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ORIGINAL ARTICLE

Inducing Polyclonal Eag1-Specific Antibodies by Vaccination with a Linear Epitope Immunogen and Its Relation to Breast Tumorigenesis

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Abstract Ether à-go-go 1 (*KCNH1*, Kv10.1) (Eag1) is a voltage-gated potassium channel, which is commonly overexpressed in tested breast cancer patients. This occurrence makes it a potential molecular marker and a promising tool for breast cancer diagnosis and therapy. In order to explore protective or specific polyclonal antibodies for further research, potential linear epitopes from Eag1 were collected by sequence alignment. The sequence was synthesized and then coupled to the carrier protein keyhole limpet hemocyanin (KLH) for animal immunization. Polyclonal antibodies against Eag1 were produced and purified from the rabbit antisera. Enzyme linked immunosorbent assay (ELISA) and western blot were performed to characterize their specificities. Immunohistochemical staining was carried out on normal and cancerous breast tissue sections using the purified polyclonal Eag1-specific antibodies. The results indicate that the overexpression of Eag1 might be associated with an increased risk of progression to breast cancer (Grade 1 tissue = 57.89%;Grade 2 tissue = 92.59%;Grade 3 tissue = 100%). These results also suggest that *Eag1* gene is a putative growth-promoting gene that might be involved in breast tumorigenesis and development. Eag1 might further be represented as a potential target for some human diseases treatment.

Keywords Eag1 · Breast cancer · Polyclonal Eag1-specific antibodies · Immunohistochemical marker

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Introduction

Breast cancer is one of the leading causes of mortality among women in the U.K., U.S., and indeed in most western countries, where women have more than a > 10% chance of developing invasive breast cancer within their lifetime [1]. In recent years, the importance of voltage-gated potassium channels in tumour biology has aroused interest with the identification of ion channels as potential novel targets for tumour therapy [2]. The first identified voltage-gated potassium channel implicated in oncogenesis and tumour progression was Eag1 [3]. This membrane protein is accessible from the extracellular side and is predominantly present in tumour cells. For several reasons, Eagl is an interesting potential target for tumour therapy. Studies indicate that the human Eag1 K^+ channel is expressed in a variety of cell lines derived from human malignant tumors and in clinical samples in several different cancers, but is otherwise absent in normal tissues. Of all the various categories of K^+ channels, the Eag voltage-dependent K^+ channel family plays the most prominent role in tumor generation, progression and metastasis [4]. It was found to be necessary for cell cycle progression and tumorigenesis [5, 6]. Eag1 is

restricted in its expression to the nervous system, indicating that the channel is not normally expressed in differentiated peripheral tissues, while the surrounding tissues are devoid of Eag1 expression. Moreover, specific expression inhibition of *Eag1* gene by antisense techniques, siRNA or antibodies leads to a reduction in tumor cell proliferation in vitro and in vivo [7–9].

To date, Eag1 is the only potassium channel that has been shown to affect tumour progression in animal models. Eag1 protein expression has been detected in several cell lines derived from human malignant tumours, such as neuroblastoma, melanoma, and breast, and cervical carcinoma [10].

For any potential clinical application it is an essential pre-requisite that samples from human tumours overexpress the target Eag1. In order to understand the biological functions of Eag1 and explore its clinical applications, in this study, potential linear epitopes from Eag1 were firstly collected by sequence alignment. Then specific polyclonal antibodies were prepared from antiserum by animal immunization using the synthetic linear epitope peptide coupled to keyhole limpet hemocyanin (KLH). The following immunohistochemical staining was performed to evaluate identification abilities of the purified polyclonal antibodies and further to understand the distribution and localization of Eag1 in normal and cancerous breast tissues including simple carcinoma, invasive ductal carcinoma, mucinous adenocarcinoma and normal hyperplasia.

Materials and Methods

Animals, Ethics Statement

New Zealand white rabbits were purchased from Changchun Institute of Biological Products Co., Ltd. The animal trials in this study were carried out in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals approved by the State Council of People's Republic of China (11–14-1988). All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Jilin University (permit number: SYXK 2014–0012).

Sequence Alignment of Eag1

The mammalian Eag subfamily comprises of two members, termed Eag1 (KCNH1) and Eag2 (KCNH5) [11]. The sequences of 7 KCNH1 and 5 KCNH5 from *Homo sapiens* were downloaded from NCBI database. Each gene ID is shown in Table 1. Alignments were performed using MEGA version 5.0.

Table 1 The Gene ID of 7 KCNH1 and 5 KCNH5 from *Homo sapiens*

Eag subfamily	Gene ID
Eag1(KCNH1)	109,731,377
	119,613,830
	119,613,831
	4,504,831
	219,520,343
	26,006,799
	27,437,001
Eag2(KCNH5)	119,601,225
	21,359,678
	21,359,694
	22,024,390
	49,257,149

Animal Immunization and Purification of Antisera

New Zealand white rabbits were immunized subcutaneously with 300 μg of the given immunogen 4 times at 2-week intervals. Immunogens were administered in 100 μl phosphate buffered saline (PBS) mixed with an equal volume of an Freund's adjuvant (Beijing Dingguo Inc., Beijing, China) per dose.

Immunized serum was harvested and purified by passing through protein A Sepharose (Amersham Biosciences) with salt elution. Purified antibodies were used for immunohistochemical staining analysis.

Enzyme Linked Immunosorbent Assay (ELISA)

Synthetic linear peptide of Eag1 epitope was used as antigen to detect the activity of the rabbit serum antibodies. 96-well plates (Jet Biofil, Guangzhou, China) were coated with the above mentioned antigen at 0.2 μg/mL in PBS and kept for 3 h at 37 °C overnight at 4 °C. Thereafter, the plates were blocked for 30 min at room temperature with 1% bovine serum albumin (BSA)/PBS and then washed 3 times with PBS/0.05% Tween-20. Antisera from individuals vaccinated as mentioned above were serially diluted in 1%BSA/PBS five times and added to the plate, after which the plates were incubated at 37 °C for 1.5 h. The plates were washed 3 times, and an anti-rabbit horseradish peroxidase (HRP)-conjugated antibody (Dingguo, China) diluted at 1:1000 was added to the wells for incubation at 42 °C for 1 h. Subsequently, 3,3,5,5-tetramethyl benzidine (TMB) solution was added to the wells for 20–30 min at room temperature. Optical density measurements (TECAN-680, Sunrise) were obtained at 492 nm. The highest reciprocal serum dilution that yielded an absorbance of >2-fold over background values was determined as the ELISA endpoint titer.

Western Blotting

Cell lysates prepared by MCF7 cells were separated by SDS-PAGE on 12% gels and electrophoretically transferred onto a polyvinylidene difluoride (PVDF) membrane (Amersham). After blocking with 5% milk in TBST (10 mM Tris pH 8.0, 150 mM NaCl, 0.05% Tween20), the membrane was incubated in 1% milk in TBST at 4 °C for 12 h with affinity purified anti-Eag1 antibody (1:600 dilution) or anti-GAPDH antibody. Then, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies. The specific proteins were detected by ECL (Beyotime Biotech, China).

Detection of Eag1 Expression in Normal and Cancerous Breast Tissue Sections by Immunohistochemical Staining

The breast tissue samples with paraffin embedded blocks were collected from Department of Pathology and Pathophysiology of Jilin University. The tissues samples included 25 cases of simplex carcinoma, 12 cases of invasive ductal carcinoma, 6 cases of breast mucinous adenocarcinoma, and 30 cases of breast hyperplasia as shown in Table 2. For immunohistochemistry staining, the 5 μm thick sections of breast tissues were prepared and mounted on poly-(L-lysine)-coated slides. After deparaffinization and epitope retrieval in 10 mM citrate buffer, the non-specific binding sites were blocked by incubating the section in 5% normal rabbit serum in PBS (pH 7.4) for 15–20 min. The sections were washed with PBS and incubated with the anti-Eag1 antibody at a 1:500 dilution in 1%BSA/PBS for 12 h at 4 °C. Following extensive washes in PBS, the biotinylated second antibody was added to the sections for 20 min incubation at room temperature. After washing in PBS, the sections were incubated with a peroxidase conjugated avidin-biotin complex for 20 min at room temperature. Then, the reaction was developed using 3,3-diaminobenzidine tetrahydrochloride and hydrogen peroxide in PBS. The sections were counterstained with hematoxylin, dehydrated in ethanol, cleared in histosol and coverslips mounted using neutral balsam. Immunohistochemical staining images of the sections were captured under a microscope and photographed.

Table 2 Characteristics of breast tumors

Characteristics	Data	
Lymph node status, n (%)	Negative	35 (42.7)
	Positive	47 (57.3)
	Unknown	0 (0)
Tumor size, n (%)	0–2 cm	20 (24.4)
	2–5 cm	48 (58.5)
	>5 cm	14 (17.1)
Tumor grade, n (%)	Grade 1	38 (46.3)
	Grade 2	27 (32.9)
	Grade 3	17 (20.8)

Statistical Analysis

All in vitro experiments were repeated at least 3 times. Each result was the reflection of the mean of three independent experiments. The statistical significance of the difference between groups analyzed was determined by Student's t-test. Comparisons resulting in a *P value* of less than 0.05 were considered statistically significant and identified in the figures with an asterisk (*). Immunoreactivity was scored on an ordinal scale of 0 (no staining), 1 (weak staining), 2 (moderate staining), and 3 (strong staining) for Eag1 protein evaluated. In an attempt to diminish interobserver variability, all of the cases were graded in a blinded fashion.

Results

Alignment Analysis of Eag1 (370–442) and Eag2 (366–438)

Both sequences used were limited, Eag1 (370–442) and Eag2 (366–438) [9], and the alignment analysis of these sequences was carried out using the alignment method (MEGA software, version 6.0). The result of the alignments is shown in Fig. 1. The binding epitopes of antibodies against Eag1: 406–419 was specific in Eag1.

Immunogen Design and Synthesis

Based on analysis of the multiple alignments, specific sequence of Eag1 (406–419) was collected and designated as immunogen epitope. The sequence, which is acetylated at the N terminus and followed by a cysteine at the C terminus, was synthesized by solid-phase chemical synthesis and purified by reversed-phase high-performance liquid chromatography and then coupled to the carrier protein keyhole limpet hemocyanin (KLH) as Eag1-KLH by GL Biochem Ltd. (Shanghai, China). Synthetic peptide masses were characterized by mass spectrometry.

Characterization of Polyclonal Antibodies Elicited by Eag1-KLH Immunogen

Strong immune response could be elicited by the Eag1-KLH immunogen. The rabbit antisera was obtained and purified for ELISA and western blot (Fig. 2). Half-maximum binding activity could be detected at a dilution of antiserum of about >1:6400.

Fig. 1 Alignment analysis of Eag1 (370–442) and Eag2 (366–438). The sequences of 7 KCNH1 and 5 KCNH5 from *Homo sapiens* were aligned using MEGA version 5.0. The binding epitopes of antibodies against Eag1: 406–419 was specific

The Specificity of the Polyclonal Antibodies

To further identify the specificity against Eag1 in breast cancer cells, the purified antibodies were applied in western blotting. The results indicated that anti-Eag1 polyclonal antibodies could specifically interact with Eag1 from the lysates of MCF7 cells as shown in Fig. 2b.

Distribution of Eag1 in Normal and Cancerous Breast Tissues

Eag1 could be expressed in both normal and malignant breast tissues. Omission of the primary antibody was used as a negative control (Fig. 3a). Enhanced staining levels could be detected in cancer tissue sections including simple carcinoma, mucinous adenocarcinoma, infiltrating ductal carcinoma and fibroadenoma (Fig. 3b–e). Moderate to weak staining levels were found in normal hyperplasia (Fig. 3f). Statistical analysis of the intensity scores indicated that Eag1 was more frequently intense in malignant tissues as shown in Table 2 (81.43% simplex carcinoma; 80% invasive ductal carcinoma; and 100% mucinous adenocarcinoma) compared with normal hyperplasia and fibroadenoma cases as shown in Table 3 (29.41% normal hyperplasia).

Discussion

The relatively newfound roles of potassium channels in cancer activity have merited further attention in recent years [12–16]. Among these channels, Eag1 is unique in its restricted expression outside the central nervous system [3, 17] while being overexpressed in tumour-derived cell lines [3, 18–22] and detectable in several cases of cervical carcinoma using the antibody [23]. However, it is yet unclear about the actual expression frequency of Eag1 in tumours. Therefore it is urgent to setup the detection of Eag1 in clinical specimens by immunohistochemical staining [24].

The first problem when investigating the expression patterns of a protein is the specificity of the available tools, especially in the case of antibodies. It's very stringent for the selection criteria during the antibody generation process. The commonly accepted method used to define the specificity of an antibody is the recognition of the target protein in a western blot without evidence of any non-specific cross-reacting bands. Experiments have been performed using rat brain extracts without non-specific bands. However, similar to many other monoclonal antibodies, Eag1.62.mAb has a very low sensitivity in western blots as compared with polyclonal antibodies [9]. It has been reported that the antibody could recognize human Eag1 in transfected cells but does not stain cells transfected with human Eag2 by immunofluorescence experiments. We therefore used alternative approaches to confirm the selectivity of the antibodies. By alignment analysis of Eag1 (370–442) and Eag2 (366–438), potential specific linear epitopes were identified by mAb62, mAb56 [9] and polyclonal antibodies elicited by vaccination using the linear Eag1 epitope KLH immunogen: 406–419 were specific in Eag1 (Fig. 1). These results strongly suggest that the ability of Eag1 could specifically recognize its own epitope instead of Eag2.

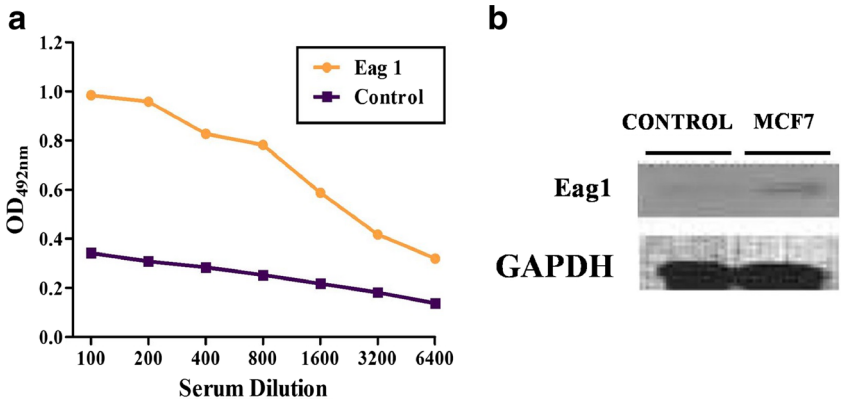

Fig. 2 Characterization of anti serum against Eag1 by ELISA and western blot. **a** Binding activity of serial dilutions of anti serum and preimmune serum were tested against linear epitope peptides. **b** MCF7 cell lysates were identified by western blotting

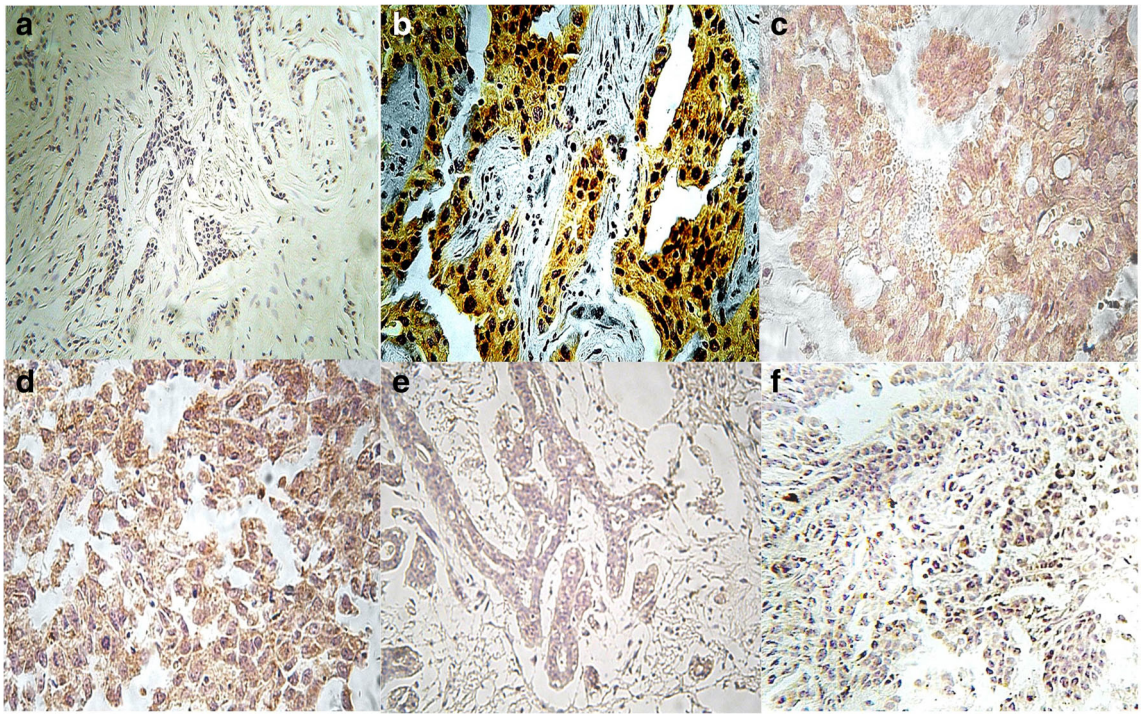

Fig. 3 Eag1 immunostaining in normal and cancerous breast tissue sections. Tissue sections stained in brown is positive for immunoreactivity. Nuclei are stained in blue. **a** Negative control treated without primary antibody. **b** Simple carcinoma. **c** Mucinous adenocarcinoma. **d** Infiltrating ductal carcinoma. **e** Fibroadenoma. **f** Normal hyperplasia. Original magnification is ×20 for all panels

Many aberrantly expressed tumour-associated proteins are useful tools for the management of cancer patients [25]. The expression of such proteins has been used to make diagnostic, prognostic and therapeutic decisions. In some cases, the aberrantly expressed protein can be used for tumour vaccination [26]. However, the high frequency (>85% in 15 of 17 tumour types; Table 2) with which Eag1 was found to be overexpressed in this study of various neoplastic tissues is unusual, especially given that most molecules overproduced in neoplastic tissues [25] are ubiquitous proteins expressed also in normal tissues.

It is important to be mentioned that although mainly cytoplasmic signals have been revealed by immunostaining, Eag1 activity could be detected in the plasma membrane by electrophysiological measurements [3, 17–19, 23, 27–29]. It's unequivocally demonstrating the channel expression on cell surface. Even in transfected cells with robust current expression, it is difficult to detect membrane staining of the channel, although biotinylation experiments have shown that the channel is exposed to the external milieu [29].

It is still unclear that at which stage of malignant transformation Eag1 expression could increase. Chromosomal aberrations affecting the long arm of chromosome 1 are relatively frequent. In fact of 1800 recurrent chromosome 1 aberrations, 280 directly affect region 1q32 and therefore Eag1 [30]. Since the channel itself is sufficient to induce transformation and can also increase both the growth rate and the invasiveness of experimental tumours [3], Eag1 expression may confer a growth advantage to tumour cells and permit a selective enrichment of Eag1-expressing cells. Given the elevated levels of Eag1 in various tumour tissues, it is interesting that inhibition of Eag1 expression leads to a reduction of DNA synthesis

Table 3 Correlations for intense hARIP2 and clinical and pathological grade of breast cancer

Tissue	Eag1 Staining Intensity		
	0, 1 (No or Weak)	2, 3 (Moderate or Strong)	Positive rate
Grade 1 tissue	16	22	57.89%
Grade 2 tissue	2	25	92.59%
Grade 3 tissue	0	17	100%

Table 4 Correlation for intense hARIP2 and lymph mode status of breast cancer

Lymph node status	Eag1 Staining Intensity		
	0, 1 (No or Weak)	2, 3 (Moderate or Strong)	Positive rate
0–3	12	29	70.73%
4–9	0	24	100%
≥ 10	0	17	100%

Table 5 Correlation for intense hARIP2 and tumor size of breast cancer

Tumor size	Eag1 Staining Intensity		
	0, 1 (No or Weak)	2, 3 (Moderate or Strong)	Positive rate
< 2 cm	8	12	60%
2–5 cm	6	42	87.5%
> 5 cm	0	14	100%

in human tumour cell lines [3, 8]. Whether this will also be the case in primary tumours remains to be elucidated.

We believe that our data justifies further studies to qualify Eag1 as a target for clinical applications. Like Her2/Neu, Eag1 is a transmembrane protein, extracellularly accessible, involved in signal transmission and expressed in neoplastic tissues such as breast cancer, although only to a limited extent in normal tissue [31]. Her2/Neu is expressed in about 30% of breast cancers, where it has proven effective as a target for immunotherapeutic approaches [32–34]. A similar scenario is conceivable for Eag1, given its high level of tumour specificity. A potential Eag1-targetted therapy presents many advantages in comparison to other established therapeutic approaches. Firstly, it could be applied to a broad spectrum of neoplasms that overexpress the Eag1 channel and thus become available to a large number of patients. Secondly, most normal cells expressing Eag1 are either protected by the blood brain barrier or represent terminally differentiated cells, thereby allowing more aggressive therapeutic intervention.

In this paper we demonstrated the production of polyclonal Eag1-specific antibodies by vaccinating rabbits using the linear epitope KLH immunization. Western blotting and ELISA analysis results confirmed that the polyclonal antibodies were specific to the Eag1 protein. Also the obtained antibodies performed remarkably in immunostaining assays on breast cancer tissues and cells. Thus, immunization with linear epitope KLH immunogen is an effective method for the generation of specific polyclonal antibodies. As mentioned above, we were able to generate polyclonal Eag1-specific antibody, which could be applied in the detection of Eag1 expression in cells and tissues and ultimately might be employed in the analysis of differential Eag1 regulation during physiological and pathophysiological processes.

In the present study five kinds of human breast tissue samples were collected, including simple carcinoma, invasive ductal carcinoma, breast mucinous adenocarcinoma, breast hyperplasia, and breast fibroadenoma. Then the expression levels of Eag1 in these tissue sections were investigated by immunohistochemistry. The results indicated that Eag1 was more frequently and much more intensely expressed in malignant tissues than that was expressed in normal hyperplasia and fibroadenoma cases (Table 3). In addition, a significant association between Eag1 staining and the presence of lymph-node metastasis in breast cancer was also observed (Table 4). However, we do not know whether Eag1 is an accelerator of metastasis or merely a correlative product during progression of breast cancer. Likewise, our result shows that the abundance of Eag1 is proportional to tumor size (Table 5). In short, the breast cancer stage correlation with Eag1 is intriguing and worth being further investigated.

Eag1 staining score was much higher in higher degree of malignancy, increased number of lymph node metastasis, and bigger tumor size. But we still do not understand the exact mechanism by which Eag1 plays its role in the progression of breast cancer.

Currently, there is great interest in the development of clinical applications for Eag1. Clinical trials of Eag1 or Eag1 antagonists have not yet started; however, the efficacy of these factors in animal disease models suggests that they might also prove beneficial in treating human diseases. According to our studies, Eag1 can also act as a marker of breast cancer development.

Although we confirmed some functions of Eag1, there are still questions to be answered. The detailed mechanism by which Eag1 exerts its role in breast cancerous development is not yet clear. Therefore, future studies of Eag1 might shed light on the exact molecular mechanisms. At the same time, further work is required in order to determine whether Eag1 has other roles or is involved in other signaling pathways.

Conclusion

The results indicate that overexpression of Eag1 might be associated with an increased risk of progression to breast cancer (Grade 1 tissue = 57.89%; Grade 2 tissue = 92.59%; Grade 3 tissue = 100%). These results also suggest that Eag1 is a putative growth-promoting gene that might be involved in breast tumorigenesis and development. Eag1 might be represented as a potential target for some human diseases treatment.

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