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LEA.135 EXPRESSION: AN INDEPENDENT AND FAVORABLE PROGNOSTIC BIOMARKER FOR PATIENTS WITH PRIMARY INVASIVE BREAST CANCER

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The prognostic significance of LEA.135 expression, detected by immunohistochemistry in formalin-fixed and paraffin-embedded tissue sections, was evaluated and compared with the widely utilized clinicopathological parameters for patients with primary invasive breast carcinomas. Pathological parameters such as tumor size, histological tumor type, histological grade, nuclear grade, lymph node (LN) status, bone marrow (BM) status, as well as age of patient at initial diagnosis together with follow-up in years were available for this group of patients ($n = 178$). Among these parameters, tumor size, histological tumor type, histological grade, LN status, and BM status were individually and significantly associated with increased probability of recurrence by univariate analysis. By multivariate analysis, however, only tumor size, LN status, and BM status remained statistically significant. LEA.135-positive patients showed a statistically significant probability of not recurring ($77 \pm 5\%$ at 5 years after surgery) compared with patients who were LEA.135-negative ($49 \pm 6\%$ at 5 years after surgery) (log-rank $p < 0.001$). Furthermore, the association remained statistically significant by multivariate analysis (log-rank $p = 0.019$), demonstrating that LEA.135 expression independently and significantly identified breast cancer patients with favorable clinical outcome. In addition, there was a statistically significant association between loss of LEA.135 expression and poor prognosis when patients were stratified by pathological parameters. Furthermore, a subgroup of patients who were LEA.135-positive/LN-negative experienced a decreased rate of recurrence compared with those who were LEA.135-negative/LN-negative (16% vs. 27%, respectively). A similar result was also obtained when BM-negative patients were stratified on the basis of LEA.135-positive or LEA.135-negative subgroups for recurrence (18% vs. 43%, respectively). Most interestingly, the patients whose cancer cells were LEA.135-positive/LN-positive experienced a much lower rate of recurrence than those whose cells were LEA.135-negative/LN-positive (29% vs. 57%, respectively). The results clearly demonstrate that LEA.135 expression was a significantly independent and favorable prognostic marker for patients with primary invasive breast carcinoma by both univariate and multivariate analyses. *Int. J. Cancer (Pred. Oncol.)* 89:224–229, 2000.

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A monoclonal antibody (MAb) was generated by immunization of BALB/c mice with extracts of normal breast tissues following prior immunotolerization with breast carcinoma cell lines. This approach facilitated the identification of molecules in normal cells or the cells that maintain some of the normal cell function during progression of cancer. Utilizing an immunotolerization/immunization technique (Imam *et al.*, 1990a, 1994a), an antibody-producing hybridoma was cloned. The antibody identified an apical plasma membrane-associated sialoglycoprotein with some of the desired properties, termed luminal epithelial antigen (LEA.135) (Imam *et al.*, 1994b). Competitive blocking and immunoprecipitation studies had shown that LEA.135 was distinct from other known epithelial cell-associated antigens, including the family of mucin, keratin, and epidermal growth factor receptor (Imam *et al.*, 1994b). However, the biological function and mode of regulation of LEA.135 expression remain unclear.

The pattern of expression of LEA.135 was determined by immunohistochemical staining in both frozen and formalin-fixed/

paraffin-embedded tissue sections (Imam *et al.*, 1993, 1996). LEA.135 expression was detectable on the apical plasma membrane of luminal epithelial cells in normal breast tissues (Imam *et al.*, 1993, 1994a). In primary breast carcinoma, LEA.135 was not detected in some cases but was present in others, irrespective of the morphological appearance of the tumor cells, suggesting that LEA.135 expression might have prognostic value. Based on the above observation, a preliminary retrospective study was conducted on a small cohort of patients with primary invasive breast carcinoma, to investigate the possible prognostic value of LEA.135 expression. LEA.135 expression was associated with a significantly decreased rate of recurrence and an increased overall survival, independent of size, histological grade of tumor, and patient age (Imam *et al.*, 1996).

Our goal was to further evaluate the prognostic value of LEA.135 expression in a large series of well-characterized breast cancer patients ($n = 178$) with a median follow-up of 5.2 years. Our results confirm and extend the conclusion of the previous studies on a smaller number of patients and clearly demonstrate that LEA.135 expression is independently and significantly associated with a favorable prognosis for patients with primary invasive breast carcinoma.

MATERIAL AND METHODS

Patient population

Breast tissue sections from 178 patients with primary invasive breast carcinoma who had undergone mastectomy were obtained from the Ludwig Institute for Cancer Research, London, UK. The following data were available for this series of patients: age at initial diagnosis, size of tumor, histological type, histological grade, nuclear grade, histological lymph node (LN) status based on detection of metastatic cells by hematoxylin–eosin (H&E) staining, bone marrow (BM) status based on detection of metastatic cells by immunohistochemical staining with anti-EMA antibody (Dearnaley *et al.*, 1981; Berger *et al.*, 1988), and time to develop recurrence. However, the data on treatment of patients were unavailable. At the conclusion of the study, the median follow-up was 5.2 years and 64 (36%) patients had developed recurrence. The distribution of clinical and pathological features of the patients is outlined in Table I. Prior to immunostaining, an adjacent tissue section of each case was stained with H&E and reviewed independently by 2 authors (WYN and SAI) for consistency of the original observation of histological tumor type, histological grade, and nuclear grade. The original observation of the characteristics of the specimens was confirmed. Histological classification of

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TABLE I – CLINICOPATHOLOGICAL FEATURES OF TUMOR FROM PATIENTS IN RELATION TO STATUS OF LEA.135 EXPRESSION

Factors	Total number of patients	Number of LEA.135-positive patients (%)	Number of LEA.135-negative patients (%)
All patients	178	85 (47.8)	93 (52.2)
Age (years)			
≤49	41	20 (48.8)	21 (51.2)
50–59	50	23 (46.0)	27 (54.0)
≥60	85	40 (47.1)	45 (52.9)
Unknown	2	2 (100)	0 (0)
Size (mm)			
≤20	72	34 (47.2)	38 (52.8)
21–50	85	43 (50.6)	42 (49.4)
≥51	12	5 (41.7)	7 (58.3)
Unknown	9	3 (33.3)	6 (66.7)
Histological tumor type			
IDC ¹	166	78 (47.0)	88 (53.0)
Other	12	7 (58.3)	5 (41.7)
Histological grade			
1	1	1 (100)	0 (0)
2	53	26 (49.1)	27 (50.9)
3	124	58 (46.8)	66 (53.2)
Nuclear grade			
1	9	7 (77.8)	2 (22.2)
2	137	63 (46.0)	74 (54.0)
3	32	15 (46.9)	17 (53.1)
LN metastasis			
No	57	31 (54.4)	26 (45.6)
Yes	98	42 (42.9)	56 (57.1)
Unknown	23	11 (47.8)	12 (52.2)
BM micrometastasis			
No	131	71 (54.2)	60 (45.8)
Yes	35	10 (28.6)	25 (71.4)
Unknown	12	4 (33.3)	8 (66.7)

¹IDC, infiltrating ductal carcinoma.

breast cancer tissue was determined according to the criteria of Bloom and Richardson (1957).

Immunohistochemical staining

Routinely processed, formalin-fixed/paraffin-embedded tissue sections were analyzed for LEA.135 immunoreactivity with the ABC indirect immunohistochemical method, as described previously (Imam *et al.*, 1996). Briefly, after deparaffinization, anti-LEA.135 mouse MAb (0.1 mg/ml) was applied to 5-μm-thick tissue sections for 1 hr (Imam *et al.*, 1996). Biotinylated horse anti-mouse immunoglobulin was added as secondary linking antibody for 30 min, followed by ABC for 30 min. Sections were rinsed 3 times with PBS after each step. Aminoethyl carbazole was used as the chromogen, and tissue sections were counterstained with Mayer's hematoxylin. For each experiment, both positive and negative controls were included. Tissue sections containing normal breast cells and the uninvolved breast cells in the experimental cases served as positive controls and internal positive controls, respectively. The primary antibody, pre-absorbed with the immunogen (1 mg/ml of purified LEA.135), was used as a negative control.

Evaluation of immunohistochemical staining

LEA.135 immunoreactivity was reviewed independently by 3 investigators (DL, SAI, and WYN) in a blind fashion. The criterion of selecting the cut-off point of 10% LEA.135-positive cells for statistical analysis was determined to be optimal, as described previously (Imam *et al.*, 1996).

Statistical analysis

Recurrence was the primary outcome of the study, measured from the date of mastectomy to the date of breast cancer recurrence or the last date of follow-up.

Probability-of-recurrence plots were drawn using the Kaplan-Meier method. For univariate analysis, log-rank tests were performed to investigate the association of LEA.135 expression and

other clinicopathological parameters with recurrence. A Cox proportional hazards model for the risk ratio was used to assess the simultaneous contribution of the following covariates: histological diagnosis, histological grade, nuclear grade, LN status, and BM status. For multivariate analysis, stepwise selection of variables was used to determine whether LEA.135 expression was an independent prognostic factor.

RESULTS

Immunohistological localization of LEA.135

Using immunohistochemical staining methods, LEA.135 expression was detected predominantly on the apical plasma membrane of luminal epithelial cells lining the ducts or lobules in normal breast tissue (Fig. 1). Normal ducts or lobules as well as breast epithelium in case of hyperplasia exhibited strong LEA.135 expression and served as internal positive controls in most specimens. LEA.135 expression was found in 85/178 (47.8%) patients (Table I). In breast cancer patients without BM micrometastasis, 54.2% cases showed LEA.135 expression, whereas in those with BM micrometastasis, 28.6% were LEA.135-positive. Similarly, the difference was also observed in patients with and without LN metastasis (42.9% vs. 54.4%, respectively).

Association of LEA.135 expression with prognosis by univariate analysis

In the cohort of 178 patients with primary invasive breast carcinomas, tumor size, LN metastasis, BM micrometastasis, and histological grade were individually and significantly associated with probability of recurrence by univariate analysis, whereas age at initial diagnosis, histological type, and nuclear grade were not associated (Table II). A comparison of the probability of recurrence was made between patients whose cancer cells were LEA.135-positive (Fig. 2a) and those who were LEA.135-negative (Fig. 2b). A statistically significant univariate association was observed between loss of LEA.135 expression and increased prob-

FIGURE 1 – Immunohistochemical localization of LEA.135 expression in normal breast epithelial cells. Formalin-fixed paraffin-embedded tissue section was stained with anti-LEA.135 MAb. The plasma membrane of normal luminal epithelial cells surrounding the ducts shows predominant expression of LEA.135, while myoepithelial cells, lymphocytes, and other stromal cells are unstained. The tissue section was counterstained with Mayer's hematoxylin. Original magnification $\times 250$.

TABLE II – ASSOCIATION OF PROGNOSTIC FACTORS WITH PROBABILITY OF RECURRENCE BY UNIVARIATE ANALYSIS

Factors	Relative risk ¹	Probability of recurring at 5 years after surgery	<i>p</i> value ²	Prognostic value of LEA.135 expression in subsets of patients (<i>p</i> value ³)
LEA.135 expression	2.38		<0.001	
Positive		0.23 $\pm$ 0.05		
Negative		0.51 $\pm$ 0.06		
Age (years)			0.906	
≥ 60		0.38 $\pm$ 0.06		<0.001
50–59	1.13	0.42 $\pm$ 0.08		0.400
≤ 49	1.01	0.33 $\pm$ 0.07		0.033
Size (mm)			<0.001	
≤ 20		0.25 $\pm$ 0.05		0.008
21–50	2.46	0.46 $\pm$ 0.06		0.081
≥ 51	5.65	0.70 $\pm$ 0.14		0.309
Histological tumor type	1.32		0.554	
IDC ³		0.38 $\pm$ 0.04		0.002
Other		0.44 $\pm$ 0.15		0.280
Histological grade	2.00		0.023	
≤ 2		0.25 $\pm$ 0.06		0.107
3		0.44 $\pm$ 0.05		0.004
Nuclear grade	1.36		0.375	
3		0.29 $\pm$ 0.08		0.013
≤ 2		0.40 $\pm$ 0.05		0.011
LN metastasis	2.49		0.004	
No		0.27 $\pm$ 0.06		0.368
Yes		0.46 $\pm$ 0.06		0.002
BM micrometastasis	2.16		0.004	
No		0.32 $\pm$ 0.05		0.001
Yes		0.54 $\pm$ 0.09		0.947

¹Relative risk can be thought of as the average increased chance of recurring at any point in time for patients in the second group compared to those in the first group. It is the ratio of the ratio in the preceding column. The group with better prognosis is listed first. ²Based on log-rank test. ³IDC, infiltrating ductal carcinoma.

FIGURE 2 – Immunohistochemical localization of LEA.135 expression in patients with primary invasive breast carcinoma. Formalin-fixed/paraffin-embedded tissue sections were stained with anti-LEA.135 Mab. The specimen is from an LEA.135-positive patient who did not experience recurrence (a), whereas patients with recurrence showed no LEA.135 expression (b). Cells in connective tissue were negative for LEA.135 expression. Sections were counterstained with Mayer's hematoxylin. Original magnification $\times 400$.

FIGURE 3 – Kaplan-Meier plot of the probability of recurrence. Probability of recurrence of patients whose breast cancer cells were LEA.135-positive vs. LEA.135-negative. Time was measured from surgery to last follow-up in years.

ability of recurrence (log-rank $p < 0.001$). The probability of recurrence among LEA.135-positive patients at 5 years after surgery was $23 \pm 5\%$, whereas among LEA.135-negative patients it was $51 \pm 6\%$ (Fig. 3).

Next, LEA.135-positive and LEA.135-negative patients were stratified according to age, tumor size, histological type, histological grade, nuclear grade, LN status, and BM status. A statistically significant association was observed between LEA.135 expression and favorable prognosis in subsets of patients younger than 49 years (log-rank $p = 0.033$) or older than 60 years (log-rank $p < 0.001$) with tumor size < 20 mm (log-rank $p > 0.008$), infiltrating ductal carcinomas (log-rank $p = 0.002$), histological grade 3 (log-rank $p = 0.004$), nuclear grade 2 (log-rank $p = 0.011$) or 3 (log-rank $p = 0.013$), LN metastasis (log-rank $p = 0.002$), or without BM metastasis (log-rank $p = 0.001$) (Table II). However, LEA.135 expression showed no statistically significant prognostic predictive value for the subset of patients with BM metastasis (log-rank $p = 0.947$). For the subgroup of patients aged 50 to 59 years with tumor size 21 to 50 mm or larger than 50 mm without LN metastasis, LEA.135 expression showed a trend associated with favorable outcome, though the association failed to reach statistical significance (Table II).

When LEA.135 expression and LN status or BM status were combined to stratify this series of patients, the most favorable group (LEA.135-positive/LN-negative or LEA.135-positive/BM-negative), the intermediately favorable group (LEA.135-positive/LN-positive, LEA.135-negative/LN-negative or LEA.135-positive/BM-positive, LEA.135-negative/BM-negative), and the least favorable group (LEA.135-negative/LN-positive or LEA.135-neg-

ative/BM-positive) with a decreased possibility of recurrence were identified. The difference was statistically significant (Table III).

Association of LEA.135 expression with prognosis by multivariate analysis

Multivariate analysis was performed by incorporating LEA.135 expression status with the clinicopathological factors, such as tumor size, histological grade, metastasis to LN, and micrometastasis to BM, that were individually and significantly associated with an increased probability of recurrence by univariate analysis (Table II). A statistically significant association was obtained between LEA.135 expression and a decreased probability of recurrence (log-rank $p = 0.019$, risk ratio 2.04), demonstrating that LEA.135 expression was an independently favorable prognostic biomarker (Table IV). Among clinicopathological parameters, tumor size of 21 to 50 mm (log-rank $p < 0.001$, risk ratio 2.45) or greater than 50 mm (log-rank $p < 0.001$, risk ratio 4.02), LN metastasis (log-rank $p < 0.001$, risk ratio 1.95), and BM micrometastasis (log-rank $p < 0.001$, risk ratio 1.45) were also independent prognostic predictors of an increased probability of recurrence (Table IV). Although histological grade, which was significantly associated with an increased probability of recurrence by univariate analysis, was not significant by multivariate analysis (log-rank $p = 0.319$, risk ratio 1.40) (Table IV).

DISCUSSION

The present study was conducted on a series of well-characterized patients with primary invasive breast cancer. The clinical and pathological records included tumor size, histological type, histological grade, nuclear grade, LN status, BM status, and age at diagnosis and follow-up in years. Consistent with the previously published reports of patients with breast cancer, tumor size (Carter *et al.*, 1989) histological grade (Henson, 1988; Elston and Ellis, 1991), LN metastasis (Nemoto *et al.*, 1980), and BM micrometastasis (Diel *et al.*, 1992; Mansi *et al.*, 1991) were significant predictors for this group of patients.

Although LN metastasis was an independent prognostic predictor of recurrence for breast cancer patients in the present study, evaluation of metastasis is laborious and time-consuming. Meanwhile, it is widely believed that some occult metastasis are overlooked in routine H&E-stained slides (Fisher *et al.*, 1978; Nasser *et al.*, 1993; Wilkinson *et al.*, 1982). Another independent prognostic marker determined by the present study is the immunolocalization of micrometastasis in BM, a procedure that is equally time-consuming and laborious, requiring special preparation of BM specimens and identification of occasional positive cells among millions of negative lymphocytes (Berger *et al.*, 1988; Diel *et al.*, 1992). Furthermore, the antibodies utilized for the detection of micrometastatic epithelial cells have been reported to cross-react with lymphocytes in BM and LN, leading to false-positivity (Diel *et al.*, 1992; Fisher *et al.*, 1978).

TABLE III – PROGNOSIS OF SUBGROUPS OF BREAST CANCER PATIENTS STRATIFIED BY THE COMBINATION OF LEA.135 AND LN STATUS OR BM STATUS

Subgroup of patients	Number of patients	Number of patients recurred (%)	Probability of recurring at 5 years after surgery	<i>p</i> value ¹
Combination of LEA.135 expression and LN status				<0.001
LEA.135 ⁺ /LN ⁻	31	5 (16.1)	0.16 ± 0.08	
LEA.135 ⁺ /LN ⁺	42	12 (28.6)	0.27 ± 0.08	
LEA.135 ⁻ /LN ⁻	26	7 (27.0)	0.29 ± 0.09	
LEA.135 ⁻ /LN ⁺	56	32 (57.1)	0.62 ± 0.07	
Combination of LEA.135 expression and BM status				<0.001
LEA.135 ⁺ /BM ⁻	71	13 (18.3)	0.18 ± 0.05	
LEA.135 ⁺ /BM ⁺	10	6 (60.0)	0.52 ± 0.16	
LEA.135 ⁻ /BM ⁻	60	26 (43.3)	0.49 ± 0.07	
LEA.135 ⁻ /BM ⁺	25	14 (56.0)	0.55 ± 0.10	

¹Based on log-rank test.**TABLE IV** – ASSOCIATION OF PROGNOSTIC FACTORS WITH PROBABILITY OF RECURRENCE BY MULTIVARIATE ANALYSIS

Factors	Relative risk	<i>p</i> value ¹
LEA.135		0.019
Positive	1.00 ²	
Negative	2.04	
Histological grade		0.318
≤2	1.00 ²	
3	1.40	
Size (mm)		<0.001
≥20	1.00 ²	
21–50	2.45	
≥51	4.02	
LN metastasis		<0.001
Negative	1.00 ²	
Positive	1.95	
BM micrometastasis		<0.001
Negative	1.00 ²	
Positive	1.45	

¹Based on 142 patients without missing values for each factor by the likelihood ratio χ^2 test. The final model includes the variables of LEA.135, LN status, BM status, and tumor size, which were significant at the 0.05 level by univariate analysis. ²Reference group.

In this report, a newly identified cell-surface sialoglycoprotein, LEA.135, was evaluated for its prognostic value in this cohort of patients. Our results clearly demonstrate that LEA.135 expression is an independent and favorable prognostic marker for patients with invasive primary breast cancer by both univariate and multivariate analyses. LEA.135-positive patients had a decreased probability of recurrence compared with LEA.135-negative patients. The discriminatory effect was particularly evident in patients with high histological grade of tumors, without BM micrometastasis, or

with LN metastasis. However, LEA.135 expression was not a significant prognostic predictor in patients without LN metastasis. This is not consistent with the results of a previous study conducted on a much smaller number of patients in this subset (Imam *et al.*, 1996). A much larger number of patients in this subset in the current study may have contributed to the contrasting results. In this cohort, only 1 patient had tumor with histological grade 1, while 53 and 124 patients exhibited grade 2 and 3 tumors, respectively. Furthermore, a rather high percentage of patients were positive for LN metastasis. Therefore, the clinicopathological features of this group were not characteristically reflective of the usual mix of histological types encountered by surgical pathologists. However, our investigation has confirmed that LEA.135 expression is independently and significantly associated with a favorable clinical outcome in this group of patients.

Cancer is a multistep, genetic process, in which the accumulation of multiple alterations produces the malignant phenotype (Fearon and Vogelstein, 1990). In this context, alterations in the LEA.135 molecule and other prognostic markers, such as p53, HER-2/neu, or Ki-67 (Friedrichs *et al.*, 1993; Marks *et al.*, 1994; Descotes *et al.*, 1993), may act synergistically in the progression of breast cancer. Therefore, analysis of a combination of contrasting biomarkers on the same series of patients may provide a more accurate assessment of their usefulness for the identification of high-risk patients.

Partial or complete loss of LEA.135 expression might result from mutation or inactivation of the *LEA.135* gene, which needs to be explored in future studies. From the functional point of view, LEA.135 expression in breast cancer cells appears to reflect the ability of these cells to maintain some degree of functional differentiation, leading to residual responsiveness to growth control. This hypothesis is indirectly supported by the observations made in this study.

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