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Differential cellular localization of CELSR2 and ING4 and correlations with hormone receptor status in breast cancer

Liejun Jiang[1]*, Xiliu Zhang[2]*, Chenglin Xiang[3], Joseph Geradts[4]Δ, Qiang Wei[5], Yuanzi Liang[6], Huayi Huang[1,7] and Jun-Fa Xu[6]

[1]Department of Laboratory Medicine, the People's Hospital of Guangxi Zhuang Autonomous Region, [2]Department of Pathology, the First Affiliated Hospital of Guangxi Traditional Chinese Medicine, Nanning, Guangxi, [3]Department of Pathology, Huayin Medical Laboratory, Guangzhou, Guangdong, China, [4]Department of Pathology, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts, USA, [5]Department of Pathology, the People's Hospital of Guangxi Zhuang Autonomous Region, Nanning, Guangxi, China, [6]Institute of Laboratory Medicine, Guangdong Medical University, Dongguan, Guangdong, China and [7]Department of Surgical Oncology, Roswell Park Cancer Institute, Buffalo, New York, USA

ΔPresent address: Multi-Scale Translational Research Core Laboratory, Department of Population Sciences, City of Hope National Medical Center, Duarte, California, USA

*These authors contributed equally to this work

Summary. CELSR2 is postulated to be a receptor involved in contact-mediated communication; however, its expression and function in cancer remain unknown. ING4 is a tumor suppresor encoded by the *ING4* gene which inhibits cell growth. The expression of CELSR2 and ING4 in breast tumors and in benign epithelial cells have been analyzed and correlated with HER2, ER, and PR status.

Immunohistochemistry was used to analyze the expression of CELSR2 and ING4 protein in breast tumors and benign epithelial cells. The differential cellular localization of both markers was analyzed and results were also correlated with HER2, ER, and PR status. CELSR2 and ING4 cytoplasmic expression was significantly stronger in tumors than in benign epithelial cells, while the nuclear expression of both markers was significantly stronger in benign epithelial cells than in tumors. When comparing the two markers in the same type of tissues, the nuclear expression of CELSR2 was significantly stronger than cytoplasmic in benign epithelial cells, while there was no significant difference in the cellular localization of CELSR2 in tumors. For ING4, the cytoplasmic expression was significantly stronger than nuclear expression in tumors, while in benign epithelial cells, ING4 was expressed at similar levels in both compartments. There was no correlation between CELSR2 expression and HER2, ER, and PR status in tumors. However, the cytoplasmic expression of ING4 was associated with HER2 positivity in tumors. Both CELSR2 and ING4 display increased cytoplasmic staining in breast cancer cells compared to benign epithelium, suggesting a possible role of both genes in the pathogenesis of human mammary neoplasia.

Key words: Cadherin EGF LAG seven-pass G-type receptor 2 (CELSR2), Inhibitor of growth 4 (ING4), Human epidermal growth factor receptor 2 (HER2), Breast cancer, Metastasis

Offprint requets to: Huayi Huang, PhD, Department of Laboratory Medicine, the People's Hospital of Guangxi Zhuang Autonomous Region, Nanning, Guangxi, China and Department of Surgical Oncology, Roswell Park Cancer Institute, Buffalo, New York, USA. e-mail: Huayi.Huang@Roswellpark.org or Junfa Xu, MD, PhD, Institute of Laboratory Medicine, Guangdong Medical University, Dongguan, Guangdong, China. e-mail: imxujunfa@163.com

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Introduction

Breast cancer is a leading cause of death in women and metastasis is the cause of death which remains a challenging issue (AACR, 2016; Chen et al., 2016a).

Alteration of expression of certain genes is associated with the metastatic process. CELSR2 (Cadherin EGF LAG seven-pass G-type receptor 2) is encoded by the *CELSR2* gene, which is a member of the flamingo subfamily, a part of the cadherin superfamily. Studies found that CELSR2 was associated with Joubert syndrome (Vilboux et al., 2017) and ciliogenesis (Tissir et al., 2010; Shaheen et al., 2016). Deletion of *CELSR2* gene on chromosome 1 was associated with abnormal lipid metabolism and cardiovascular diseases (Hopewell et al., 2013; Kjolby et al., 2015; Surakka et al., 2015). Studies also suggested that CELSR2 is a receptor involved in contact-mediated communication, with cadherin domains acting as homophilic binding regions and the EGF-like domains involved in cell adhesion and receptor-ligand interactions (Goffinet and Tissir, 2017). However, the specific function of this particular member has not been determined. We previously reported that the expression of CELSR2 was reduced in a small subset of breast cancers and in one breast cancer cell line, thus it might play a role in cancer biology (Huang et al., 2005). However, the role of CELSR2 in cancer is still unknown so far.

The inhibitor of growth 4 (ING4) is a new member of the ING family (ING1-5) and is implicated in controlling the epithelial-mesenchymal transition, suppressing cell cycle and repressing cell growth and migration, as well as invasion in different types of cancers (Guerillon et al., 2014; Qu et al., 2016; Wang et al., 2015a,b; Zhang et al., 2016). ING4 also plays an important role in the inhibition of tumor angiogenesis, induction of cell apoptosis and senescence in human osteosarcoma cells where it is frequently lost (Li et al., 2014; Xu et al., 2015). However, there is no report on the association between CELSR2 and ING4 differential cellular localization and hormone receptors status in breast cancer so far.

We analyzed the expression of CELSR2 and ING4 in breast tumors and found that the expression pattern of the two proteins were very similar. We then correlated the protein expression of CELSR2 and ING4 with HER2, estrogen receptor (ER), and progesterone receptor (PR) status which is reported in this work.

Materials and methods

Patients and samples

Sixty-four (for CELSR2) and 70 (for ING4) formalin fixed paraffin embedded (FFPE) breast cancer tissue blocks and 39 adjacent non-cancerous tissues (benign epithelial cells) from the tissue bank of Guangzhou Huayin Medical Laboratory of South China Medical University were used in this study. The tissue blocks were collected during 2011 and 2015. The study was approved by the Institute Review Board of Guangzhou Huayin Medical Laboratory for the use of human materials. The HER2, ER, and PR immunohistochemistry results were obtained from the clinical pathology records.

Immunohistochemistry

The CELSR2 and ING4 immunohistochemistry analysis was carried out by using a goat anti-CELSR2 polyclonal antibody (AF6739) from R&D Systems Inc. (Minneapolis, MN, USA) and rabbit anti-ING4 polyclonal antibody (ab113425) from Abcam (Cambridge, MA, USA). Briefly, the FFPE tissues were cut into 5 μm thick sections, followed by de-waxing according to routine histological protocol. The de-waxed slides were pretreated with 1 mM of pH 9.0 EDTA buffer in a microwave oven at 600 W for 5 min, followed by blocking with 1% H_2O_2 in methanol to block the endogenous peroxidase activity for 30 min at room temperature. The sections were stained with goat anti-CELSR2 (1:100) or rabbit anti-ING4 (1:400) antibodies and incubated at 37°C for 1 h. The rest of the procedures were performed following routine IHC protocols. The reaction was visualized by DAB and counterstaining with hematoxylin; mounting in glycerol jelly followed.

Interpretation of immunostains

The scoring criteria for the immunostains were according to Nishimura et al. (2016). For CELSR2 and ING4, the staining results were scored as 0, 1+, 2+, or 3+ after reviewing all the samples combining the criteria of Nishimura et al. Results were scored by two pathologists separately. Specifically, for either nucleus or cytoplasm, if there was no staining, the result was scored as 0 (negative); weak staining was scored as 1+, moderate staining was scored as 2+, and strong staining was scored as 3+. 1+ and above were considered positive. The HER2, ER, and PR IHC results were obtained from the medical records.

Statistical analysis

The IHC results of CELSR2 and ING4 were correlated with the HER, ER, and PR status from routine IHC analysis. The statistical analysis was performed using the SPSS version 18.0, in which the Wilcoxon one-Sample test and Mann-Whitney U-test were performed. A p value of less than 0.05 was considered significant.

Results

The basic characteristic of the expression of CELSR2 and ING4 in breast tumors and normal tissues are shown in Table 1. Of 64 tumors, 24 (37.5%) showed cytoplasmic staining only, 12 (18.8%) showed nuclear staining alone, 19 (29.7%) showed both cytoplasmic and nuclear staining, and 9 (14.1%) showed all negative staining. In benign epithelial cells, of 39 cases, only 1 (2.6%) showed cytoplasmic staining alone, 13 (33.3%)

showed nuclear staining alone, 18 (46.2%) showed both cytoplasmic and nuclear staining, and 7 (17.9%) showed no staining in cytoplasm or nucleus. For ING4, 67 of 70 tumors (95.7%) showed cytoplasmic staining alone, no cases had nuclear staining alone, 3 (11.4%) showed both cytoplasmic and nuclear staining, and no cases showed negative staining. Of 40 cases of benign epithelial cells, 4 (10.0%) showed cytoplasmic staining only, 2 (5%) showed nuclear staining alone, 30 (75.0%) showed dual staining, and 2 (5.0%) showed negative staining.

Differential cellular localization of CELSR2 and ING4 in breast tumors and benign epithelial cells

CELSR2 cytoplasmic expression was stronger in tumors than in benign epithelial cells (p=0.033), however, CELSR2 nuclear expression was stronger in benign epithelial cells than in tumors (p=0.016). For ING4, the differential cellular localization pattern was similar to CELSR2, with cytoplasmic expression stronger in tumors than in benign epithelial cells (p=0.002) and nuclear expression stronger in benign epithelial cells than in tumors (p=0.000) (Table 2). Representative staining patterns of tumors and benign epithelium are shown in Figs. 1, 2.

Table 1. Basic characteristic of the expression of CELSR2 and ING4 in breast tumors and benign epithelial cells.

	Tumors (n)	Benign epithelial cells (n)
CELSR2		
Cytoplasmic staining positive	24/64	1/39
Nuclear staining positive	12/64	13/39
Cytoplasmic and nuclear staining positive	19/64	18/39
All negative	9/64	7/39
ING4		
Cytoplasmic staining positive	67/70	6/40
Nuclear staining positive	0/70	2/40
Cytoplasmic and nuclear staining positive	3/70	30/40
All negative	0/70	2/40

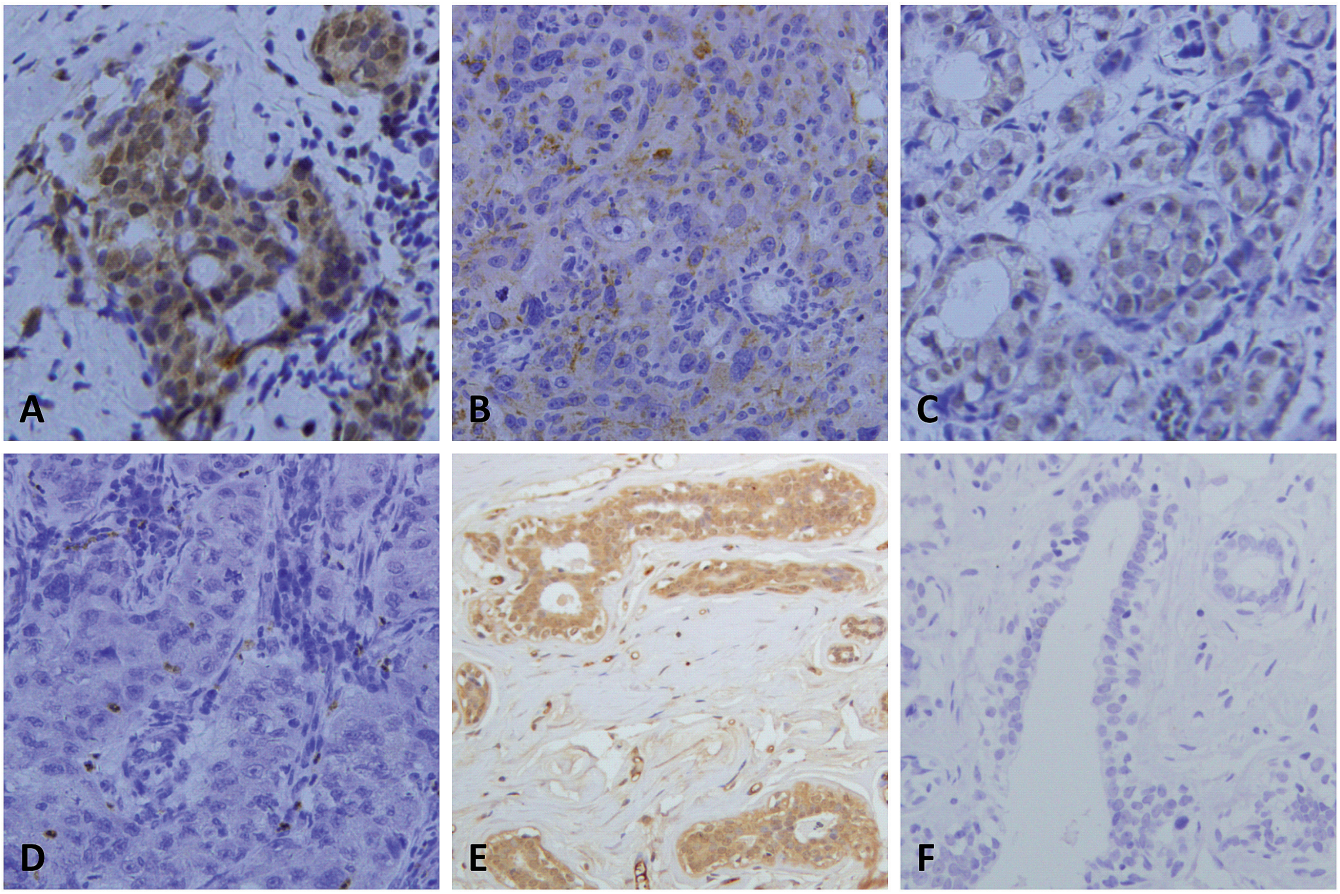

Fig. 1. Expression of CELSR2 in breast tumors and benign epithelial cells. **A.** Breast tumor with both cytoplasmic and nuclear staining. **B.** Breast tumor with moderate cytoplasmic staining. **C.** Breast tumor with moderate nuclear staining. **D.** Breast tumor with negative staining. **E.** Benign epithelial cells with moderate to strong staining in both cytoplasm and nuclei. **F.** Benign epithelial cells with negative staining. × 200.

Differential cellular distribution of CELSR2 and ING4 in breast tumors and benign epithelial cells

In order to compare the differential cellular distribution of the two markers in the same cells, the expression patterns and scores of two proteins were analyzed. We found that in tumors, there was no significant difference in CELSR2 expression between cytoplasm and nucleus (p=0.054), while in benign epithelial cells, the expression of CELSR2 in the nucleus was significantly stronger than that in the cytoplasm (p=0.018). For ING4, the cytoplasmic expression was

Table 2. Differential cellular localization of CELSR2 and ING4 in breast tumors and benign epithelial cells (Mann-Whitney U test).

	CELSR2 IHC scores (Mean Rank)		ING4 IHC scores (Mean Rank)	
	Cytoplasmic expression	Nuclear expression	Cytoplasmic expression	Nuclear expression
Tumors	56.43	47.16	61.75	40.06
Benign epithelial cells	44.73	59.95	44.56	82.53
P	0.033*	0.016**	0.002*	0.000**

*CELSR2 or ING4 cytoplasmic expression between tumors and benign epithelial cells; **CELSR2 or ING4 nuclear expression between tumors and benign epithelial cells

Table 3. Differential cellular distribution of CELSR2 and ING4 in breast tumors and benign epithelial cells (Wilcoxon one-sample test).

	CELSR2 scores (Sum of Ranks)		ING4 scores (Sum of Ranks)	
	Tumors	BECs	Tumors	BECs
Nuclear expression minus cytoplasmic expression				
Negative Ranks	522.0	22.50	2278.0	194.5
Positive Ranks	258.0	97.50	0	183.5
P	0.054*	0.018**	0.000*	0.888**

*CELSR2 or ING4 expression between cytoplasm and nucleus in tumors; **CELSR2 or ING4 expression between cytoplasm and nucleus in benign epithelial cells; BECs: Benign epithelial cells

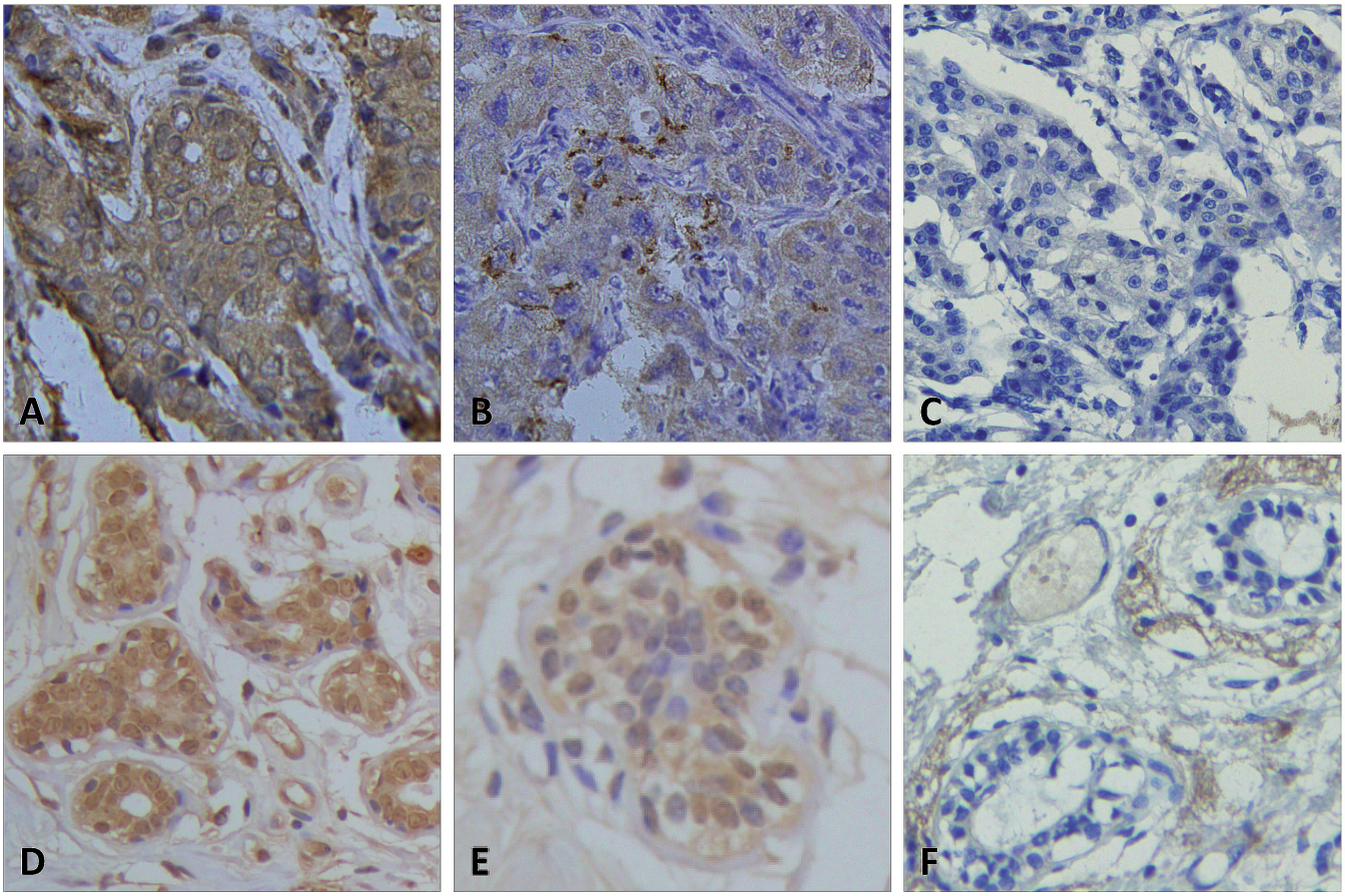

Fig. 2. Expression of ING4 in breast tumors and benign epithelial cells. **A.** Breast tumor with strong cytoplasmic staining. **B.** Breast tumor with moderate cytoplasmic staining. **C.** Breast tumor with negative staining. **D.** Benign epithelial cells with strong staining in both cytoplasm and nuclei. **E.** Benign epithelial cells with moderate staining in both cytoplasm and nuclei. **F.** Benign epithelial cells with negative staining. × 200.

significantly stronger than that in the nucleus in tumors (p=0.000), however, in benign epithelial cells, there was no significant difference in ING4 expression between cytoplasm and nucleus (p=0.888) (Table 3).

Correlation of CELSR2 expression with HER2, ER, and PR status in breast tumors

About 20% of breast cancers have *HER2* gene amplification and HER2 protein overexpression. HER2 overexpression is believed to promote breast cancer cells grow and metastasis, while ER and PR expression are favorable biomarkers of hormonal therapy response. In order to understand if there is any correlation among the CELSR2 expression and HER2, ER, and PR status in tumors, we correlated the CELSR2 expression with HER2, ER, and PR status in tumors. We found that there was no significant difference among these parameters (Table 4).

Correlation of ING4 expression with HER2, ER, and PR status in breast tumors

For the same reason as above, the expression of ING4 was correlated with HER2, ER, and PR status in tumors. We identified a correlation between the cytoplasmic expression of ING4 and HER2 positive tumors (HER2 positive tumors had more cytoplasmic expression of ING4, p=0.007) (Table 5).

A list of the differentiation of tumor cells (tumor grade) and hormone status of cases used for CELSR2 and ING4 immunohistochemistry are shown in Table 6.

Discussion

CELSR2 is a membranous protein which is believed to be associated with cardiovascular diseases and lipid metabolism disorders (Hopewell et al., 2013; Kjolby et al., 2015; Surakka et al., 2015). This protein has not been described extensively in cancers. Our previous study found that CELSR2 was expressed both in breast tumors and in benign epithelial cells (Huang et al., 2005). However, its role in cancer is not known so far. Protein trafficking and differential cellular localization are believed to play a role in cell differentiation, proliferation, and the transcription of downstream or partner genes (Gundry et al., 2017; Soeda et al., 2017). A previous report showed that CELSR2 controlled the

Table 4. Correlation of CELSR2 expression with HER2, ER, and PR status in breast tumors (Mann-Whitney U test).

	n	CELSR2 cytoplasmic staining Mean Rank	p	CELSR2 nuclear staining Mean Rank	p
HER2 status			0.635		0.201
HER2-	40	31.71		29.13	
HER2+	21	29.64		34.57	
ER status			0.076		0.496
ER-	29	34.86		29.55	
ER+	32	27.50		32.31	
PR status			0.124		0.618
PR-	35	33.74		30.13	
PR+	26	27.31		32.17	
HER2, ER, PR status			0.212		0.518
Triple negative	19	34.84		31.88	
Non triple negative	42	29.26		29.05	

Table 5. Correlation of ING4 expression with HER2, ER, and PR status in breast tumors (Mann-Whitney U test).

	n	ING4 cytoplasmic staining Mean Rank	p	ING4 nuclear staining Mean Rank	p
HER2 status			0.007		0.162
HER2-	45	30.34		34.00	
HER2+	23	42.63		35.48	
ER status			0.648		0.275
ER-	31	33.44		35.10	
ER+	37	35.39		34.00	
PR status			0.830		0.374
PR-	38	34.09		34.89	
PR+	30	35.02		34.00	
HER2, ER, and PR status			0.233		0.475
Triple negative	23	30.93		34.00	
Non triple negative	45	36.32		34.76	

Table 6. The differentiation of tumor cells (tumor grade) and hormone status of cases used for CELSR2 and ING4 immunohistochemistry.

Case number	Differentiation (grade)	ER	PR	HER2	Used for CELSR2 IHC	Used for ING4 IHC
1	3	-	-	-	Yes	Yes
2	2	+	+	+	Yes	Yes
3	2	+	+	+	Yes	Yes
4	3	-	-	-	Yes	Yes
5	2	+	+	-	Yes	Yes
6	1	+	+	-	Yes	Yes
7	3	+	+	+	Yes	Yes
8	2	+	-	+	Yes	Yes
9	3	+	-	+	Yes	Yes
10	3	-	-	-	Yes	Yes
11	3	+	+	+	Yes	Yes
12	2	-	+	+	Yes	Yes
13	2-3	-	-	-	Yes	Yes
14	3	+	+	+	Yes	Yes
15	3	+	+	+	Yes	Yes
16	3	-	-	-	Yes	Yes
17	2	-	-	-	Yes	Yes
18	2	-	-	+	Yes	Yes
19	3	-	-	-	Yes	Yes
20	3	+	+	-	Yes	Yes
21	1	+	+	-	Yes	Yes
22	2	+	+	-	Yes	Yes
23	2	-	-	-	Yes	Yes
24	3	-	-	-	Yes	Yes
25	2	-	-	-	Yes	Yes
26	2	-	-	+	Yes	Yes
27	3	-	-	-	Yes	Yes
28	3	+	-	-	Yes	Yes
29	1	+	+	-	Yes	Yes
30	1	+	+	-	Yes	Yes
31	2	+	+	-	Yes	Yes
32	1	+	+	-	Yes	Yes
33	2	+	-	+	Yes	Yes
34	1	+	+	-	Yes	Yes
35	1	-	-	+	Yes	Yes
36	3	+	+	+	Yes	Yes
37	2	-	-	-	Yes	Yes
38	2	+	+	+	Yes	Yes
39	2	-	-	+	Yes	Yes
40	3	-	-	-	Yes	Yes
41	3	-	-	-	Yes	Yes
42	3	+	-	-	Yes	Yes
43	2	+	+	-	Yes	Yes
44	2	+	-	-	Yes	Yes
45	2	+	-	-	Yes	Yes
46	1	+	+	-	Yes	Yes
47	2	+	+	-	Yes	Yes
48	1	+	-	-	Yes	Yes
49	2	-	-	-	Yes	Yes
50	2	-	-	+	Yes	Yes
51	1	+	+	-	Yes	Yes
52	2	-	-	-	Yes	Yes
53	2	+	+	-	Yes	Yes
54	3	-	-	+	Yes	Yes
55	3	-	-	-	Yes	Yes
56	3	-	-	+	Yes	Yes
57	3	-	-	-	Yes	Yes
58	3	-	-	-	Yes	Yes
59	3	+	+	+	Yes	No
60	3	-	+	-	Yes	No
61	2	-	-	+	Yes	No
62	3	+	+	+	No	Yes
63	2	-	-	+	No	Yes
64	1	+	+	+	No	Yes
65	2	+	+	-	No	Yes
66	2	+	+	-	No	Yes
67	2	-	-	-	No	Yes
68	1	+	+	-	No	Yes
69	2	-	-	+	No	Yes
70	2	-	-	-	No	Yes
71	3	-	-	-	No	Yes

migration ability of facial branchiomotor neurons (Qu et al., 2010).

Using a commercial antibody against human CELSR2 and performing IHC analysis, we were able to characterize the differential cellular localization of CELSR2 in breast tumors and benign epithelial cells.

ING4 is a tumor suppressor and plays a role in regulating p53 function (Chen et al., 2016b). ING4 inhibited estrogen receptor activity and suppressed the recurrence of breast cancer after hormonal therapy. Overexpression of ING4 increased the sensitivity of breast cancer cells to hormone deprivation (Keenen et al., 2016). Previous studies found that the *ING4* gene was shown to be deleted in 10% to 20% of breast cancers. ING4 deletion was significantly associated with HER2 overexpression. Tumors with ING4 deletion were more likely to belong to the HER2 molecular subtype (ER-/PR-/HER2+) of breast cancer. In addition, ING4 negativity is more prevalent in HER2 positive tumors, suggesting a functional antagonistic relationship between the ING4 tumor suppressor and the HER2 oncogene that may contribute to the pathogenesis of HER2 positive breast cancer (Tapia et al., 2011). However, there are no reports on the association between ING4 differential cellular localization and hormone receptor status in breast cancer so far.

In this study, we have categorized the expression of CELSR2 and ING4 in tumors and benign epithelial cells (Table 1). The staining results suggest that there was a high proportion of cases with cytoplasmic expression of CELSR2 in tumors, and 9 tumors did not express CELSR2 which a deletion of the gene as in other diseases (Tissir et al., 2010). In addition, there were high proportions of cases with both cytoplasmic and nuclear expression as well (19 out of 64), second to the cytoplasmic expression alone. In benign epithelial cells, there was a high proportion of cases with both nuclear and cytoplasmic staining. These results indicate that the differential cellular localization of CELSR2 might be of functional significance. Since the CELSR2 is a membranous protein and plays a role in contact-mediated communication of the cells. Thus, in tumor, the cellular distribution (localization) of this protein might reflect a pathological alteration of protein transportation and re-localization status, so might have clinical significance especially combining the testing with E-cadherin and other membranous proteins. Further study is needed to illustrate this hypothesis.

The immunohistochemical staining results indicate that in tumors ING4 is expressed mainly in the cytoplasm, while in benign epithelial cells, it is expressed mainly in both cytoplasm and nucleus. ING4 is reported to interact with the tumor suppressor p53 and induces apoptosis (Zhang et al., 2005; Ren et al., 2016). Zhang et al. reported that ING4 nuclear localization was involved in the binding to p53, thus, the nuclear localization signal domain of ING4 may be essential for the binding of ING4 to p53 and the function of ING4 associated with p53 (Tapia et al., 2011). In our study, we found that in benign epithelial cells, ING4 was expressed strongly in the nucleus as well as in the cytoplasm. However, the protein was mainly expressed in the cytoplasm in tumors. Our results are consistent with Zhang's report on MCF-7 breast cancer cells. Another report from IHC on 246 lung cancers showed that reduced ING4 nuclear and cytoplasmic expression was associated with tumor grade. Compared with normal tissues, the authors found more tumors with ING4 expression in the cytoplasm higher than in the nucleus, and nuclear ING4 inhibition correlated with tumor stage and lymph node metastasis (Wang et al., 2010).

When we correlated the CELSR2 and ING4 expression with hormone status in breast tumors, we found that there was no correlation between the expression of CELSR2 and the biomarker status (HER2, ER, and PR) either in cytoplasm or in nucleus (Table 4). However, we found that the cytoplasmic expression of ING4 was correlated with HER2 status in tumors, with more HER2+ cases having high cytoplasmic expression of ING4 ($p=0.007$). There was no correlation between ING4 nuclear expression and hormone status (Table 5). This result indicates that cytoplasmic expression of ING4 represents more aggressive and metastatic potential.

For the ease in understanding, a list of the differentiation of tumor cells (tumor grade) and hormone status of cases used for CELSR2 and ING4 immunohistochemistry have been shown.

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