

ORIGINAL ARTICLE

Expression of nectin 3: Novel prognostic marker of lung adenocarcinoma

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Keywords

E-cadherin; lung cancer; lung adenocarcinoma; nectin-3; prognostic factor.

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Received: 17 November 2011;

accepted 16 December 2011.

doi: 10.1111/j.1759-7714.2011.00104.x

*Yoshimasa Maniwa is an editorial board member of *Thoracic Cancer*.

Abstract

Background: We investigated the prognostic significance of the immunoglobulin-like cell adhesion molecule nectin-3, a regulator of the formation of adherens junctions, in human lung adenocarcinoma.

Methods: Tumor-tissue samples of 127 patients with surgically resected lung adenocarcinoma were used for analysis of the proteins expression by immunohistochemistry.

Results: Of the 127 patients, 25% showed membranous expression of nectin-3, and others showed negative or cytoplasmic expression. Membranous expression of nectin-3 was found to be a prognostic factor for decreased overall survival on univariate analysis ($P = 0.001$). Multivariate Cox proportional hazards model analyses also revealed that membranous expression of nectin-3 turned out to be an independent prognostic factor ($P = 0.048$). Moreover, in tumors expressing membranous nectin-3, some tumors did not co-localize with E-cadherin, and the patients of such tumors showed poorer prognosis than other patients for overall survival on univariate analysis ($P < 0.03$). Conversely, membranous expression of nectin-3 with E-cadherin co-localization was found to associate with good prognosis of patients.

Conclusion: Membranous expression of nectin-3 was an independent prognostic factor of lung adenocarcinoma, and it might play an important role in progression of the tumor.

Introduction

Lung adenocarcinoma is currently the most major tissue type of lung cancer that is the common cause of cancer death worldwide. Lung adenocarcinoma is composed of several tissue types, which are clearly divided into an invasive component and a non-invasive component.¹ Although prevention of tumor invasion is a critical aim in clinical medicine, it is still unclear what triggers tumor invasion and what occurs in such a process. Recently, in a large number of tumors, disruption of cell-cell adhesion is often observed, and causes loss of inhibition of cell movement and proliferation, eventually leading such cells to invasion into surrounding tissues and metastasis to other organs.² Such cells obtain mesenchymal-like phenotype, and thus, this process is called epithelial-mesenchymal transition (EMT).³ Many reports have demonstrated that EMT observed in primary carcinomas from clinical patients related to poor prognosis and tumor malignancy.⁴⁻⁶ It is well

known that several adhesion molecules (e.g., E-cadherin and integrins), transcriptional factors (e.g., Snail and Zinc finger E-box-binding homeobox [ZEB]), and central mediators (e.g., β -catenin and SMADs [drosophila protein, mothers against decapentaplegic (MAD) and the *Caenorhabditis elegans* protein SMA]) are involved in EMT.⁷

Nectins and nectin-like molecules (Necls) are immunoglobulin (Ig)-like cell adhesion molecules (CAMs) that have recently been shown to be essential contributors to the formation of cell-cell adhesions, especially adherens junctions (AJs) and regulators of cellular activities, including cell polarization, differentiation, movement, proliferation and survival.⁸ Nectin-3 has been identified as the third of four members of the nectin family, also described as PRR3 or PVRL3.⁹ It has been reported that nectin-3 was involved in the formation of cell-cell junctions *in vivo*, especially in synapses and testis, and in the epithelial remodeling during mouse development, especially at the site of neural morphogenesis.¹⁰⁻¹² On the

other hand, nectin-3 is ubiquitously expressed in a variety of cells *in vitro*, including epithelial cells, fibroblasts, neurons and spermatids.¹³ There are many partners of nectin-3 that *trans*-interact with; that is, nectin-1 and -2, and Necl-1, -2 and -5.⁸ Nectins including nectin-3 *trans*-interact each other at cell-cell contact sites, which introduce signals resulting in recruitment of E-cadherin to the site, resulting in the formation of AJs.⁸ Despite such important roles of nectin-3 *in vitro*, however, it is unknown whether nectin-3 is involved in malignancy of human primary carcinomas. Here, we demonstrated that nectin-3 was highly expressed at cytoplasm and cell membrane in human primary lung adenocarcinoma (frequency 81.1% [103/127]) by using immunohistochemistry for the first time, and its altered expression related with poor prognosis of patient survival. Our results suggest that the altered expression of nectin-3 causes tumor progression and malignancy.

Materials and methods

Collection of samples and patient data

One hundred and twenty-seven patients examined and treated at the Kobe University Hospital between 2001 and 2004 for lung adenocarcinoma were evaluated for this study. The project was approved by the local Institutional Review Board and consent obtained. Primary tumors and adjacent non-neoplastic lung tissue were obtained at the time of surgery. Peripheral portions of resected lung carcinomas were sectioned, evaluated by a study pathologist, and utilized for immunohistochemistry (IHC).

Physicians collected detailed clinical and demographic information on all patients enrolled in the study and patients were prospectively followed-up to determine clinical outcome and disease progression.

Immunohistochemistry

Formalin-fixed paraffin-embedded specimens were sectioned at 5 μ m thick and sections were deparaffinized with xylene and rehydrated with ethanol. Antigen retrieval was performed by placing the specimen in Dako REAL Target Retrieval Solution (DAKO) at 98°C for 20 min. For detection of nectin-3 and E-cadherin expression, rabbit anti-human nectin-3 polyclonal antibody and mouse anti-human E-cadherin monoclonal antibody (1:50 each, Santa Cruz Biotechnology) were used for the primary antibody, respectively. The DAKO EnVision/HRP Universal (DAB) Kit (DAKO, Tokyo, Japan) was used for endogenous peroxidase blocking, treatment of a secondary antibody against anti-rabbit and anti-mouse immunoglobulin antibody, and the visualization of HRP. Hematoxylin staining was used for counterstain. Pic-

tures of immunohistochemical stained sections were taken by a digital microscope (BZ-8000; Keyence, Tokyo, Japan).

Classification of immunohistochemical stained patterns

By light microscopy, immunochemical stained sections were classified. In assessment of the protein expression of nectin-3 and E-cadherin, if the ratio of stained cells in total epithelial cancer cells of a tumor tissue was more than 20%, the sample was classified as a membranous positive group. Others, the negative and cytoplasmic positive group, were classified as a non-membranous group. In assessment of co-localization of nectin-3 and E-cadherin, serial sections showing membranous expression of nectin-3 were used. If the ratio of the co-localizing region was more than 5% of total membranous positive cells, the sample was classified as co-localization (+) group. The classification of samples was done independently by N. S and Y. H. (pathologist) in a blind manner.

Statistical analysis

All statistical analyses were performed using Stata software (Stata Corp), version 10.1. The Fisher's Exact test or Student's *t*-test was used to examine the association between nectin-3 expression and various clinicopathological parameters. Univariate analysis was performed using the Kaplan-Meier method, and statistical significance between survival curves was assessed by log rank test. Overall survival was determined from the date of surgery to the time of death. To assess the independent value of different variables on survival, in the presence of other variables, multivariate analysis was carried out using the Cox proportional hazards model. Variables of significant value from the univariate analysis were entered into the Cox regression analysis, however, p factor (pleural invasion) was not contained in those variables to avoid double compensation as it is already reflected in the Tumor stage (pT). Probability values <0.05 were considered statistically significant in all of the analyses.

Results

Nectin-3 is highly expressed in epithelial cancer cells of human lung adenocarcinoma with various expression patterns

The expression of nectin-3 was examined in 127 lung adenocarcinomas and the adjacent normal lung tissues by IHC using the anti-human nectin-3 polyclonal antibody. In normal lung tissues, the expression of nectin-3 was not detected (Fig 1A a). In some tumor tissues, nectin-3 expression was observed in epithelial cancer cells, and was not observed in other tissues (Fig 1A b–c). The frequency of

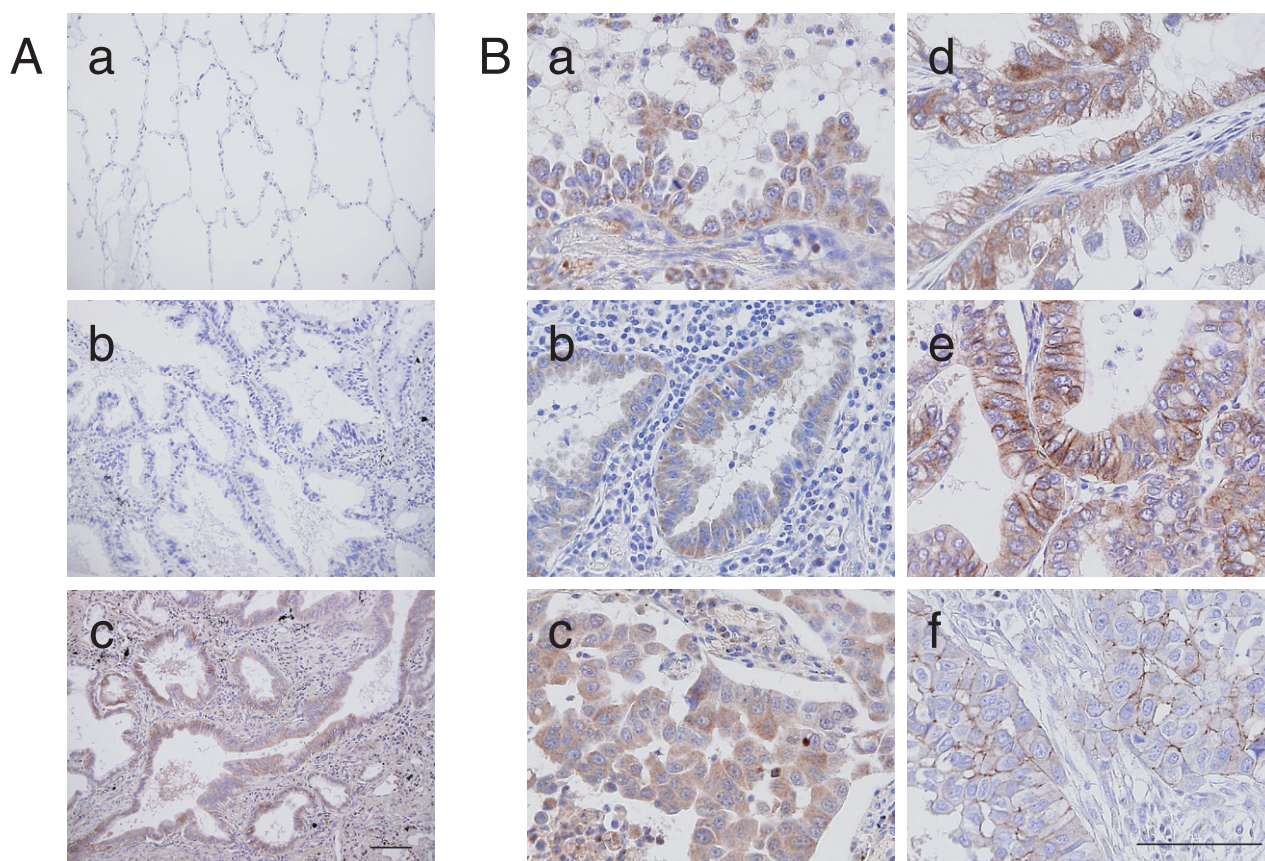


Figure 1 A. Nectin-3 is expressed in epithelial cancer cells of human lung adenocarcinoma. (a) Nectin-3-negative normal lung tissue; (b) Nectin-3-negative tumor tissue; (c) Nectin-3 widely expressing tumor tissue. B. Various expression patterns of nectin-3. (a–c) Cytoplasmic pattern. “White line” can be observed at the cell-cell contact site; (d–f) Membranous pattern. “Deep-brown line” can be observed at the cell-cell contact site; (e) Tumor tissue with cytoplasmic and membranous patterns. Pathological group: (a, d) papillary; (b, e) acinar; (c, f) solid. Bars, 100 μ m (A; x200 field, B; x600 field).

nectin-3 stained samples was 81.1% of all samples (103/127). This is the first report regarding the overexpression of nectin-3 in human primary tumor. Furthermore, various expression patterns of nectin-3 were observed; cytoplasmic (Fig 1B a–c) and membranous patterns (Fig 1B d–f). In some tumors, both cytoplasmic and membranous expression patterns were observed (Fig 1B e).

Membranous expression of nectin-3 is related to poor prognosis of patient survival of lung adenocarcinoma

Using the data collected on 127 study patients, we assessed the prognostic association with the expression patterns of nectin-3. We first assessed the overall survival and relapse-free survival of nectin-3 negative and positive groups (including cytoplasmic and membranous expression groups), but we observed no significance among them ($P = 0.675$ and $P = 0.823$, respectively; data not shown). Since nectin-3 is a member of Ig-like cell adhesion molecules, the most likely

explanation about this finding is that the membranous expression of nectin-3 is functional while others are not. The frequency of the samples with membranous (functional) expression of nectin-3 was 25.2% of all samples (32/127). The next survival analysis was performed by dividing 127 samples into membranous and non-membranous expression groups. Interestingly, we observed a significantly poorer prognosis in patients showing the membranous expression of nectin-3 (Fig 2a) compared with the non-membranous expression group. Moreover, they also showed significantly shorter time to relapse as well as death ($P = 0.006$; data not shown).

Univariate analysis and multivariate Cox regression analysis

The statistically significant clinical variables on univariate analysis are shown in Table 1. Membranous expression of nectin-3 correlated with higher p factor (pleural invasion), higher pT category, distant metastasis, and invasion to both artery and vein (all P -values < 0.05). All significant

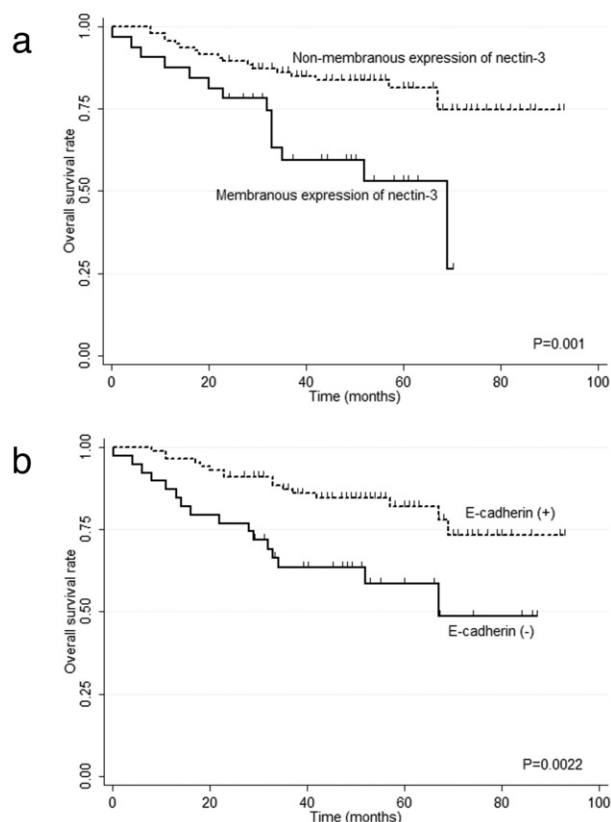


Figure 2 Kaplan-Meier plots for overall survival by the functional expression of nectin-3 (a) and E-cadherin (b). P-value by using log rank test.

clinicopathological and molecular variables from the univariate analyses were entered into the multivariate analysis. Data is presented in Table 2. The membranous expression of nectin-3 turned out to be an independent prognostic factor for survival ($P = 0.048$; hazard ratio, 2.181; 95% confidence interval [CI], 1.006–4.731). Invasion to artery tended towards a statistical significance ($P = 0.087$).

Altered expression of nectin-3 is related to tumor progression and malignancy

Next, we assessed whether the membranous expression of nectin-3 in tumor epithelial cells is physiological or not. Because nectins play a key role in the recruitment of E-cadherin to the cell-cell contact site, we analysed the expression of E-cadherin by IHC. Eighty-eight samples (69.3%) showed the membranous expression of E-cadherin, and 39 samples (30.7%) did not. The latter showed significantly poorer prognosis than the former for overall survival (Fig 2b) and relapse-free survival ($P = 0.0011$; data not shown). This result replicates previous reports.¹⁴

We then assessed co-localization of nectin-3 and E-cadherin in 127 samples by IHC. Interestingly, there were

various patterns of localization: nectin-3 co-localizes with E-cadherin (Fig 3a,d); nectin-3 is not expressed, but E-cadherin is expressed (Fig 3b,e); only nectin-3 is expressed (Fig 3c,f); and both nectin-3 and E-cadherin aren't expressed at some regions (data not shown). This result shows that some nectin-3 does not function physiologically, which suggests that such expression is pathological. Subsequently, using 32 samples that expressed membranous nectin-3, we conducted survival analysis for co-localization of nectin-3 and E-cadherin. It revealed that tumors without co-localization of nectin-3 and E-cadherin showed significantly poorer prognosis than others for overall survival (Fig 4A a) and relapse-free survival ($P < 0.05$; data not shown). Furthermore, in such samples, E-cadherin negative samples showed poorer prognosis (Fig 4A b). A schematic diagram of each group in Figure 4A b is shown in Figure 4B. These results suggest that there is an altered expression of nectin-3 in the epithelium of human lung adenocarcinoma, which is related to tumor progression and poor prognosis of patient survival.

Discussion

In this study, nectin-3 expression was observed in human tumor for the first time. Moreover, approximately 25% of

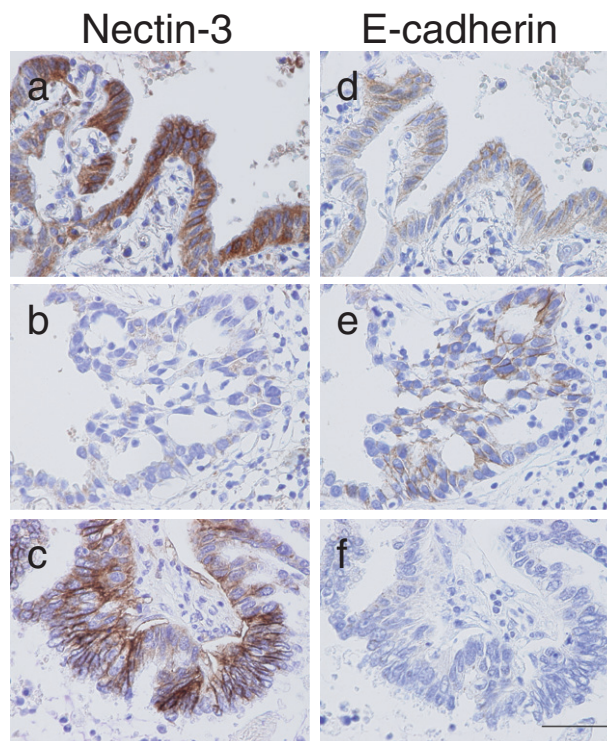


Figure 3 Co-localization of nectin-3 and E-cadherin in serial sections. (a, d) Nectin-3 co-localizes with E-cadherin; (b, e) Nectin-3 is not expressed, but E-cadherin is expressed. (c, f) Only nectin-3 is expressed. Bar, 50 μ m (x800 field).

Table 1 Prognostic clinicopathological variables as predictors for overall survival in 127 lung adenocarcinoma patients (univariate analysis; Fisher's Exact test)

Characteristics	Patients (n)	Nectin-3 expression		P-value
		Not membranous	Membranous	
Number of patients	127	95 (74.8%)	32 (25.2%)	
Age				
Mean (range)	67.8 (42–86)	67.9 (42–84)	67.5 (42–86)	0.832†
Gender				
Male/Female	72/55	51/44	21/11	0.165
Stage				
I/II/III, IV	83/12/31†	65/9/20	18/3/11	0.284
p factor				
0/1/2/3	86/21/12/8	72/13/8/2	14/8/4/6	0.001**
Tumor stage				
0/1/2/3	69/43/4/10†	56/31/0/7	13/12/4/3	0.007**
Node stage				
0/1/2/3	88/13/24/1†	68/9/16/1	20/4/8/0	0.576
Distant metastasis				
Absence/presence	123/3†	94/0	29/3	0.015*
Invasion to artery				
Absence/presence	100/25†	79/14	21/11	0.021*
Invasion to vein				
Absence/presence	73/52†	60/33	13/19	0.016*
Invasion to Ly vessel				
Absence/presence	75/50†	57/36	18/14	0.678

*P < 0.05; **P < 0.01. †P-value by using Student's *t*-test; missing †one or †two samples. Ly, lymphatic; p factor, pleural invasion.

lung adenocarcinoma specimens were classified as the membranous nectin-3 expression group by IHC staining, which showed poor prognosis of patient survival. Our results suggest that the altered expression of nectin-3 causes tumor progression and malignancy.

Recently, many groups have reported that disruption of cell-cell adhesion of tumor epithelial cells, resulting in EMT, caused tumor progression and was highly related to poor prognosis of cancer patients.^{4–6} The first step of EMT is disruption of AJs composed of the junction of E-cadherin. It has been unclear, however, whether nectins were involved in that process or not. We found that there were some tumors in which E-cadherin was absent, in spite of the presence of the membranous expression of nectin-3 (Fig 3c,f). Since nectin-3 is

involved in the formation of AJs by the recruitment of E-cadherin in physiological condition, we first considered it as “protective” factor in tumor progression. Unexpectedly, however, the survival rate of the patients who had such tumor was the poorest of the groups showing various co-localization patterns of nectin-3 and E-cadherin (Fig 4A b). This result suggests that the membranous expression of nectin-3 plays a malignant role in tumor progression. Furthermore, most interestingly, if nectin-3 co-localized with E-cadherin, such patients showed better prognosis compared with the other groups without co-localization of nectin-3 and E-cadherin, even if nectin-3 was expressed membranously. Thus, the membranous nectin-3 that doesn't have physiological function (recruitment of E-cadherin) may contribute to the tumor malignancy. Thinking about interaction partners of nectin-3, since necl-2 and necl-5 are involved in tumor suppression and tumor progression respectively, those molecules are likely to be the cause of malignant function of nectin-3.^{15,16}

It has also been controversial what causes the difference among the tissue types of human lung adenocarcinoma, such as bronchoalveolar carcinoma (BAC), papillary, acinar, and solid.¹ BAC is well known as non-invasive carcinoma, and shows better prognosis than other tissue types that are invasive carcinomas. Human lung adenocarcinoma is often composed of mixed components of these tissue types. We found that the membranous expression of nectin-3 was observed in 19.8% (17/86) of tumor specimens containing BAC

Table 2 Results of Cox regression analysis summarizing significant independent prognosis factors

Factors	Hazard ratio	95% CI	P-value
Membranous expression of Nectin-3	2.103	1.006–4.396	0.048*
p factor	1.341	0.862–2.087	0.194
Tumor stage	1.054	0.653–1.699	0.831
Invasion to artery	2.430	0.993–5.948	0.052
Invasion to vein	1.501	0.580–3.885	0.403
Distant metastasis	1.625	0.300–8.803	0.574

*P < 0.05. CI, confidence interval.

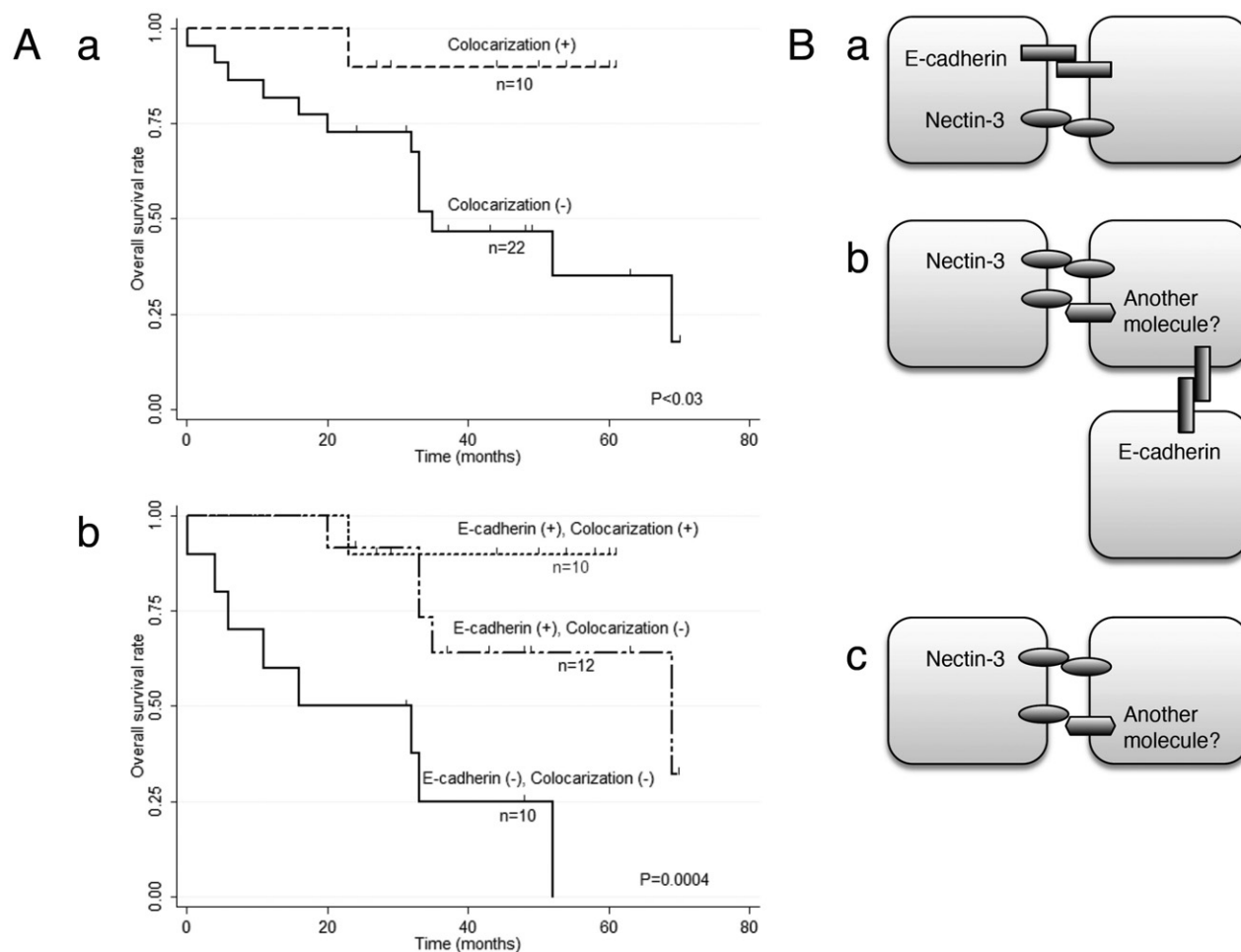


Figure 4 A. Kaplan-Meier plots for overall survival in 32 nectin-3 membranously expressing samples by (a) the colocalization of nectin-3 and E-cadherin or (b) both the colocalization and E-cadherin expression. P-value by using log rank test. B. Schematic diagram of colocalization of nectin-3 and E-cadherin in tumor epithelium. (a) Physiological junction: nectin-3 colocalizes with E-cadherin. (b) Intermediate state: both nectin-3 and E-cadherin is expressed, but the colocalization is absent. (c) Pathological state: nectin-3 is expressed, but E-cadherin is absent.

component, and in 37.5% (15/40) of tumor specimens not containing BAC component ($P=0.03$). In addition, the membranous expression of nectin-3 was significantly associated with the clinicopathological variables T stage (tumor size) and p factor (pleural invasion), $P=0.007$ and $P=0.001$, respectively (Table 1). These results suggested that membranous nectin-3 contributes to promoting tumor malignancy and progression in adenocarcinoma. However, the overall staging, a composition of the above factors, is not significantly associated with the pattern. The nodal (N) stage was also not associated with the Nect3 status. It might be the reason for the pattern.

Conclusion

Aberration of cell-cell junctions in tumor epithelium leads to tumor invasion and progression, which causes tumor malig-

nancy. In this study, the membranous expression of nectin-3 without E-cadherin co-localization was found to associate with poor prognosis of lung adenocarcinoma patients. Conversely, the membranous expression of nectin-3 with E-cadherin co-localization was found to associate with good prognosis of patients. Here, we present one hypothesis that aberration of strength of cell-cell adhesion triggers cell invasion. In epithelial cells with strong adhesion, cell motility and proliferation are suppressed, but once the adhesion is disrupted, in the case of cancer cells, epithelial cells with no shackles can move and proliferate, resulting in cancer invasion. However, if we consider that some extent of adherent strength is likely to be necessary, this will provide cells with the chance of interaction with each other and the motility along epithelium or stroma. Taken together, nectin-3 may act as a mediator on this point and contribute to tumor progression and malignancy by cooperating with other molecules

when it doesn't co-localize with E-cadherin. Although further *in vitro* and *in vivo* analyses are needed to confirm this hypothesis, this concept can contribute to understanding the basis of tumor biology and developing cancer therapies.

Disclosure

No authors report any conflict of interest.

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