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Clusterin Expression in Non-neoplastic Adenohypophyses and Pituitary Adenomas: Cytoplasmic Clusterin Localization in Adenohypophysis is Related to Aging

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Abstract Clusterin is a circulating multifunctional glycoprotein produced in several kinds of epithelial and neuronal cells. Clusterin is upregulated during different physiological and pathological states, such as senescence, type-2 diabetes mellitus, Alzheimer disease, and in various neoplasms. Herein, we investigated the immunohistochemical expression of clusterin in non-neoplastic adenohypophysis of human autopsy subjects and pituitary adenomas. We also investigated the association of clusterin increase with age in adenohypophysis of autopsy subjects. Immunohistochemically, clusterin was found positive in the cytoplasm of all adenoma cases, and in the cytoplasm of parenchymal cells, stellate cells, mixed cell follicles and in colloidal material inside of the follicles of non-neoplastic adenohypophysis as well. Clusterin expression in pituitary adenomas was found significantly higher than in non-neoplastic adenohypophyses. In addition, in non-neoplastic adenohypophysis, a significant increase in clusterin expression levels between young (≤30 years), middle aged (31 to 60 years), and older (≥61 years) subjects ($p<0.00001$, analysis of variance [ANOVA]) was found. In addition to clusterin accumulation, presence of calcification ($p<0.045$, ANOVA) and presence of large follicles with colloid accumulation ($p<0.004$, ANOVA) were also statistically significant factors related to aging in non-neoplastic adenohypophysis. In conclusion, the present study demonstrated that clusterin expression was found in non-neoplastic adenohypophysis and in upregulated amounts in pituitary adenomas. This study also demonstrated that in non-neoplastic adenohypophyses, increase of clusterin positive cells; histopathological findings of calcification or presence colloidal material accumulation in large follicles were associated with age. To our knowledge, immunohistochemical localization of clusterin in pituitary adenomas was not reported previously.

Keywords human pituitary · pituitary adenoma · aging · clusterin · immunohistochemistry

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Introduction

Clusterin is a circulating multifunctional glycoprotein produced in several kinds of epithelial cells and neuronal cells [1–7]. Clusterin (apolipoprotein J/ApoJ) was firstly identified in ram rete testis fluid as a secreted glycoprotein enhancing cell aggregation in vitro and thus named as clusterin [1]. In human, it was initially purified from serum and the cloned gene was named as complement cytolysis inhibitor (CLI) SP-40, 40 (secreted protein 40, 40) or apolipoprotein J [8]. Clusterin is upregulated during various physiological and pathological conditions, for example; during replicative senescence, stress-induced premature senescence, type-2 diabetes mellitus, Alzheimer disease, Creutzfeldt–Jacob disease, myocardial infarction, renal

diseases, drug abuse, and in a variety of neoplasms such as colonic adenomas and carcinomas, ovarian carcinomas, hepatocellular carcinomas, urothelial carcinomas, breast carcinomas, and laryngeal carcinomas [1–23]. To our knowledge, clusterin expression in pituitary adenomas was not reported previously.

Formerly, clusterin was analyzed by biochemical methods or by cloning of specific enzymes related to clusterin or by detecting clusterin mRNA [11]. After production of specific monoclonal antibodies to clusterin, immunohistochemical methods have been developed. O'Bryan et al. [4] showed increased level of clusterin in mouse brain related to aging.

It is known that dysfunction or hypofunction of neuroendocrine system occurs with age. The pituitary gland is a central neuroendocrine organ that showed some structural changes such as increase of follicles in adenohypophysis with various acute stresses and aging or age-related decrease in growth hormone secreting cells in pituitary [24]. Immunohistochemically, S-100 containing follicles and its association with aging were reported in porcine adenohypophysis previously [25].

There were only two studies reported by Ishikawa et al. related to immunohistochemical clusterin expression in non-neoplastic adenohypophysis in Japanese people [2, 3]. One of those studies was related to age-dependent clusterin increase and the other was related to clusterin increase in drug abusers' adenohypophysis. In both of those studies, the authors indicated the necessity for further studies about clusterin, its mechanism in individual differences, and its forensic application in age determination.

Herein, we investigated the immunohistochemical expression of clusterin in pituitary adenomas, and the variation in clusterin expression levels between non-neoplastic adenohypophyses and pituitary adenomas. We also investigated the immunohistochemical expression of clusterin in the parenchymal cells of human adenohypophysis besides the alteration in clusterin expression during aging and other age-related histopathological changes in non-neoplastic human adenohypophysis in a distinct population.

Material and Methods

Specimens

Paraffin-embedded archival specimens of pituitary adenomas from 15 cases (11 [73.3%] male, 4 [26.7] female) and paraffin-embedded specimens of non-neoplastic pituitaries from serial autopsy cases (n=96) within 48 h postmortem were obtained. From the 96 autopsy cases, 63 (65.6%) of them were male and 33 (34.4%) were female.

The age of adenoma subjects ranged from 16 to 77 year (mean, 48.5 y) and the age of autopsy subjects ranged from 1 month to 97 years (mean, 25 y).

The age of autopsy subjects were examined by grouping as follows; group I: subjects between 0 and 30 years (n=38), group II: subjects between 31 and 60 years (n=31), group III: subjects 61 years and older (n=27). Reasons of death were blunt injury (n=16 [16.7%]), sharp injury (n=18 [18.8%]), poisoning (n=6 [6.3%]), drowning (n=9 [9.4%]), asphyxia (n=18 [18.8%]), hyperthermia (n=1 [1%]), and natural diseases (n=28 [29.2%]).

The age of adenoma cases were also examined by grouping as in autopsy cases as follows; group I: cases ≤ 30 years (n=4 [26.7%]), group II: cases between 31 and 60 years (n=7 [47.7%]), group III: cases >60 years (n=4 [26.7%]).

Immunohistochemically, anti-prolactin (Clone SPM108, mouse monoclonal, prediluted ready to use, NeoMarkers, Lab Vision Corporation, UK), anti-luteinizing hormone (LH, Clone SPM103, mouse monoclonal, prediluted ready to use, NeoMarkers, Lab Vision Corporation, UK), anti-follicle-stimulating hormone (FSH, Clone FSH03, mouse monoclonal, prediluted ready to use, NeoMarkers, Lab Vision Corporation, UK), anti-thyroid stimulating hormone (TSH, Clone SPM104, mouse monoclonal, prediluted ready to use, NeoMarkers, Lab Vision Corporation, UK), anti-adrenocorticotrophic hormone (ACTH Ab1, prediluted ready to use, NeoMarkers, Lab Vision Corporation, UK), anti-growth hormone (GH, CloneSPM106, mouse monoclonal, prediluted ready to use, NeoMarkers, Lab Vision Corporation, UK) and histochemically reticulin stain (Bio-Optica, Milan, Italy) were performed on 4-μm thick sections of pituitary adenomas.

Clusterin Immunohistochemistry

Formalin-fixed paraffin-embedded specimens were sectioned at 4-μm thick. Three sections from three different levels were obtained from all paraffin blocks of non-neoplastic adenohypophysis and pituitary adenomas. Sections were deparaffinized in xylene and dehydrated in ethyl alcohol. After deparaffinization, sections were immersed for 30 min in 0.3% hydrogen peroxide in methanol for endogenous peroxide inactivation followed by three washes in PBS (*Phosphate buffer saline*, pH 7.4) in room temperature. Blocking with PBS containing 1% goat serum and 1% bovine serum albumin was performed for 30 min. Next, Clusterin (1:100, rabbit anti-human clusterin-α/β (H-330) polyclonal antibody, Santa Cruz, CA, USA) was reacted for 1 h at room temperature. After washing in PBS, peroxidase activity was localized with chromogen 3,3′-diaminobenzidine (DAB; DAKO Liquid DAB-Substrate-chromogen K-3466, CA, USA) and 0.03% hydrogen

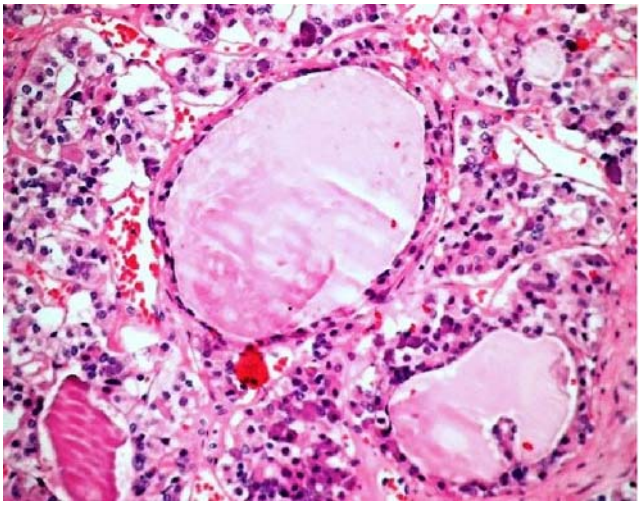

Fig. 1 Large follicles with colloidal material accumulation in adenohypophysis of a 56-year-old autopsy subject (×400; H&E)

peroxide. Sections were counterstained with hematoxylin, cleaned and mounted. Negative control studies were performed concurrently in the absence of the primary antibody. Positive control studies were performed simultaneously in the sections of an invasive, high grade, urothelial carcinoma that was reported to be clusterin positive [13, 16]. One pathologist evaluated immunoreactivity by using a digital light microscope (Leica, DM4000B, Leica-microsystems, Wetzlar, Germany). Brown staining in cytoplasm was considered as positive. Number of both negative and positive cells was counted in three serial slides of each case. In autopsy cases, the result was expressed as the proportion of the average number of clusterin-containing cells to the whole number of the follicular cells counted in adenohypophysis for each slide (percent of clusterin positive cells). In adenoma cases, the result was expressed as the proportion of the number of clusterin positive adenoma cells to the whole number of adenoma cells detected on each slide (percent of clusterin positive cells).

Statistical analyses were by *SPSS for Windows Version* 11.0 and $p<0.05$ was accepted for statistically significance. The data were analyzed by Kolmogorov–Smirnov test of normality and after confirming that the data showed normal distribution, one-factor analysis of variance (ANOVA) was performed. Then, Scheffe *F* test was performed to identify the significant difference in means. Simple linear regression analysis was used to examine the correlation between age and percent clusterin positive cells and, the correlation coefficient and regression equation (*R*) were calculated.

Results

On light microscopic examination of adenoma cases, the diagnoses of pituitary adenomas were reevaluated both morphologically and immunohistochemically. From 15 adenoma cases, eight (53.3%) of them were prolactinomas (the percents of positive immunostaining of those eight prolactinoma cases were as follows: case-I: prolactin (prl) 85% and GH 20%; case-II: prl 95%; case-III: prl 90%, ACTH 5%; case-IV: prl 100%; case-V: prl 80%; case VI: prl 95%, ACTH 10%; case-VII: prl 95%, case-VIII: prl 100%). 5 (33.3%) were FSH and LH secreting adenomas (the percents of positive immunostaining of those five FSH and LH secreting adenoma cases were as follows: case-I: FSH 50%, LH 60%; case-II: FSH 70%, LH 30%; case-III: FSH 45%, LH 65%; case-IV: FSH 80%, LH 10%; case-V: FSH 70%, LH 50%), 1 (6.7%) was ACTH secreting adenoma (ACTH was 80% positive in this case's adenoma cells) and 1 (6.7%) was null-cell adenoma.

On light microscopic examination of hematoxylin and eosin (H&E) stained slides, the histopathological alterations observed in adenohypophysis of autopsy cases were areas of calcification, presence of large follicles with colloidal material accumulation, and abscess formation (Fig. 1). Large follicles with colloidal material accumulation were present in 42 (43.8%) cases and absent in 54 (56.3%) cases. Areas of dystrophic calcification were present in 17 (17.7%) cases and absent in 79 (82.3%) cases. Abscess formation was found in 1 case (1.04%).

Immunohistochemically, in autopsy cases, clusterin was found positive in the cytoplasm of the parenchymal cells, stellate cells, mixed cell follicles and in colloidal material inside the follicles of adenohypophysis (Figs. 2, 3, 4, 5). In all pituitary adenoma cases, adenoma cells were found positive for clusterin as well (Fig. 6). In autopsy cases, mean value of percent clusterin positive cells for non-neoplastic adenohypophyses were 39.47%. The mean value of clusterin expression in pituitary adenomas was 88.33%. There was a statistically significant difference for

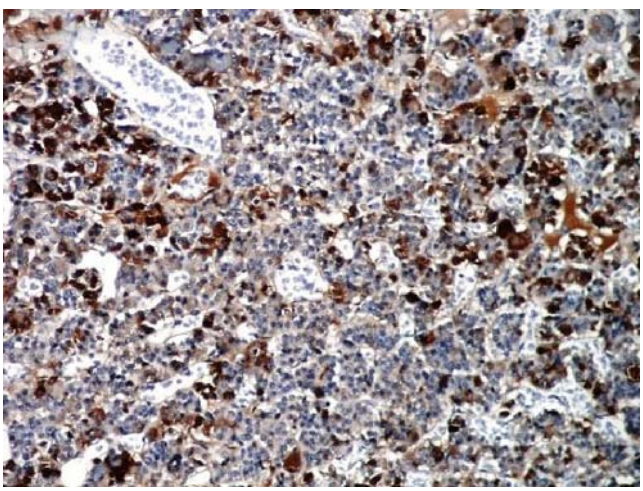

Fig. 2 Clusterin-positive parenchymal cells of non-neoplastic adenohypophysis, clusterin expression could be detected in the cytoplasm (×100; clusterin)

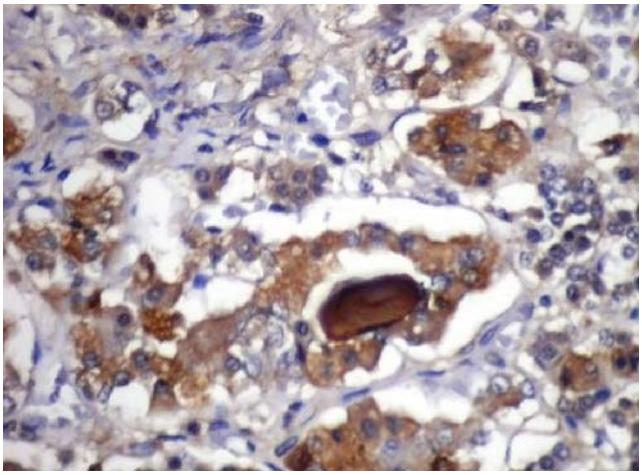

Fig. 3 Clusterin-positive colloidal material inside of the follicles of adenohypophysis. There were not many clusterin positive cells in this 23-year-old autopsy subject (×200; clusterin)

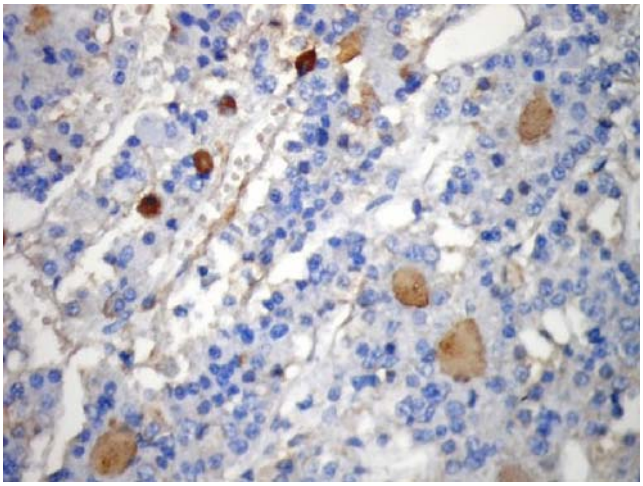

Fig. 5 Clusterin positive parenchymal cells and colloidal material inside of the follicles in a 6-year-old autopsy subject. (×200; clusterin; note the remarkable clusterin expression diversity between Figs. 4 and 5)

mean clusterin expression percents between adenomas and non-neoplastic adenohypophyses ($p<0.0000$, ANOVA, Table 1).

The mean value of percent clusterin positive cells in non-neoplastic adenohypophyses of age groups I, II, and III were 29.89%, 38.16%, and 54.44%, respectively (Table 2). There was a significant difference between age groups ($p<0.00001$, ANOVA; $R=0.456$, $p<0.00001$) in non-neoplastic adenohypophyses (The lower and upper values of percent clusterin expression for each age group are given in Table 1). In addition, histopathological alterations observed in adenohypophysis that related to aging significantly were: presence of large follicles with colloid accumulation ($p<0.004$, ANOVA) and areas of calcification ($p<0.045$, ANOVA; Tables 3 and 4).

The mean value of percent clusterin positive cells in pituitary adenomas of age groups I, II, and III were 77.5%, 89.3%, and 97.5%, respectively. In adenoma cases, we detected significantly increased expression of clusterin related to aging compared to these three age groups ($p<0.034$, ANOVA).

There was no gender predilection for clusterin expression between males and females in autopsy cases ($p=0.540$, ANOVA). No gender predilection for clusterin expression was found in adenoma cases as well ($p=0.800$, ANOVA).

Association between clusterin expression and cause of death was statistically insignificant in autopsy cases ($p>0.05$, ANOVA). No statistically significant relationship was found between clusterin expression in the autopsy cases of patients who died of hypoxia (asphyxia and drowning, $n=27$, 28.1%) and those who died of other reasons ($p=0.562$, ANOVA).

From 96 (100%) cases, 28 (29.2%) of them were died because of a chronic disease. From the medical records of

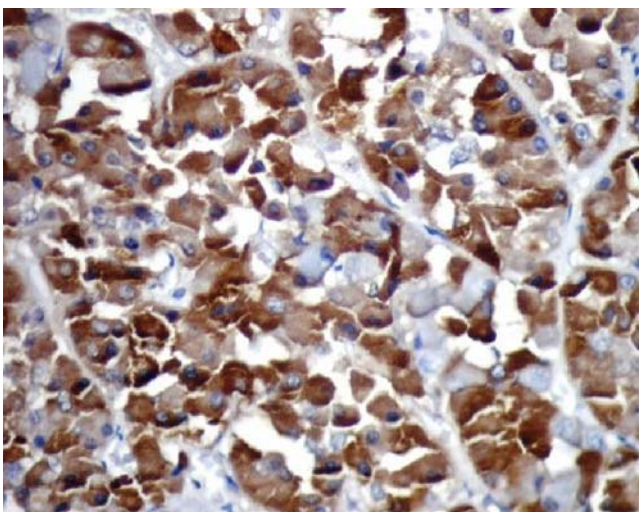

Fig. 4 Clusterin positive parenchymal cells of a 74-year-old autopsy subject (×200; clusterin)

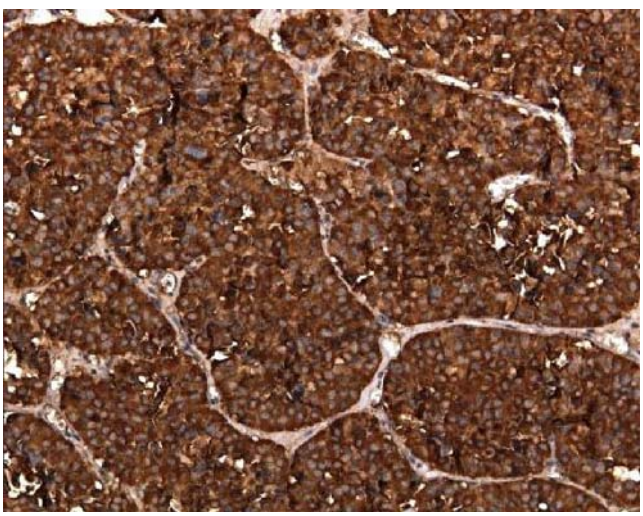

Fig. 6 Clusterin expression in a pituitary adenoma case (×200; clusterin)

Table 1 Clusterin Expression in Non-neoplastic Adenohypophyses and Pituitary Adenoma Cases

Pituitary Adenoma	Number of cases	Clusterin expression (mean)	%95 confidence interval for mean		Significance (p)
			Lower bound	Upper bound	
Absent	96	39.47	35.08	43.86	0.00000
Present	15	88.33	81.91	94.76	
Total	111	46.07	41.08	51.06	

(ANOVA, $p>0.05$ significant)

those 28 (29.2%) cases, 25 (26%) of them died because of cardiac failure and three (3.1%) because of sepsis after bacterial infection. From the 25 (26.1%) cases who died because of chronic cardiac failure, four of them had type-2 diabetes mellitus. No statistically significant difference in clusterin expression level was found between to those who died because of chronic diseases (n=28, 29.2%) and those who died suddenly because of forensic reasons (n=68, 70.1%; p=0.608, ANOVA). There was also no statistical significance in clusterin expression level between the cases with type-2 diabetes mellitus (n=4, 4.2%) and the cases without endocrine disease (n=92, 95.8%; p=0.942, ANOVA).

In adenoma cases, no significant association was detected between clusterin expression and the type of adenoma.

Discussion

In this study, we demonstrated clusterin expression in pituitary adenomas and non-neoplastic pituitaries. To our knowledge, we first showed clusterin expression in pituitary adenomas and we originally found significant increase in clusterin expression in pituitary adenomas compared to non-neoplastic adenohypophyses. We also detected a significant difference in adenohypophysial clusterin levels between young (≤30 years), middle-aged (31 to 60 years) and older (≥61 years) subjects. Besides, presence of large follicles with accumulation of colloidal material and areas of calcification in adenohypophysis were histopathological findings that were detected in adenohypophyses of the autopsy subjects with aging. Although the ethnical population and experimental procedures were different, Ishikawa et al. [2] have reported almost similar findings about clusterin expression in human adenohypophysis related to aging.

The findings related to clusterin from the previous studies showed that clusterin may increase in response to the cell damage because of degenerative diseases and stress during aging, which may increase clusterin levels in pituitary [1–3, 5, 6, 19, 23]. For example, increased levels of clusterin mRNA as a result of cells attempting to protect themselves from local stress conditions were reported in vital and morphologically normal glial cells and neuronal cells of rat brain exposed to specific neurotoxins [5]. The generation of reactive oxygen species and initiation of lipid peroxidation that precede the upregulation of clusterin protein expression in viable cells were also demonstrated [6]. Therefore, it was postulated that oxidative injury that does not alter cell viability induced increased levels of clusterin. Thus, clusterin appeared as a part of protective cellular response to increased levels of reactive oxygen species and lipid peroxidation [5, 6]. Also, in light of these observations and postulations, clusterin might be produced in the pituitary after degenerative changes and oxidative stresses during aging. Because of the significant clusterin increase in adenohypophysis during aging, the idea of its application in forensic age estimation, especially for unidentified bodies, may be considered. However, the individual factors may cause some problems. Alcohol abuse, distinct eating habits, degenerative diseases or chronic metabolic diseases may cause individual differences in clusterin accumulation. Therefore, for each age, detecting exact pituitary clusterin level immunohistochemically may not be possible. On the other hand, if it would be used concomitantly with other known procedures such as morphological, histological, and biological markers of

Table 2 Clusterin Expression in Non-neoplastic Adenohypophysis of Autopsy Cases, in Three Age Groups: Group I: Subjects between 0 and 30 Years; Group II: Subjects between 31 and 60 Years; Group III: Subjects Older than 61 Years

Age (year)	Number of cases	Clusterin expression (mean)	%95 confidence interval for mean		Significance (p)
			Lower bound	Upper bound	
≤30	38	29.89	23.28	36.51	0.000001
31-60	31	38.16	31.36	44.97	
≥61	27	54.44	46.82	62.07	
Total	96	39.47	35.08	43.86	

(ANOVA, $p>0.05$ significant)

Table 3 Presence of Large Follicles with Colloidal Material Accumulation and its Correlation with Age in Autopsy Cases

Large follicles with colloidal material	Number of cases	Age (mean)	%95 confidence interval for mean age		Significance (p)
			Lower bound	Upper bound	
Absent	54	34	27	41	0.004
Present	42	49	42	55.5	
Total	96	25	36	46	

(ANOVA, p>0.05 significant)

aging, particularly for forensic purposes, it would be helpful. Several procedures such as skeletal and dental maturation and degeneration, histological and immunohistochemical investigations of skeletal muscle, cerebrum, hippocampus, and pituitary gland were also reported previously during age estimation [2, 20, 24, 26]. Clusterin use for this purpose should be searched in larger groups of subjects further. Surprisingly, we also detected a significant association between increased clusterin expression and age of adenoma cases, but this finding might be incidental because of the limited number of our cases. Therefore, to verify this association, further studies in larger series of pituitary adenomas are required.

Increased clusterin levels were reported in various neoplasms such as colonic adenomas and carcinomas, hepatocellular carcinomas, ovarian carcinomas, urothelial carcinomas, and laryngeal carcinomas as well [12, 13, 16, 18, 23]. However, clusterin localization in pituitary adenomas was not reported previously. Upregulation of clusterin was found correlated with aggressive behavior and decreased survival in some neoplasms [12, 13, 16, 18, 19, 23]. Several authors have proposed that the anti-apoptotic activity of clusterin may account in part for the genesis and biologically aggressive behavior of some types of tumor cells [17, 18]. Clusterin has been involved in apoptosis as a proapoptotic or antiapoptotic molecule in a variety of models and under distinct experimental conditions [21]. It was reported that clusterin gene encoded for a family of different protein isoforms, which were derived by alternative posttranslational processes from the same precursor of 53-KDa protein [7, 14, 22]. Different isoforms of clusterin were reported in apoptotic and surviving cells in the regressing rat ventral prostate [9]. Nuclear clusterin was proposed as a cell death protein [15]. It was reported that apoptosis-associated isoforms of clusterin localized primarily in perinuclear regions, whereas anti-apoptosis associated isoforms were localized predominantly in the cytoplasm in breast carcinoma [19]. Data from clusterin studies in colon carcinogenesis have showed that cytoplasmic clusterin may function as an anti-apoptotic protein [17]. Those data were found during the in vitro or in vivo studies of several neoplasms. We found an increase of cytoplasmic clusterin in pituitary adenomas and in non-neoplastic adenohypophyses of older subjects and we did not detect any nuclear clusterin.

To our opinion, several questions are remaining about clusterin and its accumulation in pituitary adenomas and aging adenohypophysis:

i. Further studies are needed to clarify the mechanism of increase of clusterin and the exact function of clusterin—either antiapoptotic or antioxidant— in non-neoplastic pituitary tissue during aging;
ii. Further studies are required to clarify the mechanism of clusterin accumulation and to verify its probable age association in pituitary adenomas in larger groups;
iii. It is important to know the increase in clusterin in non-neoplastic cells may occur with age and because of this reason, further studies related to clusterin expression in certain neoplasms may be planned on the subjects in the same age group by the researchers.
iv. In conclusion, the present study originally demonstrated the cytoplasmic clusterin accumulation in pituitary adenomas. In non-neoplastic adenohypophyses, age-related increase of clusterin with other histopathological findings such as calcification and colloidal material accumulation in large follicles were found. Finally, because of individual differences, detecting exact pituitary clusterin level for each age and its forensic use during age determination of an unidentified body may not be possible, but may be helpful when used concomitantly with other known

Table 4 Presence of Calcification in Adenohypophysis and its Correlation with Age in Autopsy Cases

Calcification	Number of cases	Age (mean)	%95 confidence interval for mean age		Significance (p)
			Lower bound	Upper bound	
Absent	79	38	33	44	0.045
Present	17	52	39	64.5	
Total	96	25	36	46	

(ANOVA, p>0.05 significant)

procedures such as morphological, histological, and biological markers of aging.

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