Immunohistochemical Study of Expression of Sohlh1 and Sohlh2 in Normal Adult Human Tissues

Xiaoli Zhang1, Ruihua Liu2, Zhongxue Su3, Yuecun Zhang4, Wenfang Zhang1, Xinyu Liu1, Fuwu Wang1, Yuji Guo1, Chuangang Li5, Jing Hao1*

1 Key Laboratory of the Ministry of Education for Experimental Teratology, Department of Histology and Embryology, School of Medicine, Shandong University, Jinan, China, 2 Department of Ultrasound, Yantai Yuhuangding Hospital, Yantai, China, 3 Department of Hepatobiliary Surgery, Shandong Provincial Hospital Affiliated to Shandong University, Jinan, China, 4 Department of Gynecology and Obstetrics, Nanjing Tongren Hospital Affiliated to School of Medicine of Dongnan University, Nanjing, China, 5 Department of Anesthesiology, The Second Affiliated Hospital to Shandong University, Jinan, China

* haojing@sdu.edu.cn

Abstract

The expression pattern of Sohlh1 (spermatogenesis and oogenesis specific basic helix-loop-helix 1) and Sohlh2 in mice has been reported in previous studies. Sohlh1 and Sohlh2 are specifically expressed in spermatogonia, prespermatogonia in male mice and oocytes of primordial and primary follicles in female mice. In this report, we studied the expression pattern of Sohlh1 and Sohlh2 in human adult tissues. Immunohistochemical staining of Sohlh1 and Sohlh2 was performed in 5 samples of normal ovaries and testes, respectively. The results revealed that Sohlh genes are not only expressed in oocytes and spermatogonia, but also in granular cells, theca cells, Sertoli cells and Leydig cells, and in smooth muscles of blood vessel walls. To further investigate the expression of Sohlh genes in other adult human tissues, we collected representative normal adult tissues developed from three embryonic germ layers. Compared with the expression in mice, Sohlhs exhibited a much more extensive expression pattern in human tissues. Sohlhs were detected in testis, ovary and epithelia developed from embryonic endoderm, ectoderm and tissues developed from embryonic mesoderm. Sohlh signals were found in spermatogonia, Sertoli cells and also Leydig cells in testis, while in ovary, the expression was mainly in oocytes of primordial and primary follicles, granular cells and theca cells of secondary follicles. Compared with Sohlh2, the expression of Sohlh1 was stronger and more extensive. Our study explored the expression of Sohlh genes in human tissues and might provide insights for functional studies of Sohlh genes.
**Introduction**

**Sohlh**1 (spermatogenesis and oogenesis helix-loop-helix 1) and **Sohlh**2 are transcription factors and play a pivotal role in the transition of germ cells from primordial to primary follicles and in the differentiation of spermatogonia in *mice* [1–3]. **Sohlh**1 was detected preferentially in oocytes but not in other *mouse* cDNA libraries [1, 2, 4]. **Sohlh**2 was discovered based on the homology with **Sohlh**2 in the bHLH domains [3–5]. Later it was found that both genes are specifically expressed in germ cell clusters, primordial and early primary oocytes in females and in prespermatogonia and spermatogonia in males. The expression signals disappeared rapidly as oocytes reached the secondary follicle stage and as type A differentiate to type B spermatogonia. **Sohlh**1 or **Sohlh**2 null *mice* were sterile due to the defect in the differentiation of spermatogonia and oocytes. These findings indicate that **Sohlhs** play crucial roles in spermatogenesis and oogenesis [2, 5, 6].

Interestingly, **Sohlh**1 is down-regulated in **Sohlh**2−/− *mice*, suggesting that the expression of **Sohlh**1 and **Sohlh**2 are correlated and the two genes potentially cross-regulate each other’s transcription [2, 5]. Newborn ovaries and testes from **Sohlh**2−/− *mice* showed very similar molecular changes as those from **Sohlh**1−/− *mice*, and it was suggested that **Sohlh**1 and **Sohlh**2 could form heterodimers to regulate spermatogonial and oocyte genes to promote the differentiation of germ cells in vivo [2, 5–7].

However, very little is known about the expression of possible cross-regulating **Sohlh**1 and **Sohlh**2 in normal *human* tissues. Here we provide evidence that **Sohlh**1 and **Sohlh**2 are widely expressed in normal adult *human* tissues. Using immunohistochemical staining, we revealed an expression pattern that was different from that in *mice*. **Sohlh**s were expressed more extensively in *human* tissues. As expected, the expression pattern of **Sohlh**1 and **Sohlh**2 is very similar in normal adult *human* tissues probably due to their functional interrelationship. Our exploration of immunoexpression of **Sohlh**1 and **Sohlh**2 provides a basis for further study of the roles of *human* **Sohlh**1 and **Sohlh**2.

**Materials and Methods**

**Human tissue samples**

Normal paraffin-embedded adult *human* tissues (each type of selected tissue is from 5 people) were obtained from the Department of Pathology in Shandong University Affiliated Qilu Hospital and Shandong Provincial Hospital. All the samples are examined by licensed pathologists and histologists and confirmed to be normal. Prior written and informed consent was obtained from every patient and the study was approved by the ethics review board of Shandong University (Permission NO. 201301031).

**Reagents**

The rabbit anti- *human* polyclonal **Sohlh**1 and **Sohlh**2 primary antibodies were purchased from Abcam Inc. (Cambridge, MA, USA). Phosphate buffer solution (PBS) was a product of Gibco (CA, USA). Rabbit SABC immunohistochemical kit and DAB color development kit were purchased from Boster Bio-engineering Limited Company (Wuhan, China).

**Immunohistochemical staining**

To prepare the samples for immunostaining, 5μm sections were deparaffinized in two changes of fresh xylene in 60°C incubator, each for 30 min, followed by treatment in a series of gradient ethanol (100%:X2, 95%:X2, 90%, 80%, 70% and then PBS; each for 5 min;) Antigens retrieval were performed through incubation in sodium citrate (pH 6.0) for 30 min at 96°C. The slides
were naturally cooled down to the room temperature. The immunohistochemical staining was carried out following the procedures described below: Endogenous peroxidases were blocked with 0.3% hydrogen peroxide for 30 min at room temperature and washed three times in PBS, each for 5 min; Normal goat serum was then added and incubated with the sections for 15 min to block the nonspecific binding site; Next, the sections were incubated with primary anti-Sohlh1 and Sohlh2 antibodies overnight at 4°C. For negative control, PBS was used instead of the primary antibody. After (insert times of washes) washes with PBS. The sections were incubated with anti-rabbit secondary antibody at 37°C for 1 hour followed by (insert wash times) washes in PBS. To further enhance the staining, SABC was added to the sections and incubated at 37°C for 1 hour. The chromogen diaminobenzidine (DAB) was prepared freshly by mixing one drop of chromogen to 1 ml of buffer in a mixing vial and added to the sections and incubated for 5 min, the sections were then washed in PBS and counterstained with Harris hematoxylin. At the end of staining, the slides were air dried, cleared in xylene and mounted with Neutral balsam. The staining was viewed and photographed under the Olympus U-LH100HG microscope.

Results

1. Sohlh1 and Sohlh2 expression in adult testis and ovary

To study if the Sohlh1 and Sohlh2 expression pattern is the same as that in mice, we first stained Sohlh1 and Sohlh2 in adult human testis and ovary using immunohistochemistry. We found that the expression pattern of Sohlh1 and Sohlh2 in ovary and testis is very similar, but the staining of Sohlh1 is stronger and more extensive.

The Sohlh1 protein was primarily observed in the nuclei of oocytes in primordial and primary follicles. Among the cells of secondary follicles, Sohlh1 was found highly expressed in granular layer, theca cells and most stromal cells. Similarly, Sohlh1 signals were found in almost all seminiferous epithelium except spermatids in testis. Intensive signals were found in Leydig cells and myoid cells around seminiferous tubules as well.

Compared to Sohlh1, Sohlh2 was mainly confined to the nuclei of oocytes and very weak in the cytoplasm of theca cells and granular cells in ovary. In testis, Sohlh2 was found in spermatogonia and Sertoli cells in seminiferous tubule and Leydig cells outside of seminiferous tubules (Fig 1).

2. oih1 and Sohlh2 are expressed in adult muscle tissues

The finding that Sohlh1 and Sohlh2 were strongly expressed in smooth muscle fibers of blood vessels in ovary promoted us to investigate the expression of Sohlh1 and Sohlh2 in all three kinds of muscle tissues-skeletal muscle, cardiac muscle and smooth muscle. The staining revealed that Sohlh1 and Sohlh2 were present in all three kinds of muscle tissues. The expression pattern of Sohlh1 and Sohlh2 was very similar. The expression intensity of Sohlh1 and Sohlh2 was very strong. Sohlh1 was localized in nucleus, cytoplasm, or both, while the location of Sohlh2 is mainly confined in the cytoplasm (Fig 2). To detect if the expression is linked to developmental lineages, we also stained a variety of tissues derived from embryonic mesoderm such as kidney and uterine tube. Our results showed that Sohlh1 and Sohlh2 were detected in these tissues (data not shown).

3. Sohlh1 and Sohlh2 are expressed in adult cerebral cortex

As we found that Sohlh genes can not only be expressed in ovary and testis but also in muscle tissues, we further studied their expressions in the brain.
The results showed that Sohlh1 immunostaining was positive in both neurons and neuroglial cells of cerebral cortex. The signals were equally observed in both nucleus and cytoplasm. However, Sohlh2 signals were mainly confined to the nuclei of the neurons, while they were not detectable in neuroglial cells using immunohistochemical staining method (Fig 3). In addition to the brain, we also detected the expression of Sohlh genes in some other tissues derived from embryonic ectoderm including iris, ciliary body, and retina (data not shown).

4. Sohlh1 and Sohlh2 are expressed in epithelia of digestive system and respiratory system

As we investigated Sohlh genes expression in mesoderm derived organs and ectoderm derived organs, we then stained Sohlh genes in some embryonic endoderm derived tissues. The results showed that Sohlh1 and Sohlh2 were present in epithelia of esophagus, lung, liver and pancreas. The expression pattern was similar for Sohlh1 and Sohlh2; but the intensity of Sohlh1 was much stronger than that of Sohlh2 and the location of Sohlh1 was also much diverse than that of Sohlh2 (Fig 4).

Discussion

Sohlh1 and Sohlh2 are germ cell-specific spermatogenesis and oogenesis basic helix-loop-helix (bHLH) transcription factors [1–3]. Sohlh1 shares 50% identity with Sohlh2 in bHLH region. Mouse Sohlh2 protein shares 50% identity with its human orthologue, with the highest conservation observed in the bHLH domain. Sohlh1 and Sohlh2 were expressed in mouse spermatogonia and in primordial to primary oocytes in embryonic, neonatal or adult mice [1–3].
Sohlh1 or Sohlh2 causes infertility by disrupting spermatogonial differentiation into spermatoocytes or ovarian follicle differentiation from primordial to growing follicles [1–2, 5–7]. Seven-day-old testis lacking of Sohlh1 overexpress Sohlh2 [2]. The Sohlh2-null mice downregulated the expression of Sohlh1 indicating an interrelationship between Sohlh1 and Sohlh2 [7]. Sohlh1 and Sohlh2 can form heterodimers or homodimers [7–9]. A Sohlh2/Sohlh1/SP1 ternary complex autonomously and cooperatively regulates Sohlh1 gene transcription during early spermatogenesis and oogenesis [7, 10]. Several other spermatogonial transcriptors could also monitor spermatogenesis by regulating the expression of Sohlh1 or Sohlh2 [11–13].

We studied the expression of Sohlh1 and Sohlh2 in normal adult human tissues by immuno-histochemical staining and found that they were expressed not only in ovary and testis, but
also in many other tissues. The expression patterns of Sohlh1 and Sohlh2 were very similar, which was not surprising given the previous observations of the relationship between Sohlh1 and Sohlh2 in mice. The proteins were found in both nucleus and cytoplasm. We were able to detect Sohlh1 and Sohlh2 in testis tissues such as spermatogonia, Sertoli cells and Leydig cells and in ovary cells including oocytes, early primary follicles, granular cells, and theca cells in secondary follicles. Because we did not find any oocytes in all of the secondary follicles, it was difficult to tell if Sohlh1 and Sohlh2 were expressed in oocytes of secondary follicles.

In the mammalian ovary and testis, progressive activation of primordial follicles or spermatogonia serves as the source of fertilizable ova and sperms, and disorders in the development of primordial follicles or spermatogonia lead to various diseases [14–20]. The polymorphisms of the Sohlh 2 gene could be the genetic risk factors for nonobstructive azoospermia (NOA) in the Chinese population [16]. A splice-acceptor site mutation of the Sohlh1 gene also leads to nonobstructive azoospermia [17]. Novel variants in the Sohlh2 gene were also found in women with premature ovarian failure (POF) of both Chinese and Serbian [18]. Sohlh2 was expressed at very low levels in epithelial ovarian cancer (EOC) samples probably by the epigenetic mechanisms [21–25]. These findings strongly suggest the important roles of Sohlh2 in various diseases and promote us to study the expression patterns of these genes in normal human tissues.

The most notable finding in the current study is that Sohlh1 and Sohlh2 seem to be expressed ubiquitously and not to be associated with developmental lineages. Sohlh1 and Sohlh2 were found in various tissue types like cerebral cortex, muscle tissues and epithelial tissues of esophagus, lung, liver and pancreas. The above studies indicate that Sohlh1 and Sohlh2 may play very important roles in normal human tissues, and our exploration for the expression of Sohlh1 and Sohlh2 provides the basis for further study of functions of Sohlh1 and Sohlh2 in relevant academic fields.

The notable difference of expression pattern with that in mice is not uncommon. Bonnet A observed the constant expression of Sohlh2 in sheep granular cells and in oocytes during early follicular development until the small antral (SA) stage which is also quite different from that
They speculated that such different Sohlh2 expression pattern suggests the existence of different mechanisms that need further investigation. The difference also underlines the importance of acquiring expression data from different species and highlights certain species specificities.

As to our knowledge, this study is the first to investigate the expression of Sohlh1 and Sohlh2 in normal adult human tissues. Like the study in the rhesus monkey, we also found the difference of Sohlh1 and Sohlh2 expression between human beings and mice. For cells in the same section, some signals are confined in the nucleus, and some signals are found in the cytoplasm and some signals are found in both nucleus and cytoplasm. In regard to the location of the proteins, Suresh et al. [27] discovered that the spermatogonial Sohlh1 nucleocytoplasmic shuttling was associated with the initiation of spermatogenesis in the rhesus monkey and suggested that in the monkey, nuclear location of Sohlh1 is closely associated with spermatogonial differentiation. We surmise that it could also be the nucleocytoplasmic shuttling mechanism of Sohlh1 and Sohlh2 that determine the different state (proliferation or differentiation) of the cells in human tissues. Consistent with this, our current study confirmed that Sohlh1 and Sohlh2 in human were localized in both nucleus and cytoplasm.

The expression pattern of Sohlh1 and Sohlh2 in ovary is important to the human reproductive expert to decipher the critical molecular processes and the complexity of the communication between oocytes, granular cells and theca cells. Similarly, the different expression pattern of Sohlh1 and Sohlh2 in testis could be illuminating of scientific researchers in male reproductive field to explore the relationships among spermatogonial cells, Sertoli cells, Leydig cells, or even the myoid cells around seminiferous tubule during spermatogenesis.

We hope our study can be a starting point for further investigation of the function of Sohlh1 and Sohlh2 in human tissues, not only in the reproductive system but also in various academic fields.

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Author Contributions
Conceived and designed the experiments: X. Zhang R. Liu JH ZXS Y. Zhang. Performed the experiments: X Zhang W. Zhang X. Liu YG FW. Analyzed the data: X. Zhang R. Liu JH. Contributed reagents/materials/analysis tools: ZXS Y. Zhang R. Liu YG FWW CGL. Wrote the paper: X. Zhang R. Liu JH.

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