

行政院國家科學委員會專題研究計畫 成果報告

睪丸專一表現同源箱基因-TSX1 之分子及功能性探討(3/3)

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☐ 期中進度報告

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中、英文摘要及關鍵詞

中文摘要

除了 Y 染色體上之某些片段確知與男性不孕有關外，是否有其他染色體上的基因牽涉這類病變，並不是很清楚，同時為探討同源箱基因(homeobox gene)在睪丸與精子發育的過程所扮演的角色，我們利用 PCR 的方法找到一個新的人類同源箱基因(*Homeobox gene*)，在組織表現檢測結果中發現其在睪丸中有相當高的專一性表現，我們將之命名為 *TSX1* 基因(testis specific homeobox gene)，我們利用反轉錄 PCR(RT-PCR)及原位雜交(*in situ hybridization*)等技術，推知 *TSX1* 基因是表現在睪丸的精細胞上，我們推測 *TSX1* 基因與精子發育的過程可能有很大的關聯性。由於 *TSX1* 基因在睪丸有高專一性的表現，因此我們相當好奇是否有些男性不孕是由於 *TSX1* 基因的突變所造成。所以我們以 4 組的 PCR 引子檢測 250 位睪丸起因不孕症男性之染色體區域，但並未發現有不正常的 PCR 產物出現。我們進一步以 SSC 方式檢測是否有點突變或小片段基因被刪除，也未發現有不正常的片段出現。我們已經證明了 *TSX1* 基因並不存在於人類以外的其他物種。在過去，我們用人類的 *TSX1* 基因做為探針(probe)，對數個小鼠睪丸 cDNA libraries 進行 *TSX1* 小鼠基因之篩選，也密切注意 GenBank 是否有小鼠 *Tsx1* 基因之發現。然而一直徒勞無功，我們於是進行了 Zoo blot，果然證實 *TSX1* 只存在於 human 的 genome 中，而未存在於其他物種中。為了能更進一步探討 *TSX1* 在活體中之功能，於是我們建立了 *TSX1* 基因轉殖鼠。經由 PCR 檢測，我們在 70 隻小鼠中共找到 8 隻帶有 *TSX1* 基因的 founder 小鼠，將這些小鼠與 wild type 小鼠交配後所生的小鼠經過 western blot 分析，我們發現有一個 line 母鼠的卵巢及三個 lines 的睪丸有 *TSX1* 之蛋白表現，但在組織及生育功能上並未發現有進一步之性狀。由這些基因轉殖鼠的結果看來，我們所使用的 pEF 啟動子，雖然是可以到處都表現的啟動子，但是在絕大多數組織中，並未發現有 *TSX1* 之表現，可能是受到「position effect」的影響，而在三個睪丸表現 *TSX1* 基因之轉殖鼠中缺乏性狀的結果，顯示睪丸可能存在某些調控機制壓抑了 *TSX1* 過量表現可能造成之傷害。

關鍵詞：睪丸、精子熟成、同源箱基因、不孕症

英文摘要

There is not much genetic information about male infertility other than some segments on Y chromosome. To uncover more genetic information related to spermatogenesis and identify the function of homeobox genes in the testis, degenerated oligonucleotide PCR screening of testis cDNA libraries was performed to isolated more testis specific genes. One novel human testis-specific homeobox gene was identified and named *TSX1* (testis-specific homeobox). RT-PCR and Northern analysis confirmed that *TSX1* is expressed in the adult testis and *in situ* hybridization identified that the expression is in the germ cells. These results suggest that *TSX1* may play an important role during germ cell development. To determine whether mutations in *TSX1* are related to impaired spermatogenesis, an intensive PCR screening was performed on 250 infertile man DNA samples with 4 sets of primers across the *TSX1* gene locus. There was no unexpected PCR products identified in any of the 250 DNA samples. Further SSCP analyses were performed and no aberrant PCR fragment were identified. We have tried intensively and failed to identify the mouse *Tsx1* ortholog through frequently GenBank searching and many times mouse testis cDNA libraries screening. We then proved that the *TSX1* is only present in the human but

not other species' genome by Zoo blot analysis. This data implied that *TSX1* is a gene appeared late during evolution and may have a more specific function to human. To understand the in vivo function of *TSX1*, we generated *TSX1*-overexpression transgenic mice with a ubiquitous promoter pEF. We obtained 8 transgenic fonder lines from 70 mice. After western blot analysis, we identified 1 transgenic line with *TSX1* expression in the ovary and 3 lines with expression in the testis. However, no significant phenotype in fertility or abnormal histology in reproduction tracts was observed after analysis for 3-4 generations. From the results of transgenic mice, we concluded that the pEF promoter was suppressed in most of the mouse tissues although it was assumed as a ubiquitous promoter, and the pEF promoter was also under the regulation of mechanisms which repressed the damage might arise from the overexpression of *TSX1*.

Keywords: Testis; Spermatogenesis; Homeobox gene; Infertility

二、 報告內容

前言與文獻探討

Spermatogenesis and Male Infertility

Sperm production (spermatogenesis) is one of the most dramatic processes of differentiation. In the initial stages, spermatogonia undergo two mitotic divisions, giving rise to primary and secondary spermatocytes, which undergo two meiotic divisions to produce the haploid round spermatids. During the spermeiogenesis, the round spermatids elongate, compact their chromatin into the sperm head and produce the other sperm components (1-3). This progression from pro-spermatogonia to mature spermatozoa has been well defined and makes spermatogenesis an attractive system to study the regulation of a developmental process in an adult organism.

Male infertility, affecting as many as 10% of the adult population, is an extremely prevalent disorder. In most cases, these men are infertile either because of insufficient sperm (oligozoospermia) or lack of sperm (azoospermia). The cause of these defects in sperm production is unclear, but recent works point to both potential environmental and genetic causes (4-5). Genetic factors that might affect male infertility, other than some sequences on the Y chromosome, have not been identified. An important facet of spermatogenesis occurs at the level of transcriptional regulation, and the characterization of transcription factor genes, such as homeobox genes, expressed during spermatogenesis constitutes a necessary step toward an understanding of this level of control.

Homeobox genes

Homeobox genes encode transcription factors containing a 60-amino acid DNA binding motif, term the homeodomain. The tertiary structure of which conforms to a helix-turn-helix motif conserved in species as evolutionarily distant from yeast to human. Homeobox genes are known to be intricately involved in the development and differentiation processes (6-8). The *prd-like* class homeodomains comprise similar coding sequences as the *prd* class homeodomains, but the characteristic serine residue at position 50 in *prd* homeodomains is replaced by a

glutamine (Q) or lysine (K) in *prd*-like homeodomains; the *paired-like* genes also lack the paired domain of *prd* genes (9). Several homeobox genes have been identified expressed in the human and murine male reproductive systems, suggesting the possibility that they may regulate developmental events during male gametogenesis (10-11).

研究目的

To identify novel homeobox genes involved in spermatogenesis; we have conducted a degenerate oligonucleotide PCR screen of several human and mouse testis cDNA libraries. One novel homeobox gene *TSX1* was isolated from human testis cDNA library. To understand the functional role of *TSX1* during spermatogenesis, we characterized the expression pattern of *TSX1* in the testis. Transgenic mice were established to identify the effect of *TSX1* over-expression in vivo.

研究方法

Cloning of human homeobox gene TSX1. The 23-mer degenerate oligonucleotides were designed based on the highly conserved helix 3 of homeodomain sequences and the PCR amplification from cDNA libraries were performed as described previously (12). The 3' 0.6 kb fragment of *TSX1* cDNA was obtained from human testis cDNA library (Life Technologies). DNA sequences of this PCR product containing a putative novel homeodomain and was used to search EST databases to obtain additional cDNA sequence information. EST clones were purchased (Genome Research) and sequenced. Clone AA933590 with the 1244 bp cDNA insert was used in further studies.

Sequence analysis. The MacVector program (Kodak) was used for alignment of the cDNA sequence to the genomic DNA sequences to determine the intron/exon junctions, translation of the DNA sequences into amino-acid sequences, identification of putative functional motifs, and GeneBank search for both DNAs and proteins through the network.

RNA isolation and RT-PCR analysis. Total RNA was extracted from tissues that are fresh frozen in liquid nitrogen with Trizol reagent (Life Technologies). 1 µg of total RNA was used in reverse transcription with MMLV reverse transcriptase (Promega). Most of the human multiple tissue cDNAs were purchased from Clontech Co. The PCR amplification was further performed with primers specific to *TSX1*, RT-F, 5'-GCGGAGAAGAAAAAGATGGCG-3' and RT-B, 5'-GGGAATGTGAAAGAAG-GCACG-3'.

Multiple Tissue Array and Northern blot analysis. Human Multiple Tissue Arrays containing normalized loading of poly(A) RNA from different human tissues were purchased from Clontech. Human multiple tissue Northern blots with 2 µg of poly(A) RNA on each lane were purchased from Clontech and Ambion. These blots were hybridized to the radiolabeled *TSX1* (bps 748-1169) cDNA probe according to the manufacturer's protocol.

In situ hybridization. The *TSX1* (bps 748-1169) cDNA fragment was PCR-amplified and subcloned into pGEM-Teasy vectors (Promega). Sense and antisense riboprobes were prepared by *in vitro* transcription using T7 and SP6 polymerase, respectively, with digoxigenin-UTP (Boehringer Mannheim). The *in situ* hybridizations of testis paraffin sections were performed using standard protocols.

Screening of mouse testis cDNA libraries. *TSX1* (bps 3-1169) cDNA fragment was radio-labeled and used as probes to screen mouse testis cDNA libraries (λ gt10 library was from Clontech, SuperScript library was from Life Technology and Mouse Unamplified cDNA library was from Edge BioSystem) for several times with low stringency hybridization conditions in hybridization solution [1M NaCl, 0.1M Tris-HCl (pH 7.4), 10% dextrane sulfate and 1% SDS] at 50-55°C for more than 16 hr. The membranes were then washed twice with wash buffer (2XSSC and 1% SDS) for 1 hr. The radiographs were developed after exposure for 16-48 hr.

Zoo blot analysis. 10 μ g of genomic DNAs from human (peripheral blood), pig, chick, rat and mouse (liver) were digested with restriction enzymes (*EcoRI* or *HindIII*), size fractionated in 0.8% agarose gel and then blotted onto a nitrocellulose membrane (Hybron N⁺, Pharmacia). Another Zoo blot membrane with 8 species genomic DNA (from yeast to human) was purchased from Origene. These blots were hybridized to the radio labeled *TSX1* probe (exon 3) according to the manufacturer's protocol.

Establishment of TSX1 transgenic mice. *TSX1* coding region was digested with *ApoI* and *BspEI* and subcloned into the *NotI* site of pEF/myc/cyto expression vector (Invitrogen). The transgenic construct was digested with restriction enzymes *Acc65I* and *XmaI* to remove most of the vector sequences and the resulting fragment was microinjected into the FVB mouse pronucli. Transgenic mice were identified by PCR analysis of the tail biopsies from pups got birth from foster mother and further breeding. The primers used in PCR were localized on pEF promoter and *TSX1* cDNA. Transgenic founder mice were breed with wild type mice to produce more transgenic offspring for further phenotypic, expression and histological analyses.

SSCP analysis 3 µl of the PCR product was mixed with 3 µl of gel-loading buffer (99% deionized formamide, 20 mmol/L EDTA, 0.05% xylene cyanol FF and 0.05% bromophenol blue). The mixture was denatured for 5 min at 95°C and then plunged into ice. The mixture was electrophoresed at 8°C in a Genephor electrophoresis unit using a GeneGel Excal 12.5/24 kit (Pharmacia) under constant of 600V for 2 hr. The gel was stained by silver staining using a PlusOne DNA silver staining kit according to the manufacture's instructions (Pharmacia).

結果與討論

We have isolated *TSX1* homeobox gene from the human testis cDNA library and identified that the homeodomain belongs to a pair-type subfamily. To confirm the expression of *TSX1* in human testis, RT-PCR was performed with primers located on exon 2 and 3 individually. An expected product was amplified only from the human testis (Fig. 1A). Further Northern analysis also confirmed this specific expression pattern and revealed the transcript of *TSX1* is about 1.2 kb (Fig. 1B). A more detailed expression pattern in testis was investigated by in situ hybridization and showed major signals present in germ cells (Fig. 2). These expression patterns indicated that *TSX1* might play a role in regulation of spermatogenesis.

By GenBank sequence alignment, we identified that the *TSX1* was localized on the human X chromosome. From the alignment of *TSX1* cDNA and genomic sequences, we identified that *TSX1* has 4 exons (Fig. 3). The intron-exon boundaries listed on Table 1 are match to the splicing donor and acceptor sites. To determine whether *TSX1* is related to infertility, an intensive PCR screening was performed on infertile male DNA with 4 sets of primers across the *TSX1* gene locus (Table 2). There was no unexpected PCR products were identified in total of 250 DNA samples after repeated screening (Table 3). This PCR result revealed that there is no PCR-detectable deletion among these patients' *TSX1* locus. Since point mutation and smaller fragment (< 30 bp) deletion was unable to be detected through PCR analysis, SSCP analysis was further used to resolve this problem. However, the analyses of 4 sets of 150-200 bp of PCR products of the *TSX1* coding region were fail to identified any aberrant single strand conformation (Fig. 4).

To isolate the mouse ortholog of *TSX1* gene, we have tried intensively to screen several different mouse testis cDNA libraries for many times; on the other hand, we also tried to search GenBank frequently with human *TSX1* gene applied in the sequence alignment. Both of the above approaches gave us the negative results. To identify whether there is any mouse *TSX1* gene exists in mouse genome, Zoo blot analysis was performed by using *TSX1* probe to hybridize human, pig,

rabbit, mouse and chick's genomic DNA which were digested with *EcoRI* or *HindIII* (Fig. 5). We can only detect the signal from the human genome which explained that why we were unable to isolate any mouse *TSX1* gene from mouse testis cDNA libraries. This data further implies that *TSX1* might be involved in a human-specific spermatogenic pathway.

To study the *in vivo* function of *TSX1*, we have established the *TSX1* transgenic mouse lines. The transgenic construct and the junction sequences between *TSX1* and vector are shown in Fig. 6, *TSX1-myc* fusion gene expression is driven by pEF promoter and the myc epitope tag can be used for identification of the expression of TSX1 protein in the transgenic mice. The vector of the expression construct was removed and the *TSX1*-containing DNA fragment was microinjected into the pronuclei of zygotes. PCR analysis for the seventy F1 mice with the primers located on the transgenic fragment identified eight transgenic founder lines (Fig. 7). We crossed these F1 mice with FVB wild type mice to generate more transgenic offspring for further analysis. We have identified TSX1 expression in oviduct of one line and testes of three lines (Fig. 8). However, no specific reproduction phenotypes were identified after analyses of their histology and reproduction activity for 3-4 generation. Occasionally, we founded some mice with behavior abnormality such as continuously circling. One of the founder mouse (No. 63) was identified with a sarcoma tumor in the lower limb of the right side (Fig. 9), but no other offspring has the same phenotype observed so far. Another founder mouse (No. 31) was identified to have lung dysfunctional phenotype (Fig. 10)

In conclusion, we have identified a pair-like homeobox gene, *TSX1*. Expression analyses from both RT-PCR and Northern blot indicated that *TSX1* is testis-specific. These results implied that *TSX1*, as a homeodomain-containing transcription factor, may be involved in spermatogenesis-specific gene regulation pathway. However, intensive PCR screening and SSCP analysis were failed to polymorphism present in 250 infertile patients. To further investigated the *in vivo* function, we established the *TSX1* transgenic mice. Preliminary characterization showed that the over-expression of *TSX1* could not interfere the reproduction activity. Whether the effect is under the restrict control of gonad or the human-specific regulation is not valid in murine system remained to be further

investigated.

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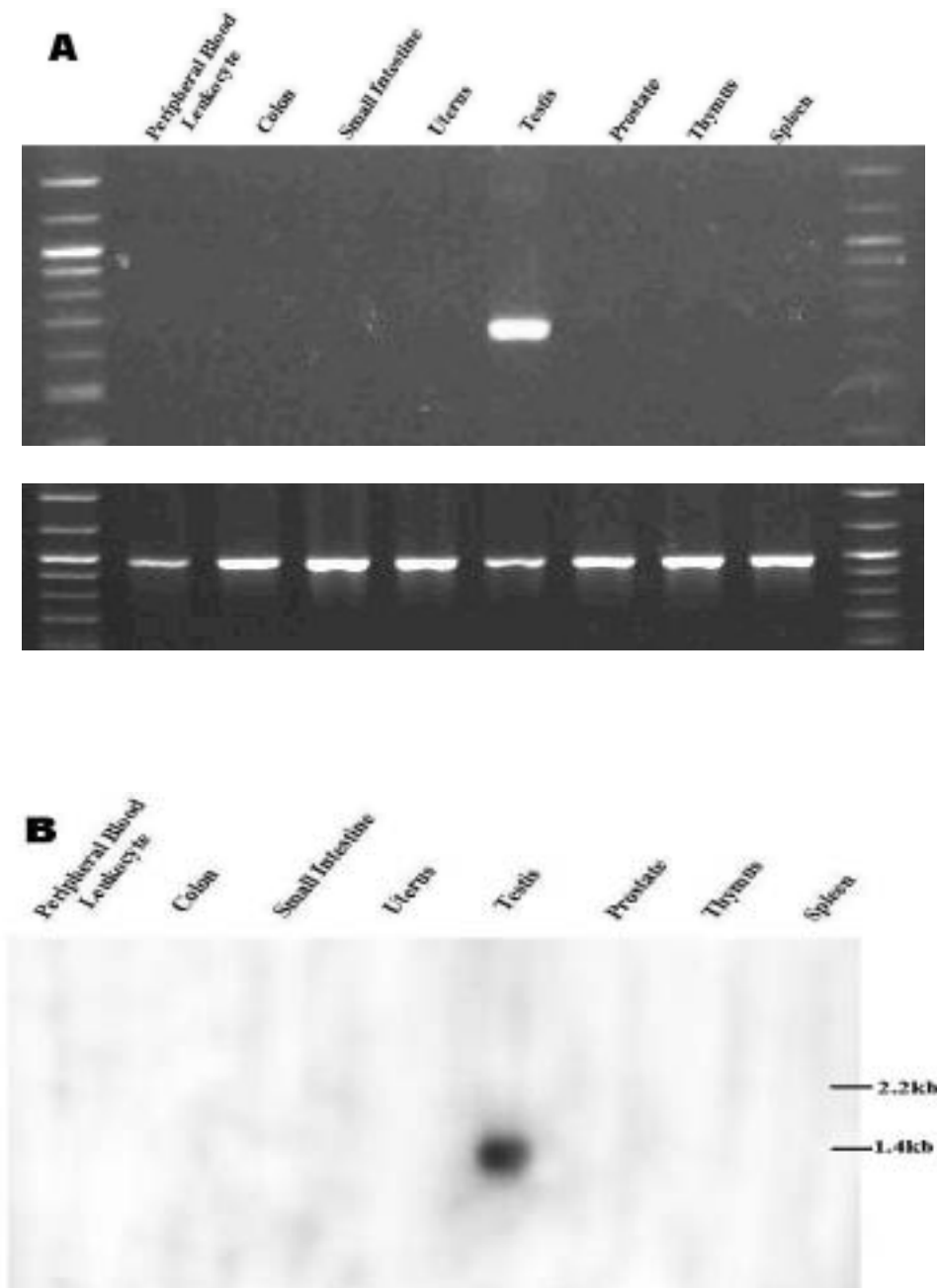


Fig. 1 Human *TSX1* homeobox gene shows testis-specific expression. (A) Expression pattern of *TSX1* in multiple tissues. PCR was performed with *TSX1*-specific primers using human cDNA from different organs. *TSX1* is specifically expressed in the testis.(B) Northern analysis of *TSX1* expression in multiple tissues with *TSX1*specific probe. A 1.2 kb transcript is only identified in human testis.

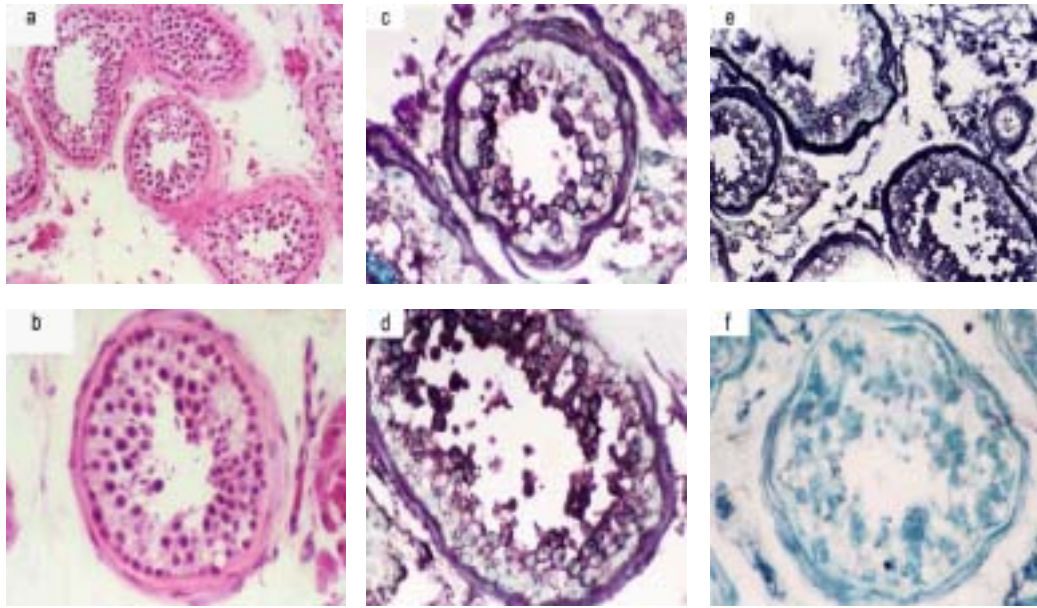


Fig. 2 Tsx1 expression pattern in testis seminiferous tubules. The cellular expression pattern of TSX1 was characterized by in situ hybridization. (a) and (b), H and E staining. The results from using TSX1 anti-sense [(c) to (e)] and sense (f) probes for the in situ hybridization analysis.

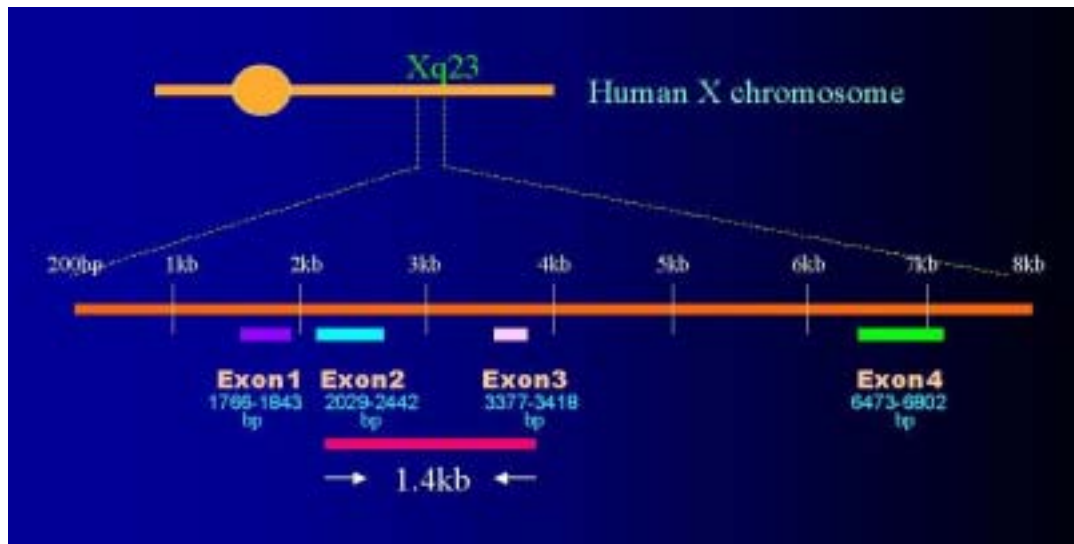


Fig. 3 The Chromosome localization and enomic structure of *TSX1*. *TSX1* gene is localized on human Xq23 and the schematic diagram of the intron-exon structure is shown in the botton.

Table 1 The exon-intron boundaries of the TSX1 gene.

No.	Splice donor	Splice donor	Intron size (bp)
1	AGAAGAACGT t gcaggcggc	gaactacag g GTCAGTACCT	1765
2	TACTTTTCCA a tatgaatgc	gttcctgc g TAAGCCCATT	933
3	CCATAGAAG G aggctggcaa	cgcagtgc a gGTCAGTAAAC	3126
4	CTGATTGAAG a tttggtttg	tcaaaccg a aTGTCACGGAC	945

Table 2. The primer pairs used to PCR screen the mutations on the exons 1-4 of TSX1 gene.

Name	Sequence	PCR product size	Annealing temp. (°C)
TsxEx1-F'	GCAGAAAAGCAAGAGAGGAGGTC	310bp	55
TsxEx1-B'	AGGGGTATCATCCAGTCTACTCGC		
TsxEx2-F'	TCACCACCTCCTTCCAACACC	480bp	57
TsxEx2-B'	ACCAGAGCGCAATGGGCTTACCG		
TsxEx3-F'	TGGCATCTCCTTTATTTGTGGC	250bp	55
TsxEx3-B'	TGAAGCGGGTTTTAGAATGGC		
TsxEx4-F'	GAAATAAGCCAAGCCAGAGCG	800bp	55
TsxEx4-B'	AGACGAGAGTCCGTGACATTCG		

Table 3 The results from PCR and SSCP analyses of TSX1 gene on 250 infertile men's genomic DNAs.

Exon no.	Number of mutants from analysis		
	1 ^o PCR	2-4 ^o PCR	SSCP
1	10	0	0
2	5	0	0
3	8	0	0
4	7	0	0

N 1 2 3 4 5 6 7 8 9 10 11 12 13 14

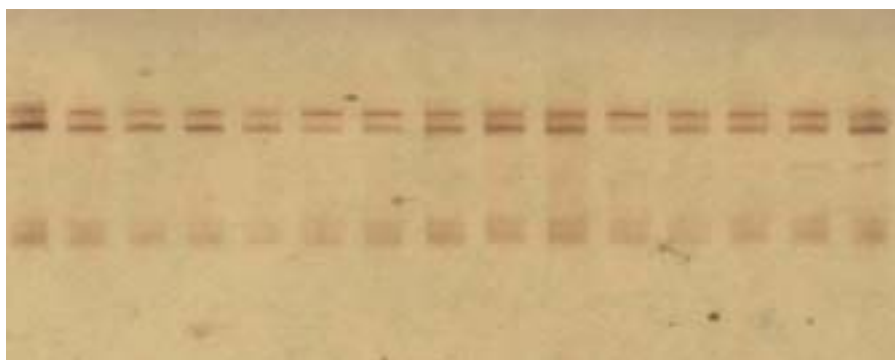


Fig. 4 SSCP analysis of the TSX1 gene mutation. Lanes 1-14, PCR products from patient DNA; N, normal control DNA.

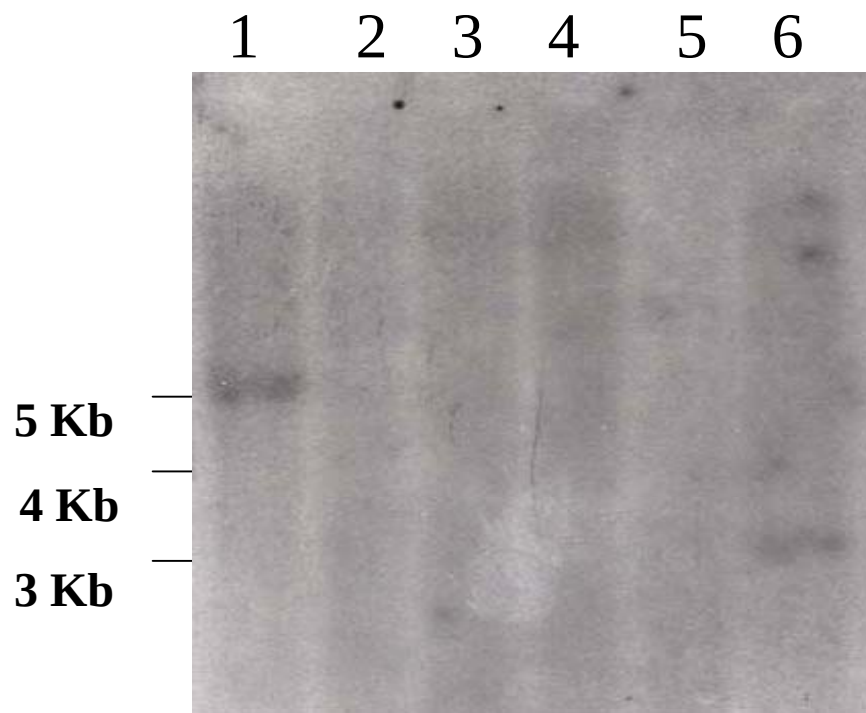


Fig. 5 Zoo blot analysis of TSX1. Genomic DNA blot hybridized with TSX1 specific probe showed that TSX1 is only present in the human genome. Lanes 1-5, genomic DNAs digested with EcoRI, lane 6, genomic DNA digested with HindIII. Lanes: 1, human; 2, pig; 3, rabbit; 4, mouse; 5, chicken; 6, human.

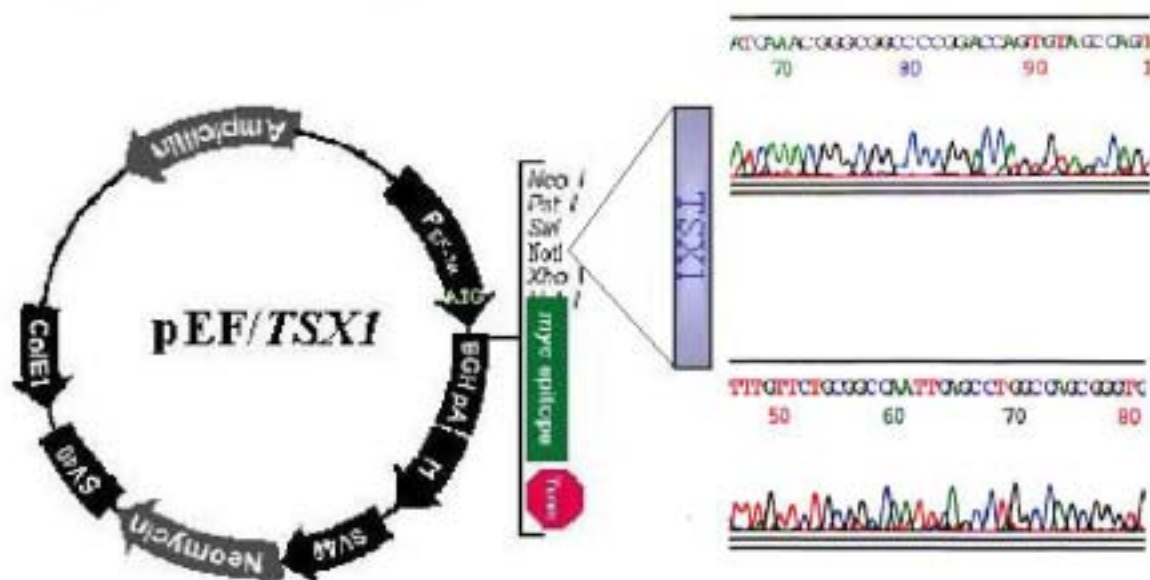


Fig. 6 The TSX1 expression construct. TSX1 was inframe-inserted before a myc epitope tag and driven by a pEF promoter. The sequences between the junction of cDNA and vector are shown on the right side.

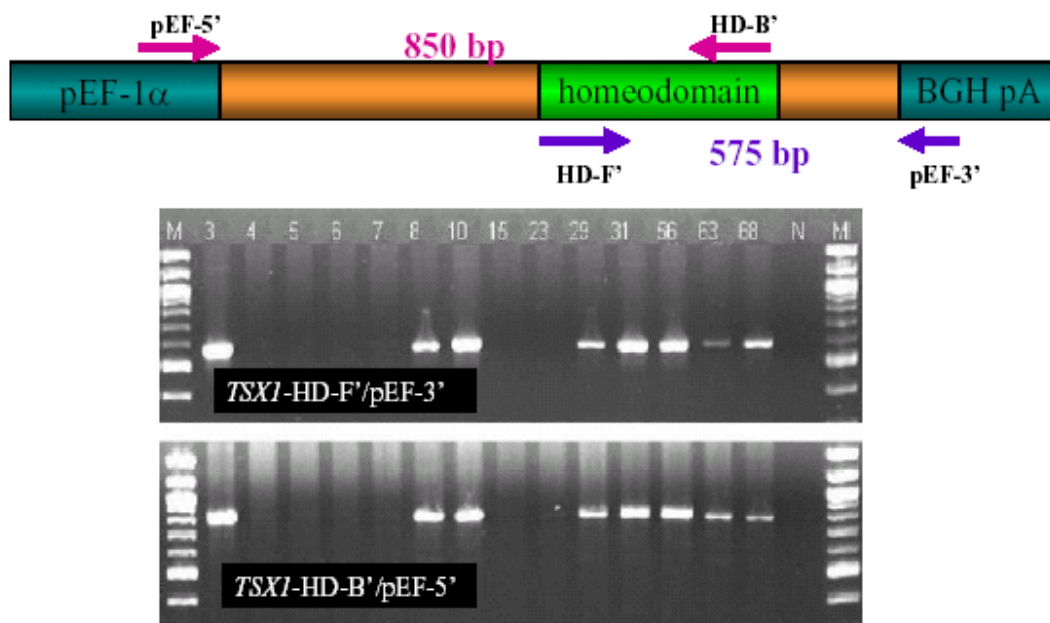


Fig. 7 Identification of TSX1 transgenic mouse founders. The PCR primer localization was shown in the top. There are total 8 transgenic mouse founder lines (3,8,10,29,31,56,63 and 68) according to the PCR analysis resultsshowed in the bottom.

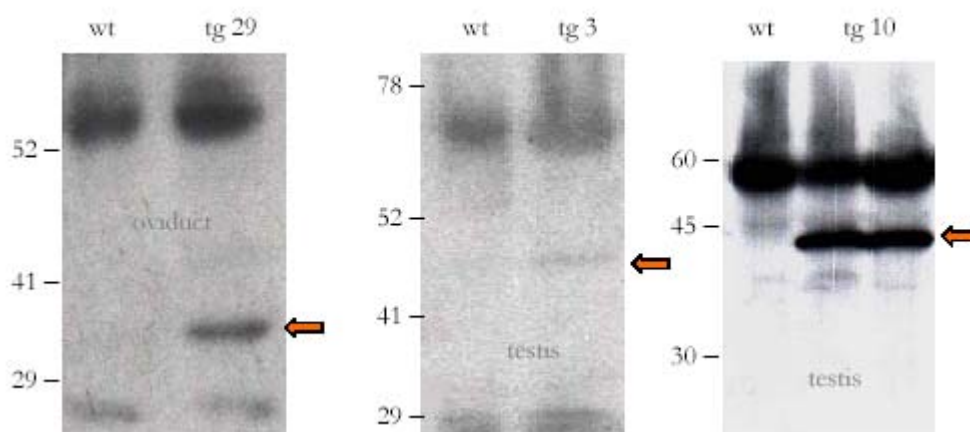


Fig.8 TSX1 transgene expression in transgenic mice. Western blot analyses of the transgenic mouse reproductive tracts show that TSX1 (arrows) are expressed in the oviduct of line 29, testes of line 3 and 10. All the other bands are endogenous myc expression or non-specific signals.

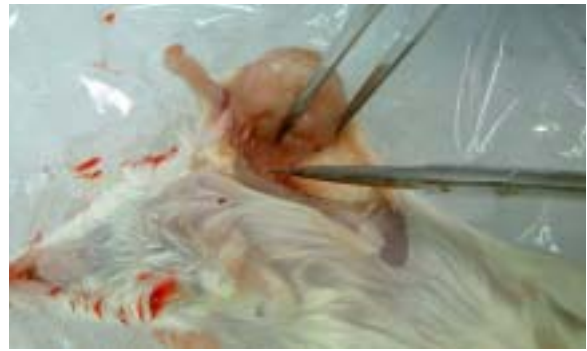


Fig. 9 Scramble carcinoma developed in line 63 transgenic founder mouse.



Fig. 10 Abnormality of TSX1 transgenic founder mouse line 31. The rotten skin around eyes and ears are shown on the top. The lung isolated from the normal littermate is pink and floating on the PBS (left), while the tissue from the transgenic mouse is pale and sink on the bottom.