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Short Communication

Copy number increase of aurora kinase A in colorectal cancers: a correlation with tumor progression

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The centrosome-associated kinase aurora A (AURKA) is involved in genetic instability and is over-expressed in several human carcinomas including colorectal cancer (CRC). The choromosome locus of AURKA, 20q13, is frequently amplified in CRC, and the functional impact of such regions needs to be extensively investigated in large amount of clinical samples. Case-matched tissues of colorectal adenocarcinomas and adjacent normal epithelium ($n = 134$) were included in this study. Quantitative PCR was carried out to examine the copy number and mRNA level of AURKA in CRC. Our results showed that copy number gains of AUKRA were detected in a relative high percentage of CRC samples (32.4%, 43 of 134). There was a positive correlation between copy number increase of AURKA and tumor progression. And copy number gains of AURKA also showed a positive correlation with mRNA over-expression in CRC. However, the expression level of AURKA mRNA was also enhanced in the group of CRC samples with unaltered copy numbers. These findings indicated that sporadic colorectal cancers exhibit different mechanisms of aurora A regulation and this may impact the efficacy of aurora-targeted therapies.

Keywords colorectal cancer; AURKA; copy number variation; gene expression

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Introduction

Aurora kinases are a family of mitotic serine-threonine kinases (S/T kinases) that function as a class of novel oncogenes and are over-expressed in several solid tumors [1,2]. Aurora kinase A (AURKA), also known as Aurora-A, S/T kinase 15 (STK15), or breast-tumor-amplified kinase, is a cell-cycle-associated S/T kinase that regulates the functions of centrosomes, spindles, and kinetochores for proper mitotic progression. AURKA over-expression is observed in various cancers including colorectal cancer (CRC) [3–5]. AURKA may not be a bona fide oncoprotein in humans, but it is a promising drug target in cancer therapy and has potent antiapoptotic functions [4], clinicopathological features and telomerase activity [5].

CRC is currently the third most common cancer and causes about 130,000 deaths per year in the Chinese population. Both genetic and environmental factors contribute to the disease etiology, and about one-third of the disease variance can be attributed to genetic factors [6]. The implication of copy-number variation (CNV) in cancers has become a hotspot over the past few years [7–9]. Studies using SNP array and array comparative genomic hybridization (aCGH) have suggested that DNA amplification at chromosome position 20q13, which is also the chromosome locus of AURKA, is common in colorectal cancers [10–12]. There might be a correlation between CNV and AURKA over-expression in CRC. However, most of the aCGH experiments focused on the genome-wide screening of CNV, and the data obtained are generally informative but not definitive. Thus, a study comprehensively examining CNV in relation to AURKA expression or prognosis should be performed using a large number of tumor samples.

In this study, we collected 134 colorectal adenocarcinoma samples with matched adjacent normal tissues from Chinese population for CNV analysis. Our results showed that the copy number gains of AURKA were in a rather high percentage of CRC samples. And mRNA was over-expressed in most of the CRC samples. There was a positive correlation between the gains of AURKA and mRNA over-expression. These findings suggested the potential role of CNV of Aurora-A in CRC.

Materials and Methods

Patients and tissue collection

Colorectal cancer samples were obtained from 134 surgical patients in the Department of Gastroenterology, Peking University Shenzhen Hospital. Adjacent normal mucosa samples located at least 2 cm from the macroscopically unaffected margins of the tumor (polyp orcarcinoma) were defined as controls. All tumors were adenocarcinomas and mucinous carcinomas (when more than 50% of the tumor volume was composed of mucin) were excluded. Colorectal cancers were staged according to the Dukes classification system: Dukes A (T1–T2, N_0, and M0; $n = 41$), Dukes B (T3–T4, N_0, and M0; $n = 36$), Dukes C (any T, N1–2, M0; $n = 43$) and Dukes D (any T and 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. All patients were informed about the aims of the specimen collection and gave signed written consent in accordance with the ethical guidelines of Peking University. Peripheral blood samples from 152 healthy people as controls were collected at Peking University People's Hospital. The study was approved by the ethical committee of Peking University Shenzhen Hospital.

DNA extraction and quantification of the copy numbers

Genomic DNA was isolated from the tissues using the Genomic DNA Extraction Kit (Innocent, Shenzhen, China) according to the manufacturer's instruction. Quantitative PCR was performed through BioRad Chromo4 real-time PCR system (Hercules, USA). The primers for RNase P are: forward: 5′-AGA CTA GGG TCA GAA CRCA A-3′ and reverse: 5′-CAT TTC ACT GAA TCC GTT C-3′. The primers for AURKA are: forward: 5′- ACRC CAC GAG AAT TGT CRCT ACT′ and reverse: 5′- TTT CRCC TCC TGT GAA GAC AC-3′.

The average copy numbers of RNase P in normal candidates (copy number = 2) were used as control. The copy numbers of AURKA were calculated using the comparative C (T) method. Cut-off values of 0.25, 0.75, 1.25, 1.75 were used to define the copy numbers as 0, 1, 2, 3, respectively. Fold change of each sample was presented as follows: 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 solutions, in which serially diluted samples (original, 2-, 4-, 8-, 16-fold diluted) were included. The slopes of Ct and efficiency of each primer were calculated by the BioRad Chromo4 real-time PCR system and Microsoft Excel 2007 for Windows. The relative quantification of AURKA was performed with the 2-ddCt method.

RNA extraction and real-time PCR

Total RNA was isolated from tissues using AxyPrepTM Blood Total RNA MiniPrep Kit (Axygen, Union City, USA) according to the manufacturer's instruction. First-strand cDNA was synthesized with RevertAidTM First-Strand cDNA Synthesis Kit (Fermentas, Burlington, Canada). Quantitative PCR was performed through BioRad Chromo4 real-time PCR system. At the end point of PCR cycles, melt curves were made to check product purity. Since the amplification efficiency of the targeted genes approached 100% (**Supplementary Fig. S1**), we used $2^{-\triangle ct}$ method to calculate the real-time PCR results. The mRNA level of AURKA was expressed as a ratio relative to the GAPDH mRNA in each sample (one of the representative calculations was shown in **Supplementary Fig. S2**). Exploratory data analysis using box plot was applied to visually identify the expression level of the target mRNA.

Statistical analysis

Statistical analysis was performed with the SPSS software version 12. Data were analyzed by the chi-square test or Fisher exact test. P values <0.05 were considered statistically significant. Results of AURKA mRNA level for normal and tumor tissue samples were compared using Two-way Repeated-measures 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 each group and control. Two-way analysis of variance (ANOVA-2) was performed after baseline subtraction, at a significance level of $P = 0.05$ to determine the differences between the groups with amplified and unaltered AURKA copy number.

Results

Gene copy number gains of AURKA in CRC samples

We first examined whether there is a distribution variance of AURKA copy number between the adjacent normal

Table 1 CNVsof AURKA between ANTs from CRC patients and peripheral blood from healthy normal controls (HNC)

Samples	*n*	Copy number					
		Deletion			Amplification		
		0	1	2	3	>3	*P* (vs. HNC)
HNC	152	2	7	135	5	3	–
ANT	134	2	6	111	6	5	0.758

tissues from CRC patients and peripheral blood from healthy people. As shown in **Table 1**, no statistical difference of the copy number distribution between adjacent normal tissues (ANTs) and healthy controls was observed. Thus, the ANTs could be used as controls for the CRC tissues in this study.

Table 2 shows the CNVs of AURKA in paired samples of CRCs and ANTs. A total of 134 CRC samples were examined. A representative diagram for the DNA CNV analysis for one CRC/ANT pair was shown in **Supplementary Fig. S2**. A relative high percentage (32.1%, 43 of 134) of CRC samples showed the amplification of AURKA. The colorectal cancer tissues from patients with low-grade CRC (Dukes A and B) contained on average $<30\%$ of the samples that had either three or four copies or more than four copies of the AURKA gene, whereas $>30\%$ of the samples that had either three or four copies or more than four copies of AURKA was observed in high-grade (Dukes C and D) CRC. There is a correlation between gene copy number gains and the tumor phenotypes ($P = 0.0379$).

Positive correlation between copy number increases and mRNA over-expression of AURKA in CRC

To find out whether there is a genotype–phenotype correlation in relation to the CNV of AURKA, we compared the mRNA expression levels of AURKA between the CRC samples and the paired adjacent normal tissues by quantitative real-time RT-PCR. As shown in **Fig. 1(A)**, increased mRNA expression level of AURKA was observed in the CRC tissues compared with the ANTs ($P < 0.001$). **Supplementary Fig. S2** shows a representative diagram for the mRNA expression analysis for one CRC/ANT pair. The results were consistent with others' findings [5,11]. Gene CNV can contribute to the qualitative and quantitative diversities of their gene products. Next, we selected the samples with increased or unaltered copies of AURKA, and tested whether the AURKA

Table 2 CNV of AURKA in CRC tissues and matched adjacent normal tissues (ANT)

CNV, population		*n*	Copy numbers		*P*-value (CRC vs. ANT)	*P*-value (Dukes C and D vs. Dukes A and B)
			Amplification			
			≤2	>2		
Total	ANT	134	123	11	–	–
	CRC		91	43	1.1E−6	–
Dukes A	ANT	77	72	5	–	–
and B	CRC		56	21	5.78E−4	–
Dukes C	ANT	57	51	6	–	–
and D	CRC		35	22	4.99E-4	0.0379

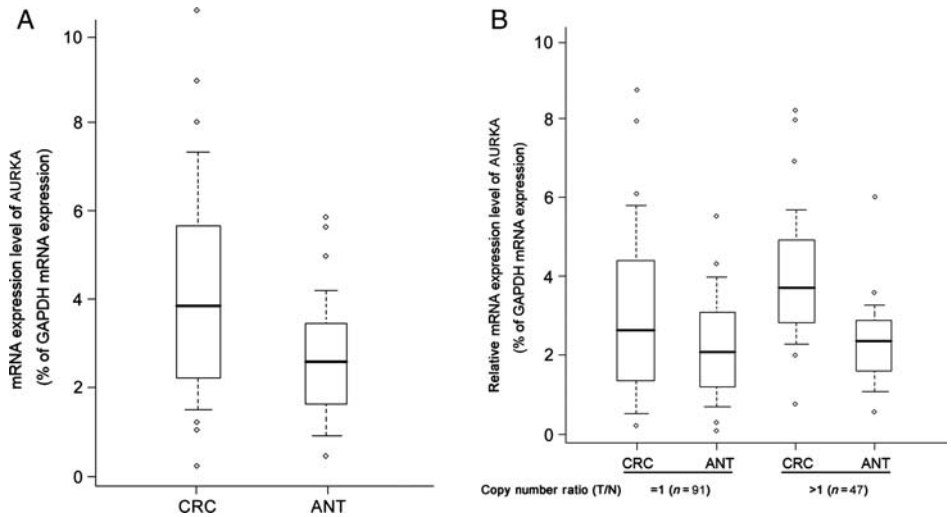


Figure 1 Expression level of AURKA mRNA in case-matched CRC samples measured by real-time PCR method Boxplots of relative copy number of AURKA mRNA measured using real-time PCR analysis showing median; box: 25th–75th percentile; bars: largest and smallest values within 1.5 box lengths; little circles: outliers. (A) mRNA expression level of AURKA in all the CRC samples compared with ANTs. (B) mRNA expression level of AURKA in groups with amplified ($n = 87$) or unaltered ($n = 43$) DNA copies.

mRNA expression was correlated with the copy number. The samples with decreased copies of AURKA (4 of 134) were not included due to the small sample size. As shown in **Fig. 1(B)**, the CRC samples in the group with amplified or unaltered copies of AURKA both showed an increased expression of mRNA compared with ANTs ($P < 0.01$). There was a statistically significant difference between the CRC samples in the groups with amplified and unchanged copies of AURKA ($P = 0.015$). Thus, the DNA copy amplification plays at least a partial role in the over-expression of AURKA in CRC. We also compared the protein level of AURKA in various CRC samples with different copy number, but the differences were not as significant as those from mRNA expression analysis (see **Supplementary Fig. S3**).

Discussion

Although cancer is ordinarily regarded as an acquired disease, familial predisposition plays a significant role in many cancer types. Thus far, several high penetrant cancer predisposing genes correlated with colorectal cancer, e.g. DNA mismatch repair genes [13], *APC*, *SMAD4*, *BMPR1A*, and *MUTYH* [14] have been identified. As yet, these genes explain only a fraction of the familial and/or hereditary cases of cancer. CNVs identified by CGH and array technology have been clearly shown to have potential influence on the healthy individual's susceptibility to cancer, for example by varying the gene dosage of tumor suppressors or oncogenes [15,16]. However, there are many discrepancies among previous studies that used high-resolution approaches to screen the CNV [17–21]. Thus, the validation of such CNV by large amount of clinical samples is required.

It is suggested that the genes present in very small regions of the CNV are excellent candidates for the evaluation of cancer pathogenesis. Examination of the CNV for such genes is a starting point for investigating the role of gene amplification in the colorectal carcinogenic process. Several most recent studies have shown that the chromosome 20q13 region encompassing AURKA was amplified in colorectal cancer samples [22,23]. In this study, we collected 134 CRC (adenocarcinomas) samples for CNV analysis of AURKA. Since there was no statistical difference of CNV between the healthy normal controls and normal tissues from CRC patients, the CNV of AURKA in CRC was more likely acquired DNA aberrations. The amplification of AURKA (32.1%, 43 of 134) was found in the collected CRC samples, and the frequency of gains was consistent with that shown in previous studies, in which the gains of chromosomes 20q13 were reported to be 30%–70% of colorectal cancers [24,25]. The results from the present study showed that the frequency of DNA copy number gains of AURKA in advanced CRC was significantly higher than that in early-stage CRC, suggesting that copy number gains play a role in CRC progression and might contribute to tumor aggressiveness. However, we found that the copy numbers of AURKA were deleted in a small percentage of samples (2.98%, 4 of 134). This discrepancy might be due to the different races and populations as well as the different methodologies used in different studies. We used gene-specific strategy to target short fragments (several hundred base pairs), and the sensitivity was increased.

The CNV is expected to have a genotype–phenotype correlation, such as the CNV are supposed to be correlated with the expression level of the gene [24,25]. In the present study, we investigated the correlation between the AURKA mRNA level and the copy number of its DNA. To our surprise, the correlation was not as positive as expected, although a statistical difference was obtained. The mRNA level of AURKA was increased in both the groups of amplified and unchanged DNA copies. There was a statistical difference of mRNA expression between the groups of amplified and unaltered DNA copies. Thus, CNV might play a role in the over-expression of the AURKA mRNA in CRC, whereas other mechanisms might also be involved. This result is consistent with two recent reports, in which the researchers assessed an over-representation of differentially expressed genes among CNV-mapping transcripts, and observed a weak but significant positive correlation between relative expression level and gene dosage [26–28]. In a small percentage of samples, the relative expression level was inversely correlated with the copy number (some of the data are shown in **Supplementary Table S1**). Similar inverse correlation was also observed in other previous studies [26,27]. The mechanism of this phenomenon is still poorly understood, but may be explained by two models: in the first model, a negative correlation between the number of copies and the relative expression is explained by immediate early genes that induce the expression of a repressor which, by a negative feedback loop, reduces or even abolishes the expression of the CNV gene; in the second model, the extra copies of a gene impair, through steric hinderance, their access to a specific transcription factory, where this particular locus should be transcribed [28].

Taken together, our findings showed that the CNV of AURKA has the potential to serve as diagnostic indicators, alone or in combination with other markers, for colorectal malignancies. However, the functional consequences of CNV, the different feature of CNV in colorectal and other gastrointestinal malignancies, as well as the underlying mechanisms of the heterogeneous expression levels need to be extensively investigated in the future.

Supplementary data

Supplementary data is available at *ABBS* online.

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