Age-dependent expression of Pten and Smad4 genes in the urogenital system of Wistar rats[1]

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ABSTRACT

PURPOSE: To analyze Pten and Smad4 gene expression in the urogenital system of Wistar rats in differents ages.

METHODS: Pten and Smad4 mRNA expression was assessed in the bladder, ventral prostate, testis, ovaries, and uterus by real-time PCR. Statistical analysis using the ANOVA ($p<0.05$).

RESULTS: Pten levels showed a progressive age-dependent increase in the bladder (male and female) and prostate and were elevated in the ovaries of the middle-aged. In the uterus, no statistically significant differences were observed; in the testis, increased and decreased levels were seen in young adult and middle-aged rats, respectively. Smad4 expression was downregulated in the ovaries of the pubertal group but increased in the middle age group. In the uterus, Smad4 expression in the oldest group was higher than the others groups. In the testis, Smad4 expression steadily declined with age; in the prostate, it was higher in middle-aged rats than in younger rats. A similar trend was observed in the bladder of male and female middle-aged rats, compared with the pubertal group.

CONCLUSION: The changes in phosphatase tensin homologue and Smad4 mRNA expression in Wistar rats appear to be associated with hormonal modifications in puberty and may be related to early follicular and testicular development.

Key words: Pten. Smad4. Urogenital system.

Introduction

Cancer is the second most common cause of death worldwide. Annually, more than 12.7 million people are diagnosed with cancer and 7.6 million die of this condition. Between 1975 and 2000, the incidence of cancer doubled, and the incidence of cancer is expected to double again by 2020 and triple by 2030, when the cancer death rate is predicted to reach 17 million per year. Lung, breast, and colon cancers are the most commonly diagnosed cancers worldwide[1].

Multiple genes are related to the control of cellular proliferation and prevention of tumorigenesis. The Pten (phosphatase tensin homologue) gene encodes a protein phosphatase that functions as a tumor suppressor and is involved in various physiological processes, including cell proliferation, migration, survival, and apoptosis. Pten is a key regulator of a signaling pathway that controls cell division, mediates apoptosis, and prevents excessive cell growth[2]. Since its discovery in 1997, Pten has been established as one of the most commonly mutated tumor suppressors in human cancer; deficiency of Pten enzymatic activity has been implicated in the development of gliomas and breast, endometrial, lung, and prostate cancers[3]. The PTEN gene has been shown to be mutated in 40–50% of gliomas and in up to 70% of prostate cancers[4].

Smad4 belongs to a family of transcription factors that mediate the transforming growth factor (TGF)-beta signaling associated with cell proliferation and differentiation, angiogenesis, and extracellular matrix remodeling[5]. A recent study has shown that Pten and Smad4 together with Spp1 and cyclin D1 form a four-gene signature that is prognostic for recurrence and lethal metastasis in human prostate cancer. The Gleason grading scale has an accuracy of 60–70% in determining prostate cancer aggressiveness, whereas the prognostic accuracy of the four gene-based analyses reaches 83%. The combination of the four biomarkers and the Gleason score has a predictive accuracy of approximately 90% and can be applied to determine the aggressiveness of prostate cancer[6]. The expression of the Pten and Smad4 genes has been extensively investigated. However, only a few studies have reported the expression of these genes in the urogenital system. In this study, we evaluated the expression of the Pten and Smad4 genes in the urogenital organs of Wistar rats at different ages.

Methods

The study was approved by the Ethics Committee for the care and use of experimental animals of the State University of Rio de Janeiro (CEUA021/2012) and followed the principles set out in The Guide for the Care and Use of Laboratory Animals.

Wistar rats from the colony of the Urogenital Research Unit were kept in polypropylene boxes in a temperature-controlled (21°C ± 2°C) 12-h light-dark cycle (07:00 h-19:00 h) environment and received food and water *ad libitum*. The animals were divided into four age groups: pre-pubertal (21 days; 10 male and 5 female rats); pubertal (50 days; 10 male and 5 female rats); young adult (90 days; 10 male and 5 female rats) and middle-aged (180 days; 10 male and 9 female rats).

Puberty was determined on the basis of the vaginal opening in female rats and the separation of the foreskin in male rats. The animals were euthanized by intraperitoneal injection of sodium pentobarbital (30 mg/kg) and the bladder, testis, prostate, uterus, and ovaries were removed, fixed in liquid nitrogen, and stored at -80°C until analysis by real-time PCR (qPCR).

Real-time PCR

RNA was extracted from the tissues using Trizol® reagent according to the protocol provided by the manufacturer (Life Technologies, Carlsbad, CA, USA). RNA concentration and purity were determined by the 260/280 nm absorbance ratio using a NanoDrop-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Total RNA (1 µg) was treated with DNase (Life Technologies) before being reverse-transcribed using the Superscript III enzyme (Life Technologies). The resulting cDNA was used as a template for qPCR analysis performed with specific Pten and Smad4 primers (Table 1) and SyBR Green reagent (Life Technologies) in the Bio-Rad CFX96 Real Time System (Bio-Rad Laboratories, Foster City, CA, USA). Relative mRNA expression was calculated by the delta-delta Ct method and normalized to that of beta-actin, which was used as a housekeeping gene. All reactions were performed in triplicate.

TABLE 1 - Sequences of oligonucleotides used for analysis of Pten and Smad4 mRNA expression by qPCR.

Gene	Sequence
Pten: forward primer	ACACCGCCAAATTTAACTGC
Pten: reverse primer	TACACCAGTCCGTCCCTTTC
Smad4: forward primer	CCCATCCTGGACATTACTGG
Smad4: reverse primer	TACACCAGTCCGTCCCTTTC
β *actina: forward primer*	CTGTCCCTGTATCGCTCTGGTC
β *actina: reverse primer*	TGAGGTAGTCCGTCAGGTCCC

Statistical analysis

The data were tested for normality and homogeneity of the variance and have been presented as the mean ± standard deviation (SD). Differences among the groups were established

by one-way analysis of variance (one-way ANOVA) followed by Bonferroni's post hoc test; p £ 0.05 was considered statistically significant. All analyses were performed using Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA).

Results

Pten mRNA expression in the bladder of middle-aged male rats was increased by 95%, 91%, and 80% relative to animals from the pre-pubertal, pubertal, and young adult groups, respectively (Table 2). Pten mRNA levels were also significantly elevated by 95%, 96%, and 90% in the prostate of the oldest age group relative to that of the pre-pubertal, pubertal, and young adult groups, respectively (Table 2). In the testes, Pten mRNA expression increased by 45% in young adults relative to pre-pubertal animals, whereas the middle-aged male rats showed a 66% reduction in Pten mRNA levels relative to the young adult group (Table 2). Pten mRNA levels in the bladder were similar in female and male rats. The middle-aged group showed an increase of 83%, 82%, and 85% in Pten mRNA levels compared with the pre-pubertal, pubertal, and young adult groups, respectively (Table 2). Pten mRNA levels in the ovaries were increased by 78% in middle-aged rats relative to young adult rats (Table 2). However, no statistically significant difference was observed in uterine Pten mRNA expression among the age groups (Table 2).

Smad4 expression in the bladder of middle-aged male rats was elevated by 78% compared to that in pubertal animals (Table 2). Similarly, in the prostate of the oldest age group, Smad4 mRNA levels increased by 74%, 51%, and 76% compared to those in the pre-pubertal, pubertal, and young adult groups, respectively (Table 2). However, in the testes, the highest Smad4 mRNA expression was observed for the youngest, pre-pubertal rats, whereas reductions of 93%, 83%, and 95% were detected for the pubertal, young adult, and middle-aged rats, respectively (Table 2). Bladder Smad4 mRNA levels in middle-aged females were higher than those in the pre-pubertal (77%), pubertal (75%), and young adult (75%) groups (Table 2). In the ovaries of the pubertal animals, Smad4 expression was reduced by 72% compared to that in the pre-pubertal group; however, the middle-aged group showed an 82% increase in Smad4 mRNA levels relative to the pubertal animals (Table 2). In the uterus, Smad4 expression in the oldest group was 94%, 82%, and 88% higher than that in the pre-pubertal, pubertal, and young adult groups, respectively (Table 2).

Data were reported as mean ± SD. The differences were tested by one-way analysis of variance (ANOVA) and the Bonferroni post hoc test, $p < 0.05$. [a] indicates difference from 21 days; [b] indicates difference from 50 days; [c] indicates difference from 90 days.

TABLE 2 - Pten and Smad4 gene expression in the urogenital organs of Wistar rats at different ages.

Pten	**21 days**	**50 days**	**90 days**	**180 days**	**p value**
Bladder male	0.59 ± 0.27	1.11 ± 1.48	2.66 ±2.83	13.21 ± 8.02[a,b,c]	0.0075
Testis	1.57 ±1.00	1.95 ± 0.48	2.88 ± 1.03[a]	0.97 ± 0.34[c]	0.0003
Prostate	0.88 ±0.66	0.67 ± 0.32	2.01 ± 1.83	19.56 ± 14.73[a,b,c]	0.0001
Bladder female	0.10 ± 0.04	0.10 ± 0.05	0.08 ± 0.03	0.58 ± 0.56[a,b,c]	0.0001
Ovaries	0.54 ± 0.53	0.83 ± 0.72	0.31 ± 0.15	1.40 ± 0.73[c]	0.0019
Uterus	0.19 ± 0.17	0.09 ± 0.04	0.25 ±0.21	0.24 ± 0.17	*0.3701*
Smad4	**21 days**	**50 days**	**90 days**	**180 days**	**p value**
Bladder male	0.21 ± 0.17	0.08 ± 0.04	0.22 ± 0.18	0.40 ± 0.23[b]	0.02
Testis	2.18 ± 1.51	0.14 ± 0.02[a]	0.37 ± 0.22[a]	0.10 ± 0.05[a]	0.0001
Prostate	0.22 ± 0.08	0.42 ± 0.30	0.20 ± 0.16	0.87 ± 0.42[a,b,c]	0.0001
Bladder female	0.25 ± 0.19	0.27 ± 0.13	0.27 ± 0.10	1.11 ± 0.58[a,b,c]	0.0016
Ovaries	0.67 ± 0.25	0.18 ± 0.05[a]	0.27 ± 0.19	1.02 ± 0.26[b,c]	0.001
Uterus	0.06 ± 0.04	0.18 ± 0.05	0.12 ± 0.19	1.03 ± 0.78[a,b,c]	0.0019

Discussion

Pten and Smad4 have been identified as tumor suppressor factors, and deletions or mutations in their respective encoding genes have been associated with various neoplasms, including gastrointestinal polyps, melanoma, glioma, and prostate, bladder, breast, and ovarian cancers[4,7,8]. Expression of Pten and Smad4 has been established as a prognostic factor for the development of metastatic prostate cancer[6]. In a murine model of prostate cancer, decreased Pten expression was positively associated with tumor aggressiveness[9]. Pten-knockout mice develop a broad spectrum of tumors, including tumors in the gastrointestinal tract, and thyroid glands, and prostate[10].

Our study demonstrated that healthy cancer-free rats showed an age-dependent increase in Pten expression in the prostate and bladder, which may indicate increased control over cell proliferation with age and is consistent with the role of the Pten gene in the inhibition of cell growth, neoplasm development, and carcinogenesis[2].

Pten activity is also closely associated with the production of follicle-stimulating hormone (FSH). Acute stimulation of rat testicular Sertoli cells, required for male sexual development, by FSH has been found to increase Pten synthesis and enzymatic activity, thus identifying a new, Pten-dependent mechanism of regulating spermatogenesis[11]. In rats, FSH plasma levels peak between 10 and 15 weeks and decline after this period[12], which agrees with our findings that, in rat testes, Pten expression increased with maturation from the pre-pubertal (21 days) to the young adult (90 days) stage, but then decreased in the older, middle-aged (180 days) group.

In the ovaries, Pten mRNA levels were higher in middle-aged rats than in young adults, thus presenting a pattern opposite to that of estrogen production, which is maximal in the proestrus stage (80–100 days)[13] and declines after 180 days[14]. The negative correlation between estrogen levels and Pten expression has been confirmed in vitro by Noh *et al*.[15] who showed that estrogen decreased Pten expression in breast cancer cells. A relationship between Pten expression and female hormonal status has been shown in estrogen-dependent human ovarian adenocarcinomas, where Pten is markedly downregulated[7].

Smad4 mRNA expression was similar in the bladders of male and female rats. Islam *et al*.[16] reported that Smad4 is expressed in all bladder layers and is important in the regulation of mouse bladder organogenesis. However, we found that Smad4 expression in the pre-pubertal rats was low and started to increase from puberty, reaching significantly increased levels in adulthood for both sexes. Given that Smad4 expression was negatively correlated with the progression of bladder carcinoma and the occurrence of metastases[17], the increase in Smad4 levels in middle-aged rats may represent a regulatory mechanism for preventing uncontrolled cellular proliferation. Indeed, it is known that Wistar rats do not spontaneously develop cancer[18].

Smad4 and Pten double-knockout mice have been shown to develop neoplasia during their lifetime[19], confirming previous findings that genetic deletion of Smad4 results in the emergence of invasive, metastatic, and lethal prostate cancers[6]. In this study, we detected an increase in Smad4 mRNA levels in the prostate of the middle-aged rats, which may indicate a protective mechanism against cancer development in the aging prostate.

Smad4 has been shown to play a critical role in testicular development in pre-pubertal animals[20]. The progressive reduction in the expression of Smad4 mRNA throughout the lifetime in the animals in the present study agrees with the findings of Narula *et al*.[21] who observed that mice overexpressing Smad4 developed hyperplasia of Leydig cells, apoptosis of germ cells, spermatogenic arrest, and degeneration of the seminiferous tubules. Our present observation that Smad4 expression in the testis decreases with age is consistent with the role of Smad4 in spermatogenesis. The reduction in Smad4 mRNA levels in pubertal and young adult rats corresponded with an increase in testicular function.

In the female gonads, Smad4 gene expression was higher in pre-pubertal animals than in pubertal animals, suggesting involvement in the early phases of folliculogenesis and ovarian development[22,23]. However, in contrast to male rats, middle-aged female rats demonstrated an increase in Smad4 mRNA expression in the reproductive organs (ovaries and uterus) relative to the pubertal and young adult groups. Given the role of Smad4 as a tumor suppressor[24], these changes may be indicative of the Smad4 related anti-proliferative mechanisms induced in aging female rats as reproductive activity declines[25].

Conclusion

The Smad4 may be a part of the regulatory mechanism controlling the initial development of the rat reproductive system, whereas the elevated expression of both Pten and Smad4 mRNA in aging animals may indicate the activation of antiproliferative events required for the protection of the urogenital system against age-related carcinogenic stimuli.

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