

Cloning and Characterization of Multiple RNA Splicing Variants of *LDH-C* Gene in Human and Rat

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Abstract: The expression of LDH-C (Lactate dehydrogenase C) gene is restricted in mature germ cells; however multiple splice variants of LDH-C expressed in human cancers and yak normal testes were reported recently. In order to know if there are any LDH-C splice variants in human normal testes, we set out to clone the putative variants in human and rat. Four splicing variants in human testes, 1 splicing variant in human spermatozoa, 6 splicing variants in rat testes and 1 splicing variant in rat non-testes tissues (liver, heart and muscle) were cloned. The putative polypeptides encoded by these variants were compared with the full-length LDH-C protein, the results showed that these putative polypeptides were truncated LDH-C proteins or truncated LDH-C proteins with a few amino acid residues different at N or C terminal. This suggested that these variants are possibly not used for translation, but targets of nonsense-mediated mRNA decay. Western blotting did not detect any bands with similar molecular weight as the putative polypeptides. RT-PCR showed that the expression levels of the splicing variants were significant during development of rat testes. The results indicate that LDH-C was not silenced by transcriptional repression in non-mature germ cells, but significantly transcribed and alternatively spliced.

Keywords: Alternative splicing, development, expression regulation, lactate dehydrogenase c, splicing variant

INTRODUCTION

Mammalian Lactate dehydrogenase (LDH; EC1.1.1.27) protein family is a kind of tetrameric NAD⁺-specific dehydrogenases and serves as the terminal enzyme of glycolysis, catalyzing reversible oxidation-reduction reaction between pyruvate and lactate (Everse and Kaplan, 1973). There are three different subunits of LDH in mammalian, comprising LDH-A, LDH-B and LDH-C, encoded by three different genes, LDH-A, LDH-B and LDH-C, respectively. Although the LDH isozymes catalyze the interconversion between pyruvate and lactate, each has presumed function and specific distribution pattern (Everse and Kaplan, 1973). LDH-A is most active in skeletal muscle and other tissues where oxygen deficiency happens frequently and glycolysis is required to satisfy metabolic needs, while LDH-B is abundantly expressed in cardiac muscle and liver that is dependent upon aerobic metabolism pathways. An exquisite tissue distribution is displayed by LDH-C, the third member of the family, which is found only in male (Goldberg, 1985) and female (Coonrod *et al.*, 2006) germ cells.

Many researchers are interested in LDH-C due to its tissue specific distribution. The restriction of LDH-C

to germ cells but not in any somatic tissues makes it an ideal model gene for studying the regulation of tissue-specific expression (Tang *et al.*, 2008). In somatic cells, the suppression of LDH-C gene expression is likely due to methylation of CpG island of LDH-C promoter, however it still remains to be illustrated solidly (Tang and Goldberg, 2009). In germ cells, transcriptional analysis of LDH-C is compromised by the lack of a reliable cell culture system. Even so Goldberg and his colleagues made progress in elucidating the regulation of LDH-C gene expression by construction of transgenic mice and transfection of germ cells in situ (Li *et al.*, 1998; Markert *et al.*, 1998). They identified some potential *cis*-regulatory elements including PAL, GC box and CRE and trans-acting factors including Sp and CREB, they suggested that the role of the CRE site is essential for spermatocyte-specific gene expression (Tang *et al.*, 2008; Tang and Goldberg, 2009; Kroft *et al.*, 2003). However, the mechanisms governing LDH-C expression and tissue specificity are largely unknown.

Koslowski *et al.* (2002) reported that the expression of multiple splicing variants of LDH-C occurred selectively in human cancer. They discovered that LDH-C escapes from transcriptional repression, resulting in significant expression level of splicing

variants in virtually all tumor types they tested (Koslowski *et al.*, 2002). They discovered the aberrant splicing patterns of *LDH-C* restricted to tumor cells and expression of LDH-C in tumors is neither mediated by gene promoter demethylation, as previously described for other germ cell-specific genes activated in cancer, nor induced by hypoxia as demonstrated for enzymes of the glycolytic pathway. The author, thus, estimated the specific expression pattern may have something to do with the unique energy metabolism of cancer cells and LDH-C activation in cancer may provide a metabolic rescue pathway in tumor cells by exploiting lactate for ATP delivery.

Recently, He and colleagues reported that LDH-C multiple splicing variants were detected in some mammals' testes (He *et al.*, 2008; Huang *et al.*, 2012). It seems that the splicing variants does not restricted to tumors.

Are there any splicing variants in human normal testes just like in yaks? To answer this question, we started to clone LDH-C cDNA from human normal testes and spermatozoa. Surprisingly, we found 5 alternative splicing variants in non-cancer human testes and spermatozoa. Six splicing variants were also cloned in rat testes and non-testes tissues and expression profiles of different LDH-C splicing variants were investigated in rat. The results indicate that LDH-C was not silenced by transcriptional repression in non-mature germ cells, but significantly transcribed and alternatively spliced.

MATERIALS AND METHODS

Materials: Human testis was obtained from The First Hospital affiliated to Chengdu Medical College (Chengdu, China) after obtaining a signed informing consent and the approval of the Ethical Committee of this college. The tissue was immediately cryostored in liquid nitrogen after surgery. Human semen samples were obtained from 8 consenting donors who met all the World Health Organization (WHO) criteria for normozoospermia by masturbation after 3-5 d of abstinence (WHO, 1999). SD rats were from the experimental animal breeding center of Sichuan University

RNA extraction and first stranded cDNA synthesis: Total RNA from liquid nitrogen frozen human testis, ejaculated spermatozoa and rat tissues were extracted with Trizol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. The purity

of RNA samples was checked by spectrophotometer at 260 nm and 280 nm and agarose electrophoresis. The first strand cDNA was synthesized using a TaKaRa RNA PCR Kit (AMV) Ver. 3.0 and 1 µg of total RNA in the presence of 9 mers random primer (TaKaRa, Kyoto, Japan). All the procedures were in accordance with the instruction manual.

Cloning of LDH-C splicing variants from human and rat: The specific primers (Table 1) for cloning were designed based on the human *LDH-C* nucleotide sequence (GenBank accession number: BC064388.1) and rat LDH-C nucleotide sequence (GenBank accession number: NM_017266). The primers were used to amplify the entire Open Reading Frame (ORF) of human and rat LDH-C cDNA. To exclude false-positive PCR products because of contaminating genomic DNA in the RNA preparation, all primer sets were evaluated by PCR reactions using either genomic DNA or non-reverse-transcribed RNA as negative controls. The PCR were performed in two rounds (Protocols listed in Table 2) and 0.5 µL PCR products of the first round were taken as the template for the second round. The PCR products were purified by purification kit (TaKaRa) to exclude DNA fragments less than 100 bp and then cloned into pMD18-T vector (TaKaRa) and direct colony PCR method was used to screen plasmids containing splicing variants with different fragment length. All selected clones were sequenced from both strand directions using BcaBEST™ Sequencing Primer RV-M and Sequencing Primer M13-47 (TaKaRa). The sequence data from this study have been deposited in GenBank under accession number HQ386000-HQ386005 (human) and JQ409364-JQ409369 (rats)

Sequence analysis: All the sequences were analyzed using the vector NTI Suite 9 software package (Invitrogen). The splicing variant cDNA sequences were aligned to the mRNA sequence of LDH-C (accession number: human, BC064388.1; rat, NM_017266) containing the full length ORF. The missing or extra sequence were then align to the genomic sequence of LDH-C (accession number: human, NC_000011; rat, NC_005100) to see which exon was skipped or which intron was spliced in.

RT-PCR analysis of LDH-C expression in rat testes: The RT-PCR was performed using primer rps and rpa and the synthesized cDNA as template. In the first round, 5 µL cDNA was taken as the template; in the

Table 1: Primers

	Sense primer	Antisense primer
Human	hps1: 5'-TCTGGTGTCACTTCTGTGCCTTC-3' hps2: 5'-ATGTCAACTGTCAAGGAGCAGCTAA-3'	hpa: 5'-TTCTGTTCTCCAAACAGTGGAACA-3'
Rats	rps: 5'-ATGTCCACTGTCAAGGAGCAGCTAAT-3' ppiaps: 5'-ATACGGGTCTGGCATCT-3'	rpa: 5'-TAATTGCAGATCTTTCTGGATG-3' ppiapa: 5'-CCATCCAACCACTCAGTCTT-3'

Table 2: PCR protocols

	PCR protocols
Human <i>LDH-C</i> variants cloning from testis and spermatozoa	Round 1: 20 cycles×(94°C, 30 s; 55.3 °C, 30 s; 72°C, 1 min) Round 2: 20 cycles×(94°C, 30 s; 55.3 °C, 30 s; 72°C, 1 min), 72°C, 10 min
Rat <i>LDH-C</i> variants cloning from testis	Round 1: 20 cycles×(94°C, 30 s; 49 °C, 30 s; 72°C, 1 min) Round 2: 20 cycles×(94°C, 30 s; 49 °C, 30 s; 72°C, 1 min), 72°C, 10 min
Rat <i>LDH-C</i> variants cloning from heart, muscle and liver	Round 1: 30 cycles×(94°C, 30 s; 49 °C, 30 s; 72°C, 1 min) Round 2: 30 cycles×(94°C, 30 s; 49 °C, 30 s; 72°C, 1 min), 72°C, 10 min
RT-PCR analysis of <i>LDH-C</i> expression in rat testis	Round 1: 20 cycles×(94°C, 30 s; 49 °C, 30 s; 72°C, 1 min) Round 2: 20 cycles×(94°C, 30 s; 49 °C, 30 s; 72°C, 1 min)
RT-PCR analysis of <i>PPIA</i> expression in rat testis	Round 1: 20 cycles×(94°C, 30 s; 49 °C, 10 s; 72°C, 10 s) Round 2: 20 cycles×(94°C, 30 s; 49 °C, 10 s; 72°C, 10 s)

second round, 0.5 µL PCR products of the first round was taken as the template. The PCR protocols were listed in Table 2. After two rounds of PCR, the products were analyzed by agarose electrophoresis. In parallel, the housekeeping gene *PPIA* (peptidylprolyl isomerase A) was amplified and set as loading control. The primers for *PPIA* were *ppiaps* and *ppiapa* (Table 1), the PCR protocol was listed in Table 2, in the first round, 1µL cDNA was taken as the template and in the second round, 0.5 µL PCR products of the first round was taken as the template. After two rounds of PCR, the products were analyzed by agarose electrophoresis.

SDS-PAGE and western blotting analysis: Fresh tissues from SD rat were homogenated in extraction buffer (100mM sodium phosphate pH 7.0, plus 1mM EDTA and 1mM DTT). After centrifugation for 20 min at 15,000 g, the supernatant was used for SDS-PAGE. Five microliter supernatant was loaded with the same volume of loading buffer (contained 0.1% bromophenol blue, 20% glycerol and 4% β-mercaptoethanol). Proteins were separated by 12% SDS-PAGE gel.

After SDS-PAGE proteins were transferred onto PVDF membrane (Millipore) immediately. The membranes were incubated in blocking buffer [TBS with 0.05% Tween-20 (TBS-T) plus 2% BSA] for 1 h at room temperature with gentle shaking. Then the membranes were washed in TBS-T for 5 min and incubated with the primary antibody H-160 (Santa Cruz, California, USA, H-160 were raised against amino acids 173-332 mapping at the C-terminus of LDH-A of human origin and can be used to detect LDH-A, B, C of rat). After several stringency washes, the membranes were incubated for 1 h at RT with the appropriate HRP-conjugated secondary antibody (BA1060, Boster, Wuhan, China). Finally the Amersham ECL kit (GE Healthcare Bio-Sciences Corp.) was used to detect the signal.

RESULTS

Cloning of LDH-C splicing variants in human and rats:

We had checked to make sure that primers were specific for LDH-C without cross-amplification of the related ubiquitously expressed isoenzymes LDH-A and LDH-B. The primers were designed to amplify the entire ORF of LDH-C gene and the expected size of

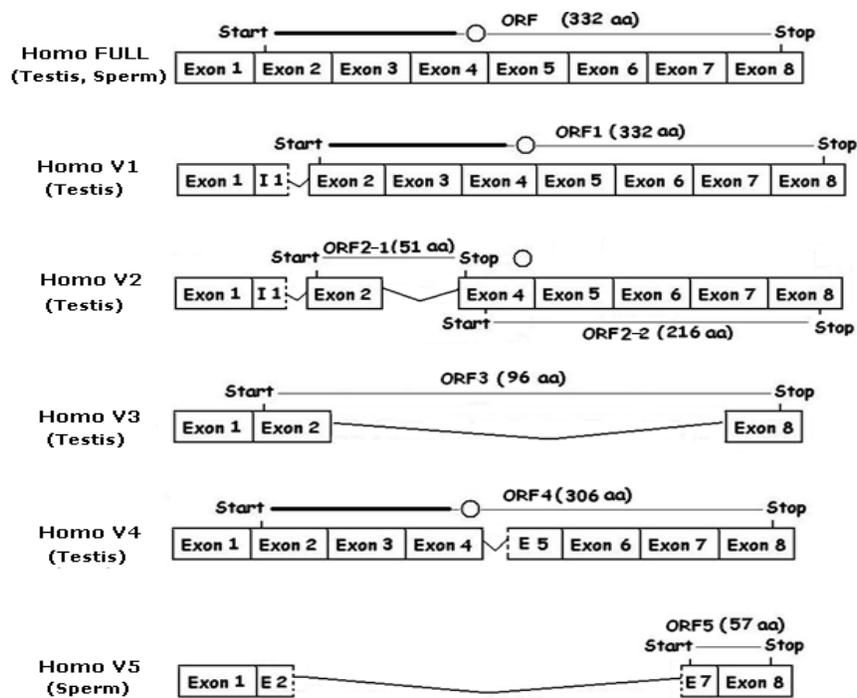
PCR amplification product was about 1 kb (full length ORF) according to human and rat LDH-C gene. Clones that show less or more than 1 kb bands were selected and sent to sequencing. Several hundreds of clones were screened and suspected clones were sequenced. Finally 4 splicing variants in human testes, 1 splicing variant in human spermatozoa, 6 splicing variants in rat testes and 1 splicing variant in rat non-testes tissues (liver, heart and muscle) were cloned (Fig. 1).

Sequence analysis of human LDH-C splicing variants:

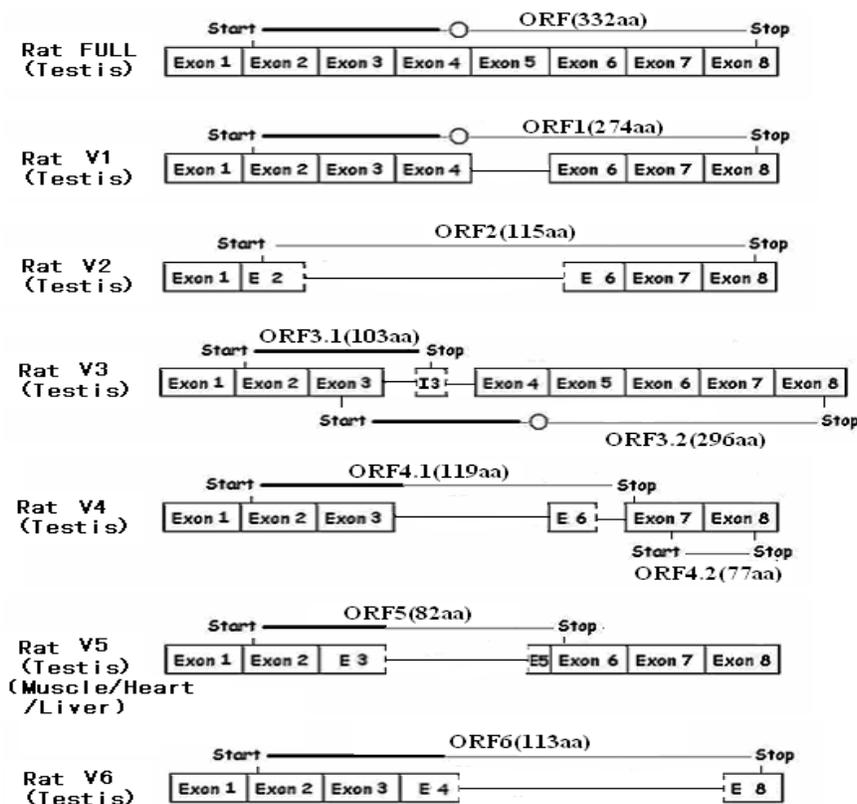
The 5 cloned human splicing variants were missing exons or certain numbers of nucleotides in different manners (Fig. 1a). In detail, variant 1 and the full length cDNA differ in their 5' untranslated region because of alternative partial insertion of intron 1 (54 bp at 5' end), but they give rise to the same protein of 332 amino acids. Variant 2 was inserted by part of intron 1 (54 bp at 5' end) and missing entire exon3 and consequently gave two ORFs, V2-1 and V2-2. V2-1 was deficient in NAD binding domain and lost the entire active site, V2-2 was deficient in the NAD binding domain, but did contain the entire active site. Variant 3 missed the entire exon 3, 4, 5, 6, 7 and lost the entire NAD binding domain and the active site. Variant 4 lacked the first 78 bp of exon 5 but contained both the entire NAD binding domain and the active site. Variant 5 (the variant found in spermatozoa) missed the partial exon 2 (71 bp at 3' end) and 7 (102 bp at 5' end) and the entire exon 3, 4, 5, 6 and lost the entire NAD binding domain and the active site. All variants except for variant 5 use the same start codon as the full length LDH-C mRNA for protein translation. The putative polypeptides (more than 50 amino-acid residues polypeptides were considered) encoded by human LDH-C splicing variants were compared with the full length LDH-C protein, the results showed that most of these putative polypeptides were truncated LDH-C proteins except the polypeptides encoded by ORF1 of variant 2 (V2-1) which was a truncated protein but with 9 amino acid residues at C-terminal different from LDH-C (Fig. 2a).

Sequence analysis of rat LDH-C splicing variants:

The 6 cloned rat splicing variants were missing exons or certain numbers of nucleotides in different manners (Fig. 1b). In detail, variant 1 lacked the entire exon 5

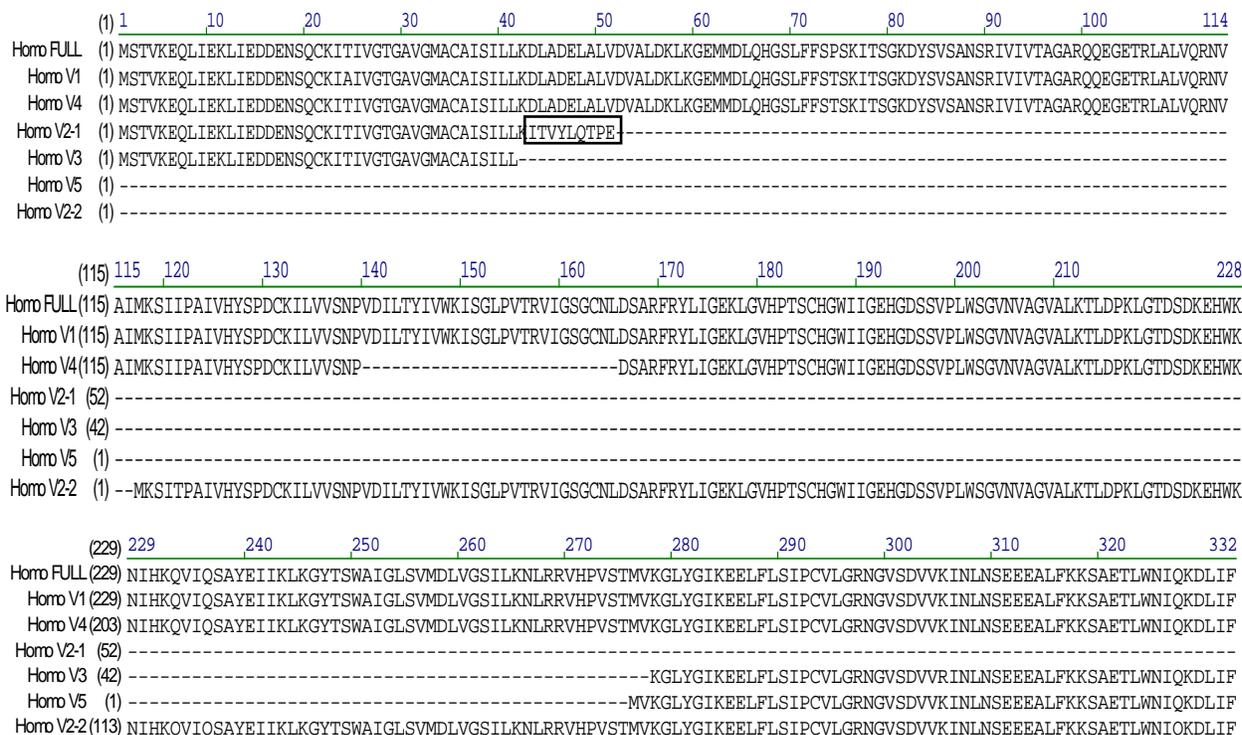


(a) Exon schematics of variants in human

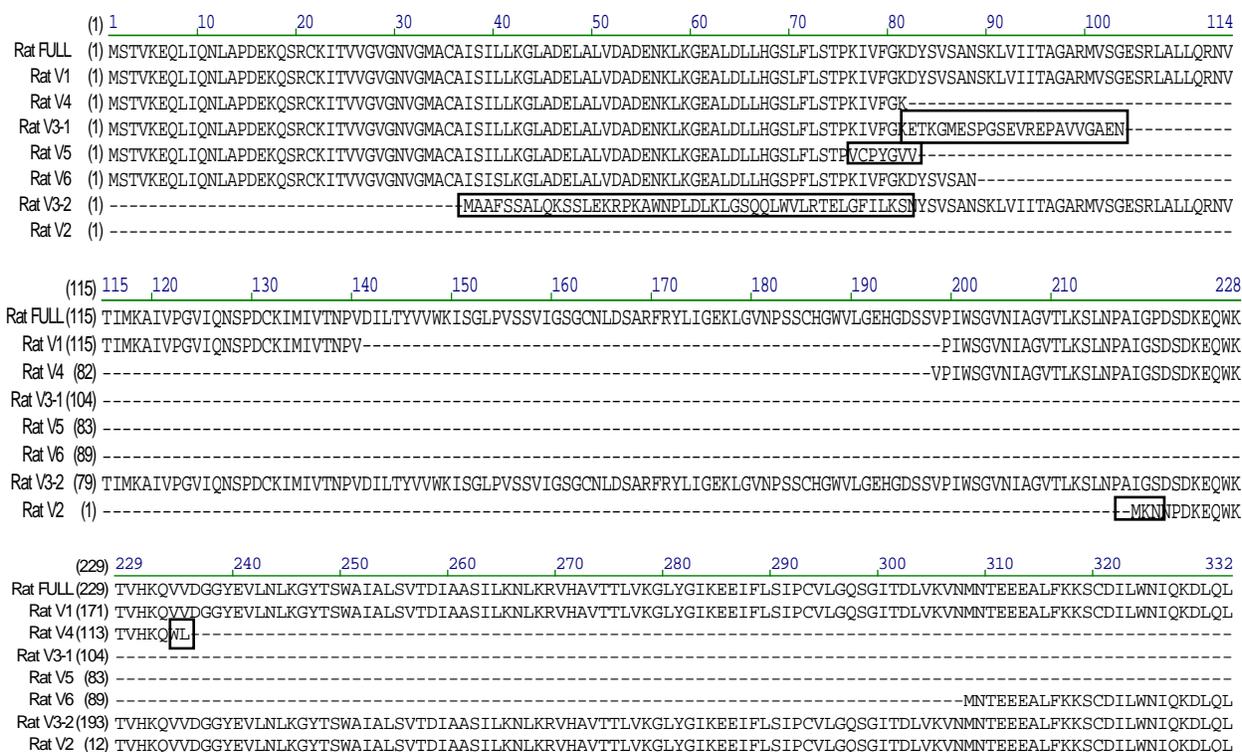


(b) Exon schematics of variants in rat

Fig. 1: Exon Schematics of LDH-C splicing variants detected in human (A) and in rat (B). The ORFs supposed to translate are marked; black bars represent lactate dehydrogenase NAD binding domain and Os represent lactate dehydrogenase active site



(a) Sequence alignments of variants in human



(b) Sequence alignments of variants in rat

Fig. 2: Amino acid sequence alignments of putative polypeptides encoded by splicing variants of human (A) and rat (B). Alignments were set to meet maximum sequences identity, gaps are represented by dashes, and amino acid residues identical at N-terminal or C-terminal were indicated by boxes

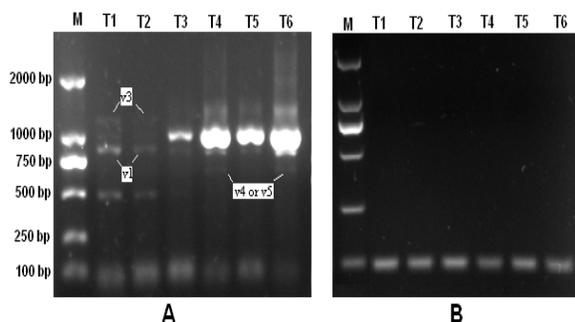


Fig. 3: Expression analysis by RT-PCR during testis development. A, expression profiles of splicing variants of LDH-C; B, expression profiles of PPIA (housekeeping gene); lane M, DNA maker; lane T1 to T6, testes of 1 week rat to 6 week rat

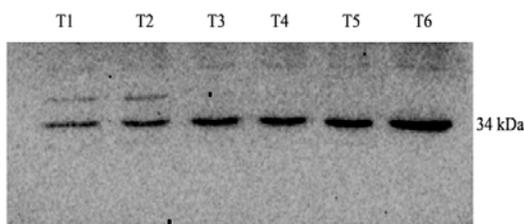


Fig. 4: Western blotting analysis of LDH-C during testis development. The bands about 34 kDa represented LDH. Lane T1 to T6, testes of 1 week rat to 6 week rat

and variant 2 lacked the partial of exon 2 (70 bp at 3' end) and 6 (72 bp at 5' end), the entire exon 3, 4 and 5; variant 3 was inserted by the partial of intron 3 (91 bp in the middle of intron 3); variant 4 lacked the entire exon 4, 5 and the partial of exon 6 (11 bp at 3' end); variant 5 lacked the partial of exon 3 (19 bp at 3' end), the entire exon 4 and kept the last 3 bp (3' end) of exon 5; variant 6 lacked the partial of exon 4 (154 bp at 3' end) and 8 (87 bp at 5' end), the entire exon 5, 6, 7; The missing of exons lead to the changes of protein structure, some variants (2, 3, 4, 5, 7) lost the entire or partial NAD binding domain, some variants (2, 4, 5, 6, 7) lost the active site and some variants (2, 4, 5, 7) lost both of them, only variant 1 contained both of them. The putative polypeptides (more than 50 amino-acid residues polypeptides were considered) encoded by rat splicing variants were compared with the full length LDH-C protein, the results show that some of these putative polypeptides were truncated LDH-C proteins (polypeptides encoded by variants 1,6) and the others were also truncated proteins with a few amino acid residues at N-terminal (polypeptides encoded by variants 3,4,5) or C-terminal (polypeptides encoded by variants 2,3) different from LDH-C(Fig. 2b).

RT-PCR analysis of rat LDH-C gene expression: In order to analysis the expression profile of these variants during testes development, RT-PCR method was

employed. Some of the variants could be detected by primer rps and rpa (Table 1). As shown in Fig. 3a, the bands in 1 kb position (the brightest band in lane T3, T4, T5, T6) reflected the mRNAs with full length ORF. The bands less than 1 kb reflected the variants. From Fig. 3 we can see that the expression of full length mRNA increased with the development of testes, this is in accordance with other reports (Thomas *et al.*, 1990). In 1 and 2 week age testes (T1 & T2), we can hardly see the 1 kb band; instead, there were some other bands less or larger than 1 kb, these bands reflected the splicing variants except the band about 500 bp in lane T1 and T2 (it turns out to be a non-special fragment, not LDH-C variant). In general, the expression levels of some variants are significant during the development of testes (T1 to T6). Figure 3b was the RT-PCR of the housekeeping gene PPIA (peptidylprolyl isomerase A); the amplification fragment was 98 bp which was the expected size.

Western blot analysis: In order to detect if the variants were translated into proteins, western blotting were performed. Because primary antibody H-160 were raised against amino acids 173-332 mapping at the C-terminus of LDH-A, the putative polypeptides beside rat V3-1 and V5 have the possibility be detected. The results were displayed in Fig. 4, the band about 34 kDa was LDH and there were no bands below 34 kDa.

DISCUSSION

Multiple splicing variants of LDH-C in human and rat normal testes were cloned. The results show that LDH-C is not totally silenced by transcriptional repression as previous reported (Tang *et al.*, 2008; Tang and Goldberg, 2009; Kroft *et al.*, 2003) in non-mature germ cells and somatic cells, but transcribed and alternatively spliced. Alternative splicing is the process by which a single pre-mRNA is spliced in different ways to generate multiple mRNA transcripts. It is thought previously that the alternative splicing variants are translated into proteins. In recent years, it becomes clearly that a substantial fraction of these variants are actually targets of NMD (nonsense-mediated mRNA decay). This process represents an important mechanism for post-transcriptional regulation of gene expression (Lejeune and Maquat, 2005; Lewis *et al.*, 2003). In this study, 5 splicing variants of LDH-C were cloned from human testes and spermatozoa and 6 splicing variants of LDH-C were cloned from rat testes and non-testes tissues. The alternative splicing events affect the reading frames and give rise to the new putative ORFs. Most of the new ORFs have the same start codon as the full length ORF of LDH-C make it likely that at least some of these putative ORFs are used for translation leading to the generation of the corresponding polypeptides. However, when the putative polypeptides encoded by human and rat

splicing variants were compared with the corresponding full length LDH-C protein, the results showed that these putative polypeptides were truncated LDH-C proteins or truncated LDH-C protein with a few amino acid residues at N-terminal or C-terminal different from LDH-C (Fig. 2). The results suggest that the variants are possibly not used for translation but probably targets of NMD (Lejeune and Maquat, 2005). Western blot were performed to analyze the putative polypeptides, all the putative polypeptides encoded by the variants were below 34 kDa and however the western blotting didn't detect any bands below 34 kDa. The reasons may be that the sensitivity of western blotting is not enough to detect these putative polypeptides or the variants can not be translated into proteins. These findings also suggest the possibility that RNA alternative splicing might involve in regulation of LDH-C expression in testes development.

From the RT-PCR results of rat testis tissues, we found that the mRNA level of the splicing variants were significant during the development of testes. This may indicate that the transcription repression of LDH-C was loosening in testes. Previous reports showed that *LDH-C* was silenced in non-testes tissues, however we did cloned one splicing variants in heart, muscle and liver of rat, so it is possible that one or several copies of LDH-C pre-mRNA may escape from transcription repression in non-testis tissues. Koslowski *et al.* (2002) reported that multiple splicing variants of *LDH-C* were expressed in significant levels in a broad spectrum of human tumors but not in normal testicular tissue. Surprisingly, our experiments showed that the alternative splicing variants of LDH-C also exist in normal human and rat testes tissues and non-testes tissues (even though their expression level is very low). We did not detect any variants with full length ORF (999 bp) in normal rat non-testes tissues, but Koslowski and his colleagues did detect in human cancers. The expression levels of splicing variants were significant in rat testes. It is interesting that the expression level of splicing variants in cancers were also significant. This may suggest that the transcription repression of LDH-C was loosen in cancers (like in rat testes). One hypothesis for ectopic expression of testis-specific genes in cancer cells (CTAs, cancer/testis-associated genes) is the induction or activation of a gametogenic program in cancer (Old, 2001). Since alternative splicing play an important role in gene regulation, we suggest that focus on RNA splicing of CTAs may provide insight into tumor development.

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