Clusterin is associated with spontaneous breast cancer in TA2 mice

Baocun Sun\textsuperscript{a,b,c,*},1, Shiwu Zhang\textsuperscript{a,c,1}, Danfang Zhang\textsuperscript{a,c,1}, Yanqing Liu\textsuperscript{a}, Yan Li\textsuperscript{a}, Zhe Rong\textsuperscript{a}, Yue Zhu\textsuperscript{a}, Xinghong Jia\textsuperscript{d}

\textsuperscript{a} Department of Pathology, Tianjin Cancer Hospital, Tianjin Medical University, Tianjin 300060, PR China
\textsuperscript{b} Department of Pathology, Tianjin Medical University, Tianjin 300070, China
\textsuperscript{c} The Key Laboratory, Breast Cancer Prevention and Treatment, The Ministry of Education, PR China
\textsuperscript{d} The Experimental Animal Center, Tianjin Medical University, Tianjin 300070, China

Received 17 May 2007; revised 28 May 2007; accepted 12 June 2007
Available online 21 June 2007

Edited by Lukas Huber

Abstract Two-dimensional electrophoresis and Matrix-assisted laser desorption ionization-time of flight-time of mass spectrometry were used to detect the differentially expressed proteins in serum of tientsin albino 2 mice with spontaneous breast cancer, normal tientsin albino 2 mice and tientsin albino 1 mice. Only nuclear clusterin (n-CLU) was expressed in tientsin albino 1. Immunohistochemistry and western blot validated that n-CLU was present in normal tientsin albino 2 and tientsin albino 1 mammary epithelium, and secretory clusterin expressed in the cytoplasm of normal tientsin albino 2 mammary epithelium and spontaneous breast cancer. n-CLU may play an important role in tientsin albino 2 spontaneous breast cancer initiation and development.

© 2007 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.

Keywords: Two-dimensional electrophoresis; Electrospray ion trap mass spectrometry; n-Clusterin; Spontaneous breast cancer

1. Introduction

With breast cancer morbidity increasing worldwide [1], breast cancer has become a significant threat to woman life. Many studies of breast cancer have focused on the levels of DNA and mRNA, but the function of a gene is actually fulfilled by the expression of its protein [2–4]. Mice are frequently used in genetic studies, as the genetic background of many particular strains has been established. After selection of many years, Tianjin Medical University has established two spontaneous breast cancer models: the TA1 strain has a low incidence of spontaneous breast cancer, while the TA2 strain has a high incidence [5]. Studies have indicated that skin transplantation is successful between F1 hybrids of TA1 and TA2, demonstrat-

\textsuperscript{1}Corresponding author. Address: Department of Pathology, Tianjin Cancer Hospital, Tianjin Medical University, Tianjin 300060, PR China. Fax: +86 022 23537796. E-mail address: zhangshiwu666@yahoo.com.cn (S. Zhang).

These authors contributed equally to this work.

Abbreviations: CLU, clusterin; s-CLU, secretory clusterin; n-CLU, nuclear clusterin; NLSs, nuclear localization signals; MALDI-TOF-MS, Matrix-assisted laser desorption ionization-time of flight-time of mass spectrometry

ing that F1 hybrids have the same histocompatibility genes. TA2 mice have a high incidence of spontaneous breast cancer without any chemical stimulus [6,7].

TA1 and TA2 mice have similar genetic backgrounds. In this study, the serum total protein extracts from TA1 mice, TA2 mice and TA2 mice that had spontaneous breast cancer were analyzed by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) with immobilized pH gradients followed by mass spectrometry to detect proteins that are absent or expressed in the serum of normal TA1 mice [8]. Immunohistochemical staining and Western blot were performed to validate the expression of proteins.

2. Materials and methods

2.1. Mice

There were four TA2 mice that had spontaneous breast cancer. Two of these mice have bred three times, and the others have bred four times. The average time it takes for is about 278 days. Four normal female TA1 mice coming from the same mother were selected, and so as the 4 normal female TA2 mice. The age of these particular TA1 and TA2 mice was almost the same, about 7–8 weeks.

2.2. Two-dimensional electrophoresis and mass spectrometric detection

Blood was collected from the epicanthic venous plexus of the mice and the serum was separated from the other blood components. Two-dimensional electrophoresis with immobilized pH gradients was performed according to the methods of Gorg et al. [9] and the Manual of Operations for the IPGphor isoelectric focusing system. The serum sample (15 μl) was loaded into the sample cup, ensuring that the IPG strip was covered with 2–3 ml of mineral oil. Isoelectric focusing was conducted with the immobilized pH 3–10 non-linear gradient strips (Amersham Biosciences) at 0–500 V for an hour, 500 V for 5 h, 500–3500 V for 5 h and 3500 V for 12 h. After IEF two SDS-PAGE gels (12%) were made to perform the second dimension. Electrophoresis was stopped when the dye front was approximately 1 mm from the bottom of the gel. After electrophoresis, the gel was removed from the gel cassette and stained [10].

2.3. Analysis of the gel image

ImageMaster 2D 5.0, Imagescanner and LabScan image collection software were used to capture, store, evaluate, and present information contained in the gel. All of the data were analyzed in SPSS10.0 and Excel. The spots absent in one sample and the spots which quantity increase or decrease three-fold were defined as the differential proteins.

2.4. Mass spectrometric detection

The differentially expressed protein spots were excised and de-

stained. Enough digestion buffer was added to just cover the gel slices and make them swell, and then the digestion buffer was removed and

0014-5793/32.00 © 2007 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/j.febslet.2007.06.021
After blocked with 10% non-fat milk, the membranes were incubated with anti-clusterin polyclonal antibody (Clusterin-z/β, clone: H-330-sc-8354, Santa Cruz Biotechnology Inc., 1:200 dilution) at 4 ℃ overnight. After washing for three times, the membranes were incubated with goat anti-rabbit IgG at room temperature for 30 min. The signals were developed with the ECL kit (Beijing Zhongshan Biotechnology Inc.) and using anti β-actin antibody (sc-1616, Santa Cruz Biotechnology Inc.) as an internal control.

5. Results

5.1. Pathological examination

The location of each spontaneous tumor in the mice is different: two of the four were located in the mammary gland near the forward limb, while the other two were located in the fourth mammary gland near the hind limb. Under the light microscope, the tumor cells formed many nests or tubules (Fig. 1A and B). The spontaneous breast cancer cells were negative for ER, PR, and Her-2.

5.2. Analysis of the 2D electrophoresis maps

Serum protein from mice with spontaneous breast cancer, normal TA1 mice and TA2 mice was separated by IPG-2DE-SDS–PAGE, with every sample tested three times. According to analysis by ImageMaster 2D 5.0, every sample had good reproducibility. The gel was silver-stained after 2D electrophoresis, and 2DE maps with good resolution and reproducibility were obtained. Silver staining was preferred over Coomassie blue staining because the resolution is higher with silver staining. Isoelectric focusing, SDS–PAGE and staining were performed identically for these six maps. The molecular mass of the proteins ranged between 10 and 100 kD, the isoelectric point was between 4 and 9, and the number of acidic points exceeded the number of basic points. Comparing the six maps, the main differentially expressed proteins were located between PI 4.5 and 6.5. Sorting of the protein spots from the three groups can be seen in Table 1.

5.3. Analysis of the differentially expressed proteins in mice with spontaneous breast cancer, normal TA1 and TA2 mice

Analysis with ImageMaster 2D 5.0 software indicated that the main differentially expressed proteins were located in the range between PI 4.5 and 6.5 and had a molecular mass between 25 and 97 kD. A difference more than 10 times was
used as the cutoff to determine if a certain protein was differentially expressed. Differential protein points were searched and compared in the three groups (Fig. 2). Eleven differential protein points were found, and the expression of these 11 proteins can be seen in Table 2.

5.4. Mass spectrographic analysis of the differentially expressed proteins and database searches

The differentially expressed proteins were identified by mass spectrometry on the basis of their peptide mass fingerprints (PMF) (Fig. 3A–C, Table 3). One differentially expressed
protein was identified by PMF and tandem mass spectrometry. Its molecular mass is 51655.71 (ID number: 2627.3033), and the protein was identified as the clusterin precursor (n-CLU) by searching the database (Fig. 4). Most of the other differentially expressed proteins are highly abundant serum proteins, and have no relation to tumorigenesis.

Table 3

<table>
<thead>
<tr>
<th>Accession number</th>
<th>Protein name</th>
<th>MW (Da)</th>
<th>Protein PI</th>
<th>Sequence coverage (%)</th>
<th>Ion score</th>
<th>Ion score C.I.%</th>
</tr>
</thead>
<tbody>
<tr>
<td>gi</td>
<td>8248739</td>
<td>Haptoglobin</td>
<td>38749.51172</td>
<td>6.08</td>
<td>56</td>
<td>88.86000061</td>
</tr>
<tr>
<td>gi</td>
<td>95108443</td>
<td>ATP-binding protein</td>
<td>70630.64844</td>
<td>5.59</td>
<td>47</td>
<td>50.40000153</td>
</tr>
<tr>
<td>gi</td>
<td>92870298</td>
<td>Hypothetical protein MtrDRAFT_AC140721g10v1</td>
<td>18858.36914</td>
<td>8.52</td>
<td>76</td>
<td>41.47999542</td>
</tr>
<tr>
<td>gi</td>
<td>77965817</td>
<td>Modification methylase HemK</td>
<td>29695.49023</td>
<td>4.86</td>
<td>55</td>
<td>53.98999977</td>
</tr>
<tr>
<td>gi</td>
<td>729152</td>
<td>Clusterin precursor</td>
<td>51655.71000</td>
<td>5.46</td>
<td>46</td>
<td>86.5552847</td>
</tr>
<tr>
<td>gi</td>
<td>1628624</td>
<td>Paraoxonase</td>
<td>39540.32031</td>
<td>5.07</td>
<td>49</td>
<td>74.31999969</td>
</tr>
<tr>
<td>gi</td>
<td>34785996</td>
<td>Pregnancy zone protein</td>
<td>16575.81255</td>
<td>6.24</td>
<td>66</td>
<td>47.95999808</td>
</tr>
<tr>
<td>gi</td>
<td>52005360</td>
<td>Putative Transaldolase</td>
<td>24397.60938</td>
<td>6.75</td>
<td>77</td>
<td>54.00999882</td>
</tr>
<tr>
<td>gi</td>
<td>44985692</td>
<td>AGL175Wp</td>
<td>43013.06641</td>
<td>5.62</td>
<td>59</td>
<td>46.49000168</td>
</tr>
<tr>
<td>gi</td>
<td>297547</td>
<td>SAP</td>
<td>26230.26953</td>
<td>5.98</td>
<td>68</td>
<td>101.7900009</td>
</tr>
<tr>
<td>gi</td>
<td>34393815</td>
<td>Peptide transporter-like protein</td>
<td>33643.53906</td>
<td>10.99</td>
<td>54</td>
<td>66.06999969</td>
</tr>
</tbody>
</table>

5.5. Immunohistochemical staining and Western blot results

Clusterin-α/β (clone: H-330) can detect the secretory clusterin (s-CLU) and n-CLU. As seen in the immunohistochemical staining, positive grains can be seen in the nuclei of breast ductal epithelial cells, which implied n-CLU is expressed in the normal mammary gland of TA1 mice. In TA2 mice positive

Fig. 5. n-CLU expression (A) n-CLU is highly expressed in the normal mammary gland of TA1 mice. Positive grains can be seen in breast ductal epithelial cell nuclei. (B) In TA2 mice positive grains are located in both the cytoplasm and nucleus of breast ductal epithelial cells. (C) TA2 spontaneous breast cancer tissue is n-CLU negative.
tein composed of 448 amino acids. Clusterin, first isolated approximately 70 kD [11]. Clusterin maps to 8q21 and encodes a pro-disulfide-linked glycoprotein with a molecular mass of approximately 55 kD. The difference in morbidity between TA1 and TA2 mice is only 1% [5]. After being inbred for 160 consecutive generations, TA1 and TA2 mice have highly consistent genotypes, and they can reproduce healthy offspring.

The morbidity of spontaneous breast cancer in parous female mice is 84.1% within an average of 280 days after birth, and the morbidity of virginial mice is 41.4% within an average of 400 days after birth. The morbidity in TA1 mice is only 1% [6]. The difference in morbidity between TA1 and TA2 mice may be because there are some mutations in the chromosomal structure of the TA2 strain. The mutations of genes may be result in different proteins expression.

In this study, we used two-dimensional electrophoresis and mass spectrometry to analyze the serum of mice with spontaneous breast cancer, normal TA1 mice and TA2 mice. Using TA1 mice as the control, we screened serum protein samples for proteins that are expressed or absent in the TA2 strain. Decreased expression of clusterin precursor protein can be seen in mice with spontaneous breast cancer and in normal TA2 mice, while it is expressed in the TA1 strain. Immunohistochemistry also indicated n-CLU, located in the nucleus, was expressed in TA1 mice.

Clusterin, also known as apolipoprotein J (APOJ), testosterone-repressed prostate message 2 (TRPM2), sulfated glycoprotein 2 (SGP2), complement-associated protein SP-40, and complement lysis inhibitor (CLI), is a secreted heterodimeric disulfide-linked glycoprotein with a molecular mass of approximately 70 kD [11]. Clusterin maps to 8q21 and encodes a protein composed of 448 amino acids. Clusterin, first isolated from testicular tissue in 1983, is a prostaglandin synthetase inhibitor. It is expressed in virtually all tissues and is found in all human body fluids [12]. Clusterin is involved in numerous physiological processes important for carcinogenesis and tumor growth [13,14], including apoptotic cell death [6,15], cell cycle regulation, DNA repair [16], cell adhesion, tissue remodeling, lipid transport, membrane recycling, and immune system regulation [17]. Clusterin can also act as a molecular chaperone to eliminate the metaproteins produced when cells are under stress [18].

There are two known clusterin protein isoforms generated in human cells: the secreted form (s-CLU) and the nuclear form (n-clusterin) [19]. s-CLU expression is initiated by translation from the first AUG codon of the full-length clusterin mRNA. This precursor protein, with a molecular mass of about 60 kD, is directed to the endoplasmic reticulum and then it is transported toward the Golgi apparatus where it is heavily glycosylated and the α and β subunits that are linked by disulfide bonds are cleaved. Under physiological conditions, the mature 70 kD protein that is secreted appears as a smear at 37–40 kD on a reducing SDS–PAGE immunoblot. Nuclear clusterin (n-CLU), with a molecular weight of about 55 kD, is synthesized from a second in-frame AUG codon using an alternatively spliced n-CLU mRNA. Unlike s-CLU, n-CLU does not undergo subunit cleavage, nor does this protein appear to be extensively glycosylated. Expression of n-CLU results in a pro-death signal that inhibits cell growth and survival, so it is also called a cell death protein [20]. Expression of n-CLU is induced by cytotoxic stress and it can lead to cell death [21]. n-CLU’s C-terminal coiled-coil domain can associate with the DNA double stranded break repair protein Ku70. This domain of n-CLU is the minimal region required for Ku70 binding and apoptosis. The binding of n-CLU and Ku70 can lead to the formation of Ku70/Ku80 dimers that can affect DNA non-homologous repair and eventually lead to genomic instability and cell death. In normal cells, the inactive of nuclear clusterin precursor (n-CLU) exists in the cytoplasm of normal cells and has two targets of nuclear localization signals (NLSs). Under damage, p-CLU can be decorated and form the 55 kDa n-CLU. With the help of NLSs, n-CLU transferred into the nucleus and induce the cell apoptosis [21].

It is indicated that clusterin is highly associated with cell apoptosis and survival [15,22]. The relationship between clusterin and the pathogenesis and progression of cancer is determined by the presence of the different isoforms, the intracellular location of the protein and the glycosylational modifications [23–26]. It is reported that clusterin takes part in the pathogenesis and progression of cancer only after glycosylation and cellular relocation [27–29]. The immature precursor and n-CLU exist together in the nucleus of tumor cells, while the mature clusterin localizes in the cytoplasm. Unlike glycosylated mature clusterin which can promote the pathogenesis and progression of cancer [30], n-CLU may be proapoptotic [31,32] and is capable of eliminating abnormally replicating cells to avert tumor progression [33,34].

The antibody we used in this study to detect clusterin α is a polyclonal antibody. It can detect both cytoplasmic clusterin and n-CLU and can therefore distinguish the glycosylated mature clusterin from n-CLU depending on the location of the positive grains. Results from our study show that clusterin in breast epithelial cells in TA1 mice is located in the cell nucleus, suggesting that n-CLU is present in the breast epithelial cells in TA1 mice.
Acknowledgements: We thank Valerie Dunmire for her expert editorial assistance with this manuscript. This work was partially supported by Tianjin Key Science and Technology Committee Foundation of China (043115211-1).

References