HER2/CHR 17 tissue microarray chromogenic in situ hybridization analysis in colon adenocarcinoma: multiple gene signals vs protein expression

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Summary

Purpose: HER2-dependent signalling pathway is deregulated in a subset of colon adenocarcinomas. Although HER2 protein expression patterns demonstrate a broad diversity in these tumors, the critical parameter for targeting the gene is the detection of gene amplification. Our aim was to investigate the correlation between HER2 protein levels and chromosome 17 (chr 17) copies.

Methods: Sixty paraffin-embedded samples of primary colon adenocarcinomas were cored at 1 mm diameter and transferred to the microarray block. Immunohistochemistry (IHC) was performed using anti-HER2 monoclonal antibody. Chromogenic in situ hybridization (CISH) was performed using HER2 gene/chromosome 17 centromeric probes.

Results: HER2 protein overexpression (score: 2+/3+) was observed in 20/60 (33.3%) samples. CISH analysis detected 11/60 (18.33%) amplified cases, whereas chromosome 17 aneuploidy (polysomy) was identified in 13/60 (21.66%) cases. Significant associations were detected correlating HER2 expression with grade of the tumors (p=0.03), Chr 17 with stage (p=0.01), gene copies with protein expression (p=0.008), and also Chr 17 centromere signals with overall gene signals (p=0.001).

Conclusion: Multiple HER2 gene copies lead to different protein expression patterns (score 1+ to 3+) but pure gene amplification is only a subset of them. Identification of chromosome polysomy is a critical parameter in detecting original gene amplification in conventional one-color CISH methods.

Key words: chromogenic in situ hybridization , colon carcinoma, genes, HER2, immunohistochemistry

Introduction

The HER2/neu protooncogene is located on the long arm of chromosome 17 (17q21), encoding for a transmembranous glycoprotein HER2 with intrinsic tyrosine kinase activity and marked sequence homology with the epidermal growth factor receptor (EGFR) [1]. HER2 protein overexpression is a frequent genetic event in a variety of solid malignancies, including breast, lung, and head and neck cancers [2-5]. Concerning gastrointestinal carcinomas, subsets of patients with gastric, pancreatic, liver and colon adenocarcinomas are characterized by different expression patterns of the receptor [6-10]. Based on extensive research in the field of HER2 clinical impact, many studies have shown that identification of gene/chr 17 deregulation mechanisms (especially, amplification) is more important parameter than simple protein expression in handling those patients, modifying also their survival [11-15]. Targeted therapeutic strategies use humanized or chimeric monoclonal anti-HER2 molecules that block the extracellular ligand-binding domain of the receptor [16,17]. Those agents block signaling pathways to nucleus such as PI3/AKT/PTEN/mTOR [18]. In sporadic co-

lon adenocarcinomas, HER2 overexpression due to pure gene amplification seem to be a relative rare event, but the role of Chr 17 aneuploidy in gene numerical modifications is under investigation [19,20].

In the current study we focused on detecting HER2 gene/Chr 17 numerical imbalances in colon adenocarcinoma tissue cores, correlating them to HER2 protein expression patterns.

Methods

Study group

For the purposes of our study, we used 60 formalin-fixed and paraffin-embedded tissue samples of histologically confirmed colon adenocarcinomas obtained by surgical resection between 2008 and 2010. The 417 VA Hospital-NIMTS Ethics Committee gave permission to use these tissues for research purposes. Oral informed consent was obtained from each patient and the study protocol conformed to the ethical guidelines of the "World Medical Association Declaration of Helsinki – Ethical Principles for Medical Research Involving Human Subjects" adopted by the 18th WMA General Assembly, Helsinki, Finland, June 1964, as revised in Tokyo 2004. Ten microscopically normal-appearing colon epithelia were used as control group for the analysis. All corresponding hematoxylin and eosin (H&E)-stained slides were reviewed by two pathologists for confirmation of diagnosis and classification according to World Health Organization (WHO 2000) grading criteria for colon adencarcinomas. Stage was assessed using the Dukes staging system which was concluded from patient medical records and follow up. Clinicopathological data are demonstrated in Table 1.

Tissue microarrays (TMA) construction

Areas of interest were identified in H&E stained slides by a conventional microscope (Olympus BX-50, Melville, NY, USA). Selection of these areas was performed on the basis of tumor sufficiency, avoiding sites of necrosis or bleeding. Using ATA-100 apparatus (Chemicon International, Temecula, CA, USA), all of the source blocks were cored twice (in order to secure the presence of each case in the final blocks) and 1-mm diameter tissue cylindrical cores were transferred from each conventional donor block to the 3 recipient blocks. After 3 mm microtome sectioning and H&E staining the final constructed TMA blocks contained 92% cores of tissue cylindrical specimens. We observed microscopically that all of the examined cases were represented by at least one or two tissue spots (confirmation of the adequacy of the examined specimens).

Antibodies and probes

Ready-to-use HER2 monoclonal mouse antibody (clone TAB250-Zymed/InVitrogen, San Fransisco, USA) recognizing predominantly the extracellular domain of HER2 protein and not reacting with other erbB receptors was applied for the identification of protein expression. HER2 gene status was determined using the ready to use SPOT LIGHT HER2 DNA Probe (Zymed/InVitrogen, San Fransisco, USA). This digoxygenin-labeled probe is located on 17q21 and covers the entire HER2 gene area. Similarly, chromosome 17 status was determined by the ready-to-use biotin-labeled chromosome 17 centromeric probe (Zymed/InVitrogen, San Fransisco, USA) recognizing the specific repetitive centromeric DNA sequences, known as α-satellite DNA.

Immunohistochemistry (IHC) assay

IHC for HER2 antigen was carried out on 3 μm serial sections of the corresponding tissue blocks. Two slides were deparaffinized and rehydrated and enzyme-digested (fiscin) for 10 min at 37ºC. The NBA kit (Zymed/InVitrogen, San Francisco, USA) was used for the following detection steps. Blocking solution was applied to the slides for 10 min, followed by incubation for 1 h using the antibodies (dilution 1:20) at room temperature. Following incubation with the secondary antibody for 10 min, diaminobenzidine-tetrahydrochloride-DAB (0.03%) containing 0.1% hydrogen peroxide was applied as a chromogen and incubated for 5 min. Sections were counterstained, dehydrated and cover-slipped. For negative control slides, the primary antibody was omitted. IHC protocol was performed using an automated staining system (I 6000 – Biogenex, San Ramon, CA, USA). Membranous predominantly and sub-membranous cytoplasmic staining was considered acceptable for HER2 expression according to the manufacturer's data sheet. Breast cancer tissue sections overexpressing HER2 protein and normal appearing lung epithelia were used as positive and negative controls, respectively. Protein expression levels were evaluated semi-quantitatively by using Zymed/InVitrogen's Evaluation Guidelines. According to the scoring guidelines, the examined cases were classified as follows: Score 0: no staining or membrane staining in <10% of tumor cells; Score 1+: faint membrane staining in >10% of tumor cells; Score 2+: weak or moderate complete membrane staining in >10% of tumor cells and Score 3+: strong, complete membrane staining in >10% of tumor cells. Scores of 0 and 1+ were considered as negative for HER2 expression while Scores 2+ and 3+ as positive (overexpression).

Chromogenic in situ hybridization (CISH) assay

CISH SPOT-Light Chromogenic ISH Detection Kit (Zymed/InVitrogen, USA) was applied. CISH for chromosome 17 and HER2 gene analysis were performed on 5 μm thick paraffin serial sections of the tissue blocks described above. Two slides were incubated at 37°C overnight followed by 2 h incubation at 60°C and then deparaffinized in xylene twice, 5 min each and in

Table 1. Clinicopathological data and IHC results on 60 patients

Clinicopathological data	HER2			HER2 gene			Chr 17		
	LL (N=40) %	OE (N=20) %	p-value	Amplified (N=11) %	Non amplified (N=49) %	p-value	Normal (N=47) %	Polysomy (N=13) %	p-value
Gender			NS			NS			NS
Female (N=32)	65.2	34.8		21.4	78.6		84.4	15.6	
Male (N=28)	67.8	32.2		15.6	84.4		71.5	28.5	
Tumor diameter (cm)			NS			NS			NS
0-3 (N=15)	62.8	38.2		29.1	70.9		48.2	13.3	
4-6 (N=30)	81.2	18.8		34.6	65.4		62.7	37.3	
≥7 (N=15)	87.3	12.7		39.2	60.8		56.6	43.4	
Grade			0.03			NS			NS
I (N=22)	77.3	22.7		71.9	18.1		77.3	22.7	
II (N=31)	44.9	45.1		25.9	74.1		81.7	19.3	
III (N=7)	85.8	14.2		14.3	85.7		71.5	28.5	
Dukes stage			NS			NS			0.001
A (N=8)	50	50		37.5	62.5		100	0	
B (N=26)	69.3	30.7		69.3	30.7		69.3	30.7	
C (N=26)	69.3	30.7		69.3	30.7		81.8	19.2	
Location			NS			NS			NS
Other than rectum-sigmoid (N=24).	81.3	18.7		31.7	68.3		71.3	28.7	
Rectum-sigmoid (N=36)	72.3	27.7		15.6	84.4		69.1	30.9	

LL: loss or low expression (score 0, 1+), OE: overexpression (2+,3+), NS: not significant. p-value derived from Spearman's rank correlation coefficient chi square test

ethanol 3 times, 3 min each. The slides were rinsed in deionised water and then placed in a coplin jar containing CISH FFPE Pre-treatment Buffer (CISH Tissue Pre-treatment Kit, Zymed). For heat pretreatment, the coplin jar was capped, loosely screwed, placed in a pressure cooker and timed for 10 min after the pressure built up. Then, the slides, were immediately washed in deionised water followed by enzyme digestion, which was performed by covering the sections with pepsin (CISH Tissue Pre-treatment Kit, Zymed) for 5 min at 37oC. The slides were washed with deionised water, dehydrated with graded ethanol and air-dried. Ready-to-use dig-labeled HER2 gene and biotin-labeled chromosome 17 centromere probes were applied to each section, respectively. Twenty μl of probe were applied to the sections. The tissue sections containing the added probe were denatured by placing the slides in a polymerase chain reaction (PCR) machine equipped with a slide block at 94°C for 5 min. The slides were then placed in a moist slide box and incubated at 37°C for overnight hybridization. These sections were stringently washed in 0.5x standard saline citrate at 75°C for 5 min. The CISH Polymer and the Horseradish (HRP) Detection Kit (Zymed/InVitrogen, San Fransisco, USA) – containing similar steps to IHC – were used. Shortly afterwards TMA sections were placed in 3% H_2O_2 and diluted with methanol for 10 min to block endogenous peroxidase. To block unspecific staining, Cas BlockTM (Zymed/InVitrogen, San Fransisco, USA) was applied and incubated for 10 min. Following incubation with

mouse anti-dig for 30 min and then polymerised HRP conjugated anti-mouse for 30 min, the HER2 probe was visualized by DAB development (CISH Polymer Detection Kit, Zymed). The biotin labeled Chr 17 centromere probe was detected by incubation with HRP conjugated streptavidin for 30 min, followed by DAB development (CISH Centromere Detection Kit, Zymed) for 30 min. Tissue sections were lightly counterstained with hematoxylin and dehydrated in graded ethanol. At the end of the process, CISH centromere signals or gene copies were easily visualized as dark brown/blue scattered or in small clusters dots, using a conventional, brightfield microscope. Interpretation of HER2 gene and chromosome 17 centromere signal results was based on Zymed's Evaluation Chart for CISH. According to this guide, two gene copies per nucleus demonstrate normal HER2 gene pattern, whereas 6-10 or small clusters characterize a low-level gene amplification. In this case, chromosome 17 status must be evaluated to exclude aneuploidy (3-5 centromeric signals per nucleus; diploid pattern demonstrates normal chromosome status). High gene amplification level is characterized by the presence of more than 10 gene copies or large clusters of them per nucleus in more than 50% of the examined cells, whereas the presence of a smaller number of HER2 copies than chromosome 17 centromeric signals is considered to be an evidence of gene deletion or "silence'' due to mechanisms, such as point mutation or loss of heterozygosity (LOH).
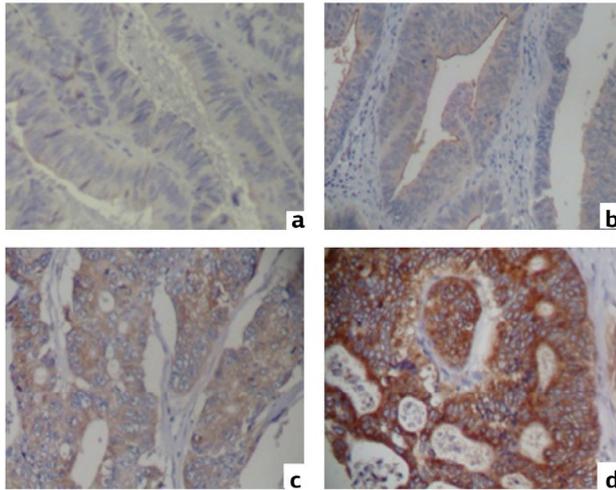
Figure 1. Different HER2 expression patterns in tissue cores of colon adenocarcinoma. Note 0 to 3+ expression score (**a-d** images: original magnification x40).
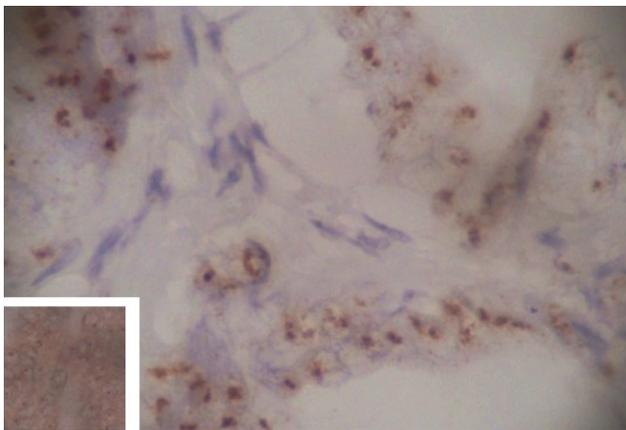

Figure 2. HER2 gene amplified case with normal (diploid) chr 17 pattern (inlet). Note multiple and clustered gene copies as brown signals inside nuclear environment (original magnification x40).

Statistics

Associations between variables including protein expression levels, gene and chromosome 17 alterations were performed using SPSS statistical software v 19 (SPPS Inc, Chicago IL, USA) . In the analysis, HER2 expression was treated as ordinal variables (0-3 scale); in the tables, however, the 0-1 (loss or low expression, "LL") and 2-3 categories have been merged for reasons of brevity. Concerning the associations between these variables and other ordinal or continuous variables, p-value was derived from Spearman's rank correlation coefficient. Regarding the associations involving HER2 gene and chromosome 17 status (binary variables), Fisher's exact test and chi-square test were appropriately performed. Total IHC and CISH results and also p-values are described in Table 1.

Results

IHC results were successfully obtained from all colon adenocarcinoma tissue cores (Figure 1).

Table 2. HER2 IHC and CISH correlation

CISH analysis	IHC 0	IHC +1	IHC +2	IHC +3
No amplification	13	27	15	5
Low amplification	-	-	6	2
High amplification	-	-	-	3
Non amplified	13	27	9	-

HER2 protein vs gene copies p=0.008. IHC: immunohistochemistry, CISH: chromegenic in situ hybridization. IHC score : 0 (loss of expression), 1+ (low expression), 2+ (moderate expression), 3+ (high/dense expression)

HER2 overexpression (overall 2+/3+ score) was observed in 20/60 (33.3%) cases, whereas the remaining (N=40) were characterized by low or negative protein expression (0/1+ score). Strong (score 3+) protein overexpression was detected in 5/20 (25%) cases.

CISH results were successfully obtained from all the examined cases (Figure 2). Multiple HER2 gene signals were observed in 18/60 (30%) cases. Based to simultaneous HER2/Chr 17 copies measurement, gene amplification was observed in 11/60 (18.33%) cases. According to CISH evaluation guidelines, low amplification (6-10 signals per nucleus) was detected in 8/60 (13.3%) cases, whereas high amplification (multiple signals or/ and clusters) in the remaining 3. Multiple gene copies combined with chr 17 polysomy (observed as 3–5 centromere dots per nucleus) were identified in 13/60 (21.6%) cases. One case demonstrated gene amplification associated with chr 17 polysomy. Significant associations were observed correlating gene copies with protein expression (p=0.008), and also Chr 17 centromere signals to overall gene signals (p=0.001), but not chromosome numerical imbalances to HER2 protein expression (Table 2). Concerning clinicopathologigal parameters, multiple HER2 copies due to chr 17 polysomy were strongly correlated with advanced Dukes stage (predominantly B2-C2) of the carcinomas (p=0.001) and also HER2 expression with tumor grade (p=0.03).

Discussion

For a long time, immunohistocytochemistry (IHC/ICC) has been performed as a routine method in almost all labs for evaluating protein expression. Modern molecular approaches show that identification of specific structural and numerical imbalances regarding genes involved in signal transduction pathways provide important data to the oncologists [21-23]. Based on an ex-

panded molecular knowledge in the last decades, HER2 high overexpression (score 3+) due to gene amplification is the critical combination for applying targeted therapeutic strategies in breast cancer patients [24,25]. In contrast to this optimal protein and gene criterion, there is a subset of cases characterized by a borderline pattern (2+ score with multiple gene signals). In these cases that are analyzed by conventional one-color CISH, chr centromere numerical analysis resolves the problem, approving or excluding pure low or high gene amplification [26,27].

In the current technical paper, we analysed simultaneously TMA colon adenocarcinoma cores by IHC and CISH regarding HER2 gene. We observed that a subset of the examined cases demonstrated protein overexpression (2+/3+), but the pure gene amplified cases were less than the overexpressed ones. Additionally, another section of the cores was characterised by low or moderate protein expression levels (1+/2+) without gene amplification, obviously due to multiple gene signals (6-10 signals per nucleus). Further CISH analysis regarding chr centromere numerical imbalances showed that in these cases a chr 17 polysomy was established. Interestingly, this combination of gene/chr 17 numerical aberrations –including also pure gene amplification- was correlated with advanced stage of the corresponding cases, leading to an aggressive phenotype. Similar studies based on HER2 IHC analyses have showed different expression patterns of the marker [28,29]. Another study group focused on investigating HER2 expression in colonic neoplasms of familial adenomatous polyposis patients (FAP), and concluded that only weak cytoplasmic expression (1+ score) was seen in a small subset of the examined cases. This diversity between sporadic and FAP–depended colon carcinomas reflects a difference in HER2 deregulation associated to the genetic mechanism of each disease case [30]. Furthermore, another study concluded that among a variety of analyzed cell cycle signaling pathway proteins, p21, p53, cyclin D1, and AURKA could possibly be used as prognostic markers to identify colon cancer patients with high risk of disease recurrence, but not HER2 expression [31]. Additionally, is seems that there is a significant gene-gene interaction between EGFR and HER2/Src in early stages regarding colorectal carcinogenesis due to specific modified single nucleotide polymorphisms (SNPs) [32]. Finally, a study published very recently focused on HER2 simultaneous genetic and phenotypic analysis of circulating tumor cells in colon cancer patients and showed the importance of this combination in identifying and selecting optimally patients who might benefit from specific targeted therapy [29].

In conclusion, HER2 gene and protein deregulation affect a subset of patients with colon adenocarcinoma. Multiple HER2 gene copies lead to different protein expression patterns (score 1+ to 3+), but pure gene amplification is only a minority of them. Identification of chromosome polysomy is a critical parameter in detecting original gene amplification in conventional one-color CISH methods, correlated also with advanced stage of the malignancy. We strongly support the idea that a combination of IHC/CISH or FISH/ CTC analysis is necessary for handling these patients with targeted therapeutic regimens earning increased response and survival rates as it happens recently in gastric adenocarcinoma cases characterized by HER2 gene deregulation [33].

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