ORIGINAL ARTICLE

Expression analysis of heat shock protein 90 (HSP90) and Her2 in colon carcinoma

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Abstract

Purpose The molecular chaperone heat shock protein 90 (HSP90) plays an important role in several types of tumors also participating in the modulation of the activity of receptor tyrosine kinases activity such as members of the Her family. We evaluated the significance of HSP90 and Her2 expression in colon cancer.

Methods HSP90 and Her2 expression was determined by immunohistochemistry and by fluorescence in situ hybridization (FISH) on 355 primary resected colon carcinomas. Results were correlated with pathologic features (Union for International Cancer Control (UICC) pTNM category, tumor localisation, tumor differentiation), additional molecular genetic characteristics (BRAF, KRAS mutational status, mismatch repair genes (MMR)), and survival.

Results HSP90 immunoreactivity was observed in various degrees. Fifty-one cases (14 %) were positive for Her2 (score 2+ and 3+) with 16/43 cases with Her2 2+ staining pattern showing amplification of Her2 determined by FISH. There was a significant correlation between high HSP90 expression and Her2 overexpression ($p=0.011$). High HSP90 expression was associated with earlier tumor stages ($p=0.019$), absence of lymph node ($p=0.006$), and absence of distant metastases ($p=0.001$). Patients with high tumoral HSP90 levels had a better survival ($p=0.032$), but this was not independent from other prognostic relevant pathologic parameters. Her2 expression was not associated with any of the investigated histopathological, molecular, or clinical parameters.

Conclusions High HSP90 levels are reflecting lower malignant potential in colon cancer. Her2 positivity can be observed in a small number of cases. Targeting HSP90 and/or Her2 may be an alternative therapeutic approach in colon cancer in a subset of patients.

Keywords HSP90 · Her2 · Immunohistochemistry · Colon cancer

Introduction

A significant number of gastric and gastroesophageal adenocarcinomas shows amplification and overexpression of Her2 [1, 2] leading to the introduction of anti-Her2-directed therapy with trastuzumab in the treatment of metastatic adenocarcinomas of the esophagogastric junction and the stomach [3, 4]. In contrast to adenocarcinomas of the upper gastrointestinal tract, studies about Her2 in colorectal cancer are less comprehensive, and the studies show a high variability of Her2 positivity rates in these tumors [5–12].

Her2 has been shown to interact with HSP90, a molecular chaperone belonging to the group of heat shock proteins. These molecules are responsible for the correct folding of proteins, prevention of protein aggregation, and protein activation [13, 14]. There are data which suggest that HSP90 expression may also support the effects of oncogenic Her2 [15] which represents a potential mechanism of resistance to Her2-directed drugs. On the other hand, HSP90 inhibitors, which have been developed recently, may potentiate the

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effects of anti-cancer drugs targeting client proteins of HSP90 [16]. The relationship between HSP90 and Her2, however, has not been investigated so far for colon carcinomas. Moreover, there are currently only scarce data about HSP90 expression in human colon cancer tissue [17, 18].

The aim of the present study was to investigate the role of Her2 in colon cancer and to get deeper insights of tumor biology and behavior regarding Her2-positive carcinomas. We furthermore investigated any possible relationship of Her2 and HSP90 as potentially targetable molecules in colon cancer.

Materials and methods

Patients and tissues

We investigated formalin-fixed and paraffin-embedded (FFPE) archival cancer tissue from 355 patients with colon carcinoma who underwent surgery between 1993 and 2005 at the Klinikum Rechts der Isar of the Technische Universität München (Germany). All patients had a primary resection. Informed consent for the use of additional molecular analysis was given at the time of surgery, and the use of archival tissue for molecular analysis was approved by the local ethical commission (No. 2136/08). Follow-up data (overall survival) were available for 349 patients.

Immunohistochemistry

Immunohistochemistry was performed on FFPE tissue on a tissue microarray (TMA) as described before [19]. Representative tumor sections were marked, and two core needle biopsy specimens of 1.0 mm in diameter were retrieved and placed in a recipient wax block using a manual arrayer (Beecher Instruments; Sun Prairie, Wisconsin, USA). For a subset of cases (n=17), where additional lymph node metastases were investigated, full slide sections were used (see below).

The paraffin blocks were freshly cut (3 μm). Slides were dewaxed and rehydrated, with subsequent heat-induced antigen retrieval using 10 mM citrate buffer (pH 6), H_2O_2 blocking using 3 % H_2O_2 in aqua dest., and avidin/biotin blocking (avidin/biotin blocking kit; Vector Laboratories, Inc., Burlingame, CA, USA). The sections were then incubated with antibodies for HSP90 (1:50; Abcam, Cambridge, UK), which recognizes both alpha and beta isoforms of HSP90, Her2 (1:300; DAKO, Glostrup, DK), and for MLH1, MSH6, MSH2, and PMS2 (1:200, 1:200, 1:1,600, and 1:75, respectively; all Leica Novocastra). Immunodetection was performed with the Dako REAL™ Detection System Peroxidase/DAB+kit (DAKO, Glostrup, DK) which resulted in a brown staining. A breast cancer tumor

sample with known Her2 status (immunohistochemistry (IHC) 3+ and high-level amplification) and high HSP90 expression served as positive control. For negative controls, staining without the primary antibody was performed.

Her2 expression was assessed according to published recommendations for Her2 evaluation in gastric carcinoma [20], and we used the criteria for assessment of Her2 on bioptic tissue due to the tumor content of the TMA core which is comparable to a biopsy. In short, immunohistochemistry 3+ staining was defined as strong complete basolateral or lateral membranous staining of cell clusters irrespective of the percentage of tumor cells, visible at low magnification (×2.5–5); immunohistochemistry 2+ was defined as weak to moderate basolateral or lateral membranous staining visible at×10–20 magnification; and immunohistochemistry 1+ staining was defined as weak membranous staining visible only with×40 magnification. Cases without visible membranous reactivity were classified as negative (Fig. 1) All cases with Her2 score 2+ were additionally investigated by fluorescence in situ hybridization (FISH) according to the criteria for assessment of Her2 status by the FDA/European Medicines Evaluation Agency (EMEA) for gastric cancer [20].

Expression of HSP90 (Fig. 2) was assessed by determination of cytoplasmic staining based on the intensity of immunostaining and the percentage of stained tumor cells. Immunostaining was scored as 0 (no immunostaining), 1 (weak immunostaining), 2 (moderate immunostaining), or 3 (strong immunostaining). The percentage of positive tumor cells was scored as 0 (none), 1 (<10 %), 2 (10–50 %), 3 (51–80 %), or 4 (>80 %). Multiplication of the scores for intensity and percentage resulted in a staining index (SI) ranging from 0 to 12. Similar to a previous study [19], we then divided the expression of HSP90 into low to moderate to high according to the terciles of the distribution of SI.

Evaluation of mismatch repair (MMR) genes MLH1, PMS2, MSH2, and MSH6 was performed following current recommendations [21]. Every nuclear immunoreactivity was regarded as positive. MMR deficiency was defined as a loss of expression of at least one marker. All immunohistochemical stainings were scored by two independent observers (KB, ED, or RL), and discrepancies were discussed on a multihead microscope with a final consent. Only cores with technically unequivocal staining results and sufficient tumor content (>50 tumor cells) were used for final analysis.

Fluorescence in situ hybridization

All cases showing an immunohistochemical Her2 score of 2+ were also tested for Her2 amplification by FISH. An assay with fluorescence-labeled locus-specific DNA probes for Her2 and chromosome-17 centromeric α-satellite (Chrombios) was hybridized onto 4-μm TMA sections as described before [22]. FISH signal evaluation was performed

Fig. 1 Examples of immunohistochemical stainings for Her2 in primary resected colon cancer. **a** Score 0 (negative). **b** Score 2+. **c** Score 3+ ; ×20; brown membranous staining of tumor cells
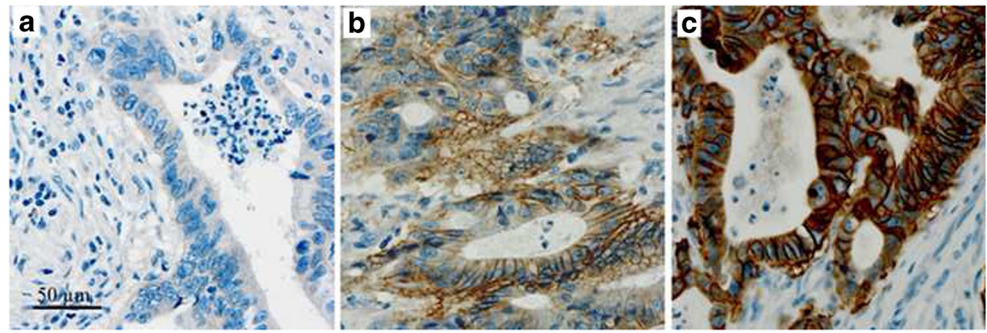
by visual counting using an epifluorescence microscope (Zeiss Axioplan 2, Carl Zeiss Microimaging GmbH). At least 50 invasive tumor cells per case with a minimum of one signal for Her2 gene and centromere 17 were randomly selected, and the mean Her2 and centromere 17 count was calculated. Cases were classified as amplified when Her2/cep17 quotient was ≥2.
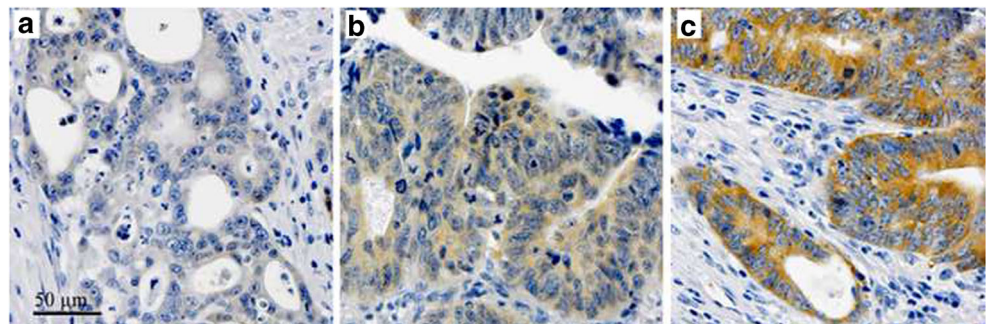
KRAS and BRAF analysis

Mutational analysis of KRAS and BRAF was performed on all cases showing positive Her2 expression (score 2+ and 3+). For this analysis, we used real-time PCR with high-resolution melting analysis (HRMA) from genomic DNA as previously described [23].

Statistical analysis

IBM SPSS 21.0 Statistics statistical software (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. Associations between immunohistochemical staining patterns, FISH analysis, and pathological features were given in crosstabs and were evaluated with χ^2 test. Kaplan-Meier estimates, log rank tests, and Cox's proportional hazards regression analysis were used for survival analysis. All tests were two-sided, and the significance level was set at 0.05.

Results

Patients and pathologic findings

One hundred fifty-five patients were female (44 %), and 200 of the patients were male (56 %). The mean age was 65±12 years (median, 66; range, 25 to 91). Mean follow-up time for surviving patients was 87±27.6 months. Complete tumor resection (R0) was achieved in 306 cases (86.2 %). All tumors were reclassified according to the current Union for International Cancer Control (UICC) TNM classification [24]. Tumor grading was done according to the WHO classifications of tumors of the digestive system [25]. The pathologic characterization of the collective is given in Table 1. The postoperative UICC staging of the tumors is given in Table 2.

Her2 expression and amplification in primary tumors

Of 355 cases, 185 tumors (52 %) showed no Her2 immunoreactivity, 119 tumors (34 %) had an immunoscore of 1+, 43 cases (12 %) showed 2+ immunoreactivity, and 8 cases (2 %) showed 3+. Sixteen cases with Her2 2+ were found to have an amplification of Her2 using FISH. Therefore, 24 cases (7 %) would have been classified as Her2-"positive status" according to the criteria of the FDA/EMEA for gastric cancer.

Fig. 2 Examples of immunohistochemical stainings for HSP90 in primary resected colon cancer. **a** Negative expression. **b** Moderate expression. **c** High expression; ×20; brown cytoplasmic staining of tumor cells

Table 1 Characterization of the case collective or primary resected colon carcinomas

Parameter	Number	Percent
pT category		
pT1	21	5.9
pT2	62	17.5
pT3	193	54.4
pT4	79	22.3
Lymph node metastases		
Absent	215	60.6
Present	140	39.4
Distant metastases		
Absent	299	84.2
Present	56	15.8
Grading		
G1	3	0.8
G2	230	64.8
G3	118	33.2
G4	4	1.1
Total		355

HSP90 expression in primary tumors

Immunohistochemical analysis for HSP90 was successfully applied on TMA cores of 353 tumors. For the remaining cases, HSP90 expression could not be studied due to too less amount of cells in the TMA cores. HSP90 expression was negative/low in 96 cases (27 %), moderate in 152 cases (43 %), and high in 105 cases (30 %).

Association between Her2 and HSP90

There was a correlation between high HSP90 expression and Her2 expression. Her2-negative tumors more frequently had

Table 2 Postoperative UICC staging of the collective of primary resected colon carcinomas

Postoperative UICC stage	Number	Percent
I	74	20.9
IIA	98	27.6
IIB	18	5.1
IIC	14	3.9
IIIA	8	2.3
IIIB	67	18.9
IIIC	21	5.9
IVA[a]	31	8.7
IVB[a]	24	6.8
Total	355	100.0

[a] Forty-three patients had liver metastases (HEP), 43 patients had peritoneal metastases (PER), and 16 patients had metastases of other location; among them, eight had pulmonary metastases (PUL)

low HSP90 expression levels, whereas the percentage of Her2-positive tumors with high HSP90 expression was comparable with lower HSP90 levels. This was statistically significant ($p=0.011$; Table 3 (a)). When using the gastric cancer criteria for Her2 status, the association between HSP90 and Her2 was not statistically significant anymore. However, only 15 cases would have fallen into this group of Her2-positive cases (Table 3 (b)).

Her2 and HSP90 in lymph node metastases

Lymph node metastases of 17 cases (7 Her2-positive and 10 Her2-negative primary tumors) were additionally investigated for Her2 and HSP90. Her2 status of the primary tumors and the lymph node metastases correlated significantly with each other ($p=0.009$), with 14/17 cases (82.3 %) showing concordant results. Two cases were Her2 positive in the primary tumor, but negative in the metastases. One case was Her2 positive in the lymph node metastasis, but negative in the primary tumor. In addition, there was a correlation between Her2 status and HSP90 expression ($p=0.035$) also in the lymph node metastases.

Clinicopathological parameters and survival analysis

Her2 expression or amplification of the primary tumors was not associated with pT category, presence of lymph node and distant metastases, or tumor differentiation. Neither an association between Her2 and tumor localization (right sided vs. left-sided carcinomas), MMR status, or survival (Table 4, Fig. 3) was present. KRAS mutations of codon 12/13 were detected in 51 % (26 cases) and BRAF mutation in 10 % (5 cases) of the Her2 IHC-positive cases, which is within the range of the usual frequency of these alterations in colorectal cancer. High HSP90 expression was associated with earlier tumor stages ($p=0.019$), absence of lymph node ($p=0.006$),

Table 3 Correlation between (a) HSP90 expression and Her2 expression ($p=0.011$) and (b) between HSP90 expression and Her2 status according to the FDA/EMEA ($p=0.58$)
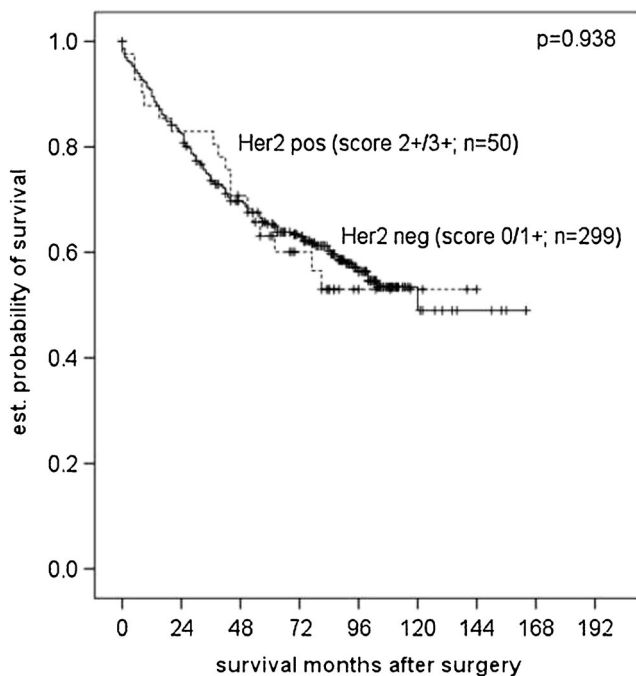
	HSP90 expression			Total
	Negative	Low	High	
(a) Her2 expression				
Score 0/1+	83	137	82	302
Score 2+/3+	13	15	23	51
total	96	152	105	353
(b) Her2 status (FDA/EMEA)				
Negative	86	145	98	329
Positive	10	7	7	24
Total	96	152	105	353

Table 4 Correlation between Her2 expression and Her2 status according to the FDA/EMEA and pathological parameters

Parameter	Her2 expression			Her2 status (FDA/EMEA)		
	Score 0/1+	Score 2+/3+	*p* value	Her2 negative	Her2 positive	*p* value
pT category						
pT1	20	1	0.16	20	1	0.54
pT2	53	9		56	6	
pT3	159	34		179	14	
pT4	72	7		76	3	
Lymph node metastases						
Absent	260	39	0.23	201	13	0.77
Present	44	12		129	11	
Distant metastases						
Absent	260	39	0.08	280	19	0.32
Present	44	12		51	5	
Grading						
1 and 2	204	29	0.32	218	15	0.88
3 and 4	100	22		113	9	
Tumor localization						
Right	52	11	0.27	56	7	0.11
Left	252	40		275	17	
MMR status						
Loss	36	4	0.29	39	1	0.22
Present	268	47		292	23	
Total	304	51		331	24	

and absence of distant metastases (*p*=0.001), but not with tumor localization or MMR status (Table 5) Patients with high tumoral HSP90 levels had a better survival compared to those with negative or low expressing tumors (*p*=0.032; Fig. 4), but

Fig. 3 No significant correlation of Her2 expression and survival in colon cancer patients

this was not independent from other prognostic relevant pathologic parameters (Table 6).

Discussion

In the present study about Her2 and HSP90 in colon carcinomas, we demonstrate that elevated HSP90 expression can be observed in a significant number of cases. It is associated with lower malignant potential in these tumors. The small proportion of tumors which showed Her2 positivity significantly more frequently had also higher tumoral HSP90 expression levels.

Her2 overexpression and amplification can be observed not only in breast cancer but also in gastrointestinal tumors. Numerous studies have investigated Her2 in malignancies of the upper gastrointestinal tract, where Her2 overexpression and amplification have been detected in significant numbers of cases [1, 2]. Works on Her2 in colon and rectal carcinomas have been published to a lesser degree and report a wide range between 2 % and up to over 40 % Her2 positivity rates [5–12]. There is a considerable variability with regard to methodical approaches (immunohistochemistry, RT-PCR, in situ hybridization) and interpretation of, e.g., immunohistochemical staining patterns of Her2 in colorectal carcinomas which may account for these diverging data. These inconsistencies are similar to many studies on gastric cancer [1, 18]. In order to gain better

Table 5 Correlation between HSP90 expression and pathological parameters

Parameter	HSP90 expression			p value
	Negative	Low	High	
pT category				
pT1	4	5	12	0.082*
pT2	15	28	19	
pT3	56	79	57	
pT4	21	40	17	
Lymph node metastases				
Absent	54	84	75	0.023**
Present	41	68	30	
Distant metastases				
Absent	71	128	98	0.001
Present	25	24	7	
Grading				
1 and 2	60	98	74	0.46
3 and 4	36	54	31	
Tumor localization				
Right	17	25	21	0.77
Left	79	127	84	
MMR status				
Loss	14	11	15	0.11
Present	82	141	90	
Total	96	152	105	

*$p=0.019$; **$p=0.006$, for high HSP90 expression vs. negative/low expression

homogenization of Her2 testing on gastric carcinomas, a proposal about standardization of immunohistochemical scoring
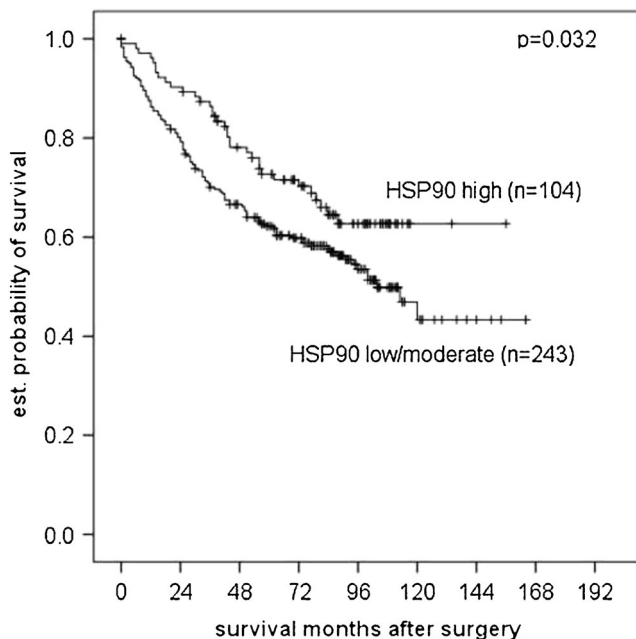
Fig. 4 Significant better survival for colon cancer patients with high tumoral HSP90 expression

Table 6 Multivariate analysis of prognostic relevant factors

Factor	HR	95.0 % CI	p value
pT category (pT1 vs. pT2 vs. pT3 vs. pT4)	1.472	1.148–1.889	0.002
Lymph node metastases (absent vs. present)	1.941	1.356–2.777	<0.001
Distant metastases (absent vs. present)	4.927	3.310–7.335	<0.001
HSP90 expression (negative/low vs. high)	0.894	0.604–1.324	0.57

was recently published by the group of Rüschoff who also were responsible for the Her2 diagnostics of the TOGA study [20]. Using these criteria, we could detect elevated membranous Her2 immunoreactivity (i.e., scores 2+ and 3+) in 14 % of the cases. However, if using the criteria for gastric carcinoma, where Her2 positivity is defined as either IHC 3+ or IHC 2+ plus positive FISH, Her2 positive status was observed in only 7 %.

Using this algorithm, the rate of Her2-positive tumors was recently reported to be 12 % of biopsies and in up to 27 % of extensively investigated resected specimens in another study on locally advanced rectal carcinomas which had been treated by neoadjuvant radiochemotherapy [26]. Our collective consisted of primary resected colon carcinomas of all stages, and we found a lower rate of Her2-positive tumors. There was no association between Her2 and relevant clinicopathologic parameters such as pT category, presence of lymph node and distant metastases, tumor localization, tumor differentiation, MMR status, KRAS or BRAF mutational status, or survival. Although we could not demonstrate any topographic differences when comparing right- to left-sided cancers, a site-specific difference between colon and rectal cancer may contribute to the difference in the rate of positive cases. However, we found similar Her2 expression and amplification rates like a previous study where the breast cancer scoring system had been applied [10]. In a subset of cases, we were also able to investigate the Her2 status in lymph node metastases. Concordant to data from gastric cancer [27, 28], we found a constant Her2 status in over 80 % of the cases. Similar to the findings from gastric carcinomas, the reason for the discrepant findings in these three cases may be either a true biological phenomenon or due to a sampling bias.

We based our investigation on a tissue microarray where we have to accept a certain amount of potentially false-negative results due to intratumoral heterogeneity, because small spots of overexpressing tumor areas may be missed in the TMA cores [29]. To take this into account, we decided to use the proposed modified criteria for Her2 testing on biopsies [20], which have comparable size and tissue content as TMA cores and which has a lower cutoff for the amount of cells whose positivity defines the Her2 status.

Despite this limitation, TMA technology has been shown to be a powerful tool for analyzing biomarkers in larger sample collections [30]. For the purpose of an explorative study, the relatively high number of cases and the advantages of homogenous reaction conditions which avoid false-positive or false-negative staining or hybridization results because of technical reasons may equilibrate limitations regarding intratumoral heterogeneity. Our approach seems to be appropriate for the purpose of an explorative study on the association between Her2 and HSP90, which also included additional immunohistochemical analysis, such as the analysis of mismatch repair genes. For clinical practice, however, the analysis of whole tissue sections should be favored since false-negative results may generate wrong therapeutic consequences.

Elevated HSP90 expression levels could be detected in a significant number of colon carcinomas with one third of tumors showing high HSP90 expression. This was observed mainly in tumors of lower stages and also associated with a better outcome of the patients. Moreover, HSP90 expression was significantly associated with Her2 expression and amplification. The biologic activity of Her2 is influenced and controlled by molecular chaperones such as members of the group of heat shock proteins (HSPs). These molecules assist in the maintenance of cellular integrity and homeostasis by assurance of accurate folding of other proteins [13, 14]. Deregulated activity of HSPs has been found in malignant diseases which may be due to intrinsic anti-apoptotic effects but also to the altered interaction with other oncogenic molecules [31, 32]. Recently, we could demonstrate that high tumoral expression levels of HSP27 and HSP70 are associated with negative outcome in colon cancer in the same case collection as in the present study [19]. HSP90 is one of the most abundant cellular HSPs. Besides the tyrosine kinases Her2 and EGFR, other molecules such as AKT, RAF, and RAS and mutated signaling proteins like p53 and v-Src have been described to interact with HSP90 [33, 34]. This strong association with oncogenes has evoked the interest on the impact of molecular chaperones, especially HSP90 as potential anti-tumoral targets. In vitro HSP90 inhibitors enhanced TRAIL-induced apoptosis in colorectal cancer cells via suppression of survival signaling [35]. Blocking heat shock protein 90 inhibited the growth of human colon cancer cells and improved the efficacy of oxaliplatin in p53-deficient colon cancer tumors in a mouse model [36]. On the tissue level, tumor HSP90α gene overexpression has been shown to be correlated with metastases and poor prognosis in a study of 56 colorectal cancer patients [18]. This is in contrast to our findings which were obtained by immunohistochemistry and, on the protein level, generated from a much larger collective of cases. Such discrepancies between gene and protein expression data, however, are not infrequent since proteomic and genomic information often does not correlate because of different posttranslational modifications or protein turnover.

Recently, we have reported a strong correlation between HSP90 and Her2 expression in gastric carcinomas which is in line with the observations of the present study [37]. Similar observations have also been made in vitro for breast and gastric cancer, where data about overcoming Her2 resistance by increasing HSP90 activity already exist [38]. Based on these data, HSP90 may represent a potential targetable molecule, and anti-HSP90 treatment alone or in combination with other drugs may offer interesting alternative therapeutic options, especially in Her2-positive tumors [39, 40]. To the best of our knowledge, there are currently no data about the co-regulation of HSP90 and Her2 in colorectal cancer yet, so that the present paper represents the first study which postulates an association between Her2 and HSP90 expression on the tissue level, in the primary tumors, and also in metastases. Although higher HSP90 levels were observed with higher frequency in the lower-stage groups, there still were a considerable number of advanced cancers showing high HSP90 as well. Since tumors of higher stages are more likely to have local or distant relapses and are therefore treated by additional cytotoxic regimes, anti-HSP90 treatment alone, or in combination with Her2 targeting could represent an additional option.

In summary, we could demonstrate an increased immunoreactivity for Her2 and corresponding gene amplification in a small subset of colon carcinomas. High HSP90 expression levels were detected in a significant number of cases and were associated with lower malignant potential in colon cancer. The observed association between HSP90 and Her2 expression may reflect a co-regulation of these molecules. Targeting HSP90 and/or Her2 may therefore be an alternative therapeutic approach in colon cancer in a subset of patients.

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