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Author
Lam, Alfred, Ong, Kate, Ho, Yik-Hong

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Aurora kinase expression in colorectal adenocarcinoma: correlations with clinicopathological features, p16 expression and telomerase activity

Running head: Aurora kinase expression in colorectal carcinoma

Alfred King-Yin Lam¹, M.B.B.S, M.D., Ph.D., F.R.C.P.A.
Kate Ong², B.Sc., M.Sc.
Yik-Hong Ho³, M.B.B.S., M.D., F.R.A.C.S.

¹ Division of Pathology (School of Medicine), Griffith University, Gold Coast, Queensland, Australia
² Division of Surgery (School of Medicine) and North Queensland Centre for Cancer Research (Australian Institute of Tropical Medicine), James Cook University, Townsville, Queensland, Australia

Correspondence and/or reprint requests:
Professor Alfred Lam
Foundation Chair Professor and Head of Pathology
Griffith Medical School
Griffith University Gold Coast Campus
Gold Coast QLD 4222
Australia

Phone +61 7 56780718; Fax +61 7 56780708; E-mail: a.lam@griffith.edu.au

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KEYWORDS: aurora kinase, expression, differentiation, colorectal adenocarcinoma, p16, telomerase.
ABSTRACT
The clinicopathological roles of Aurora kinase protein have not been studied in depth in colorectal cancer. The aim of the present study is to investigate the clinicopathological roles of Aurora kinase protein expression in a large cohort of patients with colorectal adenocarcinoma with tight methodology and close follow-up. Aurora kinase protein expression was investigated in 200 patients (110 men, 90 women) with colorectal adenocarcinomas by immunohistochemistry. The findings were correlated with the clinicopathological features, p16 expression and telomerase activity of colorectal adenocarcinomas. Aurora kinase protein was detected in 48.5% (97 of 200) of patients with colorectal carcinoma. The protein was more frequently detected in patients with well or moderately differentiated colorectal carcinomas than in poorly-differentiated colorectal adenocarcinomas (52% versus 31%, p=0.004). Mucinous adenocarcinomas were less often positive for the protein (16% versus 56%, p=0.0001). Aurora kinase protein expression was more commonly seen in carcinomas in the rectum, sigmoid and descending colon compared to more proximal colon (55% versus 36%, p=0.01). Also, aurora kinase protein expression was related to the expression of p16 protein (p=0.001) and correlated inversely with the level of telomerase activity in colorectal carcinomas (p=0.005). To conclude, aurora kinase protein is expressed in a subset of colorectal carcinoma. The expression of Aurora kinase protein was found to be related to the distal location, grade of tumour, p16 expression and telomerase activity. These findings may be important to select patients to benefit for clinical trials of aurora kinase inhibitors.
INTRODUCTION

Colorectal cancer is amongst the most common malignancy found in the Western world and usually ranks high in incidence and mortality among malignancies in those countries [1]. There is evidence that research in the molecular pathway of cancers contributes directly to improve the care of patients with colorectal cancer by more accurately refining prognosis and selecting the most appropriate adjuvant therapy for individual patients with colorectal cancer [2]. For instances, studies of inhibitors targeting the molecular pathway of cancer provide evidences of potential adjuvant treatment for cancers with aggressive behavior [3]. One of the newest classes of drugs on clinical trial is known as Aurora kinase inhibitors [4].

The Aurora kinas family is a collection of highly related serine/threonine kinases that are key regulators of mitosis. Aurora has evolved into three related kinases known as Aurora–A, Aurora-B, and Aurora-C [5]. Transfection studies have established Aurora A as a bone fide oncogene. The Aurora-A protein (also designated as STK15, BTAK, AURKA, aurora-2, AIK1 or ARK1) is a centrosome-associated protein and has been implicated in regulatory centrosome function, spindle assembly, spindle maintenance, chromosome segregation, and cytokineses [6].

The gene for Aurora-A is located on chromosome 20q13.2, a region commonly amplified in malignancies [6]. In fact, amplification of Aurora-A mRNA have been found in many human tumors [7-8]. Nevertheless, discrepancies between amplification and expression of the protein were reported [6]. Aurora-A is likely to be regulated not only by amplification, but also by other mechanisms such as transcriptional activation and suppression of protein degradation. Thus, studies of protein expression are needed to clarify the roles of the protein in human cancers. However, the antibody to the protein has become available commercially only very
recently. The roles of the Aurora kinase protein expression can therefore be studied in situ in larger number of human cancers.

In colorectal cancer, Aurora kinase mRNA have been found to be amplified. Bischoff and colleagues showed, by Northern blot, that Aurora kinase RNA expression was increased in 54% (22 of 41) of colorectal cancers [9]. Also, Gerlach and colleagues, using real-time polymerase chain reaction, noted significant difference in Aurora kinase RNA between colorectal cancer and normal tissue [10]. In addition, Takahashi and colleagues have demonstrated that 67.9% (53 of 78) of colorectal cancers were positive for polyclonal antibody HsAIRK1 [11]. Other than these, there is no study on the clinicopathological roles of aurora kinase protein expression. The latter is important as only at risk colorectal cancer patients need adjuvant chemotherapy. Therefore, we investigated Aurora kinase expression in a large consecutive cohort of patients with colorectal cancer in order to determine more definitely its relationship with the different clinicopathological parameters and molecular markers. The current series is the first study to critically determine the clinicopathological roles of expression of Aurora kinase protein in colorectal cancer. It is also the first that looked at the relationship of Aurora kinase expression with p16 protein expression and telomerase activity in colorectal cancer.
MATERIALS AND METHODS

Clinical data collection

Two hundred consecutive patients (110 men, 90 women) with primary colorectal adenocarcinoma were recruited from the Townsville Hospital in North Queensland, Australia. The age, gender, clinical presentation, management and survival data of these patients were prospectively collected in a computerized database. The median age of the patients was 65 (range, 24 to 92) years. The mean diameter of the tumours was 4.5 cm (range, 0.7 to 16.0 cm). Thirty five per cent (n=70) of the carcinomas were located in the proximal colorectum (caecum, ascending colon and transverse colon) and 65% (n=130) were noted in the distal colorectum (descending colon, sigmoid colon and rectum).

Tissue collection and pathology examination

The tissue samples were collected prospectively. Standard blocks were then taken, fixed in 10% formalin and embedded in paraffin wax. Histological sections were cut and stained for haematoxylin and eosin for light microscopic examination. The histological subtypes were classified using the World Health Organization (WHO) criteria [12]. Mucinous carcinoma was defined as an adenocarcinoma with more than 50% of the tumour composing of extracellular mucin. The carcinomas were staged according to TNM (Tumour, Lymph node and Metastases) classification adopted in American Joint Committee on Cancer [13].

In this series, 38 mucinous adenocarcinomas in the colorectum were noted. Features consistent with hereditary non-polyposis colorectal cancer (HNPCC) were noted in 16% (6 of the 38) mucinous adenocarcinomas. The other colorectal cancers were 162 conventional adenocarcinomas. Overall, the adenocarcinomas were well-
differentiated in 7% (n=14), moderately-differentiated in 75% (n=150) and poorly-
differentiated in 18% (n=36). Lymph node metastases were noted in 42% (n=83) and
distant metastases were found in 12% (n=24) of the patients. The carcinomas as
classified according to TNM were: stage I in 24.5% (n=49), II in 32% (n=64), III in
31.5% (n=63) and IV in 12% (n=24).

Patients' follow-up information

The actuarial survival rate of the patients was calculated from the date of
surgical resection of the colorectal carcinomas to the date of death or last follow-up.
Management was by a preagreed standardized multidisciplinary protocol supervised
by a senior specialist colorectal surgeon. None of the rectal cancer patients had
undergone neoadjuvant radiotherapy or chemotherapy prior to surgery. Pathological
diagnosis was also standardized and reviewed by committee criteria, chaired by a
senior academic pathologist with a special interest in the field. Follow-up was
routinely at 3 monthly intervals for the first 2 years, 6 monthly for the subsequent 3
years, and yearly thereafter. Serum carcinoembryonic antigen (CEA) levels were
taken before each visit and imaging investigations 2 years after surgery or earlier as
clinically appropriate. Colonoscopy was performed at 1 year after surgery, and
repeated as appropriate to the findings and other clinical features at follow-up.
Informed consent was obtained from patients, as per approval from the Townsville
Hospital ethics committee.

Aurora kinase staining

Four $\mu$m paraffin sections were cut from each of the 200 colorectal
carcinomas and dried at 37°C overnight. They were de-paraffinized with xylene and
rehydrated through graded alcohol. The sections were then treated with Pascal Pressure Chamber (DAKO, Gloustrup, Denmark) at 125°C in 10mM sodium citrate buffer (pH = 6.00) for 10 minutes. After sections were cool, they were washed in water, and incubated with 0.03% hydrogen peroxide (K4007, DAKO, Gloustrup, Denmark) for 10 minutes at room temperature to block the endogenous peroxidase activity. Sections were again washed with water and then with TBSB (Tris buffer saline and 0.02% bovine serum albumin). Sections were then incubated with 10% normal goat serum for 30 minutes at room temperature to block the non-specific binding. Primary mouse monoclonal anti-Aurora kinase antibody (NCL-L-AK2 at dilution 1:50; Vision BioSystems, Mount Waverley, Vic, Australia) was applied and incubated in a moist chamber overnight at room temperature. The slides were then washed three times in TBSB. Then, peroxidise labelled polymer conjugated to goat anti-mouse immuoglobulins (K4007, Dako, Gloustrup, Denmark) were applied to sections and incubated for 60 minutes at room temperature. They were washed in high salt buffer, TBS (at pH = 7.6, sodium chloride = 0.3M) and Tween 20 (polysorbate 20, a washing solution) three times, to reduce the possible nonspecific background caused by the ionic interaction of antibody with protein in tissues. Brown positive staining was developed by application of freshly prepared liquid DAB (3,3’-diaminobenzidine) and substrate-chromogen solution (K4007, Dako, Gloustrup, Denmark). Sections were then washed in plenty of water, counter-stained with Mayer’s haematoxylin, dehydrated through graded alcohol, cleared in xylene and mounted. A known case of colorectal carcinoma with strong positive for aurora kinase protein was used as positive control. Negative control was the same case with omission of the primary antibody. Brown nuclear stain was regarded as positive. The
cancerous tissues were considered positive for when ≥10% of the tumour cells showed positive staining to the protein.

**P16 protein expression**

P16 expression was done in 189 patients with colorectal cancer. The experiment was done using the method as described in our previous study [14]. In brief, p16 protein expression was tested using immunohistchemical method. The antibody p16$^\text{INK4a}$ used was a mouse monoclonal antibody supplied by Biocare Medical (Cat # CM020, Walnut Creek, CA, USA). Brown nuclear stain was regarded as positive.

**Telomerase activity**

Fresh tissues were obtained in 23 patients with colorectal carcinoma at the time of surgery to study the telomerase activity. These fresh samples were preserved in RNA	extit{later} (Ambion, Austin, TX, USA) within 10 minutes of surgery to prevent degradation of the RNA. They were stored at −80°C for the experiment. The telomerase activities of these samples were studied by reflected by hTERT levels using standard method as described in our previous study [15]. In brief, the RNAs from the tissues were extracted and cDNAs were prepared. Quantitative analysis of hTERT was done using real-time polymerase chain reaction using Rotor gene system (Corbett Research, Sydney, Australia). Absolute quantification assay was chosen to analyse both hTERT (target) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, endogenous control) genes expression. Expression level of hTERT for each sample was normalized by dividing the copy number of hTERT by the
corresponding copy number of GAPDH. (Normalized hTERT = copy number of hTERT\textsubscript{sample} / copy number of GAPDH\textsubscript{sample} \times 100)

**Statistical analysis**

Fisher’s exact test or likelihood ratio was used for categorical variables. Student t-test with Yates correction was used for continuous variables. The significance of various parameters on survival was analysed by the Kaplan-Meier method with log-rank test and multivariate Cox’s regression. Significance level was taken at p<0.05. All statistical tests were performed with the program, Statistical Package for Social Sciences (SPSS version 14.0, Chicago, IL).
Results

Aurora kinase protein expression was detected in 48.5% (97 of 200) of colorectal adenocarcinoma. The non-neoplastic mucosa and dysplastic areas (adenoma) showed no evidence of Aurora kinase protein expression. The areas with high grade dysplasia or adenomas with intramucosal carcinoma were negative for the protein. The expression was confined to the nuclei of the tumor cells (Figure 1). The percentage of tumour cells positive for the protein ranged from 10% to 50%.

The correlations of the Aurora kinase protein expression with clinicopathological features were shown in Table 1. Expression of Aurora kinase protein was related to the histology of the colorectal adenocarcinoma. In conventional colorectal adenocarcinoma, 56% (91 of 162) were positive for Aurora kinase protein whereas in mucinous adenocarcinoma, only 16% (6 of 38) were positive for the protein (p=0.0001). In mucinous adenocarcinomas, the expression has no relationship with the HNPCC status of the patient. Also, in conventional colorectal adenocarcinoma, Aurora kinase protein was more often detected in low grades (well or moderately-differentiated) colorectal adenocarcinoma than high grade (poorly-differentiated colorectal) adenocarcinoma (52% versus 31%, p=0.004) (Figure 1).

Aurora kinase protein was more often commonly found in carcinomas in the distal colorectum (the rectum, sigmoid colon or descending colon) than in proximal portions of the colon (55% versus 36%, p=0.01). The protein expression did not correlate with the age, gender of the patients, size and TNM staging of the tumors.

Overall, p16 protein expression was noted in 79% (150 of 189) of patients with colorectal carcinoma. P16 protein expression was noted in 90% of colorectal cancers positive for Aurora kinase protein. On the other hand, p16 protein expression
was found in 71% of cancers negative for aurora kinase protein. Thus, the expression of aurora kinase protein was related to the expression of p16 protein in colorectal carcinoma (p=0.001).

Telomerase activity was present in all the 23 cases tested. In the patients with positive Aurora kinase protein expression, the hTERT level was 0.66 whereas in those with negative Aurora kinase protein expression, the hTERT level was 1.53 (p=0.005).

Follow-up data were available for all the patients. Thirty percent (n=61) of the patients died of causes related to the colorectal cancer in the study period. The overall median survival rate for patients with the cancer was 60 months. By Kaplan-Meier log-rank survival and Cox-multivariate analysis, survival of the patients with colorectal cancer was only associated with the TNM stages of the tumours (p=0.0001). Survival of the patients was not significantly related to patients’ age, gender and tumours’ differentiation, location, Aurora kinase protein expression (p>0.05). In stage III cancers, patients with Aurora kinase protein had slightly poorer survival rates than other patients (Figure 2). The median survival was 60 months with negative Aurora kinase protein expression whereas the median survival was 44 months in patients with Aurora kinase protein expression. However, Kaplan-Meier analysis did not show that expression was significantly predicative of survival (p = 0.08).
Discussion

In this report, we described the first systematic survey of Aurora kinase protein expression in colorectal cancer. The results showed that the protein occurred in approximately half (48.5%) of patients with colorectal cancer. Also, in stage III colorectal cancer, there was a trend that patients with negative expression of Aurora kinase protein enjoyed slightly better survival rates than those with positive protein expression. On the other hand, positive expression of the protein may mean the cancer is more aggressive and have potential be responsive to aurora kinase inhibitors. Overall, our findings suggest that Aurora kinase protein expression is a common phenomenon in colorectal adenocarcinoma

Researchers have demonstrated that cancers located in the right (proximal) and left (distal) colorectum can be distinguished by clinical criteria [16-18]. Proximal colorectal tumours, when compared to distal tumours, are more often found in older age and females. In recent years, it has become clear that colorectal cancer evolves through multiple pathways on the basis of two molecular features: (i) DNA microsatellite instability (MSI) status stratified as MSI-high (MSI-H), MSI-low (MSI-L) and MS stable (MSS), and (ii) CpG island methylator phenotype (CIMP) stratified as CIMP-high, CIMP-low and CIMP-negative [19]. It is worth noting that MSI-high or MSS with CIMP-high colorectal cancers are more often noted in proximal colorectum, and in female patients with advanced ages. Thus, clinical difference between proximal and distal colorectal cancers may partly be explained by different involvement of these 2 molecular pathways.

Apart from the 2 molecular pathways, right sided tumours show significantly less nuclear β–catenin and p53 overexpression than left-sided tumours [16-18]. Our group noted that p16 protein expression was more often noted in mucinous
adenocarcinoma of the distal colorectum [14]. We also demonstrated that telomerase activity was higher in the distal colorectum [15]. In this study, we demonstrated for the first time that Aurora kinase protein was also more frequently noted in the distal colorectum. Thus, the differences in gene expression in different location in colorectum exist and this may have important implications with specifically targeted therapeutic regimens in the future.

In our previous study, we noted that colorectal mucinous adenocarcinoma had distinctive clinicopathological features [14]. In this study, we noted that Aurora kinase protein was less found in colorectal mucinous adenocarcinoma than in conventional colorectal adenocarcinoma. This expression profile supports the notion that the entity is different from conventional adenocarcinoma. Royce and colleagues noted that Aurora–A expression in primary breast carcinomas was correlated with nuclear grade [20]. In our study, we noted that Aurora kinase protein was more often noted in well or moderately-differentiated (grades 1 and 2) colorectal adenocarcinoma when compared to poorly-differentiated (grade 3) colorectal adenocarcinoma.

The current study is the first one documenting the correlations with p16 and Aurora kinase proteins expression in human cancer. The p16 suppressor gene is one of the most commonly studied candidates in the pathogenesis of human neoplasia [21]. p16 gene encodes p16 protein that competes with cyclin D for binding to CDK4. This inhibits the ability of the cyclin D-CDK4 complex to phosphorylate Rb (retinoblastoma) protein, thus causing cell cycle arrest at late G1 phase. As the carcinogenesis of the Aurora kinase depends on p53-Rb signaling pathway and p16 affects the Rb protein, it is logical that p16 and Aurora kinase proteins co-express in colorectal cancer [6].
Like Aurora kinase, telomerase is an important component in cell division. Telomere shortens in cell division and needs telomerase to keep the length for further cell division. It is believed that telomerase reactivation in the cell is associated with carcinogenesis and is a critical step in the tumour immortalization process. In our previous study, we demonstrated that telomerase activity was significantly higher in colorectal cancer than non-tumour tissue [15]. In human ovarian and breast lines, Yang and colleagues showed that Aurora kinase induces telomerase activity. In the current study, we also noted that telomerase activity was elevated in all colorectal cancer samples being tested. However, the telomerase activity was higher in tumours with negative Aurora kinase expression. This may indicate that other cellular mechanisms may be more important in the induction of telomerase activity in human colorectal cancer. Nevertheless, the negative association of these proteins needs further investigation.

To conclude, Aurora kinase protein is expressed in a subset of colorectal carcinoma. Also, the expression was found to be related to the distal location, grade of tumour, p16 expression and telomerase activity. These findings may be important to select patients to benefit for clinical trials of aurora kinase inhibitors.
References


Figure Legends

Figure 1.

1A. Aurora kinase staining in the nuclei of moderately-differentiated adenocarcinoma.

1B. Aurora kinase staining in the nuclei of poorly-differentiated adenocarcinoma.

Figure 2.

The survival of patient with stage III colorectal cancer in relationship with Aurora kinase protein expression (p=0.08)
Table 1:

The relationships between Aurora kinase expression and other factors in colorectal cancer

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<th>positive (n=110)</th>
<th>negative (n=90)</th>
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<td>55:48</td>
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<td>Mean age (years)</td>
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<td>0.67</td>
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<td>Mean tumour size (cm)</td>
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<td>4.5</td>
<td>0.56</td>
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<td>58</td>
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<tr>
<td>Tumour histology</td>
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