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Research article

Correlation of polyamines, acrolein-conjugated lysine and polyamine metabolic enzyme levels with age in human liver

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ABSTRACT

The polyamines spermidine, spermine and putrescine are essential for normal cellular functions. The contents of polyamines in tissue decreased in aged mice compared to young mice. In this study, the polyamine contents and their metabolic byproduct acrolein-conjugated lysine (N^{ε}-(3-formyl-3,4-dehydropiperidino)-lysine, FDP-Lys) in human liver tissue were measured and analyzed the correlation with age of the subjects. The putrescine and FDP-Lys levels were significantly increased with age. On the other hand, spermine level was decreased with age. Spermidine did not significantly correlate with age. The relative amount of spermine oxidase (SMOX) significantly correlated with the age of subjects whereas ornithine decarboxylase (ODC) and adenosylmethionine decarboxylase (AMD1) significantly reduced by the age. Our results suggested that an increase in oxidation and reduction in polyamine synthesis may cause the change of polyamine profile in the elderly.

1. Introduction

Polyamines, spermidine and spermine, and their precursor putrescine are essential factors for normal cellular functions [1]. They stimulate synthesis of a set of proteins named "polyamine modulon" which are responsible for cell growth and survival at the level of translation [2, 3]. Spermidine is specifically incorporated into the protein eIF5A as a form of hypusine and this modification is essential for the function of eIF5A protein [4]. As polyamines are implicated in cell growth, growing cells such as cancer cells contain high levels of polyamines [5]. Polyamines are also reported to extend the lifespan through inducing autophagy [6] and suppressing age associated alteration of DNA methylation and inflammation in mice [7, 8]. It is reported that tissue polyamine contents decline in old mice compared to young mice [9]. Since polyamines decline with aging, it is thought that polyamines are anti-aging agents [6]. Eisenberg *et al* showed that oral administration of spermidine and spermine extended the life span of mice [10].

Cellular polyamine level is regulated by biosynthesis, degradation and transport [11]. The synthesis of polyamines starts with the synthesis of putrescine from amino acid ornithine by an enzyme ornithine decarboxylase (ODC). Putrescine is then converted to spermidine and spermine by spermidine synthase (SPDS) and spermine synthase (SPMS), respectively, together with adenosylmethionine decarboxylase (AMD1). As for degradation of polyamines, spermine oxidase (SMOX) and acetylpolyamine oxidase (AcPAO) play central roles. SMOX catalyzes oxidation of spermine to produce spermidine and 3-aminopropanal as a byproduct. Generated 3-aminopropanal is spontaneously deaminated to produce acrolein [12]. Acrolein is a highly reactive alpha-beta unsaturated aldehyde and react with amino acid residues such as lysine in proteins to form acrolein-conjugated lysine (N^{ε}-(3-formyl-3,4-dehydropiperidino) -lysine, FDP-Lys) [13]. In another degradation pathway, spermidine and spermine are acetylated by spermidine/spermine N^1-acetyltransferase, SAT. The resulting acetylated polyamines are degraded by AcPAO. The final product of degradation of spermidine and spermine is putrescine. As for polyamine transport, a solute carrier SLC3A2 and caveolin play important roles [14, 15, 16].

Although the metabolic pathway of polyamines in animals has clarified, the mechanism of polyamine decrease with aging is not yet fully elucidated. In this report, we measured tissue polyamines and FDP-Lys levels using human liver tissue to clarify the mechanism underlying the decrease in polyamine levels with aging.

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Table 1. Distribution of the subjects by age group, postmortem intervals (PMI) and sex.

Age (y)	N	%
<30	23	15.9
30–39	14	9.7
40–49	20	13.8
50–59	13	9.0
60–69	19	13.1
70–79	22	15.2
>80	34	23.4
Total	145	100
PMI (h)		
<24	28	19.3
24–48	66	45.5
>48	51	35.2
Total	145	100
Sex		
Male	93	64.1
Female	52	35.9
Total	145	100

2. Materials and Methods

2.1. Subjects

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The use of autopsy materials without informed consent was approved by the Ethical Review Board of Kyoto Prefectural University of Medicine with approval number ERB-C-1491.

The liver specimens were corrected from 145 forensic medicine cases conducted at Kyoto Prefectural University of Medicine in the period between April 1, 2015 and March 31, 2019. The number of subjects by age group, postmortem intervals and sex were shown in Table 1. The cause of deaths include acute respiratory distress syndrome, acute subdural hematoma, blood loss, brain stem bleeding, burning, choking, drowning, fatal arrhythmia, hanging, heart tamponade, hemorrhagic shock, hypothermic death, hypoxic encephalopathy, hypoxic ischemic encephalopathy, intracranial hemorrhage, brain contusion, Ischemic heart disease, left cerebral hemorrhage, pericardial myocarditis, respiratory and circulatory failure due to massive pleural effusion, respiratory failure, sepsis, spinal rupture, subarachnoid hemorrhage, suspected epileptic seizure, suspected myocarditis, T-cell acute lymphoblastic leukemia, tension pneumothorax, traumatic subarachnoid hemorrhage. The subjects of unknown cause of death were eliminated to reduce the effect of unknown factors.

2.2. Measurement of polyamines and FDP-Lys

Tissue polyamine contents were measured as described previously [17]. In brief, 30 mg of liver tissue was homogenated in 300 μL of RIPA buffer (Nacalai tesque) containing proteinase inhibitor cocktail (Nacalai tesque). After centrifugation at 13000 g for 10 min, supernatant was treated with 5% trichloroacetic acid (TCA) at 70 °C for 30 min. Polyamines in a TCA soluble fraction were separated on a Hitachi HPLC system in which a TSKgel® Polyaminepak column (column size 4.6 mm ID × 5 cm length, particle size 7 μm, TOSOH Bioscience, Japan) heated to 50 °C was mounted. The flow rate of the separation buffer (0.35 M citric acid buffer, pH 5.1, 2 M NaCl, 20% methanol, 0.1% Brij-35) was 0.42 mL/min. Post-column detection of polyamines was performed by mixing the column effluent with an *o*-phthalaldehyde solution containing 0.06% *o*-phthalaldehyde, 0.4 M boric buffer (pH 10.4), 0.1% Brij-35, and 37 mM 2-mercaptoetahnol at 50 °C. The flow rate of the *o*-phthalaldehyde solution was 0.42 mL/min, and fluorescence was measured at an excitation wavelength of 340 nm and an emission wavelength of 455 nm. The retention times for putrescine, spermidine and spermine were 6, 11 and 22 min, respectively. One hundred picomoles of putrescine, spermidine and spermine in 10 μL of TCA were used as standards and polyamine contents were calculated using the area of individual peaks.

The FDP-Lys level was measured using FDP-Lys EIA kit (TaKaRa) according to the protocol provided by the manufacturer. The liver lysate prepared as described above was diluted 1/20 with the assay buffer provided with the kit and used for FDP-Lys assay. Protein contents were measured using BCA protein assay kit (Nacalai tesque) and bovine serum albumin as a standard.

2.3. Western blotting

Liver tissue samples taken from the body whose postmortem intervals were within 24 h for western blot analysis. Liver tissues (30 mg) were washed in PBS and homogenized in RIPA buffer (Nacalai Tesque) containing proteinase inhibitor cocktail (Nacalai tesque). Protein was separated on a 10% polyacrylamide gel and transferred electrophoretically to an Immobilon-E transfer membrane (Merck Millipore). Blots were blocked in 1% bovine serum albumin in tris-buffered saline containing 0.1% Tween 20 (TBS-T) for 30 min at room temperature. SMOX, SAT, AcPAO, ODC, SPDS, SPMS, AMD1 and GAPDH were detected by an ECL Western Blotting Detection System (GE Healthcare) using anti-SMOX (Proteintech), anti-SAT (abcam), anti-AcPAO (abcam), anti-ODC (Proteintech), anti-SPDS (Proteintech), SPMS (Proteintech), AMD1 (Proteintech) and anti-GAPDH (Sigma) antibodies. The images were captured using Amersham Imager 600 (GE Healthcare) and the bands were quantified using the ImageJ program (Schneider, C. A.; Rasband, W. S. & Eliceiri, K. W. (2012), "NIH Image to ImageJ: 25 years of image analysis," Nature methods 9 (7): 671–675), normalized to GAPDH and expressed as relative amount.

2.4. Statistical analysis

Statistical analysis was performed using GraphPad Prism version 8 for Mac, GraphPad Software, La Jolla, California, USA, www.graphpad.com. Normality was assessed by the D'Agostino & Pearson omnibus normality test. Differences between two groups were compared using Student's t-test. For comparison of multiple groups, one-way ANOVA followed by Tukey's multiple comparisons test was used. The correlation coefficient values were calculated using Spearman rank-order correlation method. A p-value < 0.05 was considered statistically significant.

3. Results

3.1. Correlation of polyamine and FDP-Lys levels in liver tissue with age

Polyamines and their metabolic byproduct FDP-Lys contents in liver tissue were measured to examine the effect of aging on polyamine levels. The normality of measured values was assessed by the D'Agostino & Pearson omnibus normality test and none of these values passed the normality test ($p < 0.0001$). Thus, the correlation with age was calculated using Spearman rank-order correlation method. As shown in Figure 1, the putrescine and FDP-Lys contents were significantly increased with age ($r = 0.26$, $p = 0.002$ for putrescine, $r = 0.17$, $p = 0.047$ for FDP-Lys, respectively). On the other hand, the spermine level was significantly decreased with age (r = -0.22, p = 0.008). The level of spermidine was not significantly correlated with age ($r = 0.13$, $p = 0.13$). There was no specific correlation between polyamine and FDP-Lys contents and the cause of death.

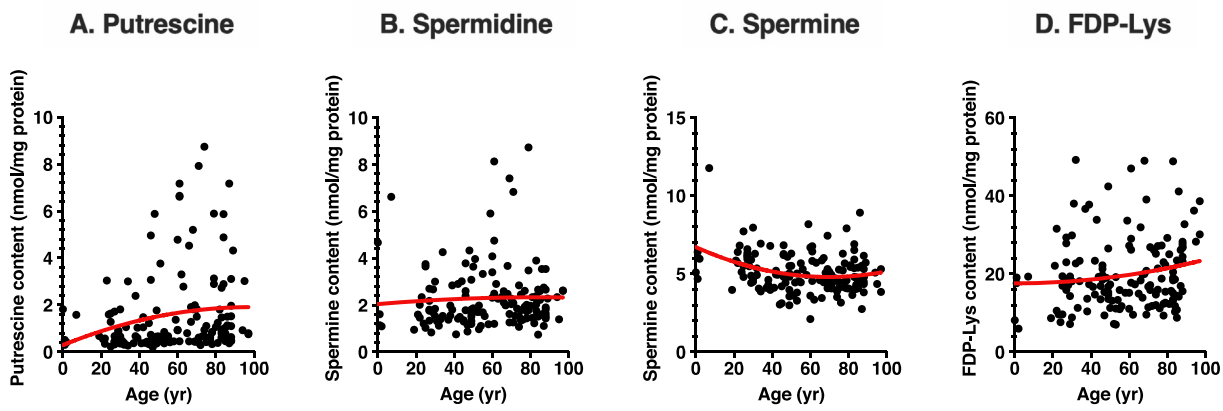

Figure 1. Age dependent change of putrescine, spermidine, spermine and FDP-Lys levels in liver. The levels of putrescine (A), spermidine (B), spermine (C) and FDP-Lys (D) were measured as described in Materials and Methods. The values were plotted against age of the subjects and correlation was calculated. Positive correlations between age and putrescine ($r = 0.26$, $p = 0.002$) and FDP-Lys ($r = 0.17$, $p = 0.047$) were observed. The spermine level was negatively correlated with age ($r = -0.22$, $p = 0.008$). The spermidine did not show significant correlation with age ($r = 0.13$, $p = 0.13$).

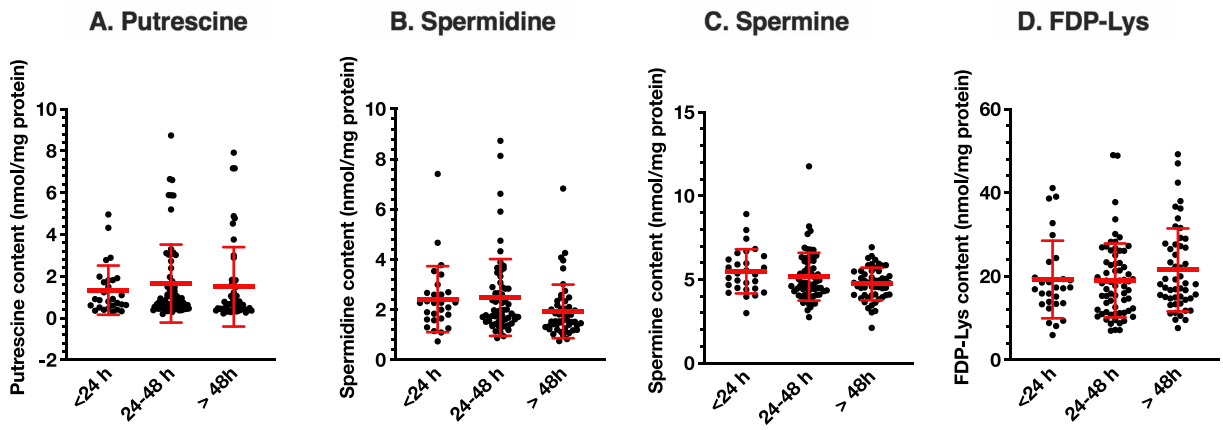

Figure 2. The effect of postmortem intervals on the levels of polyamines and FDP-Lys. The levels of putrescine (A), spermidine (B), spermine (C) and FDP-Lys (D) in different postmortem interval groups (less than 24 h, 24–48 h, longer than 48 h) were plotted. The significance of differences in three groups were assessed by one-way ANOVA followed by Tukey's multiple comparisons test. There were no significant differences in values between three groups.

3.2. Effect of postmortem intervals and sex on the polyamine and FDP-Lys levels

To test whether the postmortem intervals affected the levels of polyamines and FDP-Lys, the differences of these levels in three groups of postmortem intervals (less than 24 h, 24–48 h, more than 48 h) were assessed. As shown in Figure 2, there were no significant differences in three groups. The age was equally distributed in three groups (Supplementary Figure 1A).

Since the previous report showed that the gender-related difference in polyamine levels in rats [18], we next tested whether polyamines and FDP-Lys levels differed in a sex-dependent manner. As shown in Figure 3, there were no significant differences between male and female for polyamines and FDP-Lys. The age was equally distributed in male female (Supplementary Figure 1B). The results showed that at least for our samples, the postmortem intervals and sex were not affected the polyamine and FDP-Lys contents in liver tissues.

3.3. Correlation of age and the levels of polyamine metabolic enzymes

Age dependent increases in putrescine and FDP-Lys levels and a decrease in spermine level suggested that the polyamine degradation pathway was activated with aging. To clarify the mechanism of the change in these values, the levels of enzymes involved in polyamine catabolism were measured. Tissue samples collected within 24 h after death were picked up for western blot analysis to avoid postmortem degradation of proteins. The age of subjects ranged from 0 to 97 years old. As shown in Figure 4, the level of SMOX significantly increased by the age of subjects ($r = 0.71$, $p < 0.0001$). The ODC, which is the rate-limiting enzyme of polyamine synthesis, and AMD1, a key regulator of spermidine and spermine synthesis, declined with the age of the subjects ($r = -0.59$, $p = 0.001$ for ODC, $r = -0.42$, $p = 0.03$ for AMD1). There was no significant correlation between age and SAT, AcPAO, SPDS and SPMS levels.

4. Discussion

In this study, we evaluated polyamines and FDP-Lys levels in liver tissues and analyzed the correlation with the age of subjects. The putrescine and FDP-Lys levels were significantly correlated with the age of subjects (Figure 1). On the other hand, spermine level significantly decreased with age. The spermidine level did not significantly correlate with age. The increase of FDP-Lys and the decrease of spermine suggested that oxidative degradation of spermine was upregulated. The western blotting analysis revealed that SMOX significantly correlated with age (Figure 4). As SMOX catalyzes oxidative degradation of spermine, the upregulation of SMOX with aging can contribute to the increase in FDP-Lys and decrease in spermine levels. We have reported that SMOX promotes bile canalicular lumen formation through acrolein production and suggests that acrolein produced by SMOX can be a signaling molecule for

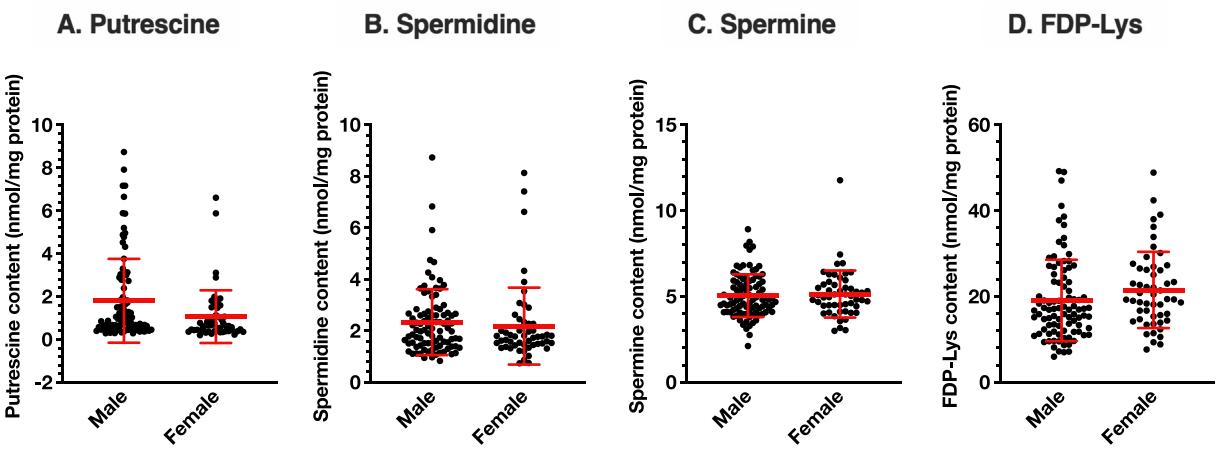

Figure 3. The sex difference in polyamines and FDP-Lys. The levels of putrescine (A), spermidine (B), spermine (C) and FDP-Lys (D) of male and female were plotted. The significance of differences in two groups were calculated by Student's t test. There were no significant differences in values between male and female.

cellular differentiation [19]. The increase in SMOX expression with age may play a role in cellular differentiation in developed tissue. Research is underway to clarify the physiological roles of SMOX upregulation. It was noted that SAT and AcPAO, both contribute to the degradation of spermidine and spermine, were not changed in an age-dependent manner. Since spermidine content was not changed with aging, it was suggested that spermidine could be converted to putrescine by the basal levels of SAT and AcPAO. It was also found that ODC and AMD1, both play significant roles in polyamine synthesis, significantly decreased with age. Our results indicate both degradation and synthesis pathways change with aging and contribute to the alteration of polyamine contents.

The postmortem intervals did not affect the polyamine and FDP-Lys values (Figure 2). The result suggested that liver tissue retained polyamines and FDP-Lys contents for a significant period of time after the death of individuals. There are no significant differences in polyamines and FDP-Lys levels between male and female (Figure 3). Tissue polyamine contents can be affected by multiple factors such as food and gut microbes [20, 21, 22]. The subjects examined in this study were not controlled in terms of these factors. The large variation in high age subjects may represent a cumulative effect of factors such as food and microbiome. It is possible that the differences in polyamine contents were not observed in our samples due to this large variation. Additional

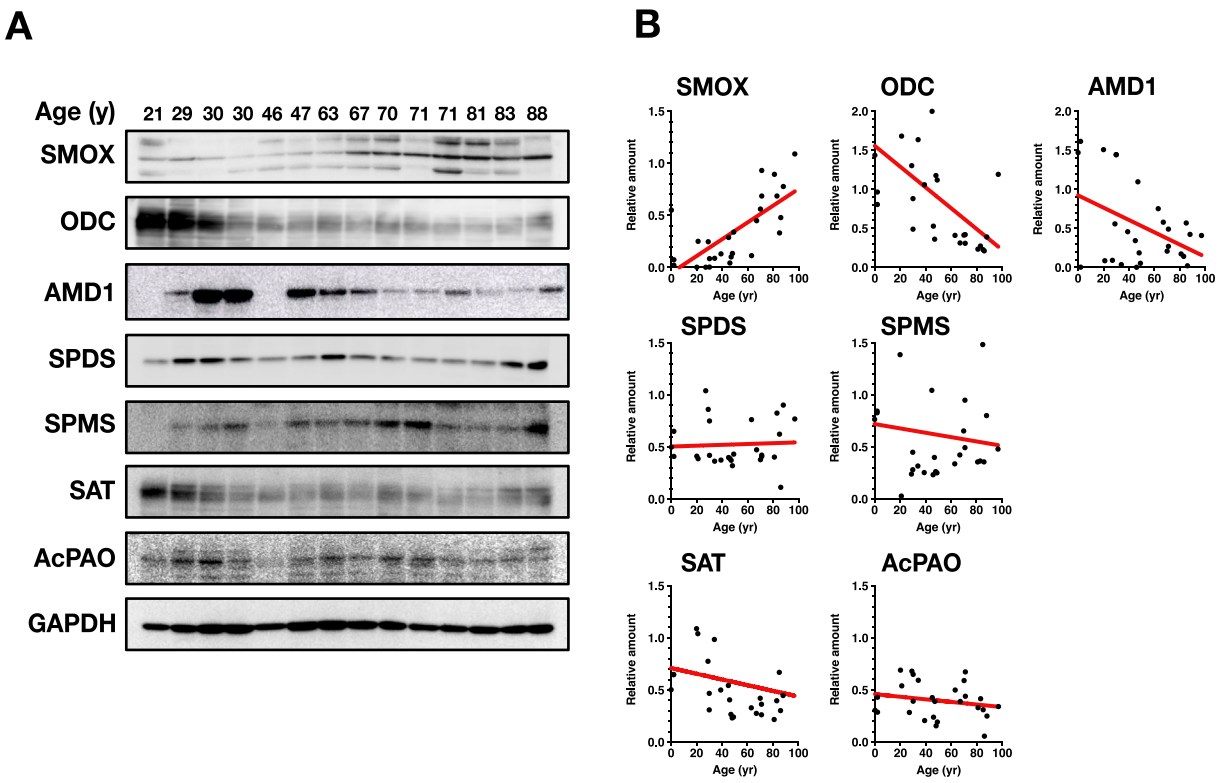

Figure 4. The effect of age on the levels of polyamine metabolic enzymes. (A) The levels of SMOX, ODC, AMD1, SPDS, SPMS, SAT, AcPAO and GAPDH were measured by western blotting as described in Materials and Methods. The subjects were picked according to their postmortem intervals (within 24 h). GAPDH was used for loading control. The result of 14 subjects were shown. The results of another 13 subjects were shown in Supplemental Figure 2. Full length, non-adjusted images are shown in Supplemental Figure 3. The bands were quantified as described in Materials and Methods, normalized to GAPDH and expressed in (B) as relative amount. The p values for the significance of slopes from zero were following: $p < 0.0001$ for SMOX, $p = 0.001$ for ODC, $p = 0.03$ for AMD1, $p = 0.80$ for SPDS and $p = 0.56$ for SPMS, $p = 0.31$ for SAT, $p = 0.31$ for AcPAO, respectively.

analysis using other tissues might reduce the data variation and give stronger correlation of polyamine levels and age. Once the solid correlation between age and polyamine profile is estimated, it will become possible to develop an efficient method to estimate the age of the subjects using polyamine profile. Also, the physiological importance of the change in polyamine profile with aging will be clarified in the future.

Declarations

Author contribution statement

T. Uemura: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

H. Ikegaya: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Y. Akasaka: Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

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