Development of biomarkers for predicting recurrence by determining the metastatic ability of cancer cells

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Abstract

Adjuvant chemotherapy has been carried out for patients with cancer who underwent curative resection, but it is basically not needed for patients without micro-metastatic lesions who undergo a perfectly curative surgical operation. The patients who need adjuvant chemotherapy are defined as those whose micro-metastases cannot be detected by imaging modalities in the other sites of the resective areas, despite curative resection for the primary sites. If biomarkers to efficiently evaluate the metastatic potential of each patient could be developed, we may be able to provide personalized adjuvant chemotherapy in the clinical setting. Actinin-4 (ACTN4, gene name ACTN4) is an actin-bundling protein that we identified in 1998 as a novel molecule involved in cancer invasion and metastasis. Protein overexpression of actinin-4 in cancer cells leads to the invasive phenotype, and patients with gene amplification of ACTN4 have a worse prognosis than patients with a normal copy number in some cancers, including pancreas, lung, and salivary gland cancers. In this review, the biological roles of actinin-4 for cancer invasion and metastasis are summarized, and the potential usefulness of actinin-4 as a biomarker for evaluation of metastatic ability is examined.
1. Introduction

The control of metastatic sites is the important factor for determining the prognosis of patients following curative resection of primary sites. Despite curative surgery, some cancers will recur in sites other than the primary lesions (1,2). Even if the primary tumor is surgically resectable at the macroscopic level, if cancer cells have high inherent metastatic ability, the presence of micro-metastases that cannot be detected by imaging modalities cannot be ruled out, and it may be difficult to determine the optimal therapeutic strategy after complete resection (3). Basically, patients who may derive potential benefit from adjuvant chemotherapy are defined as: i) having the potential for micro-metastatic lesions in the other sites of the resected area; and ii) high sensitivity to the drug used as adjuvant chemotherapy. Assuming that, at least, patients for whom adjuvant chemotherapy is potentially beneficial are defined as individuals with a high risk of metastases, we have developed a biomarker to evaluate metastatic potential, and we reported that gene amplification of ACTN4 is a potential biomarker for optimizing the therapeutic strategy of adjuvant chemotherapy.

2. Identification of actinin-4 that is associated with cancer invasion and
metastasis.

To identify the molecular mechanism of cancer invasion, we newly established a monoclonal antibody (NCC-Lu-632) that has a strong reaction to the invasive front of cancer cells, and then we carried out molecular cloning for the antigen of NCC-Lu-632 using a phage screening assay. We isolated the novel isoforms of alpha-actinin, that is the actin-bundling protein, and named it actinin-4 (ACTN4) (4). Alpha actinin consists of 4 isoforms (ACTN1-4) in humans (5). ACTN1 and ACTN4 are classified as non-muscle type, and ACTN2 and ACTN3 are muscle type. The amino acid sequences of ACTN isoforms maintain high homology. Alpha-actinin has an actin-binding domain (ABD) that is composed of two calponin homology domains (CHDs), four spectrin repeats (SRs), and two EF hand domains that are composed of calmodulin (CaM)-like domains. By alpha-actinin forming anti-parallel homodimers via the SRs, both sides of the ABD can bind actin filaments and then do actin-bundling. Moreover, non-muscle alpha-actinins interact with actin filaments to bind with the plasma membrane through beta 1-3 integrins, vinculin, and alpha-catenin (Fig 1) (3).

Immunohistochemical analysis has shown that actinin-4 protein is
histologically concentrated at the invasive fronts of several cancers, such as colorectal (6,7), lung (8-11), breast (4,12), and ovarian cancers (13-15). In addition, we have reported that patients with protein overexpression of actinin-4 had a worse prognosis than patients without overexpression in breast (4,12), pancreas (16,17), ovarian (13-15), thyroid (18), salivary gland (19), and tongue cancers (20). Moreover, involvement in malignant phenotypes for protein overexpression of actinin-4 was reported in brain tumors (21-23), head and neck cancer (24,25), lung cancer (26-28), breast cancer (29-31), esophageal cancer (32), gastric cancer (33), pancreatic cancer (34), gastrointestinal stromal tumor (GIST) (35), cervical cancer (36), ovarian cancer, bladder cancer (37), prostate cancer (38,39), melanoma (40,41), leukemia (42,43), and osteosarcoma (44,45) from not only our groups, but also other independent groups. In fact, use of an exogenous transfection technique to overexpress actinin-4 in cancer cells demonstrated that cancer cells overexpressing actinin-4 can form the protrusions that are involved in cell motility and cancer invasion and exhibit significantly increased invasive potential (6). Moreover, the reduction of protein expression of actinin-4 could decrease cell invasiveness using RNA interference in pancreatic cancer (16),
oral cancer (24), and lung cancer (10). Thus, we concluded that overexpression of actinin-4 is involved in cancer invasion and metastases.

3. Identification of gene amplification of ACTN4 in invasive phenotypes of malignant tumors.

In 2000, Kaplan et al. implied that mutations in the gene encoding actinin-4 (ACTN4) were the cause of disease in three families with autosomal dominant forms of familial focal segmental glomerulosclerosis (FSGS) by genotyping family members at markers on chromosome 19q13 (46,47). It was known that the locus of human chromosome 19q13 is often amplified in patients with several cancers, including pancreatic cancer and ovarian cancer. We confirmed the amplification status of ACTN4 in pathological specimens that were resected by surgical operations using a fluorescence in situ hybridization (FISH) probe that was newly isolated in those studies. First, gene amplification of ACTN4 was identified in pancreatic cancer (16). Overexpression of actinin-4 protein was confirmed in almost all patients with gene amplification of ACTN4 by immunohistochemistry (IHC), and a significant correlation was recognized between IHC and FISH analyses in patients with gene
amplification of \( \text{ACTN4} \). However, the cases of protein overexpression of actinin-4 were not always gene amplification cases of \( \text{ACTN4} \). Interestingly, Yamamoto et al. reported that gene amplification of \( \text{ACTN4} \) is a significant predictor for overall survival time in patients with ovarian cancer, and the copy number increase of \( \text{ACTN4} \) was a more accurate predictor for the patient’s prognosis of ovarian cancer than IHC evaluation (14). Similar phenomena have also been observed in lung adenocarcinoma (8), tongue cancer (20), and salivary gland cancer (19).

4. Development of a biomarker for efficient stratification of patients who need adjuvant chemotherapy in adenocarcinoma of the lung.

According to The Japanese Lung Cancer Society Guideline for non-small cell lung cancer (2020), complete resection of postoperative pathological stage IA/IB/II (Group 8) with an overall lesion diameter of >2 cm and lung adjuvant chemotherapy with a tegafur-uracil combination (UFT) is strongly recommended for patients with stage I adenocarcinoma of the lung (Evidence level A). The rationale for this recommendation is that a phase III trial investigating the efficacy of UFT in stage I lung adenocarcinoma showed an additive effect of 3 percentage
points (85% to 88%) overall and 11 percentage points (74% to 85%) in stage IB (T > 3 cm) (48). A meta-analysis of four additional clinical trials (2003 cases; 84% adenocarcinoma, 16% non-adenocarcinoma) showed an overall improvement in 5-year survival of 5 percentage points (77% to 82%), confirming the efficacy of UFT (49). A subgroup analysis was performed in the “patients with tumor size >2 cm and ≤3 cm” group, and an additional 6 percentage point improvement in 5-year survival was observed in the “patients with tumor size >2 cm and ≤3 cm” group, with a hazard ratio (HR) of 0.62 [95% confidence interval (95% CI) 0.42-0.90], which was good (50). On the other hand, the guideline also states that 74% of patients with complete resection of postoperative stage I disease (lung adenocarcinoma) are recurrence-free even with surgery alone, and the safety of chemotherapy should be fully considered when the clinical oncologists are carrying out adjuvant chemotherapy (The Japanese Lung Cancer Society Guideline 2020).

As mentioned in the Introduction, prediction of micro-metastases outside of the resection site by assessing the metastatic activity of the tumor is likely to be an important indicator for stratifying the group of patients who respond to adjuvant chemotherapy and implementing effective adjuvant chemotherapy.
Therefore, we confirmed actinin-4 protein expression and gene amplification in resected specimens of stage I lung adenocarcinoma that had been resected at the National Cancer Center Hospital and the National Cancer Center East Hospital without postoperative adjuvant chemotherapy using immunostaining and FISH (11). We analyzed the clinical utility of ACTN4 as a biomarker using two independent cohorts. “The cases of stage-I in adenocarcinoma of the lung with gene amplification of ACTN4 (amplification group)” (23 patients) had significantly shorter overall survival than the normal copy number group (267 patients), with a 5-year survival rate of 95% (95% CI 82%-98%) in the gene amplified group and 57% in the amplification-negative group (Fig 2A). On univariate analysis, the HR for death in the amplification group was significantly higher than that in the “normal copy number group” at 10.5 (HR 95% Cl 4.15-26.7), and multivariate analysis showed an HR of 6.78 (95% Cl 2.59-17.7), which was extracted as an independent, significant, and the strongest prognostic factor. It was also found to be the strongest independent prognostic factor. Furthermore, we classified the patients of another independent cohort into three groups for analysis of overall survival: i) ACTN4 protein-negative group/normal copy number group; ii) ACTN4 protein-positive group/normal copy number group; and iii) ACTN4 protein-positive
group/amplification group. The 5-year survival rates for the “1) ACTN4 protein-negative group/normal copy number group (98 cases),” “2) ACTN4 protein-positive group/normal copy number group (88 cases),” and “3) ACTN4 protein-positive group/gene amplification group (19 cases)” were 96% (97% CI 92-100%), 93% (95% CI 81-95%), and 63% (95% CI 45-89%), respectively (Fig 2B) (8). These findings strongly suggest that not only is ACTN4 gene amplification a strong prognostic biomarker for stage I adenocarcinoma of the lung that did not undergo postoperative chemotherapy, but also that it is a high-risk factor for death despite complete resection. Overall, this indicates that ACTN4 gene amplification is a biomarker for predicting minimal residual metastases that cannot be detected by imaging modalities (3).

In fact, when we knocked down the expression of actinin-4 protein using shRNA in A549 cells, a lung cancer cell line that showed gene amplification of ACTN4, the invasive ability and filopodia formation of the cells were markedly reduced. When the luciferase luminescent A549 cell line was injected into the tail vein of immunodeficient mice and observed for 40 days, many metastatic lesions were observed in the lung. On the other hand, no metastatic lesions in the lung were observed in mice injected with the actinin-4 knockdown A549 cell line (10).
other words, as hypothesized previously, *ACTN4* gene amplification is likely to be a surrogate biomarker reflecting the presence of micro-metastases outside the resected area due to increased metastatic activity.

The Japanese Lung Cancer Society Guideline for non-small cell lung cancer (2020) CQ29 recommends cisplatin combination chemotherapy for complete resection of stage II-IIIA postoperative pathological disease (Group 8). The rationale for this recommendation is also based on a meta-analysis showing significant prolongation with adjuvant chemotherapy (51). Using a publicly available database of results from the Canadian phase III, randomized, controlled trial of adjuvant cisplatin-vinorelbine (JBR.10) (52), a subgroup analysis of the *ACTN4* mRNA index showed that postoperative adjuvant chemotherapy prolonged overall survival in the *ACTN4* high expression group (Fig 2C), but not in the *ACTN4* low expression group. In the *ACTN4* high expression group, postoperative adjuvant chemotherapy prolonged overall survival, whereas in the *ACTN4* low expression group, no improvement in survival was observed (Fig 2D) (10). These results suggest that *ACTN4* is a potential biomarker to stratify patients who would benefit from adjuvant chemotherapy. Based on these results, an investigator-initiated study of implementation of the biomarker has been
initiated by a multi-center collaboration of Nippon Medical School, Tokyo Medical University, and the National Cancer Center with support from the Japan Agency for Medical Research and Development (AMED) (principal investigator Professor Kubota, Nippon Medical School).

5. Gene amplification of $\text{ACTN4}$ strictly predicts the prognosis of tongue squamous cell carcinoma.

Currently, the standard surgical treatment for early-stage tongue cancer (stage-I/II) without clinical lymph node metastasis is partial tongue resection or partial tongue resection plus neck dissection on the affected side. The clinical question is whether elective neck dissection is necessary for early-stage tongue cancer. A randomized, phase III trial from India in 2015 is one answer to this question, which found that elective neck dissection for early-stage oral cancer was superior to non-dissecting surgery in the primary analysis of overall survival (HR 0.64 95% CI 0.34-0.59, 3-year survival rate 80.0% vs. 67.5%) and in the secondary analysis of disease-free survival (HR 0.45 95% CI 0.34-0.59, 3-year disease-free survival rate 69.5% vs. 67.5%) (53). It is questionable whether the results can be extrapolated to daily practice in Japan.
To resolve this question, a randomized, comparative study was conducted in Japan to evaluate the value of omission of prophylactic neck dissection for stage I/II tongue cancer (JCOG 1601: Randomized Phase III study to evaluate the value of omission of prophylactic neck dissection for stage I/II tongue cancer) (17,54).

This study was designed to determine the non-inferiority in overall survival of the partial tongue resection plus prophylactic dissection group compared to the partial tongue resection group, and to establish a less invasive standard of care. The results are awaited.

On the other hand, late cervical lymph node metastasis is likely to be caused by the metastatic activity of the primary tumor. If this is the case, then amplification of the ACTN4 gene may be a potential biomarker for later cervical metastasis. In a recent study, we retrospectively examined the protein expression and gene amplification of ACTN4 by immunostaining and FISH using stage-I/II surgical pathological sections from the Department of Head and Neck Surgery, National Cancer Center Hospital. Patients were classified as follows: i) negative for immunostaining (negative for actinin-4 protein); ii) positive for immunostaining (positive for actinin-4 protein) (Fig 3A) / negative for FISH (normal copy number of ACTN4 gene); and iii) positive for immunostaining/FISH (amplified ACTN4
Disease-free survival and overall survival were then evaluated. The study included 51 patients who underwent partial resection of the tongue at the first surgery at the National Cancer Center Hospital and were excluded from the following groups: those who underwent preoperative chemotherapy, those with pathologically positive resection margins, those with local recurrence, and those who could not be evaluated by the FISH method. Disease-free survival was significantly longer in patients with i) immunostaining negative than those with ii) immunostaining positive/FISH negative (HR 2.82, 95% CI 1.04-7.64), and iii) immunostaining positive/FISH positive (HR 2.19, 95% CI 1.17-4.09) was significantly shorter. The median survival time (MST) was 1964 days and 744 days for ii) immunostaining positive/FISH negative and iii) immunostaining positive/FISH positive, respectively, and MST was not reached for i) immunostaining negative. Interestingly, when the overall survival of the same patients was evaluated, the significant difference between i) immunostaining negative and ii) immunostaining positive/FISH negative disappeared, and the difference between i) immunostaining negative and iii) immunostaining positive/FISH positive (HR 7.60, 95% CI 1.95-29.6, p=0.0008), ii) immunostaining positive/FISH negative and iii) immunostaining positive/FISH positive (HR 4.62,
CI 0.998-21.3, P=0.035) (18). These results suggest that actinin-4 protein-positive cases with normal copy numbers of ACTN4 can be rescued by second-line therapy even after recurrence. Therefore, we investigated the overall survival of 12 patients who underwent cervical dissection after recurrence at the National Cancer Center Hospital and found that the MST of ACTN4-amplified patients was 626 days, which was extremely short, and that all but one of the patients with a normal copy number could be rescued (20) (Fig 3C). Since this was a retrospective study with a small number of cases, it needs to be validated by multicenter studies including retrospective and prospective observational studies.

6. Summary

Research that began with gene cloning has shown potential as a method to determine biomarkers for the identification of high-risk groups for cancer recurrence and as indicators for the implementation of adjuvant therapy. However, there are still many hurdles to be overcome for implementation in clinical practice. We would like to aim for practical application as soon as possible through joint research, including prospective research, with clinical researchers.
Conflict of Interest.

K. H. is the inventor of a patent filing for ACTN4 biomarkers.

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Figure legends

Figure 1. Schema of actinin-4 as an actin-bundling protein (3).

(A) Domain structure of actinin-4 protein. Actinin-4 consists of the actin binding domain (ABD), spectrin repeat (SR1-4), and calmodulin (CaM)-like domain (two EF hand motifs). © 2015 Honda et al. Reprinted under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/) from Cell Biosci. 2015;5:41, Honda K et al. The biological role of actinin-4 (ACTN4) in malignant phenotypes of cancer.

(B) Actin bundling with actinin-4 homodimers. By alpha-actinin forming anti-parallel homodimers via SRs, both sides of the ABD can bind actin filaments. © 2015 Honda et al. Reprinted and modified from Ann Oncol. 2013;24(10):2594-600, Noro R and Honda K et al. Distinct outcome of stage I lung adenocarcinoma with ACTN4 cell motility gene amplification, with permission from Elsevier.

(C) Interaction of actinin-4 with the cell membrane. Actinin-4 interacts with actin filaments to bind with the plasma membrane through beta 1-3 integrins,
vinculin, and alpha-catenin. © 2017 Honda et al. Reprinted under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by-nc-nd/4.0/) from Int J Oral Maxillofac Surg. 2017;46(8):968-76, Kakuya T and Honda K et al. Prognostic significance of gene amplification of ACTN4 in stage I and II oral tongue cancer.

**Figure 2. Involvement of actinin-4 in metastatic potential of non-small cell lung cancer (8) (10).**

(A) Prognostic significance of ACTN4 gene amplification in adenocarcinoma of the lung by Kaplan-Meier analysis for overall survival of stage-I patients with adenocarcinoma of the lung. The blue line indicates the patients with normal copy number (FISH negative). The red line indicates the patients with gene amplification of ACIN4 (8).

(B) Survival prediction for patients with stage I adenocarcinoma of the lung using combination IHC and FISH. Overall survival is estimated by Kaplan-Meier analysis. The blue line indicates no expression of actinin-4 protein (IHC-
negative). The green line indicates strong expression of actinin-4 protein (IHC-positive) and normal copy number of \textit{ACTN4} (IHC-positive/FISH-negative). The red line indicates strong expression of actinin-4 protein (IHC-positive) and gene amplification of \textit{ACTN4} (IHC-positive/FISH-positive) (8).

(C) Effect of \textit{ACTN4} knockdown by shRNA on the metastatic ability of a lung cancer cell line that has gene amplification of \textit{ACTN4} in an animal inoculation model (10).

(D-E) Overall survival curves from a reanalysis of publically available data on patients enrolled in JBR.10. Subgroup with \textit{ACTN4} overexpression (D), and subgroup without \textit{ACTN4} overexpression (E). The red line indicates the overall survival curve for the patients who underwent adjuvant chemotherapy (ADJ). The blue line indicates the patients who were observed without undergoing adjuvant chemotherapy (OBS) (10).

\textbf{Figure 3. Potential biomarker for the stratification of patients with late metastatic ability of cervical lymph nodes in tongue cancer (20).}

(A,B) Representative photograph of strong expression of actinin-4 protein (A) and gene amplification of \textit{ACTN4} (B) in stage-I/II patients with tongue cancer (20).
Overall survival curves for the 12 patients who underwent therapeutic neck dissection for late cervical lymph node metastases after undergoing partial glossectomy. The blue line is the patients with normal copy numbers of ACTN4. The red line is gene amplification of ACTN4 (20).
Figure 2

Stage I (n = 290)

Overall Survival (%)

0 20 40 60 80 100

5-yr OS rate (95% CI): Months

FISH-negative (n = 267)

FISH-positive (n = 23)

$P = 1.70 \times 10^{-10}$

Stage I (Cohort 1, n = 205)

Overall Survival (%)

0 20 40 60 80 100

5-yr OS rate (95% CI): Months

IHC-negative (n = 98)

IHC-positive/FISH-negative (n = 88)

IHC-positive/FISH-positive (n = 19)

**$P = 3.4758 \times 10^{-6}$**

*I$ $P = 3.4665 \times 10^{-3}$

C

shRNA

shC

D

ACTN4 (+) subgroup

P = 0.032 (log rank test)

HR, 0.273 (95% CI 0.078 – 0.952)

E

ACTN4 (-) subgroup

P = 0.979 (log rank test)

HR, 1.008 (95% CI 0.574 – 1.767)
Figure 3

ACTN4 FISH-positive (MST, 626 days) with P=0.0145 (HR; 10.36, 95% CI; 1.05 – 102.6) compared to ACTN4 FISH-negative.