER-2-dodatnie raki żołądka znacznie częściej występowały u kobiet niż u mężczyzn (11 (14,5 %) versus 7 (5,9 %) pacjentów). Najwyższą częstość raków z nadekspresją HER-2 stwierdzono w grupie raków cewkowych (28,6%) oraz papilarnych (14,3%). W grupie raków śluzowych i śluzowokomórkowych nadeskpresję receptora HER-2 stwierdzono odpowiednio w 2,6% i 5,9%. Natomiast w rakach sklasyfikowanych jako jelitowe według Laurena zanotowano najwyższy odsetek guzów wykazujących nadekspresję receptora HER-2 (20,3%). W grupie raków HER-2-dodatnich zaobserwowano wyższy, istotny statystycznie (p=0.0021) poziom ekspresji Ki-67.

Podsumowując, ocena ekspresji HER-2 w rakach żołądka stwarza możliwości terapii tych nowotworów. Wprowadzenie do terapii raka żołądka trastuzumabu, znacznie poprawia przeżycia pacjentów z nadekspresją receptora HER-2. Ponadto, nadekspresja HER-2 w rakach o typie jelitowym według Laurena oraz w rakach wysokozróżnicowanych, które są zazwyczaj uważane za nowotworowy o lepszym rokowaniu, może stanowić niezależny czynnik gorszego rokowania w wyżej wymienionych typach nowotworów, porównywanym do raków o typie rozlanym lub niskozróżnicowanych. Konieczne są dalsze badania, na większych grupach badanych, mające na celu wyjaśnienie przyczyn obserwowanych rozbieżnych wyników.

Słowa kluczowe: rak żołądka, naskórkowy czynnik wzrostu typu 2, HER-2, czynniki predykcyjne

Introduction

Gastric cancer is one of the most common tumours in Poland. According to American data, in the last five decades, the incidence of this cancer in the United States declined, but it still remains the second leading cause of death from cancer worldwide. Over half of all stomach cancers occur in developing countries. The highest incidence is in Japan, Korea, China, the countries of Central and South America (Chile, Colombia), Russia and

the former Soviet Union, where incidence rates are at the level of 45.5-77.9 per 100 000 inhabitants a year. As for incidence, stomach cancer is sixth in men and eleventh in women. As for mortality, it is the third in men and eighth in women. The incidence rate per 100000 inhabitants in Poland is 18.9 for men and 9.5 for women (Jastrzębski et al. 2010).

Approximately half of all cases of gastric cancer in the world are diagnosed at the early stage and the rest at the advanced stage. As in Poland there is no screening of this cancer, it is often diagnosed at the advanced stage (early gastric cancer in Poland is reported merely in 5-8% of cases). Intensive screening programs were implemented only in East Asian countries. This allowed the effective diagnosis of cancers at the early stage (Jemal et al. 2011).

Thirty five – forty percentage of tumours are located in the pre-pyloric part and in the pylorus (a downward trend), 25% in the body and 30-40% in the cardia (a rising trend). Gastric cancer usually spreads by implantation of cancer cells into the peritoneum via the intraperitoneal route (about 50%) or through the lymphatic system (approximately 27%) and by the bloodstream (around 23% of cases). Thus, metastases are formed in the liver, lungs and bones. The current studies evaluating the adjuvant treatment show no significant effect of radiotherapy and chemotherapy on the prognosis. Many studies covered the heterogeneous material, thereby losing their credibility. This does not facilitates reaching the agreement on the conditions of effective adjuvant therapy (Macdonald et al. 2001, Cunningham et al. 2006; Sastre et al. 2006).

Progression of the disease is the most important prognostic factor. It is assessed according to the TNM classification; however the prognosis in patients at the same clinical stage is often different. Therefore, in these cases there is a need to evaluate additional factors in order to determine the biological course of the disease. HER-2 (epidermal growth factor receptor type 2) expression is one of the prognostic predictors recently evaluated in the clinical studies. HER2 is a transmembrane tyrosine kinase receptor involved in the conduction of signals and aimed to induce growth and differentiation of cells. It regulates cell proliferation by activating transmission pathways such as cascade associated with mitogen-activated protein kinases (MAPK). For the hitherto unknown reasons, its quantity is increased on the surface of some tumour cells (overexpression). This intensifies divisions and uncontrolled tumour growth (Badache and Goncalves 2006; Ramieri et al. 2010).

HER-2 protein overexpression is found in different types of epithelial cancers, including 10-40% of breast cancers, both in situ and invasive. Amplification and overexpression of HER-2 multiply the number of HER-2 receptor monomers on the cellular surface. It was reported that over-expression of HER-2 in breast cancer is an indicator of a more aggressive disease. Amplification of the gene is a strong and independent factor associated with shorter recurrence time. It is also connected with shorter overall survival in patients with metastases to the lymph nodes. HER-2 changes co-occur with other prognostic factors such as histological grade, histological type, absence of oestrogen receptors (ER) and progesterone receptors (PR) and the lymph node involvement. The status of HER-2 in breast cancer decides on qualifying patients to the treatment with recombinant humanized antibody against HER-2, trastuzumab (Krasińska and Jassem 2003).

The first reports on HER-2 protein overexpression in gastric cancers were published in the late twentieth century (Park et al. 1989; Tokunaga et al. 1995). Overexpression is found in about 8-35% of patients with this cancer and some studies associate it with a worse prognosis (Takehara et al. 2002; Romiti et al. 2012). However, its importance as a prognostic factor is still inconclusive and further research is needed. The aim of our study was to evaluate a relationship between the expression of HER-2 and microscopic features (histological type according to Lauren and WHO classification, grading, Ki-67 expression) in gastric cancers in patients treated at the Oncology Centre in Bydgoszcz.

Material and methods

Patients

The study included 194 patients treated at the Franciszek Łukaszczyk Oncology Centre in Bydgoszcz in the years 2010-2013. The histological material taken from patients was tested using immunohistochemical assay (IHC) for HER-2 expression. Table 1 shows the characteristics of patients.

Immunohistochemistry and fluorescence in situ hybridization (FISH)

HER-2 expression was determined using immunohistochemistry on standard sections of 4-5 □m (prepared from the histological material settled in formalin and submerged in paraffin). The assay used rabbit polyclonal Parthway anti-HER-2/neu antibody (clone 4B5), designed to the semi-quantitative evaluation of histological preparations (Ventana Medical Systems, Incorporation, F. Hoffmann-La Roche Ltd). The system based on diaminobenzidine, ultraView Universal DAB Detection Kit (Ventana Medical Systems, Incorporation, F. Hoffmann-La Roche Ltd.) was used to visualize the immunohistochemical reaction. The assays were performed using a fully automated apparatus Ventana Benchmark Ultra (Ventana Medical Systems, Incorporation, F. Hoffmann-La Roche Ltd.).

Amplification of the HER-2 gene was detected by FISH in accordance with the manufacturer's protocol (PathVysion® HER-2 DNA Probe Kit, Abbott Molecular Inc., IL, USA). Deparaffinized standard sections were treated with 0.2 N HCl at room temperature and then pre-heated in pre-treatment solution, digested with protease, fixed in formalin and dried in a hybridizer. The following fluorescently labelled probes were applied on these formulations: LSI HER-2 - complementary to the HER-2 gene (labelled with marker SpectrumOrange™) and CEP 17 - complementary to satellite DNA located in the centromere of chromosome 17 (labelled with marker SpectrumGreen™), covered with a coverglass and subjected to 5-minute denaturation at 73°C and 21-hour (overnight) hybridization at 37°C. Next, the preparations were sealed in medium containing DAPI (marking the cell nuclei) and evaluated on the next day under a fluorescent microscope at magnification of 1000x.

The assessment of Ki-67 expression used 4-5 μm sections of tissue material settled in 10% buffered formalin and sunken in paraffin, which after heating at 60°C, deparaffinization and hydration in a series of decreasing concentration alcohol solutions were heated in the citrate buffer (pH 6) for 20 minutes in a microwave oven (650W). Next, the activity of endogenous peroxidase was blocked and sections were incubated with anti-Ki-67 (1:100) (Dako, Carpinteria, CA, USA) at room temperature. Antigen-

linked antibodies were visualized using the system EnVision ™ Detection System, Peroxidase/DAB+ (Dako, Carpinteria, CA, USA), then cell nuclei were stained with hematoxylin and preparations were sealed in solid medium (Consul Mount; Thermo Fisher Scientific Inc. Waltham, MA, USA).

The evaluation of preparations

The expression of HER-2 receptor in gastric cancer was detected using immunohistochemistry and evaluated based on a four-point scale. Tumours were classified as HER-2-IHC-3+ (positive) when there was membrane expression detected (medium to strong), especially in the basolateral part of the cell. In gastroscopic specimens, expression occurred in at least 5 cancer cells, while surgical specimens contained at least 10% of tumour cells. HER-2-IHC-2+ status was indicated when total or partial membrane labelling (weak to moderate) involved more than 10% of cancer cells. HER-2-IHC 1+ status was declared in tumours where very weak membrane labelling (total or partial) occurred in more than 10% of cancer cells. There was no membrane labelling in tumours classified as HER-2-IHC-0 (negative) or it occurred in less than 10% of tumour cells (Olszewski and Olszewski 2010, Ruschoff et al. 2010).

HER-2 gene amplification detected using FISH was assessed by evaluating the ratio of HER-2 and CEP17 signals. The ratio more than 2.2 HER-2/CEP 17 indicated amplification. The FISH result showing the HER-2/CEP 17 ratio below 1.8 indicated a negative result (no amplification). The result of FISH tests HER-2/CEP17 from 1.8 to 2.2 was ambiguous.

Ki-67 expression was analyzed by assessing the percentage of gastric cancer cell nuclei that express the tested antigen.

Analysis of expression changes and statistical evaluation

Statistical analysis of the results was performed using program GraphPad Prism 5.00 (GraphPad Software, San Diego, CA). Statistically significant differences were examined using the non-parametric Mann-Whitney test; when $p < 0.05$ the results were accepted as significant.

Results

HER-2-positive gastric cancers were more common in women than in men (11 (14.5%) versus 7 (5.9%) patients). The mean age of patients in the group of HER-2-positive cancers was 62.2 years (from 36 to 84, median 65 years). Compared to the group of HER-2 negative patients, in this group median was slightly higher, but the reported differences were not statistically significant (see Table 2, Fig. 2, median 61-62 years).

The analysis also included distribution of HER-2 receptor expression in tumors grouped according to the WHO and Lauren classification. The highest incidence of cancers with HER-2 overexpression was found in the group of tubular cancers (28.6%) and papillary cancers (14.3%). Among mucinous and signet ring caners, HER-2 receptor overexpression was found in 2.6% and 5.9%, respectively (Table 3, Fig. 3A-B). However, there was the highest percentage of HER-2 receptor overexpression (20.3%) in tumours classified by Lauren as intestinal (Table 3, Fig. 4A-B).

Furthermore, a relationship was observed between HER-2 overexpression and differentiation grade. There were significantly statistically higher incidence of HER-2 overexpression ($p=0.0048$) in well-differentiated cancers (Fig. 5). Statistical analysis showed an inverse correlation of HER-2 expression and tumor grade ($r = -0.2263$, $p = 0.0017$).

There was also a significant positive correlation between HER-2 receptor overexpression and the expression of cell proliferation marker Ki-67 ($r = 0.1909$, $p = 0.0053$). There was higher statistically significant ($p = 0.0021$) level of Ki-67 expression in the group of HER-2-positive cancers (Fig. 6).

Discussion

The expression of the receptor was found in nearly 10% of patients selected for this study at the Oncology Centre in Bydgoszcz. The expression differed depending on the histological type (type according to the Lauren and WHO classification), proliferation rate and a degree of differentiation.

The importance of HER-2 as a prognostic factor is still controversial. Some published data did not indicate the existence of a relationship between HER-2 expression in gastric cancers and overall survival or disease-free time (Aoyama et al. 2013, Zhou et al. 2013). At the same time, other researchers have observed worse prognosis in patients with gastric cancers and HER-2 overexpression (Park et al. 2006, Xie et al. 2009; Liu et al. 2012). Park et al. (2006) reported worse prognosis in patients with HER-2 amplification as well as worse 5-year survival (21.4% vs. 63%). In addition, Park et al. showed that HER-2 amplification, age and TNM staging were independent prognostic factors. The similar relationships were observed by Xie et al. (2009) who noted a correlation between HER-2 overexpression and poorer survival ($p < 0.001$). However, Liu et al. (2012) reported shorter 5-year survival in patients with intestinal type of gastric cancer and early gastric cancer with HER-2 overexpression compared to cancers with no overexpression (21% vs. 47%, $p = 0.027$; 29% vs. 60%, $p=0.037$). Nevertheless, they found no such relationships in diffuse and mixed cancers.

Similar discrepancies are observed in the analysis of a relationship between HER-2 expression and pathomorphological features of gastric cancer.

Some researchers consider HER-2 expression in gastric cancers as a factor associated with such tumour characteristics as severity according to the TNM classification (depth of tumour invasion and the presence of metastases to the lymph nodes and distant metastases), histological type and a degree of differentiation. It should also be noted that other studies do not indicate the existence of such relationship (e.g.: Risio et al. 2003; Yan et al. 2011).

Some studies show higher incidence of HER-2 overexpression in the intestinal type (16%-87%) of stomach cancers than in the diffuse type (4-7%) (Uchino et al. 1993; Takehara et al. 2002; Gravalos and Jimeno 2008; Tafe et al. 2011, Yan et al. 2011; Gomez-Martin et al. 2012). Additionally, in their studies Uchino et al. (Uchino et al. 1993) found that HER-2 receptor overexpression was more often in papillary tumours or well- and medium-differentiated (14%) than in poorly differentiated or mucocellulare carcinoma (2%). It should be noted, however, that other researchers did not observe differences in the incidence of HER-2 overexpression and the type classified according to Lauren or the histological type (Sekaran et al. 2012; Tsapralis et al. 2012).

Similarly, Hayashi et al. (Hayashi et al. 2008) did not report worse prognosis in patients with HER-2 overexpression, although they found a relationship between overexpression and the type according to Lauren, with higher frequency of HER-2 overexpression in

the intestinal type (vs. diffuse type, p = 0.007). HER-2 overexpression did not depend on the clinical and pathological factors, depth of invasion, presence of metastasis or clinical stage. Yan et al. (2011) found a correlation between HER-2 overexpression and tumour infiltration depth, TNM, metastases to the lymph nodes and distant metastases ($p<0.05$), but noticed no correlation between HER-2 overexpression and age, sex, type of differentiation or location.

Our studies reported no correlation between HER-2 overexpression and the age of patients. However, there was higher incidence of cancer HER-2-positive women (14.5% versus 5.9% for men). We have also observed, like other researchers, a higher rate of HER-2 overexpression in the intestinal type cancers according to Lauren compared to the diffuse type (20.3% versus 1.5%) and tubular and papillary type in comparison with the mucinous cancers and signet ring cancers according to the WHO classification (42.9% versus 8.5%) (Uchino et al., 1993; Takehara et al. 2002; Gravalos and Jimeno 2008; Tafe et al. 2011, Yan et al. 2011; Gomez-Martin et al. 2012).

Tafe et al. [2011] noticed statistically significantly higher incidence of HER-2 overexpression in moderately differentiated tumours compared to poorly differentiated tumours ($p <0.001$). Similarly, our studies reported a negative correlation between HER-2 expression and a degree of differentiation. The highest incidence of overexpression HER-2 was observed in highly differentiated cancers (G1-25%, G2-14.9%, G3-2.8%).

Moreover, HER-2 overexpression was observed more frequently in cancers located in the esophagogastric junction than in the other parts of the stomach (25% vs. 9.5%, $p=0.01$) (Gravalos and Jimeno 2008).

The literature lacks data discussing the Ki-67 expression in relation to HER-2 overexpression, although our results indicate that there is a positive correlation between the expression of these proteins. This may suggest that HER-2 overexpression stimulates proliferation through Ki-67 by activating and mobilizing signal transduction.

Conclusions

To sum up, we can conclude that even though the studies on HER-2 overexpression in gastric cancers have been conducted for over 25 years, the prognostic and predictive value of evaluating the expression of this receptor is ambiguous. Also, some study results seem to be controversial. At the same time, it should be noted that stomach cancer therapy is associated with a number of limitations and difficulties. The introduction of trastuzumab into gastric cancer therapy significantly improves survival in patients with HER-2 receptor overexpression. Furthermore, HER-2 overexpression in intestinal and highly differentiated cancers, which are generally regarded as having a better prognosis, may be an independent risk factor for worse prognosis. This is comparable to diffuse or poorly differentiated cancers. Further research conducted on larger cohorts is needed to explain the reasons for such divergent results.

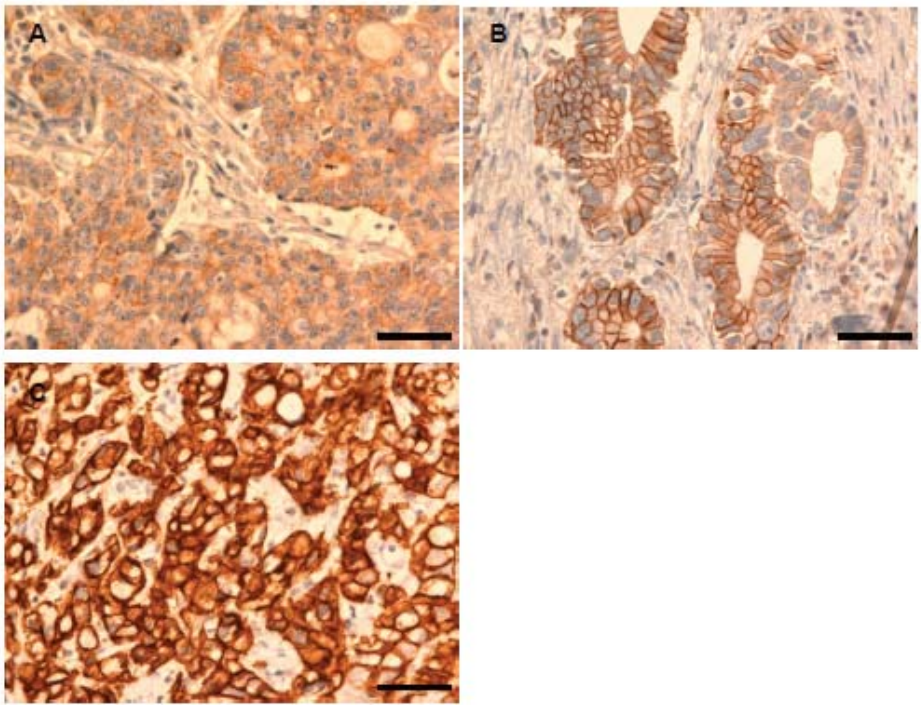

Fig. 1. A status of the HER-2 receptor in the sections labelled by immunohistochemistry in gastric cancers. A) HER-2 expression at 1+; B) HER-2 expression at 2+; C) HER-2 expression at 3+. The blue-violet nuclei are counterstained with hematoxylin. Scale bars - 50 μm.

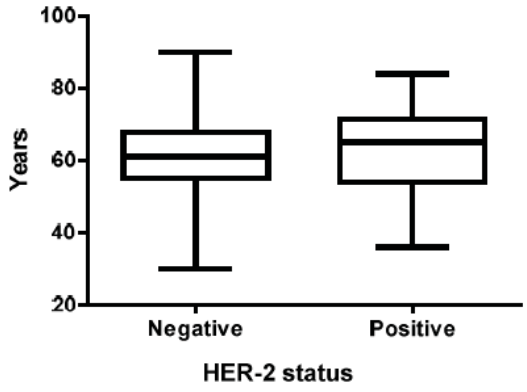

Fig. 2. The age of patients with HER-2-negative and HER-2-positive gastric cancers. Data present the median (horizontal line), 25th and 75th percentile (lower and upper limit of the rectangle) and the minimum and maximum (upper and lower vertical line) age of patients.

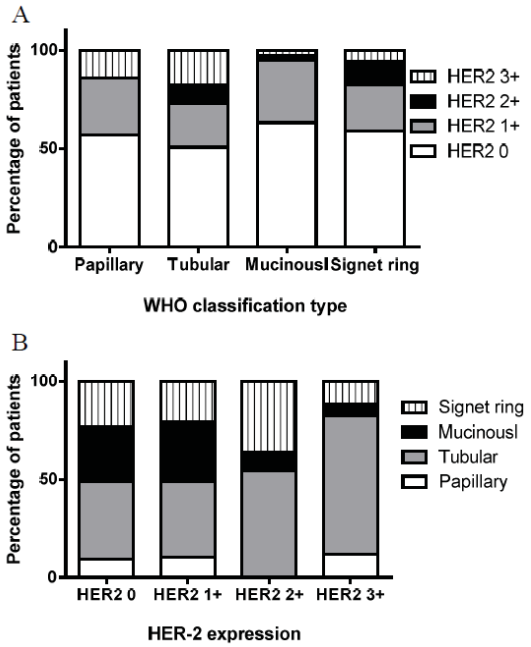

Fig. 3. HER-2 expression in gastric cancers classified according WHO classification.

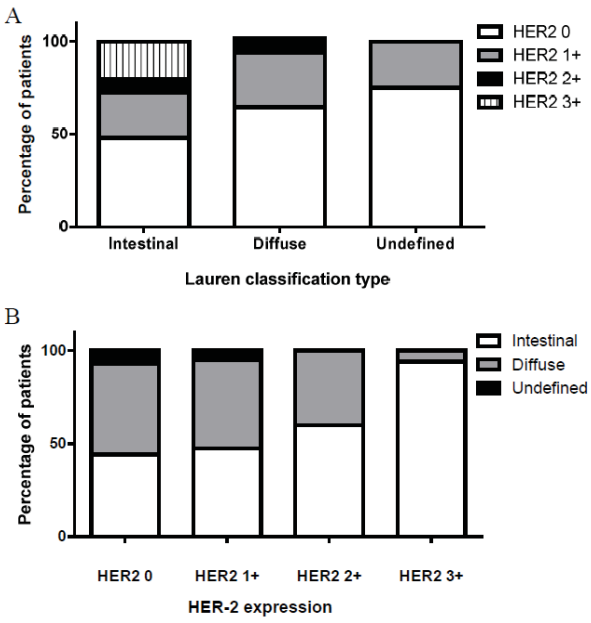

Fig. 4. HER-2 expression in gastric cancers classified according Lauren classification.

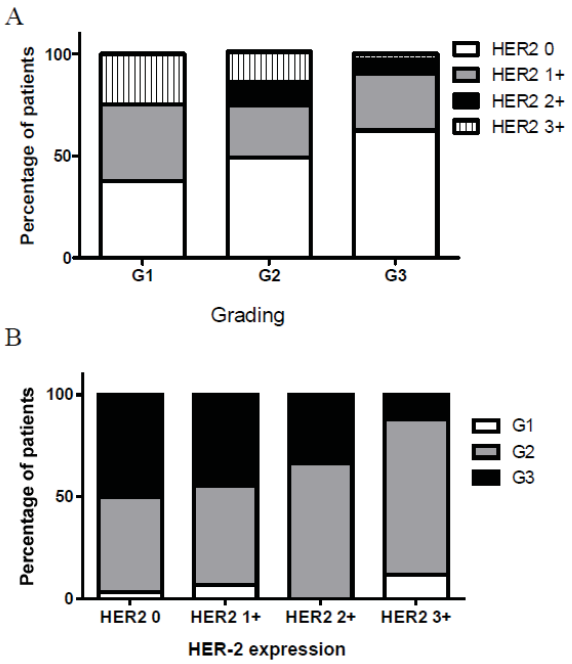

Fig. 5. HER-2 expression in gastric cancers classified according differentiation grade.

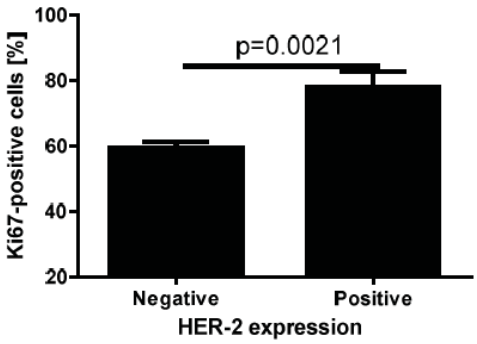

Fig. 6. Ki-67 expression in HER-2-negative and HER-2-positive gastric cancers.

Tab. 1. Clinico-pathomorphological gastric patients characteristic.

Gender	F M	n=76 n=118
Age [years]	F: Mean Median M: Mean Median	61,5 (32-84) 62 61,0 (30-90) 61
Grading*	G1 G2 G3	n=8 n=87 n=72
Lauren classification**	Intestinal Diffuse Unclassified	n=79 n=66 n=8
WHO classification***	Papillary type Tubular type Mucinous type Sygnet ring type	n=14 n=67 n=38 n=34

Due to very small gastroscopic samples or too small cancer area in sections:
* no data for 27 patients
** no data for 44 patients
*** no data for 45 patients

Tab. 2. HER-2 status in gastric cancer patients.

HER-2 status	Number of patients / percentage
HER-2-IHC-0	112 / 57,7
HER-2-IHC-1+	46 / 23,7
HER-2-IHC-2+	18 / 9,3
HER-2-IHC-3+	18* / 9,3

* HER-2 overexpression was confirmed in 16 patients using immunohistochemistry, and in 2 patients using FISH metod.

Tab. 3. HER-2 expression in gastric cancer patients classified according pathomorphological features.

Feature		Number of patients / percentage	
Age	≤ 50 r. ż.	HER2-negative HER2-positive	24/88,9 3/11,1
	>50 r. ż	HER2-negative HER2-positive	152/91,0 15/9,0
Gender	F	HER2-negative HER2-positive	65/85,5 11/14,5
	M	HER2-negative HER2-positive	111/94,1 7/5,9
Grading	G1	HER2-negative HER2-positive	6/75 2/25
	G2	HER2-negative HER2-positive	74/85,1 13/14,9
	G3	HER2-negative HER2-positive	70/97,2 2/2,8
Lauren classification	Intestinal	HER2-negative HER2-positive	63/79,7 16/20,3
	Diffuse	HER2-negative HER2-positive	65/98,5 1/1,5
	Unclassified	HER2-negative HER2-positive	8/100 0/0
WHO classification	Papillary type	HER2-negative HER2-positive	12/85,7 2/14,3
	Tubular type	HER2-negative HER2-positive	55/71,4 12/28,6
	Mucinous type	HER2-negative HER2-positive	37/97,4 1/2,6
	Sygnet ring type	HER2-negative HER2-positive	32/94,1 2/5,9

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