Yonago Acta Medica 2023;66(1):19–23 doi: 10.33160/yam.2023.02.003 *Original Article*

Clinical Significance of Subcellular Localization of Maspin in Breast Carcinoma: An Immunohistochemical Study Using Two Different Antibodies

Makoto Wakahara,* Keiko Hosoya,*† Hiroshi Ishii† and Yoshihisa Umekita†

**Division of General Thoracic Surgery and Breast and Endocrine Surgery, Department of Surgery, School of Medicine, Faculty of Medicine, Tottori University, Yonago 683-8503, Japan, and †Department of Pathology, School of Medicine, Faculty of Medicine, Tottori University, Yonago 683-8503, Japan*

ABSTRACT

Background Maspin is known to be a tumor suppressor protein: however, its prognostic value in patients with breast cancer remains controversial. The key influential factors contributing to this complexity may be the differences in antibodies used, as well as the positive criteria and sample size. To date, no study has investigated the prognostic significance of maspin expression by using two different antibodies in the same cohort. We aimed to clarify whether differences in antibodies could influence on the prognostic value of maspin in breast cancer patients.

Methods Immunohistochemical analyses using an anti-maspin antibody (clone G167-70) were performed on 164 resected specimens of invasive carcinoma of no special type (NOS). The correlation with clinicopathological factors was compared to previous results using clone EAW24, with longer follow-up duration.

Results The subcellular localization of maspin expression was as follows: cytoplasmic-only staining, 3 cases (1.8%), pancellular staining, 43 cases (26.2%); and no staining, 118 cases (72.0%). No nuclear-only staining was observed. There was no significant correlation between clinicopathological characteristics and the pancellualr expression of maspin. The pancellular expression group showed a significantly longer disease-free survival (DFS) than the other groups ($P = 0.046$). When clone EAW24 was used, the cytoplasmic-only staining group showed significantly shorter DFS than the pancellular staining group ($P = 0.003$).

Conclusion Clone EAW24 may be superior to clone G167-70 in selecting breast carcinoma with an aggressive phenotype, while clone G167-70 may be superior to clone EAW24 in selecting non-aggressive breast carcinoma.

Key words breast carcinoma; immunohistochemistry; maspin

Maspin is a unique member of the serine protease inhibitor superfamily, and was originally identified as a tumor-suppressor protein expressed in the normal human mammary epithelium but is down-regulated during the progression of breast carcinoma.[1, 2] Maspin has tumor suppressive activity, including the inhibition of tumor growth, motility, invasiveness, angiogenesis and metastasis, and pro-apoptotic activities in breast cancer cell lines and animal models.[1, 3] However, we have previously reported that maspin was frequently observed in invasive carcinoma of no special type (NOS) with an aggressive phenotype characterized by high histological grade, negative hormone receptor, positive p53 status, and triple-negative subtype, and was an independent predictor of unfavorable prognosis.[4–6] While several reports supported our findings,[7–11] Kim *et al.* reported that maspin expression was not associated with overall and disease-free survival rate.[12] Mass *et al.* reported that a significant stepwise decrease in maspin expression occurred in the sequence of ductal carcinoma in situ-invasive cancer-lymph node metastasis.[2] However, we reported that maspin expression was up-regulated during the progression of mammary ductal carcinoma.[5] The key influential factors contributing to these complexities may be the differences in antibodies used, positive criteria, and sample size.

In the present study, we focused on the difference in antibodies, and aimed to clarify whether they could affect the prognostic value of maspin in patients with invasive carcinoma of NOS. To the best of our knowledge, this is the first study comparing the prognostic significance of two different anti-maspin antibodies in the same cohort.

MATERIALS AND METHODS

Patients and tumor specimens

The cohort was the same as those reported previously,[13] except for the follow-up period. Briefly, 164 consecutive female patients underwent surgical resection of breast cancer diagnosed as invasive carcinoma of NOS according to the World Health Organization classification of

Corresponding author: Yoshihisa Umekita, MD, PhD
yume@tottori-u.ac.jp
Received 2022 October 31
Accepted 2022 November 28
Online published 2023 January 16
Abbreviations: CI, confidence interval; EMT, epithelial mesenchymal transition; TGFβ, transforming growth factor β

breast cancer[14] at Tottori University Hospital (Yonago, Japan). Estrogen receptor (ER)-positive or progesterone receptor (PgR)-positive status was defined as ≥ 1% immunoreactive cells.[13] HER2-positive status was determined based on the Hercep Test (Dako Agilent Technology, CA) scored 3+. Cases that scored 2+ were considered as HER2-positive when the presence of HER2 amplification was detected through fluorescent in situ hybridization analysis using PathVysion kit (Abott-Vysis, Inc., Downers Grove, IL). Histological grades were determined according to the criteria of Elston and Ellis.[15] Written informed consent was obtained from the patients. This study was approved by the ethics committee of the Faculty of Medicine, Tottori University (Approval No. : 20A153; December 23, 2020).

Immunohistochemistry

After the sections (4 μm-thick) were deparaffinized and endogenous peroxidase activity was blocked, they were pretreated in citrate buffer (0.01 M, pH 6.0) in a microwave oven for 15 min. Subsequently, the sections were incubated at 4 °C overnight with a monoclonal antibody against maspin (clone G167-70, diluted 1:1000; BD Pharmingen, NJ). The visualization procedure was the same as that described previously.[13]

Evaluation of the immunohistochemical findings

Immunohistochemical findings were evaluated as described previously.[5] Briefly, the subcellular localization of maspin was classified into four categories: cytoplasmic-only, pancellular (combined nuclear and cytoplasmic), nuclear-only, and no staining. All slides were evaluated independently by M. W. and Y. U., who were blinded to the patients' clinicopathological data.

Statistical analysis

All statistical analyses were performed using IBM SPSS Statistics, version 25 (IBM Corporation, Armonk, NY). The association between maspin expression status and clinicopathological factors was evaluated using non-parametric tests. The chi-square test was used when there were two categorical variables of interest. For survival analysis, cancer relapse (local recurrence or distant metastatic recurrence) was used to calculate the disease-free survival (DFS). DFS was defined as the period from the date of the initial surgery to the date of clinical or pathological cancer relapse. While calculating DFS, patients were censored at the time of their last cancer-free follow-up or at the time of death due to a reason unrelated to breast cancer. Survival curves were computed based on the Kaplan–Meier methods, and differences in DFS were tested using the log-rank test or Kruskal-Wallis test. All tests were two-sided, and statistical significance was set at < 0.05.

RESULTS

Immunohistochemistry

The subcellular localization of maspin expression was as follows: cytoplasmic-only staining, 3 cases (1.8%); pancellular staining, 43 cases (26.2%); and no staining, 118 cases (72.0%). No nuclear-only staining was not observed. Representative staining patterns of maspin expression in invasive carcinoma of NOS are shown in Fig. 1. The subcellular localization of maspin expression is shown in Table 1, compared to the result using clone EAW24 described previously.[13]

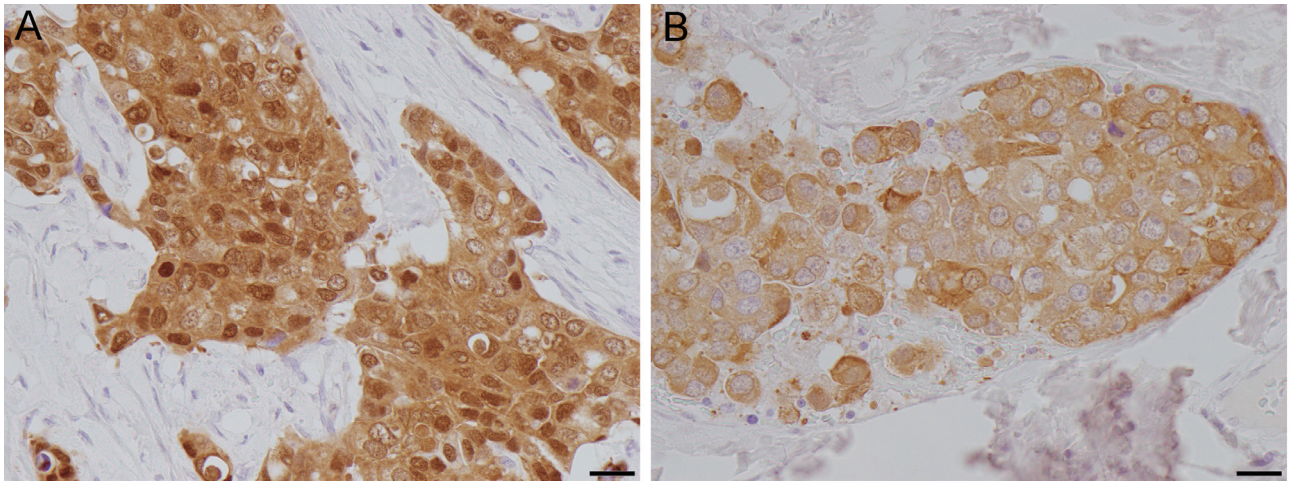

Fig. 1. Immunohistochemical staining patterns of maspin in invasive carcinoma of no special type using clone G167-70. **A**: Pancellular (combined nuclear and cytoplasmic) expression of maspin. Bar = 100 μm. **B**: Cytoplasmic-only expression of maspin. Bar = 100 μm.

Table 1. The difference of subcellular localization of maspin between clone EAW24 and clone G167-70

	Clone of anti-maspin antibody	
Subcellular localization	EAW24	G167-70
Cytoplasmic-only	50	3
Pancellular	23	43
Nuclear-only	0	0
No stain	91	118

Association between clinicopathological characteristics and subcellular localization of maspin

The relationship between the clinicopathological features and subcellular localization of maspin is summarized in Table 2. There was no significant correlation between clinicopathological characteristics and the pancellular expression of maspin.

Survival analysis according to maspin expression status

The median follow-up duration was 121 months (range = 9–162 months). During this period, 22 patients (13.4%) experienced locoregional recurrence and/or distant recurrence. For the survival analysis, we classified the expression of maspin into two groups because there were few cytoplasmic-only groups. The DFS curves are shown in Fig. 2. The 5-year DFS rates for the pancellular staining group and the combined no stain and cytoplasmic-only staining groups were 97.4% [95% confidence interval (CI) = 0.832–0.996] and 87.6% (95% CI = 0.800–0.925), respectively. According to the log-rank test, the pancellular expression group showed significantly longer DFS than the other groups (P = 0.046). The DFS curve for patients when clone EAW24 was used is illustrated in Fig. 3. The 5-year DFS rates in the cytoplasmic-only staining group, pancellular staining group and no staining groups were 86.9% (95% CI = 0.730–0.939), 95.7% (95% CI = 0.729–0.994%), and 90.5% (95% CI = 0.818–0.951%), respectively. The Kruskal-Wallis test showed that the cytoplasmic-only staining group had significantly shorter DFS than the pancellular staining group (P = 0.003).

DISCUSSION

Previously, we generated polyclonal anti-maspin antibodies using a peptide antigen consisting of a 15 amino acid peptide corresponding to amino acids 118-132 of human maspin cDNA at the same time of the generation of anti-rat maspin antibodies.[16] We started the immunohistochemical investigation of maspin protein expression in human breast cancer specimens.

Table 2. Association between subcellular localization of maspin (clone G167-70) and clinicopathological characteristics of 164 invasive carcinomas of no special type

Factors	Total	Expression of maspin		
		Pancellular	Others	P-Value
	(n = 164)	(n = 43)	(n = 121)	
Age (year)				
≤ 50	48	12	36	0.819
> 50	116	31	85	
Pathological T (mm)				
≤ 20	125	33	92	0.925
> 20	39	10	29	
Histological grade				
I	34	9	25	0.117
II	84	17	67	
III	46	17	29	
Lymph node metastasis				
Present	44	11	33	0.830
Absent	120	32	88	
Estrogen receptor				
Positive	142	38	104	0.689
Negative	22	5	17	
Progesterone receptor				
Positive	126	31	95	0.391
Negative	38	12	26	
HER2 status				
Positive	27	8	19	0.659
Negative	137	35	102	
Ki67 labeling index				
< 30%	121	28	93	0.133
≥ 30%	43	15	28	
Chemotherapy				
None	110	28	82	0.751
Performed	54	15	39	

During this investigation, a monoclonal anti-human maspin antibody (clone EAW24) became commercially available, and this antibody showed a similar staining pattern to the polyclonal antibody generated by us, and a clearer staining pattern than the polyclonal antibody. Consequently, we used clone EAW24 and showed that cytoplasmic-only expression of maspin was an independent predictor of unfavorable prognosis in patients with breast,[4, 13] lung[17, 18] and oral carcinoma.[19] In breast carcinoma, however, many investigators used different

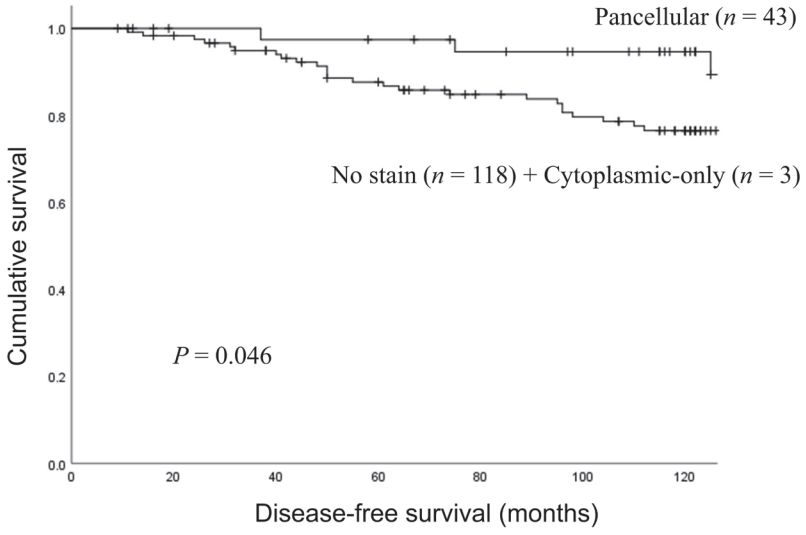

Fig. 2. Kaplan-Meier survival curve for disease-free survival of the 164 patients according to the subcellular localization of maspin expression (pancellular vs. combined no stain and cytoplasmic-only groups) using clone G167-70.

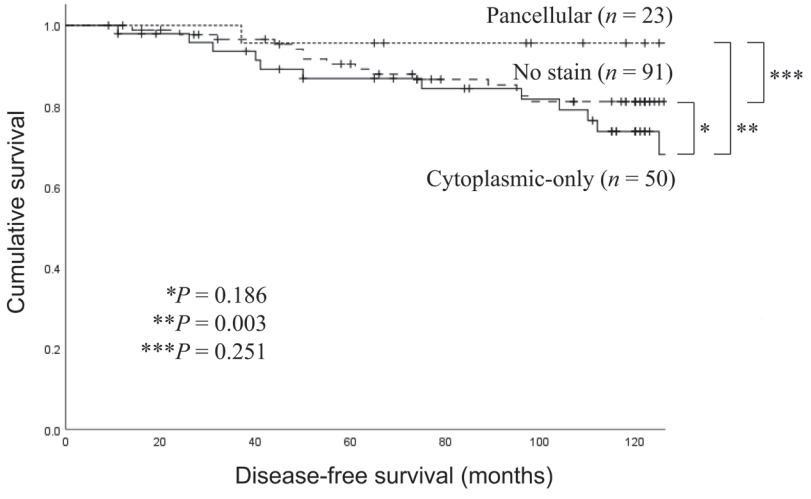

Fig. 3. Kaplan-Meier survival curve for disease-free survival of the 164 patients according to the subcellular localization of maspin expression (cytoplasmic-only, pancellular, and no staining groups) using clone EAW24.

anti-maspin antibodies (clone G167-70),[2, 7, 8, 10, 12] and only two groups used clone EAW24, except for our group.[9, 11] Therefore, we compared the prognostic value of these two antibodies in the same human breast carcinoma cohort. In the previous and present studies which used clone EAW24, the cytoplasmic-only subgroup showed significantly higher histological grade, negative PgR status and shorter DFS than the pancelllar subgroup. When clone G167-70 was used, pancellular subgroup showed longer DFS than the combined no staining and the cytoplasmic-only groups. This discrepancy may be attributable to the difference in the number between the cytoplasmic-only and pancelluar subgroups. In the largest series, Mohsin *et al*. reported that cytoplasmic staining showed some nuclear staining in all but nine of the cases using clone G167-70, and very few cases were considered to be cytoplasmic-only,[8] that was consistent with our results. On the other hand, Goulet *et al.* reported that nuclear localization of maspin is required to suppress tumor growth and metastasis, and that tumor cells expressing nucleus-excluded cytoplasmic-only maspin were more metastatic than the

controls in an *in vivo* model.[20] Their speculation that the cytoplasmic-only expression of maspin may be correlated with an aggressive phenotype is consistent with our immunohistochemical studies.[4–6, 13] Moreover, we reported that cytoplasmic maspin enhances the invasive and metastatic potential in breast cancer cells with aggressive phenotype by inducing EMT via Serglycin/TGFβ axis.[21] Considered together, these findings suggest that clone EAW24 may be superior to clone G167-70 for selecting breast carcinoma with an aggressive phenotype, while clone G167-70 may be superior to clone EAW24 for selecting breast carcinoma with a non-aggressive phenotype.

In conclusion, we demonstrated, for the first time, that the prognostic value of maspin expression was affected by the difference in antibodies in the same cohort; therefore, the characteristics of antibodies must be taken into account when evaluating the prognostic significance of maspin expression.

Acknowledgments: The authors thanks Kazuko Fukushima for their excellent technical assistance in processing of the pathological specimens. This study was supported by JSPS KAKENHI (grant number: JP21K16398).

The authors declare no conflict of interest.

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