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Laboratory of

Comparative Genomics

A Porcine Neurological Model for Ataxia-telangiectasia

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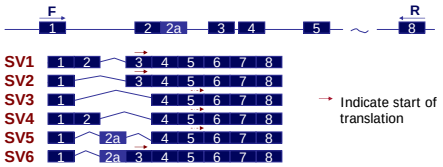
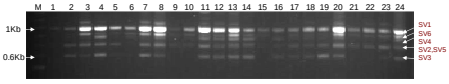
Abstract

Ataxia-telangiectasia (AT) is recessive autosomal disorder characterized by loss of cerebellar function, progressive dysarthria and choreoathetosis. In addition to neurodegeneration the typical signs of AT are immune deficiencies, sterility, cancer predisposition, chromosomal instability and radiation sensitivity. Existing mouse AT models do not reflect the extent of neurological disorders found in human. In order to develop a porcine model for AT, we sequenced ATM transcripts, characterized the 5'-untranslated region (5'UTR), identified splice variants and developed constructs for gene targeting. Similar to humans, the porcine ATM gene exhibits extensive alternative splicing. In contrast with humans it involved not only the 5'UTR but also coding regions. Six splice variants with 3 alternative exons were observed in the 5'UTR, three of them spliced out the first coding exon, altering the translation start and giving rise to the putative protein lacking the N-terminus substrate binding domain. Real time PCR analysis revealed variable levels of ATM expression in 24 different tissues with the impact of the longest splice variant to be not less than 60% of total ATM transcripts in all tissues. The majority of AT patients carry truncating ATM mutations resulting in prematurely terminated proteins that are highly unstable. Thus, to knockout the ATM gene, we developed two gene targeting constructs by introducing truncation mutations shortly after known and putative translation starts, according to splice variants detected. The similarity of the porcine to the human gene in extensive alternative splicing supports our goal of creating a more relevant model for AT than in the mouse.

5'UTR and Splice Variants

PCR amplification of ex1-ex8 cDNA fragments.





M-100bp ladder, 1-skin, 2-fat, 3-adrenal gland, 4-spinal cord, 5-muscles, 6-mandibular lymph node, 7-thyroid, 8-thymus, 9-trachea, 10-stomach, 11-brain, 12-uterus, 13-colon, 14-small intestine, 15-bone morrow, 16-heart, 17-kidney, 18-ovary, 19-bladder , 20-lung, 21-spleen, 22- testes, 23- mesenteric lymph node, 24-liver.

H. sapience MSLVLNDLLICCRQLEHDRATERKKEVEKFKRLIRDPETIKHLDRHSDSK
S. Scrota MSLALNDLLICCRQLEHDRATERRKAVENFRHLIQDPETVQHLDQHSDSK
M. musculus MSLALNDLLICCRQLEHDRATERRKEVDKFKRLIQDPETVQHLDRHSDSK

p53, BRCA1 and BLM substrate binding domain

51 100

H. sapience QGKYLNWDAVFRFLQKYIQKETECLRIAKPNVSASTQASRQKKMQEISSL
S. Scrota QGKYLNWDAAFRFLQKYIQKETECLRTAKQNVSASTQATRQKKMQEISSL
M. musculus QGKYLNWDAVFRFLQKYIQKEMESLRTAKSNVSATTQSSRQKKMQEISSL

Alternative translation starts

101 150

H. sapience VKYFIKCANRRAPRLKCQELLNYIMDTVKDSSNGAIYGADCSNILLKDIL
S. Scrota VKYFIKCANKRAPRLKCQELLNYIMDTVRDSSNNPIYGADYSNILLKDIL
M. musculus VRYFIKCANKRAPRLKCQDLLNYVMDTVKDSSNGLTYGADCSNILLKDIL

We characterized porcine ATM mRNAs (pATM). Sequence analysis of pATM cDNAs showed high homology with the human ATM (hATM) in coding regions and particularly in functional domains.

While no alternative splicing was reported within the coding region of hATM, we detected alternative splicing of exons 18, 19, 21, 22 and 25, 26 in pATM transcripts.

Six different 5' UTRs were identified as result of extensive alternative splicing of the first three exons. They varied in size, had multiply ATG codons and different secondary structure, reflecting the possibility for complex posttranscriptional regulation.

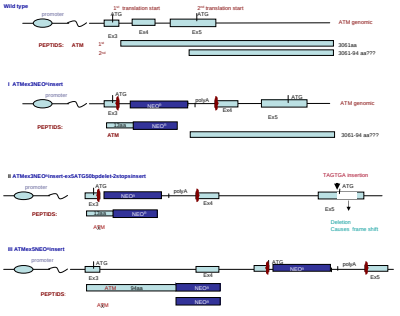
In contrast with the 12 known hATM 5'UTRs, splicing in the pig involves not only untranslated region but also the beginning of the coding region: three transcripts spliced out the first coding exon, altering the translation start and giving rise to a putative shorter protein.

The similarity of N-terminal region of mouse, human and pig suggests that only the pig transcripts that carry the same ('normal') translation start (NST) would be translated to a peptide functionally similar to mouse and human ATM. Although shorter peptides could be translated from several in frame downstream ATG codons, the possibility for translation from these alternative start sites (AST) is questionable due to the leaking translation from multiple upstream ATGs and non optimal Kozak sequence around alternative translation starts.

Even if translation occurs such peptide would lack one of the ATM key function domains, the N-terminus substrate binding domain (82-89aa) that is involved in activation of p53, BRCA1 and BLM pathways.

Targeted Vector Construction

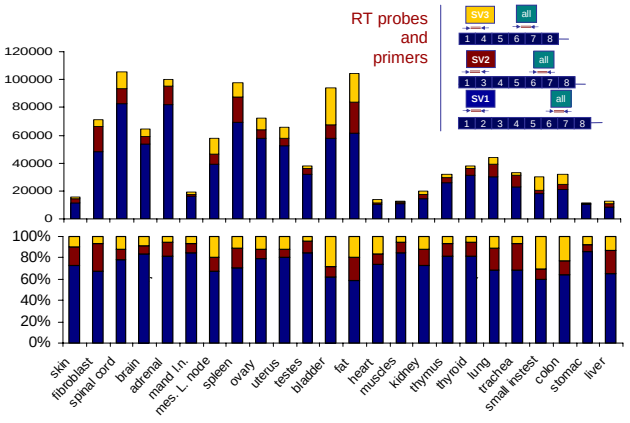
To knock-out the ATM gene by introducing truncation mutation shortly after the start codon, we developed two gene targeting constructs for both known and putative translation start sites, according to splice variants detected. Targeted vectors were constructed using a recombineering technique to target NTS site and potential ATS site, the next downstream Met codon in the main translation frame. To target 'normal' translation site Neomicin (NEO) gene was inserted in-frame into the ATM gene 40bp downstream of NTS. NEO is expected to be expressed from the ATM promoter as fusion protein with a short ATM leader sequence.To target the ATS site, NEO gene is inserted into ATS codon. Thus an expression of NEO is expected from the ATM promoter again as fusion protein or as just NEO itself depend on splicing variant transcript. NEO expression would provide drug selection for targeted porcine fibroblast. Nuclear transfer will be following the cloning and production of knock-out pigs.



AT in Human and Model Systems

The ATM gene has 66 exons spread over 150 kb of genomic DNA and produces a 13kb transcript. The protein of 3056 amino acids has a highly conserved PI-3 kinase domain, FAT, ATP-binding and N-terminus substrate binding domains. The majority of ATM gene mutations in AT patients are truncating mutations resulting in the prematurely terminated proteins that are highly unstable. The ATM is well described as a potent regulator of cell-cycle checkpoints that mediate the DNA damage response. It functions as a protein kinase that is activated by DNA damage to phosphorylate target substrates, such as p53, Chk2, Mdm2, NBS1, BRCA1, 53BP1 Smc1, FANC2, H2AX and Pin2/TRF1, that in turn control cell cycle check points, the DNA double-strand break repair pathway, and telomere metabolism. Phenotypically, patients with homozygous or compound heterozygous mutations display cerebellar degeneration, immunodeficiency, cancer predisposition, gonadal atrophy, growth retardation, and premature aging. Chromosomes of AT patients have short telomeres, and are also highly sensitive to ionizing radiation. ATM deficient mice have a variety of growth defects, meiotic defects, immunological abnormalities, radiation hypersensitivity and cancer predisposition, similar to those seen in AT patients, confirming the most common pleiotropic roles of ATM. In contrast neurological abnormalities seen in knock-out mice do not reflect the extent of human neurological abnormalities, calling for better model of human AT. *Sus scrofa* has been recognized as a powerful model for medical studies and provides insights into various human diseases. To develop a porcine AT model, we characterized the porcine analog of ATM mRNA and its splicing variants in different tissues. Alternatively spliced 5'UTRs of the pATM gene resemble complex translation regulation observed in humans and could impact in differences observed in mouse and human onset of AT.

Tissue Expression Profile



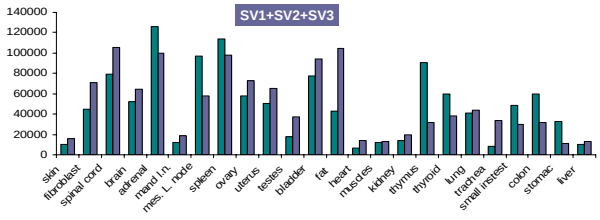
The three splice variants were ubiquitously expressed at variable levels in all tissues monitored.

SV1 is the most abundant variant and represents more then 60 % of total ATM transcripts in each tissue.

Higher total ATM expression is detected in tissues with higher basic proliferation as in tissues of reproductive and endocrine systems and in the organs of nervous system.

The estimate of total amount of ATM transcripts in tissues

all The probe for exon 6-7 junction, present in all studied splice variants.



Both SV5 and SV6 transcripts were not detected by SV1, SV2 and SV3 probes, but hybridized with 'all' probe and contributed to the difference between 2 estimates of the total amount of ATM transcripts.

Conclusions

- Six 5' UTRs of pATM were identified as result of extensive alternative splicing of the first three exons.
- Three of six transcript variants spliced out the first coding exon, altering the translation start and giving rise to a putative shorter protein.
- The similarity of N-terminal region of mouse, human and pig suggests that only those pATM transcripts that carry the 'normal' translation start would be functional. An N-terminal truncated shorter peptide is likely to be not translated at all, since it resembles N-terminal truncation mutations in AT patients who do not show any ATM protein expression. Even if it is translated, it is likely to be nonfunctional, since human N-terminal deletion mutants missing substrate binding domain fail to activate p53 pathway in AT cells exposed to bleomycin.
- pATM mRNA is ubiquitously expressed in all organs studied and has the highest impact from the SV1.
- Vector plasmids were constructed using the recombineering technique to target two translation sites.

Acknowledgements

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