Abstract. QR-32 is a regressive murine fibrosarcoma cell clone which cannot grow when they are transplanted in mice; QRsP-11 is a progressive malignant tumor cell clone derived from QR-32 which shows strong tumorigenicity. A recent study showed there to be differentially expressed up-regulated and down-regulated proteins in these cells, which were identified by proteomic differential display analyses by using two-dimensional gel electrophoresis and mass spectrometry. Cofilins are small proteins of less than 20 kDa. Their function is the regulation of actin assembly. Cofilin-1 is a small ubiquitous protein, and regulates actin dynamics by means of binding to actin filaments. Cofilin-1 plays roles in cell migration, proliferation and phagocytosis. Cofilin-2 is also a small protein, but it is mainly expressed in skeletal and cardiac muscles. There are many reports showing the positive correlation between the level of cofilin-1 and cancer progression. We have also reported an increased expression of cofilin-1 in pancreatic cancer tissues compared to adjacent paired normal tissues. On the other hand, cofilin-2 was significantly less expressed in pancreatic cancer tissues. Therefore, the present study investigated the comparison of the levels of cofilin-1 and cofilin-2 in regressive QR-32 and progressive QRsP-11 cells by western blotting. Cofilin-2 was significantly up-regulated in QRsP-11 compared to QR-32 (p<0.001). On the other hand, the difference of the intensities of the bands of cofilin-1 (18 kDa) in QR-32 and QRsP-11 was not significant. However, bands of 27 kDa showed a quite different intensity between QR-32 and QRsP-11, with much higher intensities in QRsP-11 compared to QR-32 (p<0.001). These results suggested that the 27-kDa protein recognized by the antibody against cofilin-1 is a possible biomarker for progressive tumor cells.

Although medicine has progressed and revolutionized treatment and diagnosis, malignancies are still the major cause of death in many countries. Tumor development and progression are the most crucial features of malignant tumors. The characteristics of progressive tumor cells are rapid growth, invasiveness and metastatic capacity compared to regressive benign tumor cells. Okada et al. established regressive and progressive tumor models of murine fibrosarcoma cells (QR-32 clone and QRsP-11 clone, respectively) (1). QR-32 exhibits weak tumorigenic ability and is non-metastatic. QRsP-11 was derived from QR-32, and is a progressive malignant tumor cell clone.

We have been analyzing QR-32 and QRsP-11 by proteomics combining two-dimensional gel electrophoresis and mass spectrometry. Eight nuclear proteins, including zing finger protein ZXDC, eleven cytoplasmic proteins, including heat-shock protein (HSP)-90, and 31 non-nuclear proteins, including hypoxia upregulated protein 1, were identified as differentially expressed between QR-32 and QRsP-11 cells. However, from these analyses it was difficult to elucidate the whole mechanism of tumor progression (2-4).

Cofilins, also known as actin depolymerization factors, are essential and conserved modulators of actin dynamics; they are small proteins whose molecular weights are less than 20 kDa. Their function is the regulation of actin assembly by means of depolymerization and severing F-actin (5, 6). There are three members of the cofilin family: cofilin-1, cofilin-2 and destrin. Cofilin-1 is expressed ubiquitously and regulates actin dynamics by means of binding to F-actin. Cofilin-1 plays roles in cell migration, proliferation, phagocytosis, chemotactic movement and macrophagocytosis (7, 8). Depleting cofilin-1 leads to an accumulation of F-actin, and...
defects in cell motility (9). Cofilin-2 is mainly expressed in skeletal and cardiac muscles. However, low levels of cofilin-2 are also expressed in several tissues (10). Cofilin-2 depolymerizes but does not bind to actin filaments, and was not expressed in vegetative cells (11). Cofilin-2 has a weaker actin filament depolymerization activity compared to cofilin-1 (12). Decreased levels of cofilin-2 have been associated with reduced depolymerization of actin filaments, causing their accumulation in nemaline rods (13). The role of cofilin-2 in muscle development and function is unclear, but may be involved in regulation of actin assembly during myofibrillogenesis and actin dynamics in the mature muscle. Agrawal et al. showed that cofilin-2-deficient mice died by day 8 (14). Although destrin and cofilin-1 have similar actin-dynamizing activities, destrin has more potent actin-depolymerizing ability than cofilin-1. Destrin, but not cofilin, is down-regulated with increasing actin monomer pool, and distributes with modulation by intracellular pH in the cells (5, 6).

Some reports have shown the up-regulation of cofilin-1 in tumor tissues. Cofilin-1 was up-regulated significantly in radioresistant astrocytomatas (15). Zhou et al. reported that cofilin-1 expression gradually increased in normal ovarian tissues, benign tumors, borderline tumors and carcinomas, respectively (16). We also reported that cofilin-1 was significantly up-regulated in pancreatic cancerous tissues compared to non-cancerous tissues (17). Some reports showed the relation of the cofilin-1 expression level and prognosis of tumors. The progression-free survival was longer in cofilin-1 low-expression cases than in high-expression cases in patients with epithelial ovarian cancer (18). Klamt et al. reported that patients with non-small cell lung cancer with high cofilin-1 immunoccontent had a lower overall survival rate (19, 20).

On the other hand, reports describing cofilin-2 in tumor cells and tissues, and its relation to prognosis of tumor patients are very few. Although we have reported that cofilin-2 was significantly less expressed in pancreatic cancer tissues, the regulation of cofilin-2 in tumor cells and tissues is still unclear.

In the present study, in order to compare the expression of cofilin-1 and cofilin-2 in progressive and regressive tumor cells, we performed western blot analysis of cofilin-1 and cofilin-2 in QR-32 and QRsP-11 cells.

Materials and Methods

Tumor cell lines and culture conditions. Murine fibrosarcoma cell lines, QR-32 and QRsP-11, were established at Hokkaido University. QR-32 cells cannot grow when they are transplanted in C57Bl/6 mice. However, when QR-32 cells are subcutaneously co-implanted with gelatin sponge, the cells can grow. QRsP-11 cells were obtained from the tumor tissue, and show strong tumorigenicity (1, 21). They were maintained in Eagle’s minimum essential medium (Nissui, Tokyo, Japan) supplemented with 10% fetal bovine serum, sodium pyruvate, non-essential amino acids and L-glutamine, at 37°C (22). Only cells which were passaged fewer than 15 times culture after the cells had been sent to our laboratory were used for this study.

Sample preparation. QR-32 and QRsP-11 cells were grown sub-confluently, homogenized in chilled lysis buffer [50 mM Tris-HCl, pH 7.5, 165 mM NaCl, 10 mM NaF, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1% NP40], and centrifuged at 15,000 x g for 30 min at 4°C. The supernatants were collected and used as samples (22). The samples from QR-32 and QRsP-11 cells were prepared seven times independently.

Western blotting. For western blot analysis, 20 μg of protein samples were used. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in precast gels (5-20% gradient; SuperSep™; Millipore, Bedford, MA, USA). After SDS-PAGE, the proteins were blotted to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA, USA). The blotted membranes were blocked with Tris-buffered saline (TBS) containing 5% skimmed milk for 1 h at room temperature, and then incubated with goat polyclonal antibody to cofilin-2 (1:200, #sc-32160; Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse monoclonal antibody to cofilin-1 (1:1,000, #WH0001072; Sigma-Aldrich, Saint Louis, MO, USA) or goat polyclonal antibody to actin (1:500, #sc-1616; Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. The membranes were reacted with horseradish peroxidase-conjugated secondary antibodies (dilution range 1:10,000; Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) for 1 h at room temperature after washing three times with TBS containing 0.05% Tween-20 and once with TBS. Bands of cofilin-2, cofilin-1 and actin were visualized by enhanced chemiluminescence system (ImmuNoStar Long Detection; Wako, Osaka, Japan), and recorded by using Image Reader LAS-1000 Pro (Fujifilm Corporation, Tokyo, Japan) (2), (23, 24). Progenesis PG240 software (Nonlinear Dynamics Ltd., Newcastle-upon-Tyne, UK) quantified the expression levels of the bands of cofilin-2 and cofilin-1.

Statistical analysis. Statistical significances of differences between QR-32 and QRsP-11 were calculated by Student’s t-test. p-Value of <0.05 was accepted as being significant.

Results

Western blot analysis of cofilin-2 and cofilin-1 in QR-32 and QRsP-11 cells. The intracellular proteins from QR-32 and QRsP-11 cells were analyzed by western blotting with primary antibody against cofilin-2, cofilin-1 and actin. The bands of cofilin-2 (19 kDa) showed strong intensities in QRsP-11 cells compared to QR-32 cells (Figure 1). The intensity of the bands of cofilin-2 was significantly greater by 26.6% in QRsP-11 cells compared to QR-32 cells (p=0.00002 by Student’s t-test) (Figure 2). On the other hand, the intensities of the bands of cofilin-1 (18 kDa) did not differ between QR-32 and QRsP-11 cells. However, there was a third band at 27 kDa which had quite different intensities in QR-32 and QRsP-11 cells (Figure 3), being...
significantly greater in QRsP-11 cells compared to QR-32 cells ($p=0.0000002$ by Student’s $t$-test) (Figure 4).

**Discussion**

Significant up-regulation of cofilin-2 in progressive murine fibrosarcoma cell clone QRsP-11 compared to regressive clone QR-32 was observed by western blot analysis with antibody specific to cofilin-2 ($p<0.001$ by Student’s $t$-test). On the other hand, cofilin-1 had almost the same expression in QR-32 and QRsP-11 by the western blot analysis with antibody specific to cofilin-1. However, we found an intense band in QRsP-11 cells compared to QR-32 cells ($p=0.0000002$ by Student’s $t$-test) at an unexpected position (27 kDa).

There are few reports showing the relation of cofilin-2 expression to the nature of tumor cells or distribution in tumor tissues. Although actin filament depolymerization by cofilin-2 is weaker than that by cofilin-1, cofilin-2 can act an actin-depolymerizing factor (11, 12). Albeit our recent study showed that the expression of cofilin-2 in pancreatic cancer tissues was lower than adjacent non-cancerous tissues, the present study showed overexpression of cofilin-2 in this progressive fibrosarcoma cell clone compared to regressive clone (17). How can we explain this discrepancy of the results? One possible reason is that expression is dependent on the tumor cell type. Fibrosarcoma cells are quite different from pancreatic cancer cells. Another possible reason depends on the purity of the samples. In our recent study, we used bulk human pancreatic cancer tissues, but in this study we used very pure tumor cell clones. Whether cofilin-2 is involved in tumor progression must be clarified by further study.

Cofilin-1 was not up-regulated in progressive clone cells compared to regressive clone cells, contrary to anticipation. Some research groups reported up-regulation of cofilin-1 in tumor tissues, including radioresistant astrocytoma tissues (15), ovarian cancer tissues (16) and pancreatic cancer tissues (17), and some groups also reported positive correlation of the up-regulation of cofilin-1 with poor
prognosis of tumor bearers (18-20). Although western blotting with specific mouse monoclonal antibody to cofilin-1 did not show 18 kDa bands with increased intensities, we did find a significantly intense band (27 kDa) in the progressive clone QRsP-11 alone. We analysed samples which were prepared seven times independently from QR-32 and QRsP-11 cells and this 27 kDa band was consistently obtained. From the literature, we found no information about cofilin-1 expression revealed at 27 kDa. Although we cannot completely rule out the possibility of post-translational modification, the difference in molecular weight is too great for post-translational modification. Since the antibody to cofilin-1 which we used for this study is monoclonal, the possibility of production of such a band from non-specific reaction is low. The identification of this 27 kDa protein is important because the overexpression of this protein is quite significant in the progressive clone cells. Further molecular characteristic analysis of this 27 kDa protein is required.

Figure 3. Western blot analysis of cofilin-1 in QR-32 and QRsP-11 cells. Twenty micrograms of protein from QR-32 and QRsP-11 cells were used. Bands of 18 kDa and 27 kDa are from antibody to cofilin-1, and 43 kDa bands are actin, as a loading control. The samples from QR-32 and QRsP-11 cells were prepared seven times independently.

Figure 4. Comparison of the intensities of bands for cofilin-1 between QR-32 and QRsP-11 cells. The intensity of cofilin-1 (18 kDa) was not different between QR-32 and QRsP-11 cells, but bands with 27 kDa was significantly more intense in QRsP-11 cells compared to QR-32 cells (p=0.0000002 by Student’s t-test) (n=7).
Acknowledgements

This work was supported in part by Grants-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan (no. 24501352 to Yasuhiro Kuramitsu).

References


