Up-regulation of Na$^+$,K$^+$-ATPase α3-isoform and down-regulation of the α1-isoform in human colorectal cancer

Hideki Sakai[a,1,*], Tomoyuki Suzuki[a,1], Mizuki Maeda[a], Yuji Takahashi[a], Naoki Horikawa[b], Tetsuji Minamimura[b], Kazuhiro Tsukada[b], Noriaki Takeguchi[a]

[a]*Department of Pharmaceutical Physiology, Faculty of Pharmaceutical Sciences, Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama 930-0194, Japan*
[b]*Department of Surgery II, Faculty of Medicine, Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama 930-0194, Japan*

Abstract We investigated expression levels of Na$^+$,K$^+$-ATPase α-isoforms and their ATPase activities in human colorectal cancer tissue and the accompanying normal mucosa. A decrease in expression of the α1-isoform protein was observed in all sampled cancer tissues compared with the normal mucosae. The level of ouabain (5 μM)-sensitive Na$^+$,K$^+$-ATPase activity in carcinomas was $81 \pm 5\%$ that of in the normal mucosae. The mRNA expression of α2- and α4-isoforms was decreased in almost all the carcinoma samples. Interestingly, the expression level of the α3-isoform protein in the cancer tissue was higher than that of the normal mucosa. These results indicate that a decrease in the α1-isoform expression and an increase in the α3-isoform expression may be associated with colorectal cancer.
© 2004 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

Key words: Na$^+$,K$^+$-ATPase; α-Isoform; Colon; Cancer

1. Introduction

The Na$^+$,K$^+$-ATPase is composed of two subunits, the catalytic α-subunit and the glycosylated β-subunit. So far, four different α-isoforms (α1, α2, α3 and α4) and three different β-isoforms (β1, β2 and β3) have been identified in mammalian cells [1,2].

It has been reported that expression of mRNAs of β-isoforms (β1 and/or β2) was decreased in human cancers such as renal, lung and hepatocellular cancers [3], and that expression of proteins of the β-isoforms was decreased in human clear-cell renal cell [4], gastric [5] and bladder [6] cancers. This down-regulation of β-subunits has been suggested to be associated with the loss of tight junctions and epithelial polarity in the cancer cells [7]. But an exceptional case was reported in human colon cancer: the expression level of the β1-isoform protein was increased compared with normal colonic tissue [5].

So far reported, changes in the expression of the α-subunit seem to depend on organs or tissues. No significant change in the expression of α1-isoform mRNA was observed in human renal and lung cancers [3], and no change in the expression of the protein was observed in human clear-cell renal cell cancer [4]. Decreased expression of α-subunit mRNA [8] and protein [6] was observed in human gastric and bladder cancers, respectively. To our knowledge, there is no report showing a change in the α-subunit expression in colon cancer.

Colorectal cancer is one of the major causes of cancer deaths in the Western world [9,10]. About 80% of colorectal carcinomas are histologically characterized by good gland formation (epithelial polarity) and varying degrees of differentiation, from well to moderately differentiated [11]. In the preset study, we investigated whether expression of Na$^+$,K$^+$-ATPase α-isoforms were altered in human colorectal cancers (well or moderately differentiated adenocarcinomas), and found that levels of protein expression of the α1- and α3-isoforms in the carcinoma were significantly different from those of the accompanying normal mucosa.

2. Materials and methods

2.1. Tissue procurement

Human colorectal specimens of well or moderately differentiated adenocarcinomas were obtained from surgical resection of 17 Japanese patients (11 males and six females; 66 ± 3 years) in accordance with the recommendations of the Declaration of Helsinki and with ethics committee approval. Informed consent was obtained from all patients at Toyama Medical and Pharmaceutical University Hospital. In all cases, control specimens were collected from the accompanying normal mucosae, which were 5–10 cm distant from the carcinoma. The cancer tissue and the normal epithelial layer were carefully isolated from the resected colon with scissors and forceps. These samples were free from serosa and muscularis propria. Blood vessels around the tissues were carefully removed. Clinical and histological classifications according to the general rules edited by the Japanese Research Society for Cancer of the Colon and Rectum were carried out independently by three expert pathologists.

2.2. Sodium dodecylsulfate (SDS)–polyacrylamide gel electrophoresis and Western blotting

Membrane fractions of the human colorectal tissues, human cancer cell lines and rat whole brain were prepared as described previously [12]. Membrane preparations (30 μg of protein) were incubated in a sample buffer containing 2% SDS, 2% β-mercaptoethanol, 10% glycerol and 65 mM Tris–HCl (pH 6.8) at room temperature for 2 min and applied to the SDS–polyacrylamide gel. Western blotting was carried out as described previously [12]. A polyclonal anti-Na$^+$,K$^+$-ATPase α1 antibody (N-15) and a polyclonal anti-Na$^+$,K$^+$-ATPase α2 antibody (C-16) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and were used at 1:100 dilution. For negative control, 1 μg of the anti-α1 or anti-α2 antibody was pre-incubated with 2 μg of the corresponding blocking peptide (Santa Cruz Biotechnology). A monoclonal anti-Na$^+$,K$^+$-ATPase α3 antibody (XVIF9-G10) was obtained from Affinity BioReagents (Golden, CO, USA), and was used at 1:2000 dilution. For negative control, 1 μg of
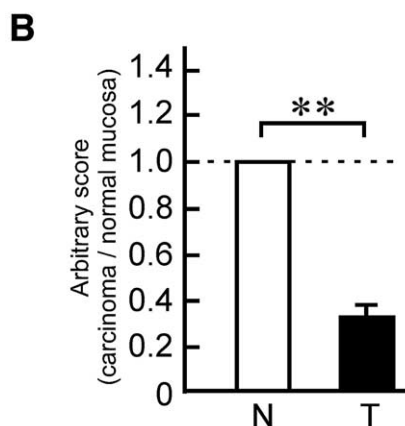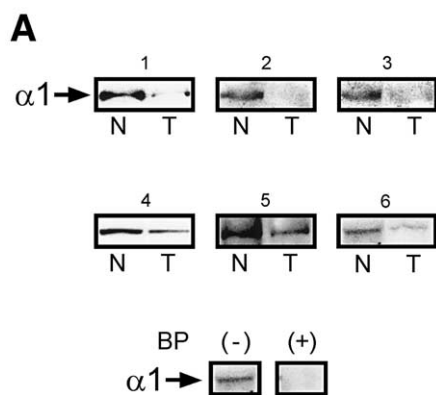

Fig. 1. Decreased expression of protein of the Na$^+$,K$^+$-ATPase α1-isoform in human colorectal carcinomas. A: Western blotting for detecting the α1-isoform protein in paired normal mucosa (N) and adenocarcinoma (T). Typical blots from six patients are shown. Age (years) and sex (M or F) of the patient, location of each carcinoma (A, ascending colon; T, transverse colon; S, sigmoid colon; R, rectum), and stage of the carcinoma according to TNM clinical classification (I, II, III or IV) are: No. 1 (43, M, A, I), No. 2 (74, M, A, III), No. 3 (70, F, T, II), No. 4 (50, F, S, III), No. 5 (73, M, R, II) and No. 6 (59, F, R, II). The specific band for the α1-isoform (100 kDa) disappeared in the presence of the blocking peptide (BP). B: Levels of protein expression of the α1-isoform in the carcinoma (T) are compared with those of the normal mucosa (N). In each case, the score was calculated using the following equation: Arbitrary score = (amount of the α1-isoform protein in the carcinoma)/ (amount of the α1-isoform protein in the normal mucosa). The score for normal mucosa is normalized as 1 in each case. Averaged scores ± S.E.M. of carcinomas ($n = 17$) are shown. **Significantly different ($P < 0.01$).

the anti-α3 antibody was pre-incubated with 3 μg of the blocking peptide that corresponds to amino acid residues 1–22 of human Na$^+$,K$^+$-ATPase α3-isoform [13]. Horseradish peroxidase-conjugated anti-goat immunoglobulin G (1:2000 dilution) or anti-mouse immunoglobulin G1 (1:5000 dilution) was used as a secondary antibody.

2.3. Measurement of Na$^+$,K$^+$-ATPase activity

ATPase activity was assayed in 1 ml of a solution containing 30 μg of membrane protein, 120 mM NaCl, 15 mM KCl, 3 mM MgSO$_4$, 3 mM ATP, 5 mM NaN$_3$, and 40 mM Tris–HCl, pH 7.4, in the presence or absence of 5 μM ouabain. After incubation for 10 min at 37°C, the reaction was terminated by the addition of ice-cold stop solution containing 12% perchloric acid and 3.6% ammonium molybdate. Inorganic phosphate released was measured from the absorbance at 320 nm as described elsewhere [14]. The Na$^+$,K$^+$-ATPase activity was calculated as the difference between activities in the presence and absence of ouabain.

2.4. RNA isolation and quantification of mRNAs

Total RNAs from the human colorectal tissues were prepared by using the RNAgents Total RNA Isolation System (Promega, Madison, WI, USA). Amounts of mRNAs of the Na$^+$,K$^+$-ATPase α2- and α4-isoforms were quantified by real-time polymerase chain reaction (PCR) (TaqMan assay) using an ABI Prism 7700 sequence detector. As a control, the amount of β-actin was measured. The specific primers and the Taqman fluorescent probes (Assays-on-Demand Gene Expression Products) were obtained from Applied Biosystems (Foster City, CA, USA).

2.5. Statistics

Results are presented as the means ± S.E.M. Differences between groups were analyzed by one-way analysis of variance, and correction for multiple comparisons was done using Dunnett's multiple comparison test. Comparison between the two groups was done using Student's t-test. Statistically significant differences were assumed at $P < 0.05$.

3. Results and discussion

The catalytic subunit of Na$^+$,K$^+$-ATPase has at least four isoforms, α1, α2, α3 and α4, each derived from a different gene. The α-isoforms exhibit a tissue-specific pattern of expression (for review, see [1]). The α1-isoform is found in nearly every tissue and is involved in a housekeeping function in all cells. The α2-isoform is predominantly expressed in adipocytes, skeletal muscle, heart and brain. The α3-isoform is abundant in nervous tissues. The α4-isoform is expressed exclusively in testis.

First, we examined the protein expression level of Na$^+$,K$^+$-ATPase α1-isoform. Interestingly, a decrease in the expression of the α1-isoform protein was observed in 17 of 17 carcinomas (100%) compared with the normal mucosae, and the average score of its expression in the cancer tissues was 0.32-fold that of the normal tissues ($n = 17$; Fig. 1).

It could be deduced that down-regulation of the α1-isoform results in a decrease of the Na$^+$,K$^+$-ATPase activity in the carcinoma. In fact, Fig. 2 shows that the ouabain (5 μM)-sensitive ATPase activities in the carcinomas were significantly lower than those of the accompanying normal mucosae. But the average activity in the carcinomas was still 81 ± 5% of that in the normal mucosae. It is noted that this value of 81% is surprisingly high when compared with the decreased α1-protein level of 32% (Fig. 1B). The possibility may be excluded that the remaining Na$^+$,K$^+$-ATPase α1-subunit in the carcinoma (Fig. 1) enhanced its ATP hydrolysis activity per mg of
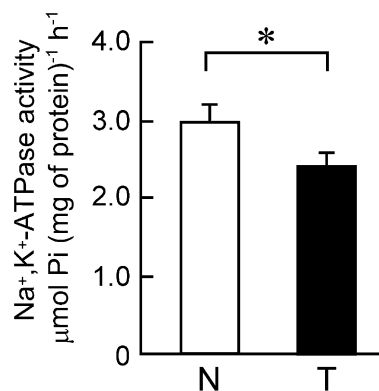

Fig. 2. Na$^+$,K$^+$-ATPase activity in the colorectal adenocarcinomas (T) and the accompanying normal mucosae (N). Averaged scores ± S.E.M. ($n = 17$) are shown. *Significantly different ($P < 0.05$).

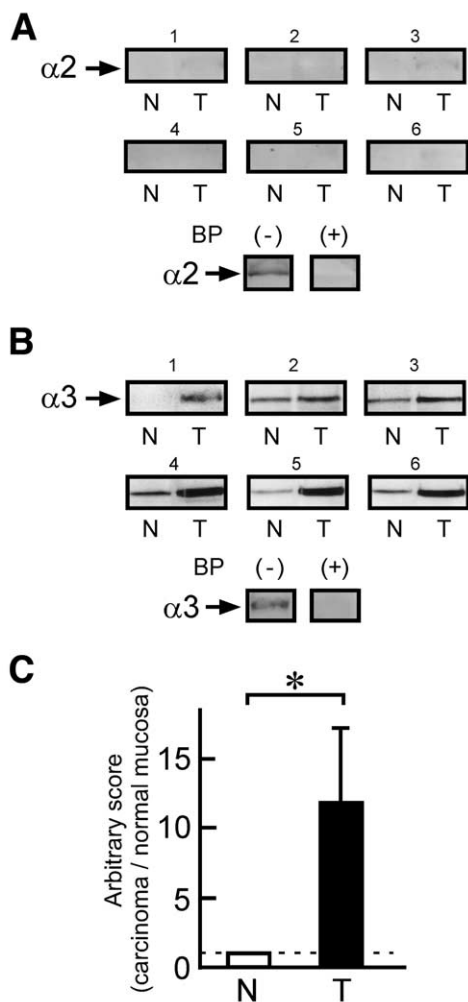Fig. 3. A: No significant expression of protein of the Na$^+$,K$^+$-ATP-ase α2-isoform in human colorectal tissues. Western blotting was performed in paired normal mucosa (N) and adenocarcinoma (T). Typical blots from six patients are shown. The number written above each panel corresponds to the number in Fig. 1A. The specific band for the α2-isoform of rat brain (100 kDa) disappeared in the presence of the blocking peptide (BP). B: Increased expression of protein of the Na$^+$,K$^+$-ATPase α3-isoform in human colorectal carcinomas. Western blotting was performed in paired normal mucosa (N) and adenocarcinoma (T). Typical blots from six patients are shown. The number written above each panel corresponds to the number in Figs. 1A and 3A. The specific band for the α3-isoform (110 kDa) disappeared in the presence of the blocking peptide (BP). C: Levels of protein expression of the α3-isoform in the carcinoma (T) are compared with those of the normal mucosa (N). In each case, the score was calculated using the following equation: Arbitrary score = (amount of the α3-isoform protein in the carcinoma)/(amount of the α3-isoform protein in the normal mucosa). The score for normal mucosa is normalized as 1 in each case. Averaged scores ± S.E.M. of carcinomas (n = 17) are shown. *Significantly different (P < 0.05).

protein, because the Na$^+$,K$^+$-ATPase molecule is known to be transformed to a regime of decreased efficiency in the presence of low ATP concentrations which is typical for tumor cells [15]. In the distal colon of mice treated with the carcinogen 1,2-dimethylhydrazine, the maximum pump rate of Na$^+$,K$^+$-ATPase was depressed [16]. This depression could result from changes such as altered molecular structure of the Na$^+$,K$^+$-ATPase, decreased membrane fluidity or decreased energy

supply or utilization by the pump [16]. Therefore, we assumed the possibility that the expression of other α-isoform(s) may be up-regulated in human colorectal carcinomas.

No significant expression of α2-isoform protein was observed in any of the colorectal carcinomas and accompanying normal mucosae (Fig. 3A). Real-time PCR (TaqMan assay) showed that expression of α2-isoform mRNA in the carcinomas decreased in 17 out of 17 carcinomas (100%) compared with the normal mucosae, and the average score of its expression in the cancer tissues was 0.19 ± 0.05-fold that of the normal tissues (P < 0.01; n = 17). Real-time PCR also showed that expression of α4-isoform mRNA decreased in 14 out of 17 carcinomas (82%) compared with the normal mucosae, and the average score of its expression in the cancer tissues was 0.56 ± 0.11-fold that of the normal tissues (P < 0.01; n = 17). Expression of the α4-isoform was not investigated at the protein level for lack of available antibody.

On the other hand, we found that expression of α3-isoform protein increased in 13 of 17 carcinomas (76%) compared with the accompanying normal mucosae. The average score of its expression in the cancer tissues was 11.8-fold higher than that of the normal tissues (n = 17; Fig. 3B,C). These results suggest that the α3-isoform but not the α2- or α4-isoform is up-regulated in colorectal carcinoma. In addition, a significant level of α3-isoform protein was consistently detected in human colonic adenocarcinoma cell lines such as KM12-L4, T-84, HT-29 and WiDr (Fig. 4). No significant expression of the α3-isoform protein was observed in the tissues of human normal kidney and renal carcinoma (not shown). Concerning the expression of α3-isoform in epithelial cells, human lens epithelial cells showed a significant expression of the protein [17].

Our present results suggest that a decrease in the α1-isoform expression and an increase in the α3-isoform expression may be associated with human colorectal cancer. It is likely that Na$^+$,K$^+$-ATPase α3-isoform is up-regulated to make up for the decreased expression of the α1-isoform in colorectal cancer cells. Crambert et al. [18] have reported that the catalytic α3-isoform can be assembled with the β1-isoform which is up-regulated in human colon carcinoma [5], and that the α3–β1 complex expressed in Xenopus oocytes is functionally active. Intracellular Na$^+$ homeostasis plays a crucial role in generation of the polarized phenotype of epithelial cells [19]. Taken together, this switching mechanism (α1 to α3) may be

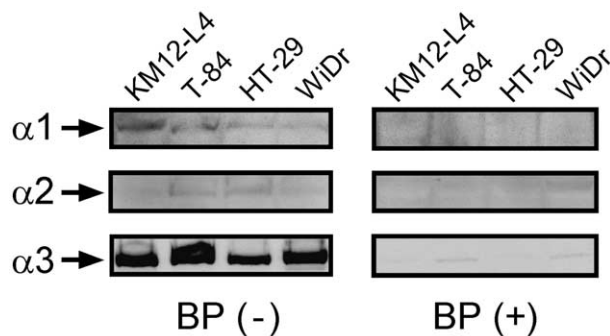Fig. 4. Expression of proteins of the α1-isoform (upper panel), α2-isoform (middle panel) and α3-isoform (bottom panel) in human colonic adenocarcinoma cell lines (KM12-L4, T-84, HT-29 and WiDr). In left panels, specific bands for the α1-isoform (100 kDa), α2-isoform (100 kDa) and α3-isoform (110 kDa) are observed. These bands disappeared in the presence of the corresponding blocking peptide (BP) (right panels).

important to maintain the gland structure (epithelial polarity) of well or moderately differentiated adenocarcinomas in the human colorectum. At present, the molecular mechanism for the switching is unknown and is an interesting subject to be clarified in a future study.

References

[1] Blanco, G. and Mercer, R.W. (1998) Am. J. Physiol. 275, F633–F650.

[2] Woo, A.L., James, P.F. and Lingrel, J.B. (1999) J. Membr. Biol. 169, 39–44.

[3] Akopyanz, N.S., Broude, N.E., Bekman, E.P., Marzen, E.O. and Sverdlov, E.D. (1991) FEBS Lett. 289, 8–10.

[4] Rajasekaran, S.A., Ball Jr., W.J., Bander, N.H., Liu, H., Pardee, J.D. and Rajasekaran, A.K. (1999) J. Urol. 162, 574–580.

[5] Avila, J., Lecuona, E., Morales, M., Soriano, A., Alonso, T. and Martín-Vasallo, P. (1997) Ann. NY Acad. Sci. 834, 633–635.

[6] Espineda, C., Seligson, D.B., Ball Jr., W.J., Rao, J.-Y., Palotie, A., Horvath, S., Huang, Y., Shi, T. and Rajasekaran, A.K. (2003) Cancer 97, 1859–1868.

[7] Rajasekaran, A.K. and Rajasekaran, S.A. (2003) Am. J. Physiol. Renal Physiol. 285, F388–F396.

[8] Jung, M.H., Kim, S.C., Jeon, G.A., Kim, S.H., Kim, Y., Choi, K.S., Park, S.I., Joe, M.K. and Kimm, K. (2000) Genomics 69, 281–286.

[9] Peto, J. (2001) Nature 411, 390–395.

[10] Grothey, A. and Schmoll, H.-J. (2001) Curr. Opin. Oncol. 13, 275–286.

[11] Riddell, R.H., Petras, R.E., Williams, G.T. and Sobin, L.H. (2003) in: Epithelial Neoplasia of the Intestines. Tumors of the Intestines, pp. 133–189, Armed Forces Institute of Pathology, Washington, DC.

[12] Asano, S., Tega, Y., Konishi, K., Fujioka, M. and Takeguchi, N. (1996) J. Biol. Chem. 271, 2740–2745.

[13] Arystarkhova, E. and Sweadner, K.J. (1996) J. Biol. Chem. 271, 23407–23417.

[14] Yoda, A. and Hokin, L.E. (1970) Biochem. Biophys. Res. Commun. 40, 880–886.

[15] Boldyrev, A.A. (1993) Mol. Chem. Neuropathol. 19, 83–93.

[16] Davies, R.J., Sandle, G.I. and Thompson, S.M. (1991) Cancer Biochem. Biophys. 12, 81–94.

[17] Cui, G., Dean, W.L. and Delamere, N.A. (2002) Invest. Ophthalmol. Vis. Sci. 43, 2714–2720.

[18] Crambert, G., Hasler, U., Beggah, A.T., Yu, C., Modyanov, N.N., Horisberger, J.-D., Lelièvre, L. and Geering, K. (2000) J. Biol. Chem. 275, 1976–1986.

[19] Rajasekaran, S.A., Palmer, L.G., Moon, S.Y., Soler, A.P., Apodaca, G.L., Harper, J.F., Zheng, Y. and Rajasekaran, A.K. (2001) Mol. Biol. Cell 12, 3717–3732.