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ORIGINAL PAPER

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Expression of mitotic Aurora/Ipl1p-related kinases in renal cell carcinomas: an immunohistochemical study

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Abstract The Aurora/Ipl1p-related kinases, AIRK1, AIRK2 and AIRK3, are members of a novel family of oncogenic serine/threonine kinases regulated by cell cycle progression and involved in chromosome segregation and cytokinesis. In this study, we examined expression of members of the AIRK family in human renal cell carcinomas. Expression of AIRK subfamilies was examined in 64 renal cell carcinomas by immunohistochemistry. Immunostaining of AIRK1, AIRK2 and AIRK3 was observed in 95%, 47% and 98% of specimens, respectively. Moreover, in specimens from the same patient, staining of AIRK2 was correlated with proliferating cell nuclear antigen labeling. Here we provide the first description of AIRK isozyme immunostaining in human renal cell carcinoma. Although the precise physiological functions of these kinases are not known, AIRK subfamily expression may play a role in renal cell carcinoma tumorigenesis.

Keywords Kinase · Aurora · Mitosis · Proliferating cell nuclear antigen · Renal cell carcinoma

Introduction

Neoplastic diseases are proliferative disorders characterized by uncontrolled cell growth. For cells to complete chromosome replication and cell division, precise regulation of centrosome function is essential. Centrosomes are thought to play important roles in bipolar spindle formation and maintenance during M phase. Centrosome amplification is often observed in cancer cells, and this abnormality is thought to cause chromosomal missegregation, which is important in the progression of malignancy [11].

Members of the Aurora/Ipl1p family of mitotically regulated serine/threonine kinases appear to be key regulators of chromosome segregation and cytokinesis [4]. Recent studies by our group and others revealed three functional subfamilies of Aurora/Ipl1p-related protein kinases (AIRK) in vertebrates: the AIRK1 subfamily (AIK1, AUR2, ARK1, BTAK, AYK1, IAK1, STK6 and STK15), the AIRK2 subfamily (AIK2, AUR1, AIM1, ARK2, STK-1 and STK12) and the AIRK3 subfamily (AIK3, AIE2 and STK13) [4]. AIRK1 is localized in the centrosome during mitosis from prophase to anaphase [7]. AIM-1/AIRK2 is localized along the plane between central spindles during late anaphase and at the midbody during telophase and cytokinesis [15]. AIRK3 is localized in the centrosome during mitosis from anaphase to cytokinesis [9]. Although the biological functions of these kinases are not well understood, overexpression of AIRK1 in fibroblasts has been shown to induce centrosome amplification [16], and a dominant-negative mutant of AIM-1 blocks cytokinesis, resulting in cell cycle arrest and multinucleation [15]. Moreover, recent studies have revealed that Aurora2/AIRK1 can transform both Rat1 fibroblasts and mouse NIH3T3 cells in vitro; Aurora2-transformed NIH3T3 cells grew as tumors in nude mice [2]. We recently showed by immunohistochemistry that AIRK1 is overexpressed in human invasive breast cancer [13]. Katayama et al. [6] showed levels of AIM-1/AIRK2 mRNA to be elevated in high-grade colorectal cancers. In the present study, we investigated expression patterns of AIRK1, AIRK2 and AIRK3 proteins in renal cell carcinoma (RCC) for

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the purpose of identifying the possible role of AIRKs in the pathogenesis of RCC.

Materials and methods

Tissue samples

The study cohort consisted of 64 Japanese patients who underwent radical nephrectomy for renal tumor. Archival tissue was obtained from radical nephrectomy specimens. Histopathologically, these renal tumors were all RCCs and comprised 59 clear cell renal cell carcinomas, 4 papillary renal cell carcinomas and 1 chromophobe renal cell carcinoma. Tumor grade was 1 to 3 in 19, 41 and 4 patients, while stage was T1 to T4 in 9, 40, 15 and 0, respectively. At operation, 10 patients had distant metastases. Cancer specific survival rate in the patients overall at 10 years of follow-up was 63.7%. All tumor tissues were evaluated for pathological stage and histological grade according to the TNM and WHO classifications, respectively [12].

Tissues were fixed in 10% neutral-buffered formalin and embedded in paraffin. Ten serial 3-μm sections were prepared from specimens from each patient. One section was stained with hematoxylin and eosin for histological examination, and the remaining sections were stained immunohistochemically with antibodies reactive with AIRK1, AIRK2, AIRK3 and proliferating cell nuclear antigen (PCNA).

Antibodies

Polyclonal antibodies reactive with human AIRK1, AIRK2 and AIRK3 proteins were raised and affinity-purified as described previously [7, 8, 9]. The same procedure was used with AIRK2 and AIRK3 proteins [8, 9]. Anti-PCNA monoclonal antibody PC10 was purchased from DAKO A/S (Glostrup, Denmark).

Immunohistochemistry

Immunohistochemical staining for AIRK1, AIRK2 and AIRK3 proteins was carried out according to the modified avidin-biotinylated peroxidase complex (SABC) method. Paraffin-embedded sections were dewaxed in toluene and rehydrated in a graded ethanol series. Prior to staining, all specimens were treated with 3% hydrogen peroxide to block endogenous peroxidase activity. Specimens stained with anti-AIRK1 and anti-AIRK2 antibodies were subjected to antigen retrieval treatment by autoclave prior to the endogenous peroxidase blocking step. After sections were rinsed with 0.01 M phosphate-buffered saline (PBS, pH 7.2), they were blocked with 10% normal goat serum in PBS for 15 min. The sections were incubated with primary antibody diluted 100-fold in PBS with 1% bovine serum albumin at 4°C for 12 h in a moist chamber. After being washed with 0.075% Brij 35 (Sigma Chemical Co., St. Louis, MO, USA) in PBS three times, the sections were reacted with the appropriate prediluted, biotinylated goat anti-rabbit IgG + IgA + IgM for 10 min in a moist chamber. Sections were subsequently washed and reacted with prediluted, horse radish peroxidase-labeled streptavidin for 5 min. After another washing, the sections were incubated with DAB, H_2O_2, Co^{2+} and Ni^{2+} without counterstaining. Two controls were performed for each specimen. One section from each specimen was subjected to the staining protocol, but primary antibody was replaced with PBS. For the other control, nonimmune rabbit serum replaced the primary antibody. Immunohistochemical staining of AIRK subfamilies was assessed by two independent observers to determine the level of expression in each specimen. AIRK expression was semi-quantified with the use of a visual grading system based on the intensity of immunoreactivity as follows: grade 0, no immunoreactivity; grade 1, weak immunoreactivity slightly above background staining level; grade 2, clear immunoreactivity in at least 50% of cancer cells; grade 3, strong immunoreactivity in the majority of cancer cells.

The proliferative activity of RCC was determined by quantification of PCNA-labeled nuclei. For determination of the number of PCNA-labeled nuclei, deparaffinized sections (3 μm thick) were immunostained with PC10 by the SABC method, and color photographs (200×) were taken from histologically representative areas (three fields per tumor, depending on cellularity) of each carcinoma. Cells displaying strong nuclear PCNA immunoreactivity were considered PCNA-positive. The number of PCNA-positive cells among a minimum of 100 cells per specimen were counted by two independent observers and averaged to determine the mean PCNA-labeling index.

Results are shown as mean ± SD. Where appropriate, significant differences between staining intensities were determined by Student's *t* test. Kaplan-Meier survival curves for cancer specific death were estimated. The Cox proportional hazards model was used for univariate survival comparison. Statistical calculations and tests were carried out with StatView ver. 5 software (Abacus Concepts, Inc., Berkeley, CA, USA) on a Macintosh computer.

Results

AIRK Immunohistochemistry

In 61 of the 64 (95%) RCCs, AIRK1 staining was detected throughout the cytoplasm (Fig. 1A). Moreover, 30 of 64 (47%) RCCs displayed prominent nuclear staining for AIRK2 (Fig. 1B). In 63 of the 64 (98%) RCCs, cancer cells had predominant cytoplasmic staining for AIRK3, but some nuclear staining was also detected (Fig. 1C). Among the three types of RCCs examined, no preferential immunostaining of any AIRK subfamily was detected. In addition, the staining patterns of all three AIRK proteins, especially AIRK2, showed both intra- and intertumor variation.

In adjacent nonneoplastic kidney tissue, weak AIRK1 immunostaining was detected in specific proximal tubule cells of a few specimens. Other cellular components of nephrons, including glomeruli, distal tubules and collecting ducts and the stroma, had no detectable AIRK1 expression. The AIRK3 expression pattern was similar to that of AIRK1 expression in normal kidney tissue. In the normal tissue of some specimens, low-level AIRK2 staining was observed in scattered inflammatory cells and mesangial cells. Negative control specimens incubated with normal rabbit serum contained low levels of nonspecific nuclear and cytoplasmic staining.

Expression of one or more AIRK subfamilies was detected in all specimens. Expression of only one AIRK subfamily was detected in three (5%) specimens, expression of two AIRK subfamilies was detected in 33 (51%) specimens and expression of all three AIRK subfamilies was detected in 28 (44%) specimens.

PCNA immunohistochemistry

All of the examined cancer specimens displayed definitive, positive nuclear staining for PCNA. Positive

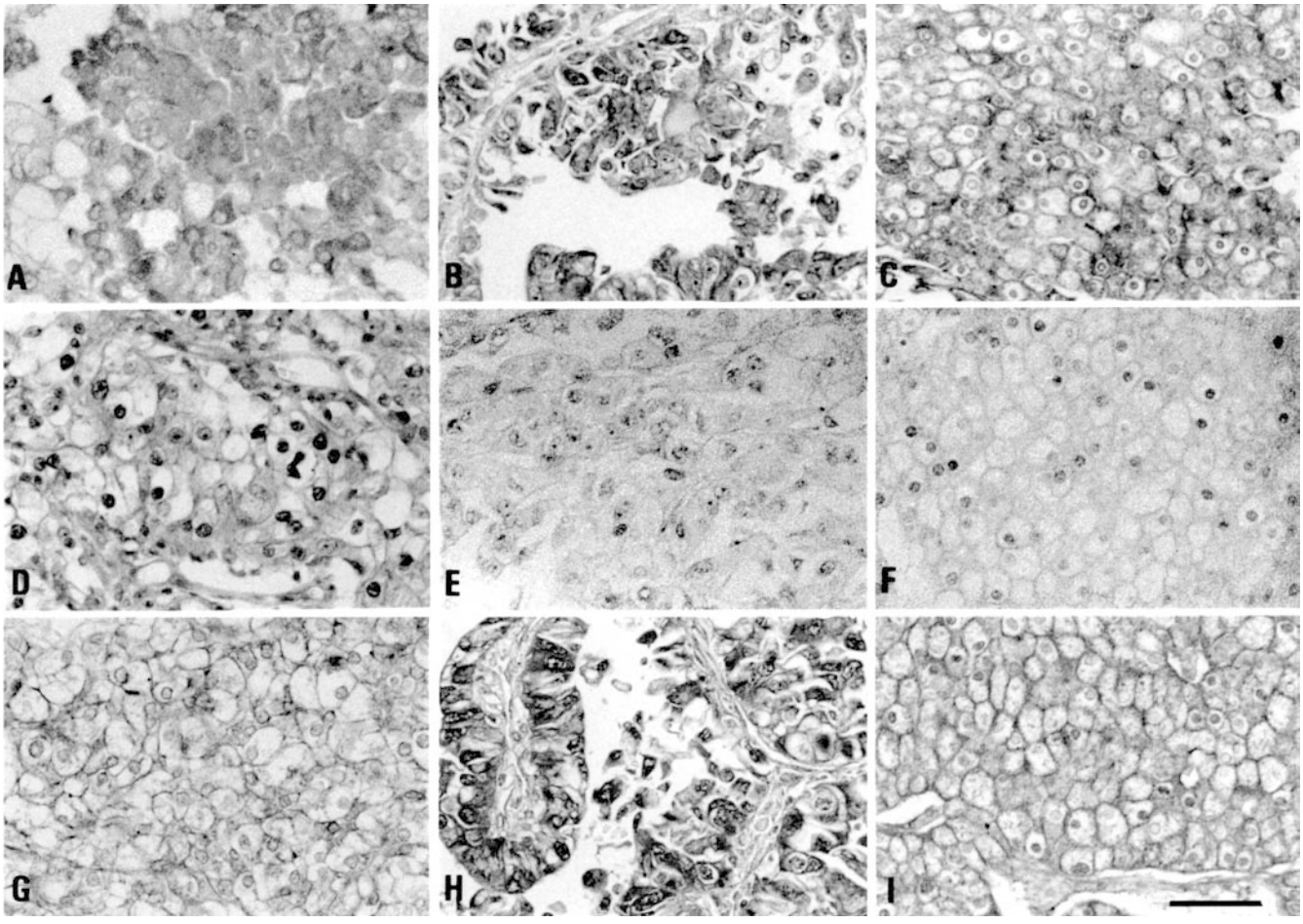

Fig. 1A–I AIRK immunostaining of RCCs. AIRK1 staining (**A–C**), AIRK2 staining (**D–F**) and AIRK3 staining (**G–I**) of clear cell renal cell carcinoma (**A, D, G**), papillary renal cell carcinoma (**B, E, H**) and chromophobe renal cell carcinoma (**C, F, I**) are shown. AIRK1 staining and AIRK3 staining were predominantly cytoplasmic, whereas AIRK2 staining was almost entirely nuclear. This figure shows weak AIRK2 staining in papillary and chromophobe carcinomas. Bar = 50 μm

staining was not present in the cytoplasm of carcinoma cells or in negative control specimens. The PCNA-labeling index was 28.0 ± 20.3% ($n = 59$) in clear cell RCCs, 32.0 ± 36.5% ($n = 4$) in papillary RCCs and 26.8% ($n = 1$) in chromophobe RCCs. No statistical differences in PCNA-labeling indices were detected between histological types.

The PCNA-labeling index of AIRK2 grades 0–3 was 19.8 ± 13.1% ($n = 34$), 36.2 ± 25.6 ($n = 24$), 44.2 ± 20.4 ($n = 5$) and 46.6 ($n = 1$). The PCNA-labeling index of AIRK2-positive specimens (grades 1–3) was significantly higher than that of AIRK2-negative specimens (37.8 ± 24.2% vs 19.8 ± 13.1%, $p = 0.0004$). In evaluating AIRK1 staining, the PCNA-labeling index of grade 3 ($n = 5$) was significantly higher than that of grade 0 ($n = 3$) (37.6 ± 13.5% vs 7.8 ± 7.2%, $p = 0.0134$). However, no significant differences in the PCNA-labeling index were detected between AIRK3 grade 2 and grade 3 and AIRK3 grade 0 and grade 1 specimens (Fig. 2).

Association of AIRK with clinicopathological features

No correlation was detected between expression levels of any of the three AIRK subfamilies regardless of clinical parameters: tumor grade, tumor stage and metastatic disease. Although univariate analysis demonstrated that significant factors associated with shortened cancer specific survival included tumor stage ($p = 0.0004$), distant metastasis ($p = 0.0005$), tumor grade ($p = 0.0013$) and high PCNA-labeling index ($p = 0.0019$), none of the three AIRK subfamilies was a significant prognostic factor in patients with RCC.

Discussion

Recent studies revealed the Aurora/Ipl1p-related serine/threonine kinase family as a fourth group of mitotic kinases [1]. These serine/threonine kinases are localized to the centrosome or midbody throughout mitosis [7, 8, 9, 15]. Overexpression of wild-type human AIRK1 transformed rat fibroblast, indicating that AIRK1 is oncogenic [2]. In addition, overexpression of AIRK1 and AIRK2 proteins induced centrosome amplification and aneuploidy [14, 16]. Loss of chromosome integrity and genomic stability act as a driving force during the processes of tumorigenesis and tumor progression [3, 10].

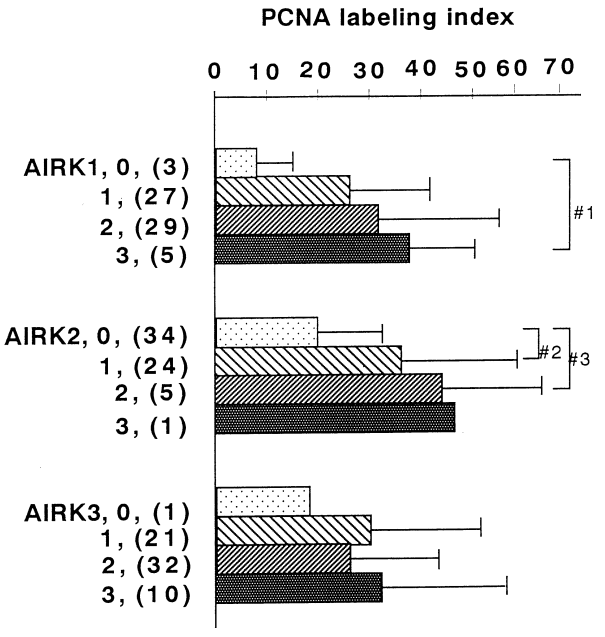

Fig. 2 Relation between PCNA-labeling index and AIRK expression in RCC specimens. At least 100 nuclei were examined for PCNA immunoreactivity in specimens displaying negative (*0*), weak (*1*), moderate (*2*) or strong (*3*) AIRK expression as defined in the Methods. PCNA-labeling index is presented as the mean ± SEM of the number of specimens examined, which is indicated in parentheses below each bar. Statistical differences are: #1 ($p = 0.0134$), #2 ($p = 0.0023$) and #3 ($p = 0.0009$)

Here, we report that malignant primary epithelial cells overexpress mitotic kinases of the AIRK subfamilies. We found that 95% of RCCs of different histological types expressed AIRK1 protein. Moreover, we show that the positive staining rate of AIRK3 is equal to that of AIRK1. AIRK1 and AIRK3 immunoreactivities were detected in the cytoplasm of cancerous cells, whereas AIRK2 protein was predominantly nuclear and detected in only 47% of RCCs. Previous immunofluorescence studies showed that AIRK1 and AIRK3 are concentrated at or near centrosomes during mitosis [7, 9]; however, our immunohistochemical analysis revealed distinct expression of these two kinases in the cytoplasm of RCC cells. Consistent with this observation, overexpression of AIRK1 was observed in the cytoplasm of breast cancer cells [13]. Terada et al. [15] reported that AIM-1/AIRK2 is expressed along the equator of central spindles during late anaphase and at the midbody during telophase and cytokinesis. Thus, it is likely that overexpression of both AIRK1 and AIRK3 is indicative of the pathological state of cancer cells. Under normal conditions, the Aurora2/AIRK1 protein is turned over rapidly with a half-life of approximately 2 h [5], whereas the half-life of AIRK1 in cancer cells is not known. Amplification of the AIRK gene or abnormal turnover of AIRK mRNAs or proteins in cancer cells may influence levels of and subcellular localization of proteins.

To examine how AIRK expression relates to cell proliferation, we stained renal cancer specimens with PCNA. The mean PCNA-labeling index was 28.3%. Less than one-third of the cancer cells were PCNA-positive, whereas nearly all of the cancer cells in more than half of the specimens were AIRK1-positive or AIRK3-positive. In contrast to expression of AIRK1 and AIRK3, AIRK2 expression correlated significantly with PCNA-labeling index. Therefore, AIRK2, but not AIRK1 and AIRK3, may be markers for cell proliferation.

In the present study, expression levels of the AIRK subfamilies were similar regardless of histopathology of RCC; however, we observed no correlations between AIRK immunostaining levels and clinicopathological characteristics of specimens. Moreover, no significant association was observed for AIRK immunostaining with cancer specific death. These findings suggest that abnormalities in AIRK proteins may be unrelated to progression of RCC.

In summary, we showed that AIRK proteins are expressed at high levels in RCC. Overexpression of AIRK2 correlates with PCNA-labeling index. Perturbations in expression of proteins that comprise the mitotic apparatus-associated kinase cascades may lead to genomic instability and defects in chromosomal segregation, which may ultimately lead to cancer. Further investigation is required to assess the possible role of AIRKs in the pathogenesis of RCC.

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