

AT cikk

by

Olga Ruzsnavszky¹, Kornél Kistamás¹, Andrea Telek¹, Livia Kosztka², Ilona Kovács²
Beatrix Dienes¹, László Csernoch¹, Tamás Józsa^{3,4}

from

¹ Department of Physiology, Medical and Health Science Center, University of Debrecen,
Debrecen, Hungary

² Department of Pathology, Kenézy Gyula Hospital, Debrecen, Hungary.

³ Department of Pediatrics, Medical and Health Science Center, University of Debrecen,
Debrecen, Hungary

⁴ Karolinska

Address all correspondence to:

Dr. Tamás Józsa

Department of Pediatrics

University of Debrecen

P.O. Box 32

Debrecen

Hungary

E-mail: jozsa_tamas@freemail.hu

Fax: +36-52- 255-335

Abstract

The female internal sex organs develop from the paramesonephric (Mullerian) duct. In male embryos, during the embryonic development, the regression of the Mullerian duct is caused by the anti-Mullerian hormone (AMH). The appendix testis (AT) is a physiological remnant of the Mullerian duct in males. Our knowledge about its physiological function is limited.

In this report the expression of anti-Mullerian hormone receptor subtype 2 (AMHR2) was investigated on AT, in correlation with different urological disorders, such as hernia inguinalis, torsion of AT, cysta epididymis, varicocele, ectopia testis, testis retractile, retention testis, hydrocele testis. The correlation between the age of the patients and the expression of AMHR2 was also examined. RT-PCR and immunohistochemistry was used to detect the receptor at mRNA and protein level.

In our report we demonstrate, at the first time, that AMHR2 is expressed in the ATs, and the expression pattern of this receptor is not connected with the examined urological disorders or with the age of the patients.

Keywords: appendix testis, anti-Mullerian hormone receptor

Abbreviations: anti-Mullerian hormone (AMH), appendix testis (AT), anti-Mullerian hormone receptor subtype 2 (AMHR2)

1. Introduction

The female internal sex organs – such as oviducts, uterine horns, cervix and the anterior vagina – develop from the paramesonephric (Mullerian) duct. In male embryos, during the embryonic development, the regression of the Mullerian duct is caused by the anti-Mullerian hormone/substance (AMH). The vestigial remnant of the Mullerian duct is called appendix testis (hydatid of Morgagni; AT), and is located at the upper pole of the testis or in the groove between the testis and the epididymis (Atkinson, 1992). The AT is not present in everyone, it can be found in 92% (Rolnick, 1968) or 76% (Józsa 2008) of

the males. Some diseases, like cryptorchidism decrease the incidence rate of AT (Józsa 2008). Our knowledge about its physiological function is limited. According to one hypothesis, the surface epithelium of the AT, with its subepithelial capillaries and lymphoid vessels form a functional unit (Posinovec J, 1969), but its function is unknown. The AT has a clinical importance because its torsion causes acute scrotum, and tumor can be developed from it.

AMH is secreted by immature Sertoli cells. The serum level of AMH stays high in males until puberty (Aks glaede, 2010). Defects of the AMH and AMH receptors (AMHR) cause persistent Mullerian duct syndrome (Lane, 1998, Josso, 2005), where some of the internal female sex organs can be found in a male. Anti-Mullerian hormone (AMH) has been evaluated by several groups as a potential novel clinical marker for ovarian reserve. Serum AMH concentrations show a progressive decline with female aging (La Marca, 2012). Serum AMH level is high from fetal life until mid-puberty. Testicular AMH production increases in response to follicle-stimulating hormone and is potently inhibited by androgens. Serum AMH is undetectable in anorchid patients. In primary or central hypogonadism affecting the whole gonad established in fetal life or in childhood, all testicular markers are low. Conversely, when hypogonadism only affects Leydig cells, serum AMH is normal. In males of pubertal age with central hypogonadism AMH is low (Grinspon, 2012).

In third month of fetal life the testes start to move from the abdomen into the future scrotum. The descent is completed around the birth. The complete process of the normal descent of testis is still unknown. The investigation of the descent is one of the most important research areas of the recent pediatric urology. It is supposed that the testis and its accessories develop together, and this progress requires a complex interaction between them. Although several theories have been proposed to explain the descent of testis, none has provided a satisfactory explanation that covers the whole spectrum of events. Recent studies show that the transabdominal phase of testicular descent is under the control of Insulin-Like Peptide 3 and AMH, and suppose a role of the cremaster muscle (Lie, 2011). The cremaster muscle is a striated muscle with some differences with other skeletal muscles, just like its firing frequency and involuntary control (Kayalioglu, 2008). Recent new theory of testicular descent suggests the role of sympathetic nerves,

that innervates the gubernacular smooth and striated muscles (Tanyel, 2001, 2004). In mice AMH is not essential for gubernacular development or testicular descent, although it is important in determining the thickness of the cremaster muscle (Bartlett, 2002).

In patients with undescended testis the presence of AT is significantly lower (Józsa, 2008), suggesting that AT might play a role in the process of testicular descent. There is no information about the expression of AMHR in AT, although it is supposable that the cells of AT express this receptor during the embryonic development, and probably after birth, as well. Mutations of the AMH and AMH receptor type 2 (AMHR2) genes lead to the persistence of the uterus and Fallopian tubes in males.

Torsion of the AT is painful, and the patients show similar symptoms to those with the torsion of testis, so it is medically indicated to remove the AT when it appears during intrascrotal operations (Anderson, 1995). Appendices from such operations were used in our research. Correlation between several urological diseases and the expression of AMH receptor in ATs were investigated.

2. Materials and methods

Collection of the samples

109 ATs and 9 cremaster muscles were collected from male patients undergoing surgical exploration at the Surgical Ward of the Department of Pediatrics, University of Debrecen (MHSCUD). The study protocol was approved by the Human Ethics Committee of MHSCUD. Written informed consent was obtained from the parents or guardians before the children entered the study. The samples used for the PCR experiments were collected from patients suffering from the following diseases: hernia inguinalis, torsion of AT, cysta epididymis, varicocele, ectopia testis, testis retractile, retention testis, hydrocele. The samples used for the immunohistochemistry experiments were obtained from patients suffering from the following diseases: hydrocele, retention testis, varicocele, cysta epididimys, hernia inguinalis.

Immunohistochemistry

4 µm thick, formaline fixed sections were used for immunohistochemistry. First the sections were rehydrated (4x5 min xilol, 4x1 min ethanol), and to uncover the antigens the sections were boiled in citrate-buffer (2 min, 0.1 mol/l, pH = 6.0). Activity of endogenous peroxidases was inhibited with hydrogen-peroxide (10 min, 3%). After that the aspecific binding places were blocked with 2.5% horse serum (20 min, room temperature, ImmPRESS kit; Vector Laboratories, Burlingame, CA, USA). Then the AMHR2 specific primary antibody (diluted in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA), produced in mouse; AbCam, Cambridge, UK) was used in an 1:300 dilution. After washing the sections (PBS, 3x5 min), they were incubated with horse radish peroxidase conjugated secondary antibody (30 min, room temperature, ImmPRESS kit), then the sections were washed again (PBS, 3x5 min). The reaction was visualized by ImmPACT DAB kit (Vector Laboratories, Burlingame, CA, USA), according to the manufacturers protocol. Nuclei were visualized by hematoxilin (10 sec), and sections were dehydrated (2x1 min ethanol, 3x1 min acetone, 2x1 min xilol) before covered by Shandon Consul Mount medium (Thermo Scientific, Budapest, Hungary). Endometrium sections were used for positive control. Negative controls were made by omitting the primary antibody.

RT-PCR analysis.

For RT-PCR analysis, samples were stored in RNAlater (Ambion, Life Technologies, Budapest, Hungary) at -70 °C. Total RNA was isolated from tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Tissues were sonicated in 1 ml TRIzol reagent (3x10 sec) then 0.2 ml chloroform was added to the samples. Samples were mixed well and centrifuged (12000 g, 15 min, 4°C). The upper, colorless phase which contains the RNA was used. Then 0.5 ml isopropanol was added, gently mixed, and incubated for 1 hour in -20 °C and centrifuged again (12000 g, 10 min, 4 °C). The sediment was washed with 75% ethanol and dissolved in nuclease free water. The assay mixture (20 µl) for reverse transcriptase reaction (Omniscript, Qiagen, Budapest, Hungary) contained 500 ng RNA, 0.25 µl RNase inhibitor, 0.25 µl oligo(dT), 1 µl dNTP (200 µM), 1µl M-MLV RT in 1 × RT buffer. Amplifications of specific cDNA sequences were performed with specific primers (Integrated DNA Technologies, Coralville, IA, USA) that were designed based on published nucleotide sequences (sequences of primer pairs: GAPDH forward primer: 5'-AAGGTCGGAGTCAACGGATTTGG-3', reverse

primer: 5'-AATGAGCCCCAGCCTTCTCCAT-3'; AMHR forward primer: 5'-TCGGGAAGATGGATCGTGT-3', reverse primer: 5'-GGAAGGGTGGTGGACTGCT-3'. PCR reactions were allowed to proceed in a final volume of 50 µl (containing 1 µl forward and reverse primers, 1 µl dNTP [200 µM], and 5 units Promega GoTaq® DNA polymerase in 1 × reaction buffer) in a programmable thermocycler (Eppendorf Mastercycler, Netheler, Hinz GmbH, Hamburg, Germany) with the following settings: 2 min at 95 °C for initial denaturation followed by repeated cycles of denaturation at 94 °C for 1 min, primer annealing for 60 sec at an optimized temperature, and extension at 72 °C for 1 min 30 sec. After the final cycle, further extension was allowed to proceed for another 10 min at 72 °C. PCR products were analyzed using a 1.5 % agarose gel. EZVision (Amresco, Solon, OH, USA) was used as loading dye and the results were observed in UV light. Image J software (Wayne Rasband, National Institutes of Health, USA) was used for densitometry analysis. Together with the anti-Mullerian hormone receptor, GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA expression was also determined and used as internal control.

3. Results

ATs, and pieces of cremaster muscles were collected from patients suffering from various urological diseases. RT-PCR reaction was used for the detection of the mRNA expression of AMHR2 on these samples. GAPDH was used as an internal control. In case of the ATs, we found the following results: In patients with retractile testis (n=9), the mRNA of the receptor was detected in only one sample (11%). In patients with torsion of AT (n=3) and cysta epididymis (n=1), the mRNA of this receptor was detected in most of the samples (67% and 100%, respectively). The mRNA of the AMHR2 was not detectable in the ATs from the patients suffering from ectopia testis and varicocele (n=2 and n=1, respectively). In the samples of hernia inguinalis (n=14) the mRNA of the receptor was detectable in 50% of the samples, while in the case of hydrocele testis (n=13) only 15% of the samples were positive for the mRNA of the receptor. In the samples with retentio testis (n=17) the mRNA was detectable in 35% of the ATs (Fig.1A).

Immunohistochemistry was used for the analysis of the protein level expression of AMHR2. The presence of the receptor was observed as a brown staining in the interstitium of the AT in immunohistochemistry sections (Fig.2). The protein of the receptor was detectable in 25% of the samples of patients with cysta epididymis (n=12), 67% of the samples of hernia inguinalis (n=6), 64% of the samples of hydrocele testis (n=25), and 100% of the samples of retentio testis (n=3). In cases of varicocele (n=3) the protein of AMHR2 was detectable in only one sample (33%) (Fig.3A).

The age dependence of the mRNA and protein expression of AMHR2 in AT was also investigated. No correlation was found between the ages of the patients and the mRNA expression of this receptor, some of the samples contained the mRNA of the AMHR2, while others from the same group did not (Fig.1B). On the other hand, the protein level expression of AMHR2 has shown some age dependence. Under the age of 12 all of the AT samples expressed the AMHR2 protein. Over the age of 12 there was no correlation between the presence of the AMHR2 and the age of the patients (Fig.3B).

In case of the cremaster muscles, the mRNA of AMHR2 was not detectable in the patients suffered from hernia inguinalis (n=3), in retentio testis one of the samples was positive for AMHR2, while the other was negative (n=2). AMHR2 was detectable in 3 of the 4 samples in case of hydrocele testis (n=4) (Fig.4).

4. Discussion

The investigation of the descent of testes is one of the most important research areas of the recent pediatric urology. It is supposed that the testis and its accessories develop together, and this progress requires a complex interaction between them. Although several theories have been proposed to explain the descent of testis, none has provided a satisfactory explanation that covers the whole spectrum of events. Recent studies show that the transabdominal phase of testicular descent is under the control of Insulin-Like Peptide 3 and AMH, and suppose a role of the cremaster muscle (Lie, 2011).

In patients with undescended testis the presence of AT is significantly lower (Józsa, 2008), suggesting that AT might play a role in the process of testicular descent. Since yet, there was no information about the expression of AMHR in AT, although it is supposable that the cells of AT express this receptor during the embryonic development, and probably after birth, as well. For the first time we have shown that both the mRNA and

the protein of the AMHR2 is detectable in the AT. Although the mRNA of AMHR2 was detectable in several samples, the lack of the mRNA expression of the receptor did not show any correlation with the most common urological disorders, just like the hernia inguinalis, retention testis or hydrocele testis. In case of retractile testis, the mRNA of the receptor could be detected in only one case from the examined 9 samples, which can indicate a correlation between the lack of AMHR2 and the development of this urological disorder. In case of the torsion of AT, and cysta epididymis, the mRNA of this receptor could be detected in most of the samples, although the number of the samples were quite low (n=3 and n=1, respectively). The protein of the receptor was present in 25% in the samples with cysta epididymis. The mRNA of the AMHR2 was not detectable in the AT in cases of ectopia testis and varicocele, although the number of the investigated samples were also very low (n=2, n=1, respectively). In cases of varicocele the protein of AMHR2 was present in only one sample (n=3). In the samples with hernia inguinalis, the mRNA of the receptor was detectable in 50% (n=14), and the protein in 67% (n=6). In cases of hydrocele testis the mRNA was detectable in 15% (n=13), the protein in 64% (n=25). In the samples with retention testis the mRNA in 35% (n=17), the protein in 100% (n=3) could be detected.

There was also no correlation between the mRNA expression of the AMHR2 and the age of the patients. We found some correlation between the protein expression of AMHR2 and the patients' ages – patients younger than 12 years expressed the AMHR2 in their AT. Although the number of the samples were low (n=12), and the PCR results unfortunately did not show the same correlation. In case of the population over 12 years, we could not find any correlation between the expression pattern and the ages (n=37).

The expression of mRNA of AMHR2 in cremaster muscles also did not show correlation with the urological disorders although in these experiments the number of the samples were limited.

According to one hypothesis, the surface epithelium of the AT, with its subepithelial capillaries and lymphoid vessels form a functional unit (Posinovec J, 1969), but its function is still unknown.

Acknowledgements

The authors are indebted to Ms Róza Óri and Ms Ibolya Kiss for their excellent technical assistance. The work was supported by the TÁMOP-4.2.2/B-10/1-2010-0024 project. The project was co-financed by the European Union and the European Social Fund.

References

Aksglaede L, Sørensen K, Boas M, Mouritsen A, Hagen CP, Jensen RB, Petersen JH, Linneberg A, Andersson AM, Main KM, Skakkebaek NE, Juul A. Changes in anti-Müllerian hormone (AMH) throughout the life span: a population-based study of 1027 healthy males from birth (cord blood) to the age of 69 years. *J Clin Endocrinol Metab*. 2010 Dec;95(12):5357-64.

Anderson PJ, Kincaid R, Orr JD: Testicular appendages: incidence and clinical significance. *Pediatr Surg Int*. 1995;10:118-119

Atkinson GO Jr, Patrick LE, Ball TI Jr, Stephenson CA, Broecker BH, Woodard JR: The normal and abnormal scrotum in children: evaluation with color Doppler sonography. *AJR Am J Roentgenol*. 1992 Mar;158(3):613-7.

Bartlett JE, Lee SM, Mishina Y, Behringer RR, Yang N, Wolf J, Temelcos C, Hutson JM: Gubernacular development in Müllerian inhibiting substance receptor-deficient mice. *BJU Int*. 2002 Jan;89(1):113-8.

Grinspon RP, Loreti N, Braslavsky D, Bedecarrás P, Ambao V, Gottlieb S, Bergadá I, Campo SM, Rey RA: Sertoli cell markers in the diagnosis of paediatric male hypogonadism. *J Pediatr Endocrinol Metab*. 2012;25(1-2):3-11.

Josso N, Belville C, di Clemente N, Picard JY: AMH and AMH receptor defects in persistent Müllerian duct syndrome. *Hum Reprod Update*. 2005 Jul-Aug;11(4):351-6.

Józsa T, Csízy I, Kutasy B, Cserni T, Flaskó T: Decreased incidence of appendix testis in cryptorchidism with intraoperative survey. *Urol. Int*. 2008;80:317-320

Kayalioglu G, Altay B, Uyaroglu FG, Bademkiran F, Uludag B, Ertekin C: Morphology and innervation of the human cremaster muscle in relation to its function. *Anat Rec (Hoboken)*. 2008 Jul;291(7):790-6.

La Marca A, Spada E, Grisendi V, Argento C, Papaleo E, Milani S, Volpe A: Normal serum anti-Müllerian hormone levels in the general female population and the relationship with reproductive history. *Eur J Obstet Gynecol Reprod Biol.* 2012 May 10. [Epub ahead of print]

Lane AH, Donahoe PK: New insights into mullerian inhibiting substance and its mechanism of action. *J Endocrinol.* 1998 Jul;158(1):1-6.

Lie G, Hutson JM: The role of cremaster muscle in testicular descent in humans and animal models. *Pediatr Surg Int.* 2011 Dec;27(12):1255-65.

Posinovec J: Is the unstalked hydatid a functioning organ? *Verh Anat Ges.* 1969;63:751-759

Rolnick D, Kawanoue S, Szanto P, Bush IM: Anatomical incidence of testicular appendages. *J Urol.* 1968;100:755-756

Tanyel FC, Müftüoğlu S, Dagdeviren A, Kaymaz FF, Büyükpamukçu N: Myofibroblasts defined by electron microscopy suggest the dedifferentiation of smooth muscle within the sac walls associated with congenital inguinal hernia. *BJU Int.* 2001 Feb;87(3):251-5.

Tanyel FC: The descent of testis and reason for failed descent. *Turk J Pediatr.* 2004;46 Suppl:7-17.

Figure legends

Figure 1. Detection of mRNA of AMHR with RT-PCR. Panel A represents the age dependence of the appearance of AMHR's mRNA in the AT. Panel B represents the appearance of AMHR's mRNA in different urological disorders. Grey parts of the columns represent the ATs are positive for AMHR's mRNA, while black parts of the columns represent the ATs are negative for AMHR's mRNA. n=60

Figure 2. Detection of AMHR protein expression with immunohistochemisry. Panel A represents the age dependence of the appearance of AMHR in the AT. Panel B represents the appearance of AMHR in different urological disorders. Grey parts of the columns represent the ATs are positive for AMHR's mRNA, while black parts of the columns represent the ATs are negative for AMHR's mRNA. n=49

Figure 3. Detection of AMHR2 on paraffin embedded, 4 µm thick sections. Brown colour (DAB) represents the AMHR2.