Extracellular Superoxide Dismutase, a Potential Extracellular Biomarker Candidate for Prolactinoma

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ABSTRACT

Aim: *To investigate whether the extracellular superoxide dismutase (EC-SOD) and manganese superoxide dismutase (Mn-SOD) level changes during prolactinoma (PRL) development.*
Methods: *Surgical tissues from 37 female patients with PRL were tested for Mn-SOD and serum samples from such PRL patients were tested for EC-SOD level changes with Western Blot. The Mn-SOD level from blood cells was also investigated to show whether the Mn-SOD variation could locate tumorigenesis tissues.*
Results: *According to the patients' age analysis, age 20−40 years is high risk for getting PRL. There is a positive relationship between the PRL severity and EC-SOD. The Mn-SOD level from surgical tissues, but not blood cells, also shows a corresponding positive relationship to PRL severity, which indicates that elevated Mn-SOD might only happen in PRL tumorigenesis tissues.*
Conclusions: *Extracellular superoxide dismutase is an extracellular protein and the serum EC-SOD could be a good candidate for the diagnoses of prolactinoma.*

Keywords: Extracellular superoxide dismutase (EC-SOD), manganese superoxide dismutase (Mn-SOD), prolactinoma

Superóxido Dismutasa Extracelular – Candidato Potencial a Biomarcador Extracelular del Prolactinoma

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RESUMEN

Objetivo: *Investigar los cambios de niveles del superóxido dismutasa extracelular (EC-SOD) y el superóxido dismutasa de manganeso (Mn-SOD) durante el desarrollo del prolactinoma (PRL).*
Métodos: *Los tejidos quirúrgicos de 37 pacientes hembras con PRL fueron examinados para investigar los niveles de cambio de Mn-SOD, mediante la técnica de Western Blot. El nivel de Mn-SOD de las células sanguíneas fue investigado para ver si la variación de Mn-SOD puede indicar la localización de tejidos de tumorigénesis.*
Resultados: *Según el análisis de la edad de los pacientes, la edad de 20-40 años presenta un alto riesgo de desarrollar PRL. Hay una relación positiva entre la severidad del PRL y el EC-SOD. El nivel de Mn-SOD en los tejidos quirúrgicos – a diferencia de lo que ocurre en las células sanguíneas – muestra una relación positiva con respecto a la severidad del PRL, lo cual indica que un Mn-SOD elevado, sólo podría tener lugar en los tejidos de la tumorigénesis del PRL.*
Conclusiones: *El superóxido dismutasa extracelular (EC-SOD) es una proteína extracelular, y el EC-SOD sérico podría ser un buen candidato para diagnosticar el prolactinoma.*

Palabras claves: Superóxido dismutasa extracelular (EC-SOD), superóxido dismutasa de manganeso (Mn-SOD), prolactinoma

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INTRODUCTION

Pituitary adenomas are the most common tumours of the pituitary gland and almost 10−25% of all intracranial tumours are caused by pituitary adenomas (1). The prevalence of diagnosing a pituitary adenoma has increased over the past 20 years (2). Studies using computed tomography (CT) or magnetic resonance imaging (MRI) reveal that 20% of pituitary glands harbour an incidental lesion (2). However, whether the diagnostic strategy so far used clinically is accurate enough is still debated. Pituitary adenomas are classified as either clinically functional adenomas or nonfunctioning pituitary adenomas (NFPAs). Functioning pituitary adenomas represent approximately 68% of pituitary adenomas and are characterized by a hormone-related clinical syndrome as compared with nonfunctional adenomas (3).

Among all these functional pituitary adenomas, prolactinoma (PRL) is the most common tumour which affects females, leading to bitemporal hemianopsia (due to pressure on the optic chiasma), vertigo, nausea and vomiting. Prolactinoma is also found in male patients and might result in hypogonadism, gynaecomastia and erectile dysfunction. Almost 80−85% of pituitary adenomas are PRLs and females have a five-fold higher risk to this tumour than males (4).

Although the pathogenesis of this tumour is still not clearly known, enhanced glycoprotein secreting hormones are commonly observed (5). According to the proteomic analysis reported recently, several glycoproteins are shown to be related to the development of non-functional PRL in humans (6). Extracellular superoxide dismutase (EC-SOD) is one kind of glycoprotein which is the principal enzymatic scavenger of superoxide in the extracellular space (7).

Superoxide dismutase is an antioxidative enzyme involved in the defense against reactive oxygen species (ROS). Three mammalian superoxide dismutases had been biochemically and molecularly characterized: SOD1, or Cu/Zn SOD, exists as a homodimer with Cu/Zn at its active site exclusively localized in the intracellular cytosol; Mn-SOD or SOD2 exists as a tetramer with Mn at the active site. Manganese superoxide dismutase is only localized in the mitochondria. Extracellular superoxide dismutase, SOD3, is the most recently characterized SOD. It has been shown that more than 99% of the enzyme is bound to heparan sulfate proteoglycans in vascular walls and to a lesser extent within the interstitium, and less than 1% is contained within the circulation in equilibrium between the plasma phase and the glycocalyx of the endothelium (8). Extracellular superoxide dismutase is now known to play an important role in maintaining vascular tone, attenuating age-related cognitive decline, lung function, and the metabolism of nitric oxide [NO] (9). Extracellular superoxide dismutase is secreted into the extracellular space and forms a glycosylated homotetramer that is anchored to the extracellular matrix (ECM) and cell surfaces through an interaction with heparan sulfate proteoglycan and collagen (9, 10). The overexpression of EC-SOD showed positive inhibition for breast cancer cell growth and invasion (11).

In our research, we analysed the serum EC-SOD level and Mn-SOD level from surgical tissues in 37 female patients with PRL of varying severity. Patients with PRL are confirmed by prolactin staining and only the patients with prolactin hypersecretion positive staining were selected. The tumour severities are divided into four grades as I (diameter < 1 cm), II (1 cm < diameter < 2 cm), III (2 cm < diameter < 3 cm) and IV (diameter > 3 cm), according to the tumour size. A positive correlation between the severity and both the EC-SOD level and surgical tissue Mn-SOD level were detected. Our results indicate that Mn-SOD variation might only happen in tumorigenesis tissues since no Mn-SOD change was detected in blood cells. However, on the contrary to Mn-SOD, EC-SOD is a secreted protein which makes it easier for diagnostic use.

SUBJECTS AND METHODS

Chinese patients diagnosed with PRL were recruited sequentially from Hospital of Baotou Medical College. The patients underwent basal and dynamic evaluation of the hypothalamic-pituitary axis. Diagnosis of PRL was based on the presence of a sellar lesion and the absence of hormonal hypersecretion and was confirmed by histological examination of the surgically removed tissues. All patients underwent a CT or MRI scan. Age- and sex-matched healthy volunteers attending the Hospital of Baotou Medical College for health examination were recruited as controls.

Blood was collected from each study participant into 5 ml serum separator vacuum tubes, kept at 4°C for 1 hour and then centrifuged at 2000 g for 30 minutes at 4°C. The serum was divided into 100 µl aliquots and stored at -80°C. The pellet was collected for blood cell analysis.

The serum was 10 times diluted with 0.9% sodium chloride (NaCl) water solution and spun down at 210 000 g at 4 °C for 10 minutes, the protein concentrations were determined with brad-ford assay (Bio-Rad). After denaturation by heating and adding DTT, the serum dilutions were subjected to SDS–PAGE (10%) electrophoresis and proteins were transferred onto nitrocellulose membrane (Whatman). The primary antibodies used included rabbit polyclonal-SOD (ADI-SOD-106, Enzo Life Sciences) for EC-SOD detection, mouse polyclonal Mn-SOD antibody (AM05804PU-N, Acris) for Mn-SOD probing. Horse radish peroxidase anti-rabbit or anti-mouse (GE Healthcare/Amersham) with 1:50 000 dilutions were used to probe rabbit or mouse IgG, respectively. Bands were visualized using the ECL Dura reagent for SODs analysis (Thermo). Signals corresponding to target bands were quantified by using the Fuji film Image gauge (Fuji) analysis software.

For Western Blot, the target protein intensities were normalized with corresponding control. The average of control values was set to 100% and statistical analysis was done

by one-way analysis of variance (ANOVA) followed by Tukey's calculation with Prism graph pad 5.0.

RESULTS

According to all patient samples collected, 37 female patients were diagnosed as having PRL (Table).

Table: Classification of prolactinoma (PRL) patient samples studied in this research

Age (year)	20−30	30−40	> 40
Total patient number	15	17	5
I	6	3	0
II	7	4	1
III	1	8	3
IV	1	2	1

A total of 37 female patients diagnosed with PRL were studied in the hospital. All the PRL cases are classified into three groups according to the patient's age. For each age group, PRL severity is further classified into four degrees according to the tumour size. I: (diameter < 1 cm), II (1 cm < diameter < 2 cm), III (2 cm < diameter < 3 cm), IV (diameter > 3 cm).

Statistical analysis shows that most PRLs occur around age 30−40 years, which is almost 46% of the total cases collected. Being age 20−40 years is a high risk for PRL. For the severity of pituitary adenomas, age 30−40 years showed relatively more serious tumour level than age 20−30 years. Similar trends were detected in patients older than 40 years also. Statistical severity analyses of PRL are shown in Fig. 1 according to patients' age.

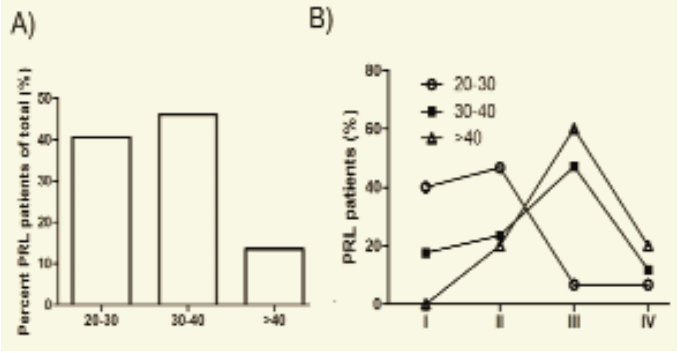

Fig. 1: The statistical analysis shows prolactinoma (PRL) could be age related.
(A) Among all the PRL patients, PRL was detected most in patients aged 30 to 40 years. Those aged 20−30 years were also at high risk for PRL. There was less possibility of PRL being detected in the older age group, 40−50 years.
(B) The severity of PRL in different ages showed different trends. Most of the patients in their 20s had a relative tumour size smaller than 2 cm in diameter. The PRL patients older than 30 years mainly had tumour bigger than 2 cm in diameter. Rarely, large tumour in grade III or IV was detected in an age younger than 20 years.

The Mn-SOD level increased according to PRL severity

In order to test the Mn-SOD variation, surgical tissues were analysed by Western Blot. The Mn-SOD probing results showed that Mn-SOD level was elevated according to the tumour size, and the more serious the PRL, the more Mn-SOD that was detected and this corresponding relationship between tumour size and Mn-SOD level was detected through all the ages of patients with PRL studied. For patients aged 20−30 years, Mn-SOD showed significant increase although the tumour size was less than 1 cm (Fig. 2A). The larger the tumour detected, the higher the Mn-SOD level. When tumours are getting larger than 3 cm in diameter, an even higher Mn-SOD level was detected. The relationship between Mn-SOD level and tumour size in patients in their twenties could be further confirmed if more patient samples were available. This hypothesis could be confirmed by the Mn-SOD level in patients in their thirties (Fig. 2B). The consistent increase in the level of Mn-SOD correlates with the PRL severity. A plateau level of Mn-SOD was also detected after grade III tumour severity, which might show the saturation level that the PRL could reach. This hypothesis would be difficult to prove because of the patients lack of such advanced tumours. Patients in their forties or older are rarely detected with small tumours and most of the patients significantly shown with increased Mn-SOD level have relative larger tumours above 3 cm in size (Fig. 2C). All our results for Mn-SOD probing strongly showed that PRL size was positively correlated to the Mn-SOD level of the tumour cells.

The SOD levels in blood cells are not affected by the severity of PRL

In order to test whether elevated Mn-SOD could only be detected in tumorigenesis cells, the Mn-SOD levels in blood cells of patients aged 30−40 years were analysed with Western Blot. No significant Mn-SOD level was detected in patients' blood cells (Fig. 4) which might indicate that besides tumorigenesis cells, Mn-SOD in other tissues might not be affected. In this experiment, we only showed the results from age 30−40 years. This age group covered the most severe PRL grades and each grade has more than one sample, which made it easier for statistical analysis. We did similar Mn-SOD level analysis on patients in other age groups; no significant difference was detected though the tumour sizes were different (data not shown).

Serum EC-SOD level elevated according to the severity of PRL

The Mn-SOD is located in mitochondria inside cells and it will be difficult for diagnostic application. Proteomic analysis showed that EC-SOD level varies in pituitary tumour. This gives a clue whether this glycoprotein also varies in PRLs and the secreting property might be very useful for diagnosis of this tumour. The EC-SOD from the patients at different ages with PRL was also detected by Western Blot. In patients in their twenties (Fig. 3A), a slightly increased trend was detected during tumour development. When the PRL tumour was detected, the EC-SOD level almost doubled that of the control. A more significant EC-SOD level was

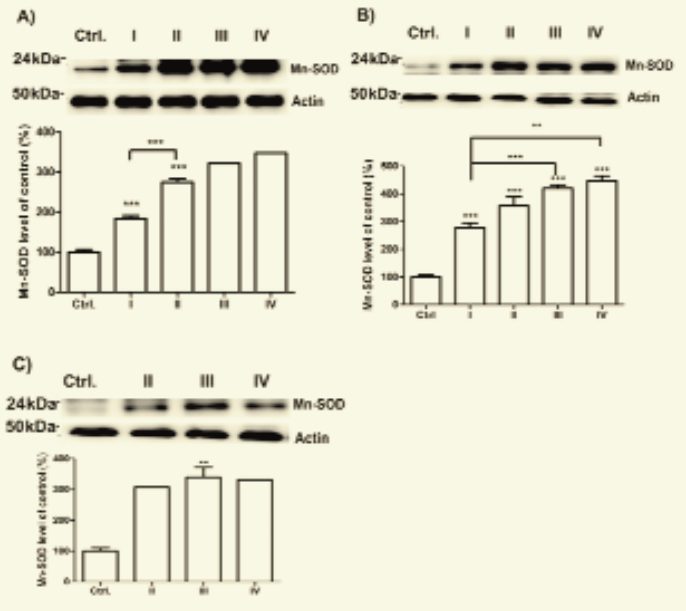

Fig. 2: The cellular Mn-SOD level is upregulated corresponding to the tumour size.

According to the different age groups, the surgically removed tissues were homogenized and prepared for Western Blot analysis. The patient samples were taken from (A) 20 to 30 years, (B) 30−40 years and (C) 40−50 years. The columns show the statistical analysis for Western Blot bands intensity. The columns with error bars show the sample size bigger than three. The relative Mn-SOD intensity obtained by normalizing Mn-SOD with Actin and the average value of negative control was set to 100%. One way ANOVA followed by Tukey's analysis were carried out. For all of the graphs, data are shown as means ± SEM for more than three separate experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

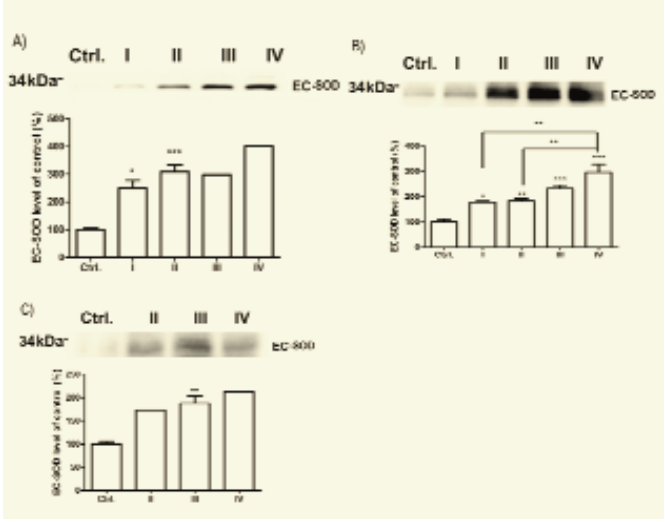

Fig. 3: The serum EC-SOD level is upregulated corresponding to the tumour size.

According to the different age groups, the sera were diluted 10 times and prepared for Western Blot analysis. The patient samples were taken from persons (A) 20 to 30 years, (B) 30−40 years and (C) 40−50 years. The columns show the statistical analysis for Western Blot bands intensity. The columns with error bars show the sample size bigger than three. The relative EC-SOD intensity obtained by normalizing EC-SOD with the total protein loaded and the average value of negative control was set to 100%. One way ANOVA followed by Tukey's analysis was carried out. For all of the graphs, data are shown as means ± SEM for more than three separate experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

detected when the tumour size was more than 1 cm in size. A larger scale EC-SOD level increase was found in PRL patients with larger tumours. When the tumour was around 4 cm or more in size, the EC-SOD level was almost three-fold in the patient's serum (Fig. 3B). A significant EC-SOD level increase was detected in patients in their forties with tumour size more than 3 cm (Fig. 3C).

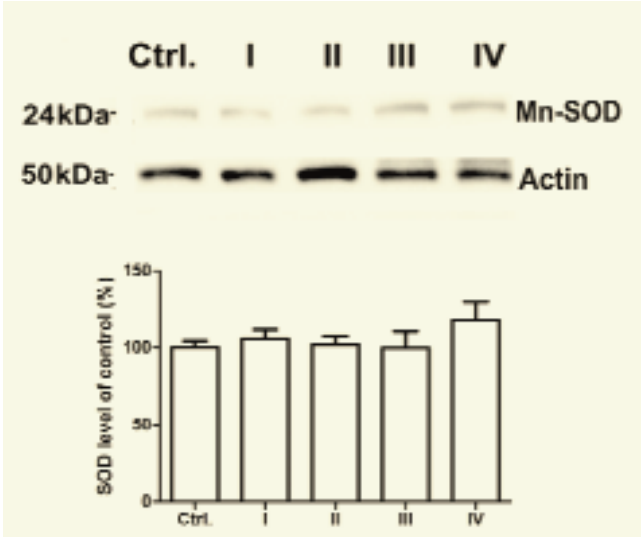

Fig. 4: The Mn-SOD level in blood cells does not change during tumour development.

The Mn-SOD level of patients aged 30−40 years was detected by Western Blot. No significant difference was detected corresponding to the PRL severity. One way ANOVA followed by Tukey's analysis was carried out. For all of the graphs, data are shown as means ± SEM for more than three separate experiments.

DISCUSSION

Pituitary adenoma is a common tumour woldwide, which is difficult to diagnose before clinical symptoms manifest. The adenomas could be classified into many types according to different criteria. Originally, these adenomas were mainly classified by size (microadenomas and macroadenomas), however, specific hormone staining techniques give a more objective way to describe the pathological mechanism of tumorigenesis. At present, proteomic methods are used to find out the variation of specific proteins during PRL development in patients. These proteins could potentially become specific biomarkers for diagnostic use and EC-SOD is one of these biomarker candidates (12). In our research, we used surgical tissues to detect the Mn-SOD variation and the serum of patients to evaluate EC-SOD level. The increased trends of EC-SOD and Mn-SOD correspond to the PRL severity. Although we had a limited number of patients with PRL, age 20−40 years seem a high risk period for PRL and

the larger the tumours, the higher the levels of Mn-SOD and EC-SOD detected.

The serum EC-SOD could be a possible diagnostic marker for PRL, but it does not mean that only EC-SOD level can be used as an absolute criterion for PRL prediction. A good biomarker needs to show both high specificity and sensitivity. Extracellular superoxide dismutase was reported to be elevated in Type 2 diabetes and other tumours (13). The EC-SOD is believed to be affected by zinc nutritional status (14). The overexpression of EC-SOD also showed protective effects on tumours, which could also be applied to therapeutic usage (15). It will be very interesting to see if EC-SOD is functioning in the development of PRL or just as a downstream feedback result from tumour development.

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