Copy number increase of HER-2 in colorectal cancers

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Abstract. HER-2 is involved in genetic instability and is overexpressed in a number of human carcinomas, including colorectal cancer (CRC). The choromosomal locus of HER-2, 17q21, is frequently amplified in breast cancer, but the correlation between copy-number variations and HER-2 over-expression in CRC has yet to be elucidated. The functional impact of such regions requires extensive investigation in large numbers of CRC samples. Case-matched tissues of colorectal adenocarcinomas and adjacent normal epithelia (n=134) were included in this study. Quantitative PCR was performed to examine the copy number and mRNA expression of HER-2 in CRC. The results showed that copy number gains of HER-2 were detected in a relatively high percentage of CRC samples (35.1%, 47 out of 134). A positive correlation was noted between the copy number increase of HER-2 and tumor progression. Furthermore, copy number gains of HER-2 showed a positive correlation with mRNA overexpression in CRC. However, the expression levels of HER-2 mRNA were also enhanced in the group of CRC samples with unaltered copy numbers. In conclusion, the findings suggest that a copy number increase of HER-2 is a potential diagnostic indicator for CRC; whether alone or in combination with other markers.

Introduction

As a member of the epidermal growth factor receptor family, HER-2 (also termed ErbB-2 or neu), encodes a 185-kDa type I transmembrane receptor tyrosine kinase (1), operates a complex signal transduction cascade and modulates prolif-eration, migration, adhesion, differentiation and apoptosis of cancer cells (2,3). HER-2 protein overexpression has been iden-tified in a number of human carcinomas, including colorectal cancer (CRC) (4-7). Clinical data showed that Trastuzumab (Herceptin) affected survival in approximately 20% of patients who overexpressed HER-2 in breast cancer (8). These data indicated that HER-2 amplification is one of the genes to be considered in the therapeutic management of CRC.

In China, CRC is one of the three cancers whose incidence increased most (together with lung and female breast cancer) between 1991 and 2005 (9,10). Genetic and environmental factors contribute to the disease etiology, with approximately one-third of disease variance attributed to inherited genetic factors (11). The role of copy-number variations (CNVs) in various types of cancer has become a hot spot over the past few years (12,13). Studies using SNP arrays and aCGH have suggested that DNA amplification at chromosome position 17q21, the chromosomal locus of HER-2, is common in breast cancers (14-17). However, the correlation between CNVs and HER-2 overexpression in CRC has yet to be determined. Furthermore, the majority of aCGH experiments focused on the genome-wide screening of CNVs and the data obtained are generally informative, but not definitive. Thus, a study comprehensively examining CNVs in relation to HER-2 expression or prognosis should be performed using a large number of tumors.

In this study, 134 colorectal adenocarcinoma samples, with matched adjacent normal tissues, were collected from the Chinese population to assess CNVs. Results showed that the copy number gains of HER-2 were observed in a high percentage of CRC samples. Furthermore, mRNA was overexpressed in the majority of the CRC samples. A positive correlation was found between gains of HER-2 and mRNA overexpression. These findings suggested the potential role of CNV of HER-2 in CRCs.

Materials and methods

Patients and tissue collection. CRC samples were obtained from 134 surgical patients of the Department of Gastroenterology, Shenzhen Hospital, and the Peking University, China. Adjacent normal mucosa samples located at least 2 cm from the macroscopically unaffected margins of the tumor (polyporcarcinoma), were defined as the normal controls. The tumors that were adenocarcinomas and mucinous

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Table I. Comparison of CNVs of HER-2 between adjacent normal tissues and healthy normal controls from peripheral blood.

| Samples | n | Copy number | | | | | P-value[a] |
| | | Deletion | | | Amplification | | |
		0	1	2	3	>3	
ANT	134	2	6	120	3	3	0.971
HNC	152	2	8	133	5	4	-

[a]P-value vs. HNC. CNV, copy-number variations; ANT, adjacent normal tissues; HNC, healthy normal control.

Table II. CNVs of HER-2 in CRC tissues and matched adjacent normal tissues.

| Samples | n | Copy number | | P-value[a] | P-value[b] |
| | | Deletion | Amplification no. | | |
		≤2	>2		
Total	134			3.22E-10	-
CRC		87	47		
ANT		128	6		
Duke's A and B	77			6.56E-05	-
CRC		58	19		
ANT		75	2		
Duke's C and D	57			2.88E-06	0.011
CRC		31	26		
ANT		53	4		

[a]CRC vs. ANT; [b]Duke's A and B vs. Duke's C and D. CNV, copy-number variations; CRC, colorectal cancer; ANT, adjacent normal tissues.

carcinomas (when >50% of the tumor volume was composed of mucin) were excluded. The CRC samples were staged according to the Duke's classification system: Duke's A (T1-T2, N0 and M0; n=41), Duke's B (T3-T4, N0 and M0; n=36), Duke's C (any T, N1-2 and M0; n=43) and Duke's D (any T, any N and M1; n=14). Matched samples of colorectal adenocarcinomas (n=134) and normal colonic mucosa (n=134) were subjected to real-time PCR analysis. CRC samples were collected from patients undergoing bowel resection. The collected samples were stored in liquid nitrogen. The patients were informed about the aims of specimen collection and gave signed written consent in accordance with the ethics guidelines of Peking University, China. Peripheral blood samples from 152 healthy controls were collected at Peking University People's Hospital, China. The study was approved by the ethics committee of Peking University Shenzhen Hospital, China.

DNA extraction and quantification of copy numbers. Genomic DNA was isolated from the tissues using the Genomic DNA Extraction kit (Innocent, Shenzhen, China) according to the manufacturer's instructions. Quantitative PCR was performed using the BioRad Chromo4 real-time PCR system. The primers for RNAse P were: forward, 5'-AGA CTA GGG TCA GAA CRCA A-3' and reverse, 5'-CAT TTC ACT GAA TCC GTT C-3'. The primers for HER-2 were: forward, 5'-CCA GCC TTC GAC AAC CTC TA-3' and reverse, 5'-ACG TCC AGA CCC AGG TAC TC-3'. Average copy numbers of RNAse P in normal candidates (copy numbers = 2) were used as controls. The copy numbers of HER-2 were calculated using the comparative Ct method. Cut-off values of 0.25, 0.75, 1.25 and 1.75 were used to define the copy numbers as 0, 1, 2 and 3, respectively. The fold change of each sample was presented: fold change = relative expression level/average expression level in the group with two copies of DNA.

A standard curve was prepared using 2 μl of crude DNA solution, in which serially diluted samples (original, 2-, 4-, 8- and 16-diluted) were included. The Ct slopes and efficacy of each primer were calculated via the BioRad Chromo4 real-time PCR system and Microsoft Excel 2007 for Windows. Relative quantification of HER-2 was performed using the $2^{-\Delta\Delta Ct}$ method.

RNA extraction and real-time PCR. Total RNA was isolated from tissues using an AxyPrep™ Blood Total RNA MiniPrep kit (Axygen) according to the manufacturer's instructions. First strand cDNA was synthesized with a RevertAid™ First Stand cDNA Synthesis kit (Fermentas, Burlington, ON, Canada). Quantitative PCR was performed via the BioRad Chromo4
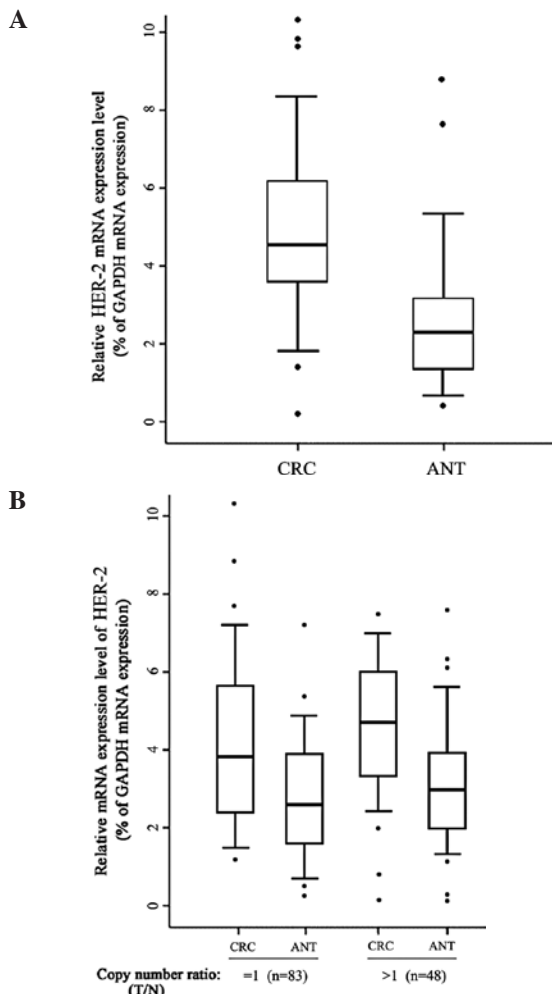
Figure 1. Real-time PCR assay was carried out as previously described, and the results were obtained from the indicated group of samples. (A) mRNA expression level of HER-2 in the CRC samples compared to ANTs. (B) mRNA expression level of HER-2 in groups with amplified (n=83) or unaltered (n=48) DNA copies. Box plots of relative copy number of HER-2 mRNA measured with real-time PCR analysis show the median; box, 25th-75th percentile; bars, largest and smallest values within 1.5 box lengths; •, outliers; CRC, colorectal cancer; ANT, adjacent normal tissues.

real-time PCR system. At the end point of the PCR cycles, melt curves were generated to check the product purity. Since the efficacy of amplification of the targeted genes was almost 100% (data not shown), the $2^{-\Delta ct}$ method was used to calculate the real-time PCR results. The mRNA level of HER-2 was expressed as a ratio relative to the GAPDH mRNA in each sample. An exploratory data analysis using box plots was applied to visually identify the expression level of target mRNA.

Statistical analysis. Statistical analysis was performed by the SPSS Software, version 11.5. Data were analyzed by the Chi-square or Fisher's exact tests. P<0.05 was considered to be statistically significant. The results of HER-2 mRNA expression for normal and tumor tissue samples were compared using two-way repeated measurement analysis of variance (ANOVA). One-way repeated measures analysis of variance (ANOVA-RM) was performed at a significance level of P=0.05 to determine the differences between the controls within

each group. ANOVA-2 was performed following baseline subtraction, at a significance level of P=0.05, to determine the differences between the groups with amplified and unaltered HER-2 copy numbers.

Results

Gene copy number gains of HER-2 in CRC samples. The distribution variance of HER-2 copy numbers between the adjacent normal tissues (ANTs) from CRC patients and peripheral blood from healthy controls was examined. As shown in Table I, no statistical difference of copy number distribution between ANTs and healthy normal controls was observed. Thus, the ANTs were used as controls for the CRC tissues in this study.

Table II shows the CNVs of HER-2 in paired samples of CRCs and ANTs. A total of 134 CRC samples were examined. A relatively high percentage of CRC samples showed amplification of HER-2 (35.1%, 47 out of 134). The CRC tissues from patients with low-grade CRC (Duke's A and B) comprised on average <30% of the samples that had either 3 or 4 copies or >4 copies of the HER-2 gene, whereas >30% of samples that had either 3 or 4 copies or >4 copies of HER-2 were observed in high-grade CRCs (Duke's C and D). A correlation was found between the gene copy number gains and the tumor phenotypes (P=0.011).

Positive correlation between copy number increases and mRNA overexpression of HER-2 in CRCs. To determine whether a genotype-phenotype correlation exists in CNVs of HER-2, the mRNA expression levels of HER-2 between the CRC samples and paired ANTs by quantitative real-time RT-PCR were compared. As shown in Fig. 1A, an increased mRNA expression level of HER-2 was observed in the CRC tissues compared to that of the ANTs (P<0.001). Gene CNVs contribute to qualitative and quantitative diversities in their gene products. Samples with increased or unaltered copies of HER-2 were selected for testing to determine whether the HER-2 mRNA expression was correlated with the copy numbers. The samples with decreased copies of HER-2 (3 out of 134) were not included due to the small sample size. As shown in Fig. 1B, the CRC samples in the group with amplified or unaltered copies of HER-2 showed an increased expression of mRNA compared to the ANTs (P<0.01). A significant statistical difference was noted between the CRC samples in the groups with amplified and unchanged copies of HER-2 (P=0.023). Thus, DNA copy amplification plays at least a partial role in the overexpression of HER-2 in CRCs.

Discussion

CNVs have recently gained considerable interest as a source of genetic variation likely to play a role in phenotypic diversity and evolution. They have been clearly shown to directly or indirectly affect a healthy individual's susceptibility to cancer, for example by varying the gene dosage of tumor suppressors or oncogenes (18,19). However, numerous discrepancies are found among previous studies that used high-resolution approaches to screen CNVs (20-24). Thus, validation of such CNVs by a substantial amount of clinical samples is required.

It is suggested that the genes present in very small regions of CNVs are ideal candidates for evaluation in cancer pathogenesis. Evaluation of the CNVs for such genes is a starting point for investigations into the role of gene amplification in the colorectal carcinogenic process. Although a number of studies showed that the chromosome 17q21 region encompassing HER-2 was amplified in breast cancer samples (14-17), the correlation between CNVs and HER-2 overexpression in CRC has yet to be elucidated. In this study, 134 CRC (adenocarcinomas) samples were collected for CNV analysis of HER-2. Since no statistical difference not noted in CNVs between the healthy normal controls and normal tissues from CRC patients, the CNVs of HER-2 in CRCs were presumably acquired DNA aberrations. The amplification of HER-2 (35.1%, 47 out of 134) was found in the collected CRC samples. Results of the present study showed that the frequency of DNA copy number gains of HER-2 in advanced CRCs was significantly more than in early-stage CRC (Table II), suggesting that copy number gains played a role in CRC progression and may contribute to tumor aggressiveness. However, we found that copy numbers of HER-2 were also deleted in a small percentage of samples (2.24%, 3 out of 134). This discrepancy may be due to the various ethnicities and populations used in the studies. Another reason involves the various methodologies used in the studies. We used a gene-specific strategy to target short fragments (several hundred base pairs) and the sensitivity was increased.

CNVs have also been shown to induce phenotypes. Phenotypic effects of genetic variations, both at the single nucleotide polymorphisms and CNVs, are presumably caused by changes in the expression levels, either by directly affecting the genes concerned via the genetic change, or indirectly through position effects or downstream pathways and regulatory networks (25). In the present study, the correlation between the HER-2 mRNA expression and the copy numbers of its DNA was investigated. The correlation was not as positive as anticipated, although a statistical difference was obtained. Furthermore, the mRNA expression of HER-2 was increased in both the groups of amplified and unchanged DNA copies. A statistical difference was noted in the mRNA expression between the groups of amplified and unaltered DNA copies. Thus, CNVs played a role in the overexpression of the HER-2 mRNA in CRCs, while another mechanism was also involved. This is consistent with two recent studies which assessed an over-representation of differentially expressed genes among CNV-mapping transcripts and observed a weak yet significant positive correlation between the relative expression level and gene dosage (26,27). However, in certain samples the number of copies had no effect on the relative expression levels, suggesting either dosage compensation mechanisms or the incomplete inclusion of regulatory elements in the deletion/duplication event (26,27). The mechanism of this phenomenon has yet to be elucidated. Investigators determined that immediate early genes may play a role in this process. These genes are initially expressed at levels proportional to the number of copies by directly or indirectly inducing the expression of a repressor, which reduces or even abolishes the expression of the CNV gene (26,27). In the second model, the extra copies of a gene impair, through steric hindrance, the access of the copies to a specific transcription factory, where this particular locus should be transcribed (28).

In conclusion, the findings suggest that copy number increasing of HER-2 serves as a diagnostic indicator for CRC, whether alone or in combination with other markers. The mechanism of the heterogeneous expression levels, however, has yet to be elucidated. Whether methylation of DNA or immediate early genes plays a role requires extensive investigation in the future.

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