PO Box 2345, Beijing 100023, China
www.wjgnet.com
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RAPID COMMUNICATION

ECA39 is a novel distant metastasis-related biomarker in colorectal cancer

Reigetsu Yoshikawa, Hidenori Yanagi, Chun-Shen Shen, Yoshinori Fujiwara, Masafumi Noda, Toshihiko Yagyu, Makoto Gega, Tsutomu Oshima, Takehira Yamamura, Haruki Okamura, Yoshiro Nakano, Tomonori Morinaga, Tomoko Hashimoto-Tamaoki

Reigetsu Yoshikawa, Hidenori Yanagi, Yoshinori Fujiwara, Masafumi Noda, Toshihiko Yagyu, Makoto Gega, Tsutomu Oshima, Yoshinori Fujiwara, Takehira Yamamura, Second Department of Surgery, Hyogo College of Medicine, Nishinomiya, Hyogo 663-8501, Japan
Reigetsu Yoshikawa, Haruki Okamura, Tomoko Hashimoto-Tamaoki, Institute for Advanced Medical Sciences, Hyogo College of Medicine, Nishinomiya, Hyogo 663-8501, Japan
Chun-Shen Shen, Department of Dermatology, Hyogo College of Medicine, Nishinomiya, Hyogo 663-8501, Japan
Yoshiro Nakano, Tomonori Morinaga, Tomoko Hashimoto-Tamaoki, Department of Genetics, Hyogo College of Medicine, Nishinomiya, Hyogo 663-8501, Japan
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Correspondence to: Reigetsu Yoshikawa, MD, PhD, Second Department of Surgery, Hyogo College of Medicine, 1-1 Mukogawa-cho, Nishinomiya, Hyogo 663-8501, Japan. yosikr2s@hyo-med.ac.jp
Telephone: +81-798-456372 Fax: +81-798-456373
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Abstract

AIM: To investigate the possible role of polysaccharide-K (PSK) -related markers in predicting distant metastasis and in the clinical outcome of colorectal cancer (CRC).

METHODS: Firstly, we used protein microarrays to analyze the *in vitro* expression profiles of potential PSK-related markers in the human colorectal adenocarcinoma cell line SW480, which carries a mutant *p53* gene. Then, we investigated the clinical implications of these markers in the prognosis of CRC patients.

RESULTS: ECA39, a direct target of c-Myc, was identified as a candidate protein affected by the anti-metastatic effects of PSK. Immunohistochemistry revealed that ECA39 was expressed at significantly higher levels in tumor tissues with distant metastases compared to those without ($P < 0.00001$). Positive ECA39 expression was shown to be highly reliable for the prediction of distant metastases (sensitivity: 86.7%, specificity: 90%, positive predictive value: 86.7%, negative predictive value: 90%). A significantly higher cumulative 5-yr disease free survival rate was observed in the ECA39-negative patient group (77.3%) compared with the ECA39-positive patient group (25.8%) ($P < 0.05$).

CONCLUSION: Our results suggest that ECA39 is a dominant predictive factor for distant metastasis in patients with advanced CRC and that its suppression by PSK might represent a useful application of immunotherapy as part of a program of integrated medicine.

Key words: ECA39; Distant metastasis; Colorectal cancer; Polysaccharide-K; Integrated medicine

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INTRODUCTION

Colorectal cancer (CRC) is one of the three most frequent malignancies in Western countries. CRC patient survival rates are delineated by local recurrence and lymphatic and hematogenous dissemination[1,2] and, once metastatic disease is diagnosed, the 5-year survival rate is less than 5%. In the majority of cases, chemotherapy is the recommended treatment for patients with advanced metastatic disease. Recently, we have achieved good clinical results in the treatment of CRC using 'Pharmacokinetic Modulating Chemotherapy (PMC)', which was designed as a hybrid of lower metronomic and higher shorter plasma 5-FU concentration. The cumulative 5-year survival rate of Dukes' C CRC patients was 95% in the group treated with PMC, compared with 67% in the non-PMC group ($P = 0.003$)[3,4]. Our PMC regimen also significantly decreased liver metastasis, resulting in a median liver metastasis-free time after hepatectomy of 34.2 mo in the PMC group compared with 18.4 mo in the non-PMC group ($P = 0.00002$)[5]. PMC offers the advantages of reduced toxicity and lower costs through outpatient treatment. However, the prognosis of some patients remains poor despite vigorous anticancer therapy. Extrahepatic recurrence, mostly in the lung, cannot always be reduced by PMC alone, although PMC turned out well for the risk reduction of liver metastasis. Indeed, intensive chemotherapy has been shown to

cause a comparatively higher risk of extrahepatic distant metastases such as the lung, bone, brain and peritoneum, with an overall recurrence rate in CRC patients receiving PMC of 13.1%[6]. While chemotherapy with hepatic arterial infusion or second-look liver resection can control the prognosis of liver metastasis to some extent, extrahepatic recurrence is refractory to any known treatment. The management of extrahepatic recurrence is a major problem as the prognosis of patients suffering extrahepatic recurrence is significantly worse (mean survival of 2 mo from diagnosis) compared with those with liver metastasis alone (mean survival of 26 mo from diagnosis) ($P < 0.05$)[6].

The integration of immunopotentiating agents with the extant treatment regimens of surgery, chemotherapy, and radiation therapy has gained popularity as an adjuvant therapy for cancer during the last three decades. The use of complementary and alternative medicine (CAM) is a growing field in health care, particularly among cancer patients in the advanced stages of disease. However, recent reports have shown that while expenditure on CAM is high, 44.6%-66.7% of cancer patients receiving palliative care use CAM without sufficient information[7,8].

Polysaccharide-K (PSK), or Krestin, is a protein-bound polysaccharide biological response modifier prepared from the mushroom *Coriolus versicolor*, that has been used in traditional Chinese medicine for centuries. PSK is widely used in adjuvant therapy after surgery or radiotherapy in Japan and other Asian countries, and the Japanese National Health Insurance scheme covers the use of PSK for gastric, colorectal, and lung cancers. Randomized, controlled clinical studies have revealed that the use of PSK in adjuvant therapy for gastric, colorectal, esophageal, and lung cancers significantly extends the 5-year survival rates of patients by 10% to 20%[9-12]. Compared with CRC patients who did not receive PSK, PSK-treated patients showed a higher 5-year survival rate (73.0% in PSK group *vs* 58.8% in non-PSK group in stage Ⅱ or Ⅲ, and 60.0% *vs* 32.1% in stage Ⅲ only) and lower rates of local recurrence (OR 0.74) and systemic recurrence (OR 0.52); lung metastases (OR 0.27), lymph node recurrence (OR 0.16), and peritoneal dissemination (OR 0.86)[10]. Based on the findings of these studies, we have been administering PSK to patients with advanced CRC since 2001. Compatible with the findings of Ohwada *et al*[10], we have observed that extrahepatic recurrences are significantly decreased when PSK therapy is used in combination with the standard PMC regimen (manuscript in preparation).

PSK produces very few adverse side effects, and its characteristics allow long-term oral administration. *In vitro* studies have confirmed that PSK induces the expression of several cytokine genes including *TNF-a*, *IL-1*, *IL-1R*, *IL-2*, *IL-4*, *IL-6*, *IL-7*, and *IL-8*[12-15]. Anti-neoplastic effects of PSK have also been reported in animal models, and involve the radical trapping and modulation of cytokine production and effector cell functions[16,17]. Recently, we have shown that PSK may have an additional anti-tumor effect on the cancer cells *per se* without disturbing cell-cycle progression[18]. PSK might also alter the local characteristics of tissue-specific factors as well as their host-mediated activities[18].

We hypothesized that some PSK-related markers could be influential in predicting the occurrence of distant metastasis in CRC patients. In order to identify those molecular markers associated with the transition from primary CRC to distant metastases, we used a protein array containing 500 human antibodies in the human colorectal adenocarcinoma cell line SW480 to screen for alterations in protein expression that are potentially required for the direct action of PSK. SW480 carries a mutant *p53* gene; such mutations have been found in approximately half of all colorectal cancers and are associated with lymphatic dissemination and poor prognosis[19,20]. We examined the expressions of candidate marker proteins in cancerous tissue obtained from CRC patients and accordingly assessed their usefulness as prognostic markers for distant metastasis.

MATERIALS AND METHODS

Cell culture and PSK treatment

The colorectal adenocarcinoma cell line, SW480, carrying a mutant *p53* gene was obtained from the Human Science Research Resource Bank (Tokyo, Japan). Cells were grown in RPMI 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 100 mL/L fetal bovine serum (FBS; HyClone, Logan, UT, USA), 2 mmol/L glutamine, 100 000 U/L penicillin, 100 mg/L streptomycin, and 40 mg/L gentamycin at 37℃ in a humidified atmosphere of 50 mL/L CO_2. For the cell growth study, 10^6 cells were plated per 60-mm dish and treated with various concentrations of PSK (Kureha Chemical Co., Tokyo, Japan). Cells were counted using a hemocytometer on the days indicated. Then they were prepared for protein extraction and Ab arrays (Clontech, Palo Alto, CA, USA).

Identification of protein expression profiles by antibody microarray

Extraction of whole cellular protein, microarray hybridisation, scanning, grid-assisted spot identification, and analysis were performed according to the manufacturer's instructions (Clontech). Briefly, 25 μg whole cellular protein was extracted, labelled with the same volume of Cy3 and Cy5, hybridized with Antibody (Ab) Microarray, and the level of radioactivity was measured by scintillation counting. Sample and control-labelled probes were mixed together and hybridized to Ab Microarray slides containing 500 human antibodies in the Ab Microarray (No. 3080600). The names of these proteins are available at http://www.clontech.com/clontech/products/families/abarray/nanoscale.shtml. Hybridized slides were scanned and the scanner output images were analyzed using AtlasImage™ software, following localization by the overlaying of a grid on the fluorescent images. Fluorescent signal intensities were normalized by the Ab Microarray Analysis Workbook. Both final reported intensities were filtered, and those spots with intensities less than 0.75 or more than 1.32 were eliminated. The results were obtained from two independent experiments.

Patients

Sixty-three patients (31 women and 32 men) with a mean age of 60 years (range 37-83 years) and with surgically excised Dukes' C lower rectal carcinomas beneath the peritoneal reflexion were studied in the Hyogo College of Medicine between April 1986 and March 1995. Thirty-five of these patients (12 women and 23 men) with a mean age of 62.9 years (range 41-83 years) were enrolled in this study. The histological grades of carcinomas are as follows: 6 were well differentiated, 27 were moderately differentiated, and 2 were poorly differentiated or mucinous carcinomas. Follow-up information was obtained from office charts and hospital records. All patients were followed up for 60 mo after the initial operation. Local recurrence was defined as any tumor recurrence within the pelvis or anal canal. Distant recurrence was defined as any tumor recurrence outside the pelvis and included metastasis to the liver, lung, bone or the abdominal cavity. No recurrence was observed in 22 patients, distant recurrence was observed in 13 (3 cases of recurrence in the lymph nodes, 3 in the liver and 7 in the lungs), and local recurrence in 2 patients. The Ethics Committee of the Institution approved the study protocol.

Immunohistochemistry

CRC tissue specimens were processed using conventional procedures for paraffin embedding, cut into 4-μm sections, and mounted onto poly-l-lysine-coated slides. Sections were dewaxed in xylene, rehydrated in a descending alcohol series, heated twice in a microwave oven for 5 min for antigen retrieval, blocked for endogenous peroxidase activity with 30 mL/L H_2O_2 in methanol, and then blocked for non-specific antibody binding with normal rabbit serum. They were incubated overnight at 4℃ with a mouse monoclonal Ab against human ECA39 (BD Biosciences Pharmingen, San Jose, CA, USA) followed by treatment with a standard avidin-biotin-peroxidase complex. The slides were developed with 3, 3-diaminobenzidine tetrahydrochloride solution containing 1 mL/L H_2O_2 and were lightly counterstained with hematoxylin. Normal mouse IgG was substituted for the primary antibody as a negative control. The sections were examined microscopically by three of the authors (Y.F., R.Y., and T.H-T.) without knowledge of their clinicopathologic features. ECA39 expression was categorized according to staining intensity compared with interstitial infiltrates as follows: score 3 (strong), staining intensity more than interstitial infiltrates; score 2 (moderate), staining intensity equal to interstitial infiltrates; score 1 (mild), staining intensity less than interstitial infiltrates; and score 0 (negative), no staining. We then categorized ECA39 expression according to ECA39 expression scores: score 1 to 3, ECA39 positive; score 0, ECA39 negative.

Statistical analysis

Disease-free survival (DFS) and overall survival (OS) curves were generated by the Kaplan-Meier method, and the Cox-Mantel test was used to compare the curves. Death without recurrence was excluded from the analysis. Values of $P < 0.05$ were considered statistically significant. Statistical analyses were carried out using STATISTICA statistical software, version 06J (STATISTICA, Tulsa, OK, USA).

Table 1 Differential protein expression in SW480 cell line following exposure to PSK, defined by a 1.32-fold or greater change

No.	Antidody/antigen name	Normalized average INR
102	HDJ-2	1.47
236	TNIK	1.46
376	TRAX	1.46
98	C-NAP1	1.35
225	Moesin	1.32
410	ECA39	0.65
403	Caspase-9/ICE-LAP6/Apaf-3	0.67
461	Synaptotagmin	0.71
406	ERK2 (MAPK2)	0.74
382	PMCA2	0.75

RESULTS

Suppression of cell growth by PSK

The effects of various concentrations of PSK (0 to 1000 mg/L) on the growth of SW480 cells was examined 96 h after treatment, and exposure to 10, 100, 500 and 1000 mg/L PSK was shown to suppress growth by 92.1%, 83.6%, 69.5% and 60.8%, respectively. Each figure represents the mean of more than three independent experiments.

Analysis of expression profiles

Protein expression in SW480 cells was analyzed following 24 h treatment with 500 mg/L PSK using an Ab Microarray (No. 3080600). Under basic selection conditions, a total of 10 proteins were selected from the 500 human proteins available on the array slide. These proteins were identified on the basis of their altered expression following exposure to PSK, with 1.32-fold or higher ratios, and included 5 upregulated and 5 downregulated proteins (Table 1). Proteins showing upregulated expression, in the order of decreasing ratio, are HDJ-2, TNIK, TRAX, C-NAP1, and Moesin. Proteins showing downregulation, in the order of increasing ratio, are ECA39, Caspase-9/ICE-LAP6/Apaf-3, Synaptotagmin, ERK2 (MAPK2), and PMCA2.

Correlation of ECA39 expression with CRC patient prognosis

ECA39 was selected from these 10 candidate proteins as a distant metastasis-related marker in CRC after immunohistochemical analysis of CRC tissue specimens (Figure 1). ECA39 was expressed at significantly higher levels in tumor tissues with distant metastases (13 of 15 expressed positive ECA39) compared to those without metastases (2 of 20 expressed positive ECA39, $P < 0.00001$). Positive ECA39 expression was also shown to be highly reliable in predicting distant metastases (sensitivity: 86.7%, specificity: 90%, positive predictive value: 86.7%, and negative predictive value: 90%). Kaplan-Meier analysis revealed a significant

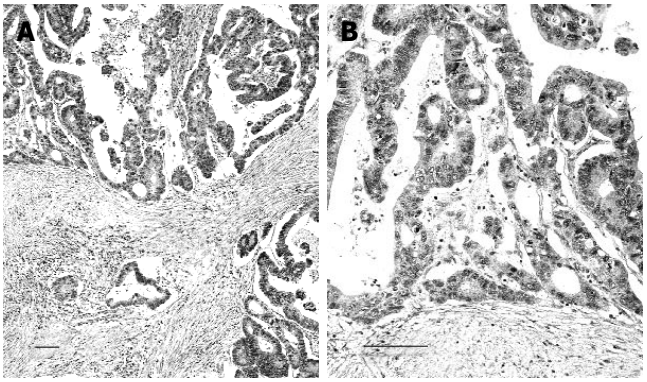

Figure 1 Immunohistochemical detection of ECA39 in rectal cancers (**A**: × 40; **B**: × 100. Bars indicate 100 μm).

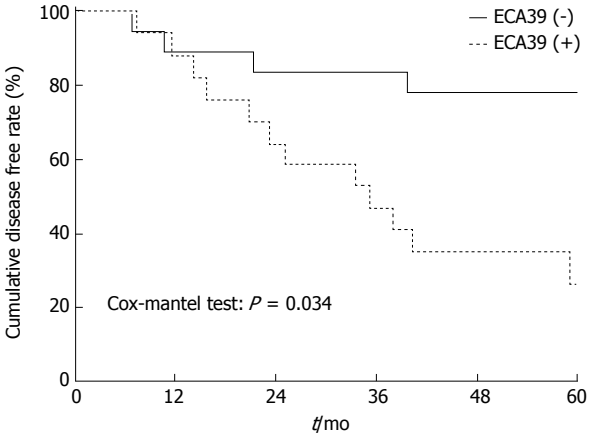

Figure 2 Five-year disease-free survival curves for eligible patients with pathologic stage Ⅲ cancer in the ECA39-negative group and ECA39-positive group.

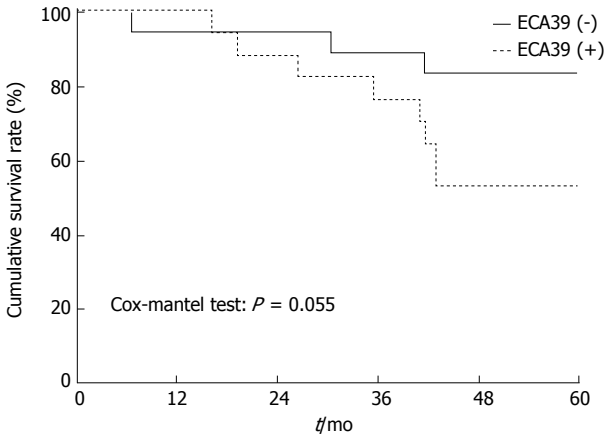

Figure 3 Five-year overall survival curves for eligible patients with pathologic stage Ⅲ cancer in the ECA39-negative group and ECA39-positive group.

decrease in DFS of patients with positive ECA39 expression (25.8%) compared with patients with negative ECA39 expression (77.3%) (P = 0.034; Figure 2). The 5-year OS rate of ECA39-positive patients was 53.0%, compared with 83.3% for ECA39-negative patients, although this difference was not statistically significant (P = 0.055; Figure 3).

DISCUSSION

Normal cells undergo certain changes during their transformation into invasive malignant clones with metastatic potential. Molecular determinants occurring during the development of sporadic CRC include mutations in certain tumor suppressor genes (*APC*, *DCC*, *Smad-2*, *Smad-4*, *p53*) and oncogenes (*K-ras*) that have been summarized in the adenoma-carcinoma sequence initially proposed by Fearon and Vogelstein[21]. However, because only 8% of CRC harbor concomitant mutations of *APC*, *K-ras*, and *p53*, it seems likely that additional pathogenic alterations are instrumental in the mediation of the progression and metastasis of CRC[22]. Cellular transformation provokes tissue remodeling inside neoplastic lesions and in the periphery of the tumor. Disorders in the local characteristics of tissue-specific factors play an essential role in cancer progression. In the present study, we have demonstrated that the ECA39 expression, which can be suppressed by PSK, has the potential to predict distant metastasis.

The ECA39 protein was originally identified by the overexpression of its mRNA in an undifferentiated mouse teratocarcinoma cell line[23]. The *ECA39* gene harbors a functional *c-Myc* binding sequence located 3' of its transcription initiation site, and has been shown to be a direct target for c-Myc activity in both mice and humans[24,25]. The functional implications of individual *c-Myc* target genes including ornithine decarboxylase[26,27], p53[28], and cdc25A[29], are now complemented by large surveys of the c-Myc network as a therapeutic target in cancer[30]. The *c-myc* oncogene is essential for cell proliferation but, paradoxically, also promotes cell death. The biological rationale for this dual signal is that c-Myc intrinsically regulates malignant transformation. ECA39 shares significant homology with the prokaryotic protein branched-chain amino acid aminotransferase (BCAT), and is highly expressed during the log phase and is down-regulated during the stationary phase of growth[31]. Thus, ECA39 might be involved in the regulation of the cell cycle. Disruption of the *ECA39* gene results in an increased growth rate in comparison to wild type[25]. As shown in the present study, ECA39 is a highly reliable marker in the prediction of distant metastasis and, in combination with other biomarkers, might produce an even higher predictability of poor prognosis. Despite significant progress in the identification of markers predicting CRC patient prognosis, there remains a need for clinical predictors of distant metastasis in order to strengthen patient surveillance. This would allow the tailoring of treatment to individual patients and the application of evidence-oriented integrated medicine, thus maximizing the probability of optimal response to the therapy.

Previous reports have demonstrated that c-Myc acts as a biomarker in the prediction of patient response to treatment with 5-FU and camptothesin[32,33]. Evidence of ECA39-based administration of PSK could also favor some CRC patients by sensitizing their response to chemotherapy, protecting normal cells during treatment as well as having an anti-metastatic effect. Such possibilities need to be confirmed in larger clinical studies, which are war-

ranted both in Japan and world-wide. We believe that the acquisition of knowledge of integrated medicine by physicians, especially oncologists, is essential and should not be underestimated. Moreover, oncologists should discuss the role of integrated medicine with their patients and encourage patients to participate in well-organized research on integrated approaches to therapy.

Here, we identified ECA39 as a biomarker that predicts distant metastasis in CRC patients. ECA39 is thought to play a role in metastasis, and could represent a potential diagnostic, prognostic, or even therapeutic target. Furthermore, the metastasis-tumor associated ECA39 profile could be of use in the selection process of tumors that are likely to develop metastases, thus optimizing the application of immunotherapy by PSK and improving clinical outcome.

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