ORIGINAL ARTICLE

Expression of calcium sensing receptor and E-cadherin correlated with survival of lung adenocarcinoma

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Abstract

Background: It has been reported that the calcium sensing receptor (CaSR), a widely expressed G protein-coupled receptor, can stimulate cell differentiation and proliferation. However, in malignant tumors, loss of CaSR expression has been associated with tumorigenesis, metastasis, and progression. Recent studies have indicated that the CaSR could promote the expression of E-cadherin, which was considered a tumor suppressor. However, in human lung adenocarcinoma, the importance of the CaSR and E-cadherin has not been sufficiently investigated.

Methods: Expression levels of CaSR and E-cadherin in paraffin sections from 117 resected lung adenocarcinoma patients were evaluated by immunohistochemistry. We analyzed the correlation between our target proteins and clinical variables. Clinical significance was analyzed by multivariate Cox regression analysis, Kaplan–Meier curve, and log-rank test.

Results: Expression of the CaSR in lung adenocarcinoma tissue was significantly lower than in the normal sample ($P = 0.003$). Kendall tau-b analysis showed that, in a lung adenocarcinoma sample, the expression of CaSR positively correlated with a high level of E-cadherin ($P < 0.001$). Lung adenocarcinoma patients with a strong expression of CaSR ($P = 0.034$) or E-cadherin ($P = 0.001$) had longer overall survival. Multivariate Cox proportional hazards model analysis showed that the combined marker was an independent prognostic indicator of overall survival (hazard ratio = 0.440, confidence interval = 0.249–0.779, $P = 0.005$).

Conclusions: We identified the CaSR as a new prognostic biomarker in lung adenocarcinoma. These results also suggested that the CaSR may become a new therapeutic target of lung adenocarcinoma.

Introduction

The calcium sensing receptor (CaSR) is a widely expressed G protein-coupled receptor (GPCR), which was first found in abundance in cells of the parathyroid glands.[1–3] The function of the CaSR is to maintain systemic calcium homeostasis by sensing changes in extracellular Ca^{2+} (Ca^{2+}_o) concentration.[1,4] Calcium molecules bind to the CaSR, which allows this protein to monitor and regulate parathyroid hormone (PTH) secretion from the parathyroid.[4,5] The receptor can be activated when a certain concentration of calcium is reached, and the activated receptor leads to an inhibition of PTH secretion, PTH gene expression, and parathyroid cell proliferation.[4,6] However, the CaSR was also found in a wide range of normal tissues and malignant tumors. Recently, many studies have shown that the CaSR is a robust inducer of differentiation.[7–9] Inhibiting the expression of the CaSR can suppress Ca^{2+}_o – induced keratinocyte differentiation.[10] It can also stimulate cell proliferation.[11–13] Recently, studies have focused on the role of the CaSR in tumor suppression. In a variety of benign tumors and malignancies, such as breast and prostate cancers, the expression levels of the CaSR differed from those in their healthy counterparts.[1] In colon cancers, loss of CaSR expression was associated with tumorigenesis, metastasis, and progression.[14–16] However, the relationship between the CaSR and lung adenocarcinoma remains largely unknown.

E-cadherin, a single-pass transmembrane protein, was first discovered as a Ca^{2+} dependent cell surface protein

that mediated cell–cell adhesion in early mouse embryo blastomeres.[17–19] It contains five extracellular repeats and mediates Ca^{2+} dependent hemophilic interaction with neighbouring cells.[19,20] It can also maintain the integrity of the epithelium.[15] It is well known that tumor cells can invade with fully intact and functional cell–cell adhesions as collective groups of cells, and that a loosening of cell–cell contacts is sufficient to permit this collective migration and invasion.[19] As a key mediator of cell–cell adhesion in epithelial tissue, loss of E-cadherin can promote invasive and metastatic behavior in many epithelial tumors.[19,21] Some believe this is a result of the downregulation of E-cadherin, leading to the occurrence of epithelial to mesenchymal transition (EMT), which plays an important role during invasion or metastasis of carcinoma cells.[19,22–24] Exactly how E-cadherin acts to suppress the transformed phenotype at the molecular level, however, is not fully understood.

Recent studies have indicated that the CaSR is a key regulator for E-cadherin-mediated cell–cell adhesion. Moreover, it could promote the expression of the tumor suppressor E-cadherin.[14,15,25] Inhibiting the expression of the CaSR in colon carcinoma blocked the formation of E-cadherin-catenin complex.[26,27] Therefore, we postulated that the CaSR and E-cadherin might play significant roles in the tumorigenesis, metastasis, and progression of lung adenocarcinoma, and the expression of CaSR and E-cadherin might be significant prognostic biomarkers. To confirm our hypothesis, we immunohistochemically examined the expression of CaSR and E-cadherin in surgically resected lung adenocarcinoma specimens, and studied the relationships between the CaSR, E-cadherin, and clinicopathological characteristics of lung adenocarcinoma, aiming to investigate the prognostic value of the CaSR and E-cadherin in lung adenocarcinoma.

Materials and methods

Tissue samples and patients

Formalin-fixed paraffin embedded (FFPE) specimens were collected from 117 lung adenocarcinoma patients undergoing surgery between January 2009 and October 2010. Primary cancers were evaluated in accordance with the 7th edition American Joint Committee on Cancer (AJCC) staging system. All patients were followed until death or the study closing date (27 December 2013). The median follow-up duration was 44.23 months (range 1.30–59.23 months). No patient received chemotherapy or radiotherapy prior to surgery. Three to four cycles of platinum-based adjuvant chemotherapy were administered to pathological stage IB patients with high risk, or stage II and III patients. This study was approved by the institutional review board of the Harbin Medical University Cancer Hospital and was

carried out in accordance with established national and institutional ethical guidelines regarding the use of human tissues for research.

Immunohistochemistry

Immunohistochemical (IHC) analysis was performed to detect the expression of CaSR and E-cadherin in paraffin sections. In brief, the sections were deparaffinized in xylene and rehydrated in a graded series of ethanol solutions. The sections were subsequently submerged in ethylenediamine-tetraacetic acid (pH 8) and autoclaved at 121°C for five minutes to retrieve the antigenicity. Endogenous peroxidase was quenched with 3% H_2O_2 for 15 minutes. After washing with phosphate buffered saline (PBS), the sections were incubated with anti-human CaSR monoclonal antibody (diluted 1:20, Santa Cruz Biotechnology, Dallas, TX, USA; sc-47741), and anti-human E-cadherin polyclonal antibody (diluted 1:50, Santa Cruz Biotechnology, sc-31020), overnight at 4°C. After washing, the slides were incubated with biotinylated secondary antibodies (Zhongshan Biotechnology Company, Beijing, China) for one hour, followed by further incubation with streptavidin-horseradish peroxidase complex. Finally, staining was visualized using diaminobenzidine. For negative controls, the antibody was replaced with PBS. The percentage of positive cells was determined by counting 500 cells in five random selected fields per section. IHC staining was scored according to the following criterion: −, 0%–5%; +, 6–25%; ++, 26–50%; and , 51–100% of the cells stained. To acquire the optimum balance ratio between low and high expression, a cut-off of 25% was used for the CaSR and E-cadherin. Hereinafter, "high" means that the positive staining cell rates were higher than the cut-off value, while "low" means lower or equal to the cut-off value. Two independent observers determined the percentage of cells being stained and interpreted the results in a blinded fashion.

Statistical analysis

All statistical analyses were performed using SPSS software version 19.0 (SPSS Inc., Chicago, IL, USA). Differences were considered statistically significant when P values were ≤0.05. The association between the CaSR, E-cadherin, and clinicopathologic parameters was tested using the chi-square test and the Mann-Whitney U test for age. The Kaplan–Meier method was used to plot cumulative overall survival (OS) curves, and the relationship between each of the variables and survival was assessed by log-rank. Prognostic factors were examined by univariate and multivariate analysis (Cox proportional hazards regression model). Covariates with $P \leq 0.2$ in univariate analysis were adopted into multivariate analysis.
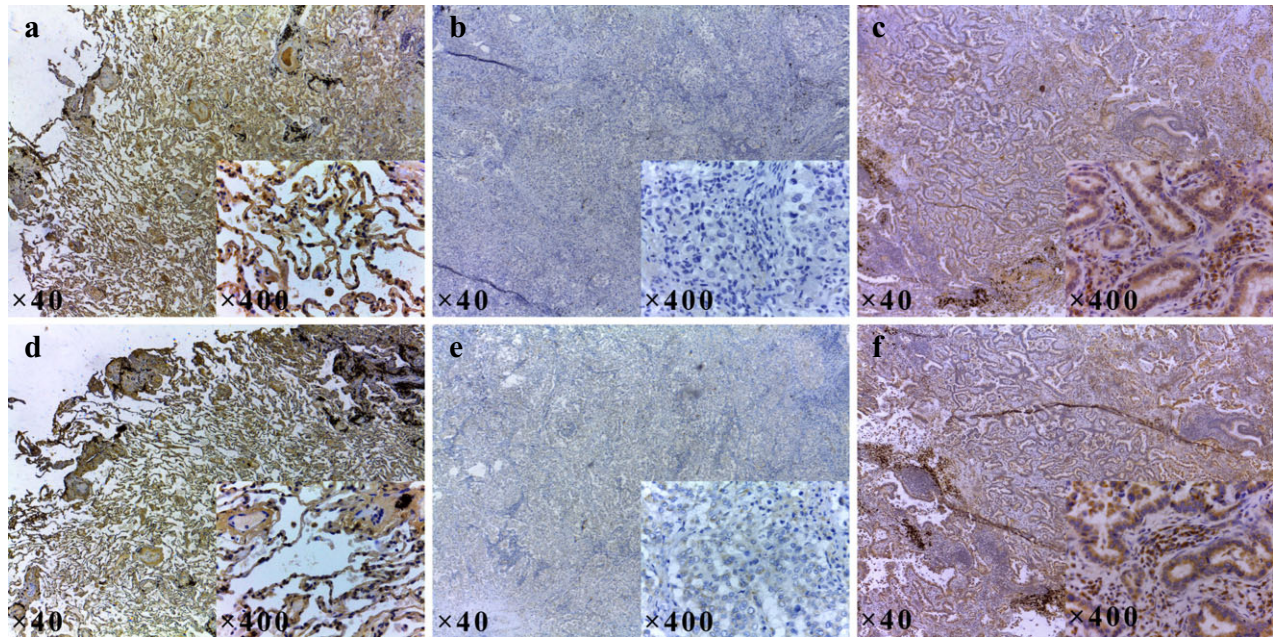
Figure 1 Immunohistochemical staining of protein expression in lung adenocarcinoma and adjacent normal alveolar tissues. A representative normal sample expressed both high (**a**) calcium sensing receptor (CaSR) and (**d**) E-cadherin, and two representative tumor tissues respectively expressed low (**b**) CaSR and (**e**) E-cadherin or high (**c**) CaSR and (**f**) E-cadherin.

Results

Expression of the calcium sensing receptor (CaSR) and E-cadherin in lung adenocarcinoma tissue

To clarify the relationship between the CaSR, E-cadherin, and lung adenocarcinoma progression, we analyzed the protein expression levels of CaSR and E-cadherin in 117 lung adenocarcinoma tissues and 43 adjacent normal alveolar tissues by immunohistochemistry. IHC analysis showed that positive staining for CaSR and E-cadherin were found mainly in the cytoplasm and membrane (Fig 1). Compared with the cancer sample, normal tissue expressed significantly high CaSR (34.2% vs. 60.5%, $P = 0.003$). There was no difference in the expression of E-cadherin between normal and lung adenocarcinoma tissues (60.5% vs. 59.0%, $P = 0.865$) (Table 1).

Relationship between the CaSR, E-cadherin, and clinicopathological characteristics

In order to study the relationship between the CaSR, E-cadherin, and clinicopathological characteristics, protein levels of the CaSR and E-cadherin in 117 lung adenocarcinoma tissues were measured. There was no statistically significant correlation between the CaSR and known clinicopathological factors; however, E-cadherin expression correlated with gender ($P = 0.011$) and tumor size ($P = 0.013$) (Table 2). Women tended to have a stronger expression of E-cadherin than men. The stronger the expression of E-cadherin, the smaller the tumor size. Kendall tau-b analysis showed that the expression of CaSR positively correlated with the expression of E-cadherin (r = 0.354, $P < 0.001$) (Table 3).

Table 1 Expression of CaSR and E-cadherin in tumor and normal alveolar tissues

Tissue	CaSR			E-cadherin		
	Low (%)	High (%)	*P*	Low (%)	High (%)	*P*
Normal Alveolar Tissue	17 (39.5)	26 (60.5)	0.003*	17 (39.5)	26 (60.5)	0.865
Tumor Tissue	77 (65.8)	40 (34.2)		48 (41.0)	69 (59.0)	

Note: Immunohistochemical analysis of protein expression in lung adenocarcinoma (n = 117) and adjacent normal alveolar tissues (n = 43). Compared with the tumor tissue, the normal tissue expressed significantly high calcium sensing receptor (CaSR) ($P = 0.003$).

Table 2 Correlation between protein expression levels and clinicopathological characteristics

| Variables | Numbers | CaSR | | | E-cadherin | | |
		Low	High	P	Low	High	P
Gender				0.259			0.011*
Male	64	45	19		33	31	
Female	53	32	21		15	38	
Age (years)	117	77	40	0.989†	48	69	0.844†
Tumor size (cm)				0.622			0.013*
≤3	49	31	18		14	36	
>3	68	46	22		34	33	
Lymph node status				0.555			0.370
Negative	57	36	21		21	36	
Positive	60	41	19		27	33	
AJCC stage				0.844			0.331
I	51	33	18		18	33	
II	24	15	9		9	15	
III	42	29	13		21	21	
Differentiation				0.622			0.118
Good/moderate	68	46	22		32	36	
Poor	49	31	18		16	33	
Adjuvant chemotherapy				0.272			0.009*
No	40	29	11		23	17	
Yes	77	48	29		25	52	

Note: [0 (negative) ≤ score ≤ 1+] and [2 + ≤ score ≤ 3+] represents low and high positive scores, respectively, for calcium sensing receptor (CaSR). [0 (negative) ≤ score ≤ 1+] and [2 + ≤ score ≤ 3+] represents low and high scores, respectively, for E-cadherin. All of the cut-off points contributed to acquiring the optimum balance ratio between low and high expression. *$P < 0.05$. †Mann-Whitney U test was used for age. AJCC, American Joint Committee on Cancer.

Clinical significance of the CaSR and E-cadherin in lung adenocarcinoma

To avoid other factors, such as postoperative complications, influencing the results, we used the data of patients whose survival time was longer than nine months in order to analyze whether the CaSR or E-cadherin could predict the OS of patients who had received lung adenocarcinoma resection. Patients with low CaSR had a shorter OS than those with high CaSR (median survival time [MST] 52.2 months vs. NA, $P = 0.034$)(Fig 2). Patients with high E-cadherin expression had a better prognosis than those with low expression (MST 56.4 vs. 36.2 months, $P = 0.001$). Thus, patients tended to have a

Table 3 The correlation between CaSR and E-cadherin in 117 lung adenocarcinoma tissues

| CaSR | E-cadherin | | | |
	Low	High	Kendall taub	P
Low	38	39	0.354	<0.001
High	10	30		

Note: Kendall tau-b analysis was used to determine the correlation between calcium sensing receptor (CaSR) and E-cadherin. The expression of CaSR positively correlated with the expression of E-cadherin ($P < 0.001$).

poorer prognosis when both the CaSR and E-cadherin were negative. The 110 patients were divided into two groups: Cluster A and Cluster B. Patients with concordant low CaSR and E-cadherin expression were assigned to Cluster A (n = 35), while the remainder were assigned to Cluster B (n = 75). Patients with either CaSR or E-cadherin expression had a longer OS (MST 56.4 vs. 29.5 months, $P < 0.001$).

Clinical significance of "both CaSR and E-cadherin low expression" in lung adenocarcinoma

Cox regression analysis was performed to determine whether "both CaSR and E-cadherin negative (the combined marker)" is an independent prognostic factor of OS in lung adenocarcinoma. As shown in Table 4, lymph node involvement ($P = 0.001$), tumor stage ($P = 0.001$), CaSR expression ($P = 0.038$), E-cadherin expression ($P = 0.001$), and the combined marker ($P = 0.001$) were significantly associated with OS in univariate Cox regression models. Multivariate Cox regression analysis revealed that tumor stage (hazard ratio [HR] = 1.649, confidence interval [CI] = 1.159–2.346, $P = 0.005$) and the combined marker (HR = 0.440, CI = 0.249–0.779, $P = 0.005$) were the only two independent prognostic indicators for lung adenocarcinoma.
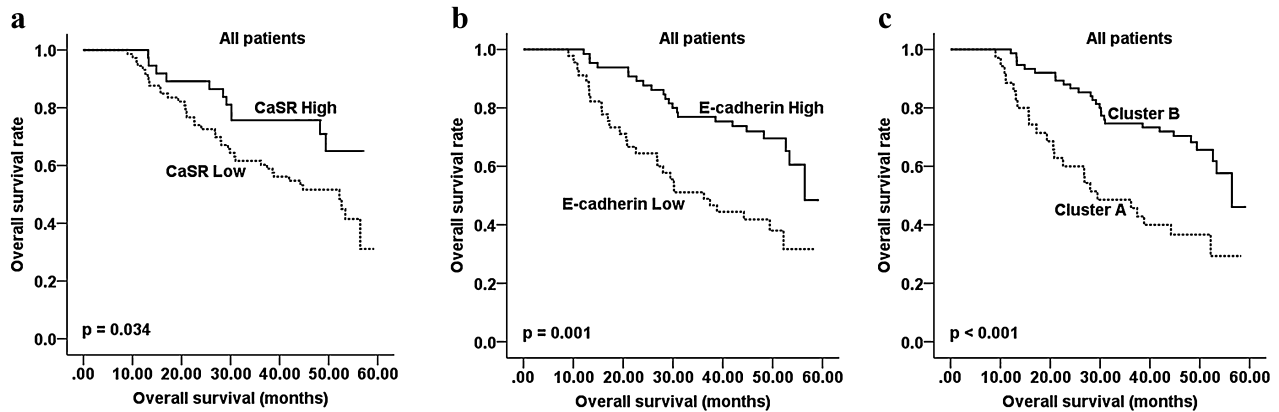
Figure 2 Overall survival (OS) periods in lung adenocarcinoma. Kaplan–Meier analysis demonstrated that (**a**) the high calcium sensing receptor (CaSR) group was associated with significantly longer OS than the low CaSR group (median survival time [MST] NA vs. 52.2 months, $P = 0.034$). (**b**) Patients with a high level of E-cadherin had significant longer OS (MST 56.4 vs. 36.2 months, $P = 0.001$). (**c**) Cluster A [CaSR (-) E-cadherin (-)] group showed a significantly shorter OS than cluster B [non-CaSR (-) E-cadherin (-)] group (MST 29.5 vs. 56.4 months, $P < 0.001$).

Discussion

In the current study, we observed that the expression of CaSR in lung adenocarcinoma cells was downregulated dramatically compared with non-tumor tissues. This phenomenon has also occurred in breast, prostate, and other carcinomas.[1] Several studies have demonstrated that the expression levels of CaSR messenger ribonucleic acid and protein were decreased in parathyroid adenomas compared with normal glands.[28–30] Chakrabarty *et al.* also found that CaSR expression in colon cancer cells was lost during colonic tumorigenesis and progression; therefore, loss of the CaSR may promote the progression of colon cancer cells.[14,15] We speculated that the loss of CaSR plays an important role in the progression of normal lung tissue to lung adenocarcinoma. However, the mechanism of the CaSR in lung adenocarcinoma carcinogenesis and progression still requires

analysis. Several reports have discussed the relationship between E-cadherin and the clinicopathological factors of lung cancer. Some of these studies demonstrated that a reduced expression of E-cadherin was associated with lymph node metastasis, advanced AJCC stage or poor differentiation of NSCLC.[31–34] Interestingly, in studies on lung adenocarcinoma analysing the relationship between E-cadherin and clinicopathological factors, no correlation was found between E-cadherin and lymph node status, AJCC stage or differentiation.[35–38] This suggests that the loss of E-cadherin plays a more important role in squamous cell carcinomas than in adenocarcinomas. Our study results strengthen previous observations.

The expression of CaSR was positively correlated with the expression of E-cadherin in our research. It has been reported that Ca^{2+} stimulates CaSR promoter activity and protein expression in human colon carcinoma CBS cells, which

Table 4 Univariate and multivariate cox regression of overall survival in 117 patients with lung adenocarcinoma

Variables	Univariate analysis		Multivariate analysis‡	
	HR (95% CI)	*P*	HR (95% CI)	*P*
Gender	0.616 (0.348, 1.093)	0.098		0.827
Age	0.975 (0.948, 1.003)	0.082		0.224
Tumor size	1.451 (0.863, 2.441)	0.160		0.457
Lymph node status	1.623 (1.211, 2.174)	0.001**		0.683
AJCC stage	1.800 (1.268, 2.557)	0.001**	1.649 (1.159, 2.346)	0.005**
Differentiation	0.741 (0.488, 1.126)	0.160		0.722
Adjuvant chemotherapy	0.713 (0.403, 1.264)	0.247		
CaSR	0.492 (0.252, 0.960)	0.038*		0.410
E-cadherin	0.385 (0.219, 0.675)	0.001**		0.317
The combined marker†	0.379 (0.217, 0.662)	0.001**	0.440 (0.249, 0.779)	0.005**

**P < 0.01 and *P < 0.05. †The combined marker, both calcium sensing receptor (CaSR) and E-cadherin low expression. ‡The covariates with $P \leq 0.2$ in univariate analysis were adopted into multivariate analysis. AJCC, American Joint Committee on Cancer; CI, confidence interval; HR, hazard ratio.

possess a functional CaSR.[15] Moreover, increasing E-cadherin expression can be induced by an elevated Ca^{2+}_o.[14,15,27] Human colon carcinoma cells cultured in Ca^{2+}-free supplemented minimum essential medium did not express E-cadherin, while the expression of E-cadherin could be restored by a supplementation with 1 mM Ca^{2+}_o.[14] Furthermore, it was observed that E-cadherin expression could be blocked by CaSR inhibition in human epidermal keratinocytes and colon carcinoma cells, which suggested that the expression of E-cadherin might be induced by Ca^{2+}_o via activating CaSR.[10,26] Although the positive correlation between CaSR and E-cadherin was observed in our study, the result was weak (r = 0.354).

We also found that the expression of CaSR and E-cadherin can predict OS in resected lung adenocarcinoma. Kaplan–Meier analysis confirmed that lung adenocarcinoma patients with low expressions of CaSR or E-cadherin had a significantly poorer OS compared with the patients with high expressions of CaSR and E-cadherin. It is well known that E-cadherin can predict OS in many carcinomas, and a meta-analysis published recently also supported this result.[39] CaSR may also predict OS in other carcinomas. In breast cancer, a loss of CaSR was significantly associated with poor OS, and Cox multivariate analysis showed that the CaSR was an independent prognostic indicator for both OS and cause-specific survival of breast cancer patients.[40] In colon carcinoma, both the percentage of positive cells and the staining intensity of the CaSR in poorly differentiated tumors were significantly reduced when compared with well to moderately differentiated tumors.[15] This may indicate that colon cancer patients with a strong expression of CaSR would live longer than those with a low expression. We have concluded that there is a link between the expression of E-cadherin and the activity of the CaSR. Therefore, logically, the lower the CaSR expressed, the lower E-cadherin expressed, which leads to the occurrence of EMT and, ultimately, causes the invasion and metastasis of lung adenocarcinoma. This might be a reasonable explanation for how the expression of CaSR can predict better OS in lung adenocarcinoma.

Although only patients with resected lung adenocarcinoma were studied here, our results also suggest a prognostic value for advanced lung adenocarcinoma, which should be validated by further studies.

Conclusions

In conclusion, the present study identified a new prognostic biomarker that may predict OS in lung adenocarcinoma. These results also suggest that the CaSR may be a therapeutic target of lung adenocarcinoma. The mechanism of how CaSR acts in lung adenocarcinoma still requires intensive study.

Disclosure

No authors report any conflict of interest.

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