

Full Length Research Paper

Human epidermal growth factor receptor (HER 2)/neu expression and gene amplification in colorectal cancer

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To investigate the relationship between the expression/amplification of human epidermal growth factor receptor (HER 2)/neu and its clinical significance in colorectal cancer, in this study, clinicopathological data and paraffin-embedded specimen were collected on 192 consecutive patients who underwent colorectal resections from 2001 to 2005 at Renmin Hospital of Wuhan University. Using immunohistochemistry and fluorescence *in situ* hybridization, HER-2/neu protein overexpression and gene amplification were detected. HER 2/neu was overexpressed in 32.3% of cases (62/192), of which 38 (19.8%) were 2⁺ and 24 (12.5%) were 3⁺, over expression/amplification of HER 2/neu was associated with the differentiation and stage of the carcinoma. No association between the expression/amplification of HER 2/neu and the tumor size, location, age, gender and lymphatic invasion was found. HER 2/neu overexpression was significantly associated with HER 2/neu gene amplification according to univariate analysis; there was a significant difference in survival rates when cases with and without HER 2/neu overexpression or amplification were compared. These results indicate that HER 2/neu could be a prognostic variable in colorectal cancer; patients with colorectal cancer exhibiting high levels of HER 2/neu might benefit from the anti-HER-2 /neu therapy.

Key words: HER 2/neu, colorectal cancer, immunohistochemistry, fluorescence *in situ* hybridization.

INTRODUCTION

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in males and the second in females, with over 1.2 million new cancer cases and an estimated 608,700 deaths annually (Jemal et al., 2011). It is usually associated with poor prognosis because it is often at an advanced stage when diagnosed. Treatment for advanced colorectal cancer remains a challenge. Recently, targeted therapy has been applied to several

tumors, opening new potential avenues for cancer treatment. The increased awareness of oncogenes as therapeutic targets has escalated the need to access oncogene in tumors.

Human epidermal growth factor receptor family (EGFR, HER 2/neu, HER-3/neu, HER-4/neu) is closely related to the development of malignant tumors (Coussens et al., 1985). Among them, HER 2/neu (c-erb-2) is one of the most studied molecules. Studies have shown that 15 to 20% of breast cancers have an amplification of the HER 2/neu gene or overexpression of its protein product. Over-expression of this receptor in breast cancer is associated with increased disease recurrence and worse prognosis (Slamon et al., 1987; Press et al., 1997). Trastuzumab, the humanized anti-HER 2/neu monoclonal antibody has been effectively used in women with HER 2/neu overexpression metastatic breast cancer demonstrating longer median response duration and

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Abbreviations: CRC, Colorectal cancer; DAPI, 4',6'-diamidino-2-phenylindole; FISH, fluorescence *in situ* hybridization; IHC, immunohistochemistry; TGF, transforming growth factor; EGFR, epidermal growth factor receptor family; HER 2, human epidermal growth factor receptor.

survival than previous chemotherapy modalities (Goldenberg, 1999).

Recent studies have shown that HER 2/neu overexpression is also seen in other tumors such as non-small cell lung cancer, esophageal cancer, gastric cancer, bladder cancer, pancreatic cancer, ovarian cancer and skin cancer, etc (Marx et al., 2009; Cappuzzo et al., 2005; Tapia et al., 2007). Trastuzumab will likely be applied to other tumors that overexpress HER 2/neu. Data from a recent study of trastuzumab in use in combination with chemotherapy compared with chemotherapy alone in nearly 4000 patients with HER 2/neu positive advanced gastric cancer (ToGA trial, protocol number: BO18255) showed that adding trastuzumab to standard chemotherapy prolongs survival in advanced gastric cancer by a median of nearly three to 13.8 months (Reichelt et al., 2007).

In CRC patients, HER 2/neu overexpression has been described in 2 to 87% of the cases (Ramanathan et al., 2004; Park et al., 2007; Kavanagh et al., 2009; Ooi et al., 2004). Some authors have reported an association between HER 2/neu overexpression and shorter survival (Park et al., 2007; Ooi et al., 2004), whereas other authors did not find such a correlation. HER 2/neu gene amplification was found in only 1 to 3% of colorectal carcinomas (Ramanathan et al., 2004).

The objectives of this study were (1) to determine the frequency of HER-2/neu amplification and overexpression in CRC, (2) to investigate the relationship between HER-2/neu amplification/overexpression and the clinicopathological characteristics of tumors, including survival rates.

MATERIALS AND METHODS

Tumor tissue collection and human subjects' approval

Specimens were selected from archive paraffin embedded blocks in Renmin Hospital of Wuhan University by two pathologists, 351 Chinese patients with CRC who underwent surgery at the Department of Surgery, Renmin Hospital of Wuhan University were eligible to be selected for the period of 2001 to 2005. Only those patients whose clinical data (include diagnosis, age, sex, address, disease history, etc) intact were included. 192 Chinese patients with CRC were ultimately selected in this study. None of the patients had received preoperative radiation or chemotherapy. Normal colorectal mucosa adjacent to tumor was analyzed whenever present (n = 102).

In all cases, we reviewed age, gender, tumor size, tumor location, histological grade, clinical stage, and lymphatic invasion. The ages of the 192 patients selected for this study ranged from 29 to 85 years, with a mean of 57.9 years. Tumors had been staged according to the Duke's classification (Beahrs, 1992) and the reference point to distinguish the proximal and distal colon was the splenic flexure. The clinicopathological data are summarized in Table 1.

The follow-up time ranged from 0 to 120 months with an average of 47.9 months. The causes of death were ascertained from medical records or autopsy, if performed. Patients who had died within 4 weeks of radical surgery were excluded from our analyses.

Deaths due to other causes resulted in censored observations beginning at time of death. The institutional review board at the Renmin Hospital of Wuhan University approved this study, and informed consent was obtained from all patients.

Immunohistochemistry (IHC)

All colorectal cancer and normal colorectal specimen were fixed in 10% buffered formalin and embedded in paraffin according to standard procedures. Serial sections (4 μ m thickness) placed on positively charged slides (MENZEL-GLASSER, GERMAN) were used for hematoxylin and eosin staining, immunohistochemistry, and fluorescence *in situ* hybridization (FISH) detection of HER 2/neu.

Immunohistochemistry (IHC) for HER 2/neu was performed using the Hercep test kit (DakoCytomation, Denmark), according to the manufacturer's instructions. Antibody binding was visualized by the EnVision detection kit (DakoCytomation, Denmark).

Sections were scored by the percentage of positive cells (membranous and cytoplasmic) and the intensity of immunostaining [that is, negative (0), weak (1+), intermediate (2+) and strong (3+)]. Immunostaining in >10% of tumour cells was considered positive. Staining was scored independently by two pathologists who were blinded to each other's findings. A score of 0 and 1+ were considered as low expression, A score of 2+ and 3+ were considered as overexpression.

Fluorescence *in situ* hybridization (FISH)

HER2/neu amplification was analyzed using FISH HER 2/neu PharmDx (Dako, Denmark), which contains both fluorescently-labeled HER2/neu gene and chromosome 17 centromere probes. In brief, the sections were incubated at 56°C overnight and deparaffinized by washing in xylene, ethanol and distilled water. After incubation in 0.2 M HCl at room temperature for 20 min, they were heat-pretreated in citrate buffer (2 \times SSC, pH 6.0) at 80°C for 1 to 1.5 h. They were then digested with pepsin at room temperature for 8 to 14 min, rinsed in 2 \times SSC at room temperature for 2 min and dehydrated in ascending concentrations of ethanol (75, 80 and 100%) for 2 min. After the HER2/CEN17 probe mix was applied to the dry slides, the tissue area was coverslipped and sealed with rubber cement. The slides were then incubated in hybridizer (Hybridizer Instrument for *in situ* hybridization, DAKO, Denmark) for denaturation at 82°C for 5 min and hybridization at 45°C for about 18 h. Posthybridization washes were performed in urea/0.1 \times SSC at 45°C for 30 min and in 2 \times SSC at room temperature for 2 min. The slides were dehydrated in graded ethanol, and after application of 15 μ L of mounting medium containing 4',6'-diamidino-2-phenylindole (DAPI), the tissue area was coverslipped.

FISH analyses were performed according to the HER 2/neu FISH PharmDx (Dako, Denmark) criteria. In each case, 100 non-overlapped, intact interphase tumor nuclei identified by DAPI staining were evaluated, and gene (red signal) and CEN17 (green signal) copy numbers in each nucleus were assessed. The cases were considered to be amplified when the average copy number ratio, HER2/CEN17, was \geq 2.0 in all nuclei evaluated or when the HER 2/neu signals formed a tight gene cluster.

Statistical analyses

All statistical analyses were performed using SPSS for Windows 15.0 (SPSS Inc). Categorical variables were compared by the Pearson Chi-square test or Fisher's exact test, depending on the expected values found in the contingency table. The overall survival

Table 1. Correlation between the HER 2/neu protein expression, amplication and clinicopathologic feature in 192 colorectal cancer.

Variable		HER2 protein expression			P value	HER2 amplication			P value
		Low expression	Over expression	Total		No amplication	Amplication	Total	
Age	<60	73 (70.9)	30 (29.1)	103	0.313	85 (82.5)	18 (17.5)	103	0.771
	≥60	57 (64.0)	32 (36.0)	89		72 (80.9)	17 (19.1)	89	
Gender	Female	58 (68.2)	27 (31.8)	85	0.889	68 (80.0)	17 (20.0)	85	0.571
	Male	72 (67.3)	35 (32.7)	107		89 (83.2)	18 (16.8)	107	
Tumor size(cm)	S < 3	19 (76.0)	6 (24.0)	25	0.404	23 (92.0)	2 (8.0)	25	0.114
	3≤S < 5	66 (69.5)	29 (30.5)	95		80 (84.2)	15 (15.8)	95	
	S≥5	45 (62.5)	27 (37.5)	72		54 (75.0)	18 (25.0)	72	
Tumor location	Proximal	51 (64.6)	28 (35.4)	79	0.719	64 (79.0)	17 (21.0)	79	0.692
	Distal	47 (69.1)	21 (30.9)	68		55 (83.3)	11 (16.7)	68	
	Rectal	32 (71.1)	13 (28.9)	45		38 (84.4)	7 (15.6)	45	
Histological grade	G ₁ -well	40 (81.6)	9 (18.4)	49	0.03	45 (91.8)	4 (8.2)	49	0.007
	G ₂ -Moderate	71 (65.1)	38 (34.9)	109		90 (82.6)	19 (17.4)	109	
	G ₃ -Poor	19 (55.9)	15 (44.1)	34		22 (64.7)	12 (35.3)	34	
Dukes' stage	A	20 (87.0)	3 (13.0)	23	0.039	21 (91.3)	2 (8.7)	23	0.027
	B	45 (73.8)	16 (26.2)	61		54 (88.5)	7 (11.5)	61	
	C	41 (63.1)	24 (36.9)	65		53 (81.5)	12 (18.5)	65	
	D	24 (55.8)	19 (44.2)	43		29 (67.4)	14 (32.6)	43	
Lymphatic invasion	No	60 (69.0)	27 (31.0)	87	0.735	70 (80.5)	17 (19.5)	87	0.668
	Yes	70 (66.7)	35 (33.3)	105		87 (82.9)	18 (17.1)	105	

rates were calculated using the Kaplan-Meier method and the curves were compared by the log-rank test. In all statistical tests, the alpha error was set at 5%. The survival period was calculated from the date of hospital admission to death or the date of last follow-up.

RESULTS

HER 2/neu immunohistochemistry (IHC)

In this study, we observed that HER 2/neu protein was overexpressed in many CRC tissues (62/192, 32.3%). When compared with normal colorectal mucosa (4/102, 3.92%), cancer tissues had higher expression, 38 (19.8%) were 2⁺ and 24 (12.5%) were 3⁺ respectively (Figure 1 and Table 2).

HER 2/neu positivity as estimated by IHC of well-differentiated G1 CRC specimen was 18.4% as against 34.9% in moderately differentiated G2 CRCs, and 44.1% in poorly differentiated G3 CRCs. Similar results were obtained for the tumor stage in 13.0% of Dukes' A CRCs,

26.2% of Dukes' B CRCs, 36.9% of Dukes' C CRCs, and 44.2% of Duke's D CRCs. Whilst in all the samples more than 55% remained HER 2/neu negative. Statistical analysis revealed a significant association between the expression of HER 2/neu and the differentiation, stage of the carcinoma (Table 1). At the same time, no association between the expression of HER 2/neu and the tumor size, location, age, gender and lymphatic invasion was found.

HER 2/neu fluorescence in situ hybridization (FISH)

Having observed differences in HER 2/neu protein expression between normal colorectal mucosa and cancer tissues as mentioned previously, we went further to see whether they could be differences in HER 2/neu gene expression characteristics. The same cases evaluated by IHC were also examined using FISH. Gene amplification was found in 8.2% of G1 CRCs, 17.4% of G2 CRCs and 35.3% of G3 CRCs. In 8.7% of Dukes' A

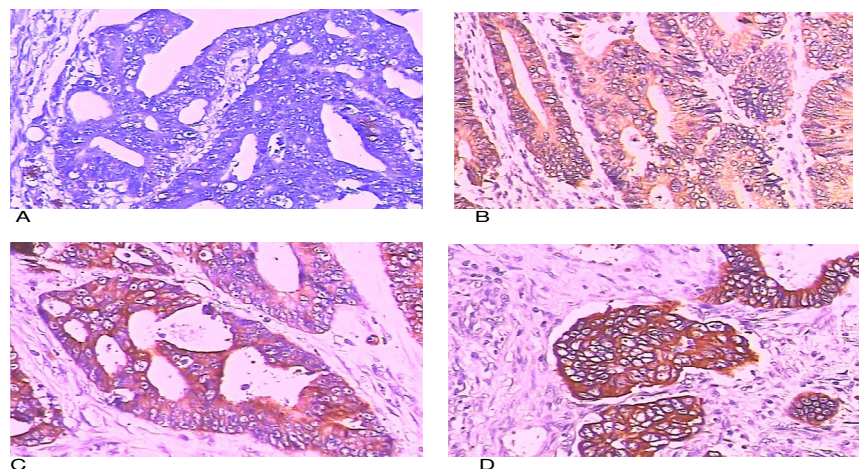


Figure 1. Immunohistochemistry showing HER 2/neu staining in CRC. a, negative case 0, 200 \times ; b, negative case 1+, 200 \times ; c, positive case 2+, 200 \times ; d, positive case 3+, 200 \times .

Table 2. Expression of HER2 protein in normal colorectal epithelium and colorectal cancer.

Variable	HER2 expression			P value
	0/1+	2+	3+	
Normal colorectal epithelium	89 (87.3%)	8 (7.8%)	5 (4.9%)	0.001
Colorectal carcinoma	130 (67.7%)	38 (19.8%)	24 (12.5%)	

CRCs, 11.5% of Dukes' B CRCs, 18.50% of Dukes' C CRCs and 32.6% of Dukes's D CRCs. Whilst in all the samples 18.2% exhibited HER 2/neu gene amplification (Figure 2).

Similar to the results of IHC, HER 2/neu amplification was significantly associated with the differentiation of the carcinoma and the tumor stage. No correlation between the amplification of HER 2/neu with the tumor size, location, age, gender and lymphatic invasion (Table 1) was found. Statistical analysis revealed a significant association between HER2 overexpression and HER2 amplification ($p = 0.007$) (Table 3).

Survival analysis

Finally, we want to know whether HER 2/neu expression has any relationship with patient survival. Survival analysis was performed on 192 patients who had survived for more than 4 weeks after surgery. The survival curves, according to HER 2/neu amplification and overexpression are shown in Figures 3 and 4. In contrast, tumors associated with HER 2/neu amplification exhibited poor mean survival rates (53.5 vs. 68.9 months). As might be expected, tumors associated with HER 2/neu overexpression also exhibited poor mean

survival rates (57.6 vs. 70.0 months).

DISCUSSION

HER 2/neu is known to form heterodimers with the EGFR, HER 3/neu and HER 4/neu members of the EGFR family. It is unique within the EGFR family of receptor tyrosine kinases as HER 2/neu appears to function primarily as a heterodimerisation partner for other EGFR family members (Akiyama et al., 1986). Over the last 20 years, elevated levels of the EGFR and its cognate ligands (which include EGF and transforming growth factor (TGF)- α) have been identified as a common component of numerous cancer types. EGFR expression has been associated with tumour grade and stage of colorectal cancer, relapse-free survival and overall survival (Kluftinger et al., 1992; Steele et al., 1990). In some studies, HER2 expression showed a better correlation with survival than EGFR expression did (Osako et al., 1998; Kapitanovic et al., 1997). Recently, a significant association was found between the coexpression of EGFR, HER 2/neu and response to treatment with afatinib (Cunningham et al., 2006).

In this study, overexpression and amplification of HER 2/neu were observed in 32.3 and 18.2% of the studied 192 CRC cases, respectively. The overexpression and amplification of HER 2/neu in CRC patients may be

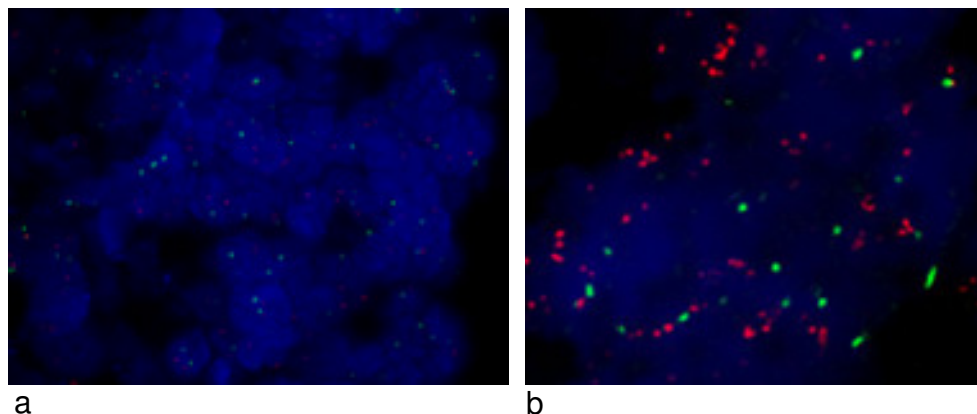


Figure 2. Analysis of HER-2/neu gene amplification in CRC. a, With no amplification; b, case considered amplified. In detail, nucleus shows ratio ≥ 2 1,000 \times .

Table 3: Relationship between HER2 amplification and HER2 protein expression.

HER2 IHC	HER2 FISH		Total
	Negative	Positive	
Lowexpression	113	17	130
Overexpression	44	18	62
Total number	157	35	192
P value	0.007		

Positive, HER2 amplification; Negative, No HER2 amplification.

important molecular events in the carcinogenesis of colorectal cancer. High levels of HER 2/neu in colorectal cancer could make HER 2/neu a good target for monoclonal antibody-based immunotherapy.

The three largest studies (100 to 293 patients per study) have reported a significant range of HER 2/neu overexpression rates (32 to 59%) (Osako et al., 1998; Tannapfel et al., 1996; Essapen et al., 2004). These rates were similar to our results. Other investigators have described lower frequencies of HER 2/neu overexpression/amplification in CRC. Nathanson et al. (2003) found HER 2/neu overexpression in only 3.6% of American patients (Nathanson et al., 2003). Marx et al. (2010) found overexpression in 2.7% of German patients (Marx et al., 2010). Al-Kuraya et al. (2007) found amplification in 0% of 98 Saudi patients and in 2.8% of 316 Swiss patients (Al-Kuraya et al., 2007).

The variability of these data may be due to different experimental methods. Technical variables in the performance of IHC staining are the most likely explanation. It is widely appreciated that IHC analysis is vulnerable to differences in tissue fixation and processing, in choice of primary antibodies, in detection systems, in epitope retrieval, in interpretation and in reporting. In addition, the biological characteristics of malignant tumors may vary between different races. For example, there is p53 gene mutation in breast

cancer, but the types of gene mutations are different among white Americans, black Americans, Australians and Japanese (Hartmann et al., 1995; Hartmann et al., 1996).

Previous studies to determine whether HER 2/neu expression was associated with the clinicopathologic characteristics of CRC have been controversial (Park et al., 2007; Kavanagh et al., 2009). We found no significant association with tumor size, location, age, gender and lymphatic invasion. This is especially interesting for tumor size and lymphatic invasion, as tumors with higher malignant potential might be larger, and might be expected to have more invasion ability. To make clear the role of HER 2/neu in CRC tumor cell proliferation, invasion, and metastasis, further studies with more cases (that is, all of our 351 cases) and additional target genes including the EGF-R (HER-1), KRAS mutation analysis, insulin like growth factor receptor 1 (IGFR1), and the proliferation rate (Ki 67), as well as studies of the expression of proteins of the intercellular signalling pathway should be included.

However, significant association between HER 2/neu expression and the clinicopathological findings, such as histological grade and tumor stage were found. HER 2/neu amplification positively correlates with tumor stage and grade, being more frequent in the advanced and less differentiated samples. At the same time, HER 2/neu

Survival Functions

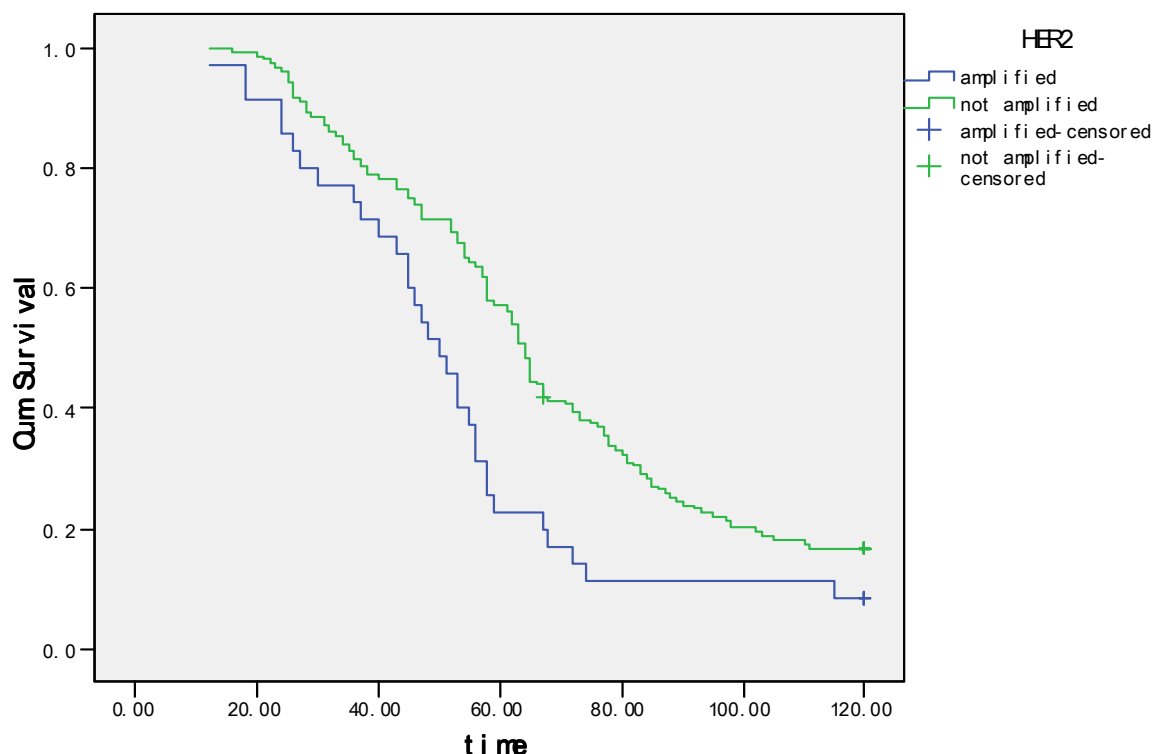


Figure 3. Kaplan-Meier plot for overall survival in 192 CRC patients according to detection of HER-2/neu amplification.

overexpression and amplification are associated with lower rates of survival. These data indicate that HER 2/neu overexpression and amplification could constitute a reliable indicator of poor prognosis in colorectal cancer, patients with colorectal cancer exhibiting high levels of HER 2/neu might benefit from the anti-HER-2 /neu therapy.

In recent studies, HER 2/neu is expressed constitutively in colon cancer cell lines (Half et al., 2004), antitumor effect had been demonstrated by the blockade of HER 2/ neu on two colon cancer cell lines (DLD-1 and Caco-2), but the changes in HER 2/neu protein and mRNA levels seem to be partly independent of the antitumor effect (Giannopoulou et al., 2009). In the only published clinical report, Ramanathan et al. (2004) found 8 out of 138 CRC patients eligible for a combination therapy with trastuzumab and irinotecan based on an IHC analysis using the Hercep test (DAKO). Although, this phase II study was prematurely closed due to low patient accrual, partial responses were described in 5 of 7 evaluable patients (Ramanathan et al., 2004). Further studies should attempt to identify the subpopulation of colorectal cancer patients more likely to benefit from erbB-directed therapy.

FISH is currently regarded as the "gold standard" for

the detection of HER-2/neu amplification: it is associated with both high sensitivity (96.5%) and high specificity (100%) (Pauletti et al., 1996). FISH can be conducted with small tumor samples. Both formalin-fixed and paraffin-embedded tissue samples can be used since tissue preparation has little or no effect on the testing. It also allows for the direct visualization of gene amplification in the nuclei and provides an objective count of genes and chromosomes on a cell-by-cell basis. However, the time and costs associated with this technique make it an impractical clinical tool for primary screening. According to the concordance between the overexpression by IHC and the amplification by FISH, we think that HER-2 /neu expression must be evaluated initially by immunohistochemistry and, if the results are not conclusive, FISH should be performed. Such a practice has been standard procedure to assist in making therapeutic decisions in patients with breast and lung cancer (Cappuzzo et al., 2005; Tsuda, 2006).

Our study demonstrates the concordance between the expression data and the amplification data, this phenomenon implicates that gene amplification is a probable mechanism by which the HER 2/neu protein is overexpressed in colon cancer. It is similar to findings in breast cancer (Field et al., 2001; Lebeau et al., 2001).

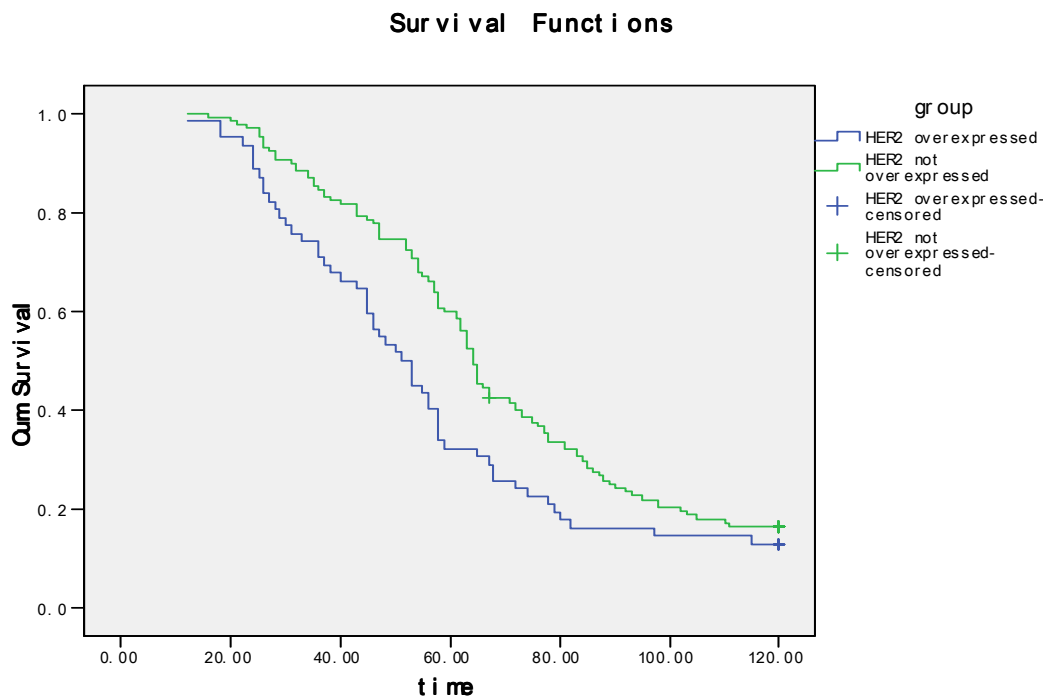


Figure 4. Kaplan-Meier plot for overall survival in 192 CRC patients according to detection of HER-2/neu overexpression.

Presence of HER 2/neu amplification is a prerequisite for response to trastuzumab in breast cancer. In recent studies, a positive correlation was even shown between the level of HER 2/neu amplification assessed by FISH and the rate of complete pathologic response to trastuzumab-based neoadjuvant treatment (Arnould et al., 2007; Gullo et al., 2009). In gastric cancer, tumors with both amplification and overexpression have recently demonstrated better response to trastuzumab than cancers with overexpression alone, these factors indicate that colorectal cancer with both amplification and overexpression may have better response to trastuzumab.

Conclusion

In summary, our results have shown that HER 2/neu amplification/overexpression may constitute an independent prognostic factor in colorectal cancer patients. Patients exhibiting HER 2/neu amplification/overexpression might benefit from the anti-HER-2/neu therapy.

However, the incidence, development, invasion and metastasis of CRC are a multi-step and multi-factor complex process. To better understand the growth pattern of CRC, further studies with more cases (that is, all of our 351 cases) and additional target genes including the EGF-R (HER-1), KRAS mutation analysis, insulin like growth factor receptor 1 (IGFR1), and the proliferation

rate (Ki 67), as well as studies of the expression of proteins of the intercellular signalling pathway should be included. In parallel, studies of tumor epigenetics and tumor microenvironment should be included too. In the future, multiple analyses will therefore be needed, to find the best individual therapy for each patient.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

WD designed the study, evaluated the immunohistochemistry and FISH results and wrote the manuscript. WGD designed the study, assisted in analysis of immunohistochemistry result. NZ carried out all the immunohistochemistry and FISH reactions, and assist in writing of the manuscript. FL performed all the statistical analyses. HXW performed the surgical procedures and evaluated the clinical records. All authors read and approved the manuscript.

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