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Identification of a novel circularized transcript of the *AML1* gene

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The *AML1* gene is an essential transcription factor regulating the differentiation of hematopoietic stem cells into mature blood cells. Though at least 12 different alternatively spliced *AML1* mRNAs are generated, three splice variants (*AML1*a, *AML1*b and *AML1*c) have been characterized. Here, using the reverse transcription-polymerase chain reaction with outward-facing primers, we identified a novel non-polyadenylated transcript from the *AML1* gene, with exons 5 and 6 scrambled. The novel transcript resisted RNase R digestion, indicating it is a circular RNA structure that may originate from products of mRNA alternative splicing. The expression of the novel transcript in different cells or cell lines of human and a number of other species matched those of the canonical transcripts. The discovery provides additional evidence that circular RNA could stably exist *in vivo* in human, and may also help to understand the mechanism of the regulation of the *AML1* gene transcription. [BMB Reports 2013; 46(3): 163-168]

INTRODUCTION

The Runt-related transcription factor 1 gene (*RUNX-1* alias *AML1*), located on chromosome 21q22, consists of 12 exons and spans 261 kb of genomic DNA. It plays an important role in establishing hematopoiesis and the generation of hematopoietic stem cells in the embryo (1). Transcription of *AML1* is initiated at two distinct 5′ regions, a distal region-promoter-1 (P1) and a proximal region-promoter-2 (P2). The two promoters generate at least 12 alternatively spliced mRNAs, which differ within the 5′ and 3′ untranslated regions (UTR) and coding regions. Among these variants, the predominant transcripts *AML1*b and *AML1*c code for a 453-amino acid protein and a 480-amino acid protein, respectively, while the minor *AML1*a codes for a protein, which has a higher affinity for DNA binding and suppresses the transcriptional activation of *AML1*b. To date, 55 partner chromosome bands and 21 translocations have been described at the molecular level, which are considered as contributors to leukemia, especially acute myeloid leukemia (AML), using different pathways (1).

In our previous study, we used a pair of outward-facing primers to identify a novel partner gene of *AML1* in an acute myeloid leukemia patient. We randomly selected 3 healthy individuals as negative control, and to our surprise, three unexpected bands of the same size could be amplified in all samples. By sequencing, we accidentally discovered the sequence of the exons was arranged in a different order compared to the genomic DNA, which consisted of a 5-6-5 exon alignment. These findings raise several fundamental questions: Where does it originate from? What does its whole structure look like?

In this study, we have presented evidence from RT-PCR using outward-facing primers that further examine its presence. Also, through several experiments, we investigated its structure. Finally, the expression of this transcript in a number of human cell types, and several other species was studied.

RESULTS

AML1 produces the abnormal transcript with the 5-6-5 exon alignment

To examine the abnormal transcript consisting of the 5-6-5 exon alignment, we performed PCR with total RNA from bone marrow or peripheral blood of 10 AML patients and 10 healthy individuals using a pair of outward-facing primers (oF/oR) from exon 5 (as in GenBank D43969). Fragments were observed in all samples at approximately 305 bp, and absent in the negative and blank controls. It was noteworthy that the 305 bp fragment contained an abnormal exon 5 following the original exons 5 and 6 (Fig. 1A).

To further examine whether the product with the abnormal exon order was an artifact, we used additional pairs of primers (6F/5R2 and 5F2/5R1). Also, primers F65 and R65 were designed to cover the boundary of the exons 6-5 and these were used in further PCRs with the primer pairs F65/5R3 and 5F1/R65. We confirmed that these amplified products contained the expected sequences (Fig. 1B).

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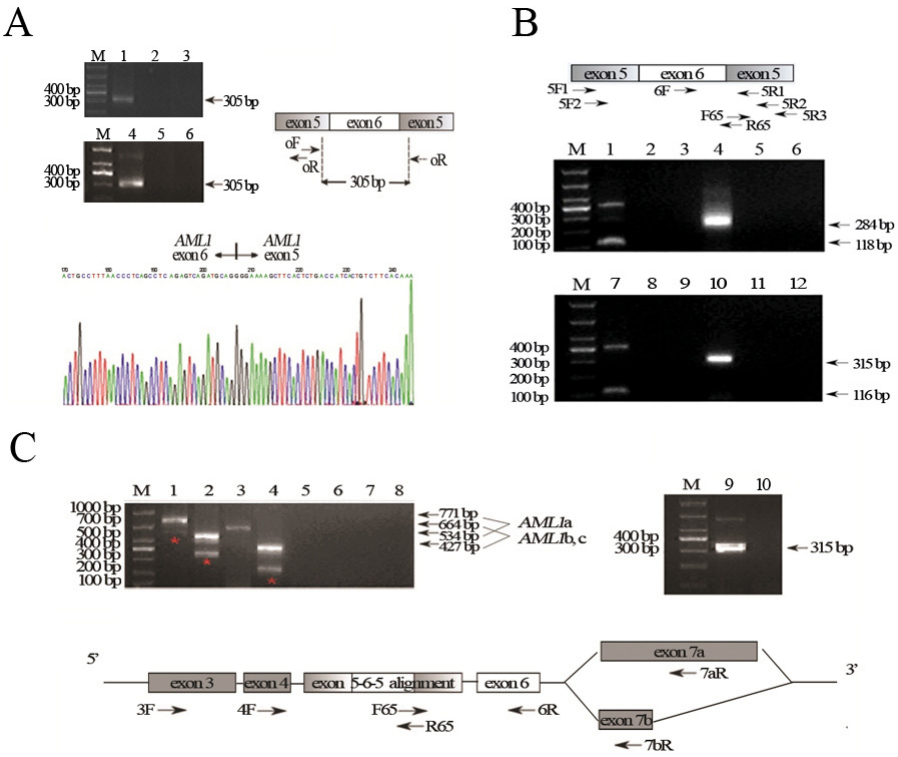

Fig. 1. Analysis of RT-PCR results. (A) Detection of the novel *AML1* transcript with the exon 5-6-5 alignment using RT-PCR and sequence analysis. Lanes 1 and 4 show the amplification products of the novel transcript in an AML patient and a healthy individual respectively, Lanes 2 and 5 are negative control, and Lanes 3 and 6 are blank control. The band above 305 bp in Lane 4 is nonspecific amplification product. The positions and directions of outward-facing primers (oF and oR) in the cDNA structure are indicated by arrows. The sizes of the expected fragment and the junction sequence of the 3′ end of exon 6 and 5′ end of exon 5 are also shown. (B) Detection of exon 5-6-5 alignment using different pairs of primers. Positions and directions of different pairs in the cDNA structure are indicated by arrows. The amplification products of primers 6F/5R2, 5F2/5R1, F65/5R3 and 5F1/R65 are shown. By sequencing, the bands of 118 bp and 284 bp in Lanes 1 and 4 and the bands of 116 bp and 315 bp in Lanes 7 and 10 respectively are the products, Lanes 2, 5, 8 and 11 are negative controls, Lanes 3, 6, 9 and 12 are blank controls. (C) Analysis of RT-PCR results and schematic diagram of the positions and directions of primers used to seek the neighboring exons of the exon 5-6-5 alignment. Lanes 1 to 4 are products of RT-PCR using 3F/7aR, 3F/7bR, 4F/7aR and 4F/7bR, respectively. Asterisks show the canonical transcripts without exon 6. Lanes 5 to 8 are products of 3F/R65, 4F/R65, F65/7aR and F65/7bR, respectively. Lane 9 shows the amplification product of F65/6R, Lane 10 is negative control.

Identification of the neighboring sequences upstream and downstream of the 5-6-5 exon alignment

To explore whether the novel transcript possesses the same neighboring sequences upstream and downstream as the canonical sequences, PCR was performed using a further set of primers. The fragment could be amplified using primers F65/6R (Fig. 1C), while the expected band was not present using the primers 3F/R65, 4F/R65, F65/7aR and F65/7bR. The canonical transcripts *AML1*a and *AML1*b were successfully amplified using 3F/7aR, 3F/7bR, 4F/7aR and 4F/7bR (Fig. 1C). Hence, we concluded that the 5-6-5 alignment was followed with an exon 6, but it did not contain exons 3 or 4 upstream or exons 7a or 7b downstream.

Poly(A) tail is absent in the exons 5 and 6 scrambled *AML1* molecule

We performed Rapid Amplification of cDNA Ends (RACE) to obtain either the 3′ or 5′ end of the novel transcript, but were unable to form any fragments (data not shown). To investigate whether a polyadenylated tail was present in this scrambled exon alignment, total RNA was reverse-transcribed with either random primers or oligo(dT) primers. PCR was then performed using the primers shown in Table 1. Only the random primers, and not the oligo(dT) primers, produced the scrambled transcript (Fig. 2A). Also, poly(A)+ and poly(A)- RNA was prepared from total RNA, and the quantity of poly(A)+ and poly(A)- RNA were equal when reverse transcribed using random primers. Canonical transcripts were amplified to testify the purity of the three samples using the primers 4F/5R1. As a

result of amplification using the different pairs of primers shown in Table 1, the expected bands were present in the total RNA and poly(A)- RNA Lanes while absent in the poly(A)+ Lane (Fig. 2B).

The novel *AML1* transcript is probably circular

As the transcript lacked the poly(A) tail, we used the One Step PrimeScript® miRNA cDNA Synthesis Kit (Takara, Catalog No. D350A) to add a segment of polyadenylate to it when it was reverse transcribed, allowing its 3′ end to be amplified using our special forward primer and the Uni-miR qPCR Primer which is reverse to the adaptor sequence supplied in the kit. We were unable to add a tail to the novel transcript, while the reference gene U6 formed the expected mono peak after real-time PCR and the expected band on an agarose gel after conventional PCR (data not shown).

To further testify the conformation of the non-canonical transcript, we determined its sensitivity to the RNA exonuclease R (RNAse R), which specifically digests linear RNA, both structured and non-structured, but does not digest circle or lariat RNA (2, 3). We performed real-time PCR to detect the change of its expression after RNase R digestion using cDNAs of five healthy individuals. The results showed a significant increase in the circular transcript. The result of calculation using the equation $2^{-\Delta Ct}$ showed there was on average a 282-fold increase (Table 2), confirming its circular nature.

The expression of the novel transcript in different cells or cell lines of human and several other species matched those of the canonical transcripts

To determine the ubiquity of the novel transcript, we employed RT-PCR using cDNAs from different cells or cell lines of human and several other species (Fig. 2C). It turned out that the novel circular transcript of *AML1* existed not in lower organisms or plant, but in higher animals we selected.

Table 1. Nucleotide sequence of primers

Primer	Sequence
3F	5′-TGCCCATCGCTTTCAAGGTG-3′
4F	5′-ACCGCAGCCATGAAGAACCAGGTTG-3′
5F1	5′-GGAAAAGCTTCACTCTGACCATC-3′
5F2	5′-CGCCACCTACCACAGAGCCATCA-3′
oF[a]	5′-GTCTTCACAAACCCACCG-3′
oR[a]	5′-GTGAAGACAGTGATGGTCAG-3′
5R	5′-GATGGCTCTGTGGTAGGTGGCGACT-3′
6F	5′-AACCACTCCACTGCCTTTAAC-3′
6R1	5′-CAGTGGAGTGGTTCAGGGAG-3′
6R2	5′-CTGCATCTGACTCTGAGGCTG-3′
F65	5′-CTCAGAGTCAGATGCAGGGGA-3′
R65	5′-GTCAGAGTGAAGCTTTTCCCCT-3′
7aR	5′-ATGCTTCCCATGTGCTGTGC-3′
7bR	5′-CAGGTATTGGTAGGACTGATCGTAG-3′

[a]Primers oF and oR are designed outward facing.

DISCUSSION

In this manuscript, we have successfully identified a novel *AML1* transcript containing the donor site of exon 6 linked to the acceptor site of exon 5. Our investigations have gained an insight into this novel transcript, characterizing its sequence and conjecturing on its structure. The observation of the novel transcript prompted experimental analysis of several possible interpretations of this phenomenon. Originally, this transcript was thought to arise from either the expression of a duplicate exon 5 to exon 6 genomic segment or an intermolecular splicing (trans-splicing) of two *AML1* pre-mRNAs. When reverse

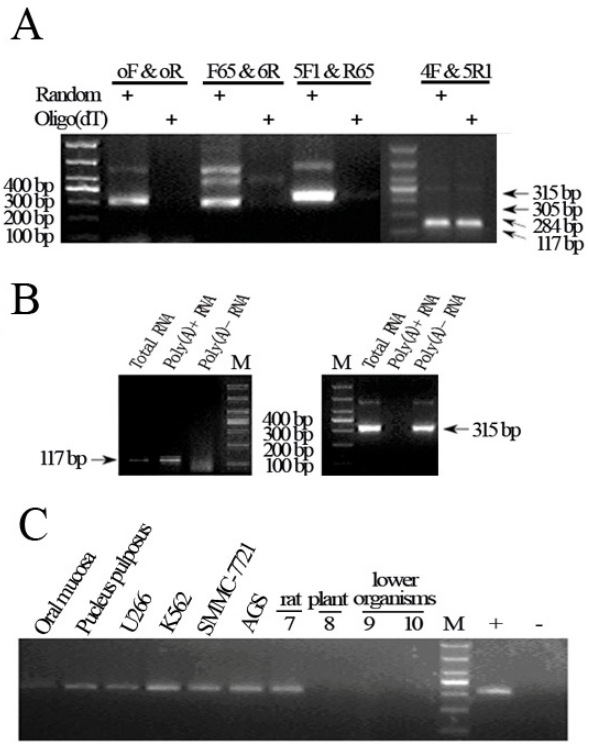

Fig. 2. Observation of poly(A) tail in the novel transcript and expression of the novel transcript in human normal cells, cancer cell lines and several other species. (A) RT-PCR results. 1 μg total RNA was subjected to reverse transcription using either random primers or oligo(dT) primer and amplified by PCR using three pairs of primers oF/oR, F65/6R, and 5F1/R65. The canonical transcript was amplified using 4F/5R1. (B) RT-PCR results of total RNA, poly(A)+ RNA and poly(A)- RNA. Canonical transcripts were amplified using primers 4F/5R1 (shown on the left). Results of PCR amplifying the novel transcript are shown on the right. (C) Lines 1 to 2 respectively represent the amplification product of Oral mucosa and nucleus pulposus cells of an intervertebral disc using primers 5F1/R65; Lines 3 to 7 respectively represent U266, K562, SMMC-7721, AGS and rat NRK52E cells; Line 8 represents tetrahymena; Lines 9 to 10 respectively represent Euplotes elegans and Tartary buckwheat. The positive control represents cDNA from bone marrow of healthy individuals, followed by the negative control.

Table 2. The enrichment of the novel transcript after RNase R digestion

Sample	CT value				$2^{-\Delta t}$	Mean ± standard deviation
	R+[a]		R-[a]			
	Gene	ABL1	Gene	ABL1		
1	19.1111	18.1087	27.3767	17.1736	588.4190	282.6934 ± 207.8469
2	29.3284	20.1606	35.2505	19.0501	130.9253	
3	20.336	19.6205	26.3639	18.8059	114.7619	
4	24.9551	21.1963	32.4336	20.4433	300.5581	
5	20.9352	19.1885	28.8849	19.0151	278.8026	

[a]R+ and R- represent the groups with or without RNase R digestion respectively.

transcriptase was omitted from the protocol, the product was not detected, as shown in Lane 2 in Fig. 1A, indicating that the amplified products did not result from contamination of the genomic DNA. We also excluded the possibility that it was produced by the self-amplification of a single primer (data not shown). Additionally, the novel transcript was non-polyadenylated, and resisted RNase R digestion, a 3′-5′ exoribonuclease that digests linear RNA. These results indicated that the novel *AML1* transcript with scrambled exons was a stable, naturally occurring, circular RNA species, as previously reported for other human genes. (2, 4-13).

Miyoshi *et al*. (4) identified three authentic transcripts of *AML1* using Northern blotting, but did not identify any circular forms. Our group also performed Northern blotting and we were also unable to detect the circular transcript (data not shown). The following two explanations may account for the negative result of the Northern blotting: (i) the content of the circular transcript is indeed insufficient to be detected by Northern blotting; (ii) circular RNA possesses unique secondary structures to give it stability in the cytoplasm, leading to an obstacle in detection via probe labeling. At present, the only techniques to identify that a molecule is circular RNA, are the results of RT-PCR using outward-facing primers and RNase R digestion. If a molecule is linear, it could not exhibit enrichment after RNase R digestion and the value of $2^{-\Delta Ct}$ is approximately 1. However, by detecting the change of the novel *AML1* transcript after RNase R treatment in five healthy individuals using SYBR Green based real-time PCR, we observed that there was approximately a 282-fold increase on average (Table 2).

Circular RNA, first reported in Viroids (14), has a particular structure where the 3′ donor splice site is joined precisely to the incongruous 5′ acceptor splice site. This type of structure did not attract great attention from researchers until circular transcripts were identified from the tumor suppressor gene, *DCC*, in rodent and human cell lines (5). Eukaryotic circular RNA was then reported in the human *ets-1* gene (6, 7) and subsequently in the mouse testis-determining gene, *sry* (8), the rat cytochrome P-450 2C24 gene (9), the human cytochrome P-450 2C18 gene (10), the Na/Ca exchanger gene 1 (12), the dystrophin gene (11, 13) and the *INK4/ARF*-associated noncoding RNA (2). Noteworthily, recently Salzman *et al*., by means of RNA-seq, has found circular transcripts of hundreds of human genes in diverse cell types (15). Here, we report a novel circularized transcript from the *AML1* gene, and provide additional evidence that circular RNA exists in humans.

Hitherto, it was reported that circular RNA may consist of several contiguous exons (2, 9-11), exons not in succession or only a single exon (8, 10, 12, 13). The intriguing mechanism of circularization of scrambled exons was inferred to be a spliceosome-mediated alternative splicing event. It seems reasonable to suppose that they may simply represent errors during the normal splicing process, namely mis-splicing (7), or lariat splicing and inverse splicing (9), or trans-splicing (10), though *in vivo*-generated trans-spliced molecules are always polyadenylated as are canonical mRNAs (16, 17). Exon skipping was considered to be associated with the formation of circular transcripts (10), by using 'molecular patches' to mask the skipped exon. We propose that there exists a particular structure which favors RNA circularization, a nuclease that recognizes this structure and a ligase that bridges the splice sites. It might be similar to the process of the generation of microRNA, which possess a hairpin structure, in which inverted repeat transcripts are precisely cleaved by RNase III enzyme(s) in the Dicer and/or Drosha protein families (18).

What does the circular species exist for? Is it trash or treasure? Since circular transcripts are reported from different genes, and are stable in the cytoplasm (1, 7, 8, 10), we support the hypothesis that they have a biological function beyond the splicing process. Chen and Sarnow (19) have demonstrated that if circular transcripts contain an internal ribosome entry site (IRES) element, they can be translated into proteins. Moreover, the circular *NCX1* exon 2 transcript was deduced to encode a truncated 602-amino acid protein (12), though the efficiency of translation might be affected by its circular structure as well as by the lack of a cap or poly(A) tail (20, 21). However, the majority of circular transcripts, like the circular *AML1* transcript, does not possess internal initiation sites for translation and probably do not code for a protein.

The non-coding characteristic of the circular RNA species are reminiscent of the group of non-coding RNAs, which are also products of the splicing process and challenge the central dogma. Recent studies indicate that members of this non-coding RNA family are far more abundant and important than considered initially (22). What is the relationship between circular RNAs and non-coding RNAs? Could we suppose that these circular RNA transcripts belong to the non-coding RNA species? Since the discovery of microRNA and the reporting of their meaningful function, more attention has been paid to these non-canonical mRNAs.

Whether the circular transcripts encode protein or not, study on their biological function has been going through a bottleneck period. To date, due to the limited approach and technology, there is no way to figure out what physiological or pathological functions circular RNA may have. Therefore, it still requires us to seek new approach or develop new technology to further disclose the mysterious mask of these circular transcripts in the future.

In summary, we identified a novel transcript from the *AML1* gene, which is likely to be a circular RNA molecule. We concluded that the novel circular transcript of AML1 is just consisted of exon5 and exon6. In other words, in the novel transcript exon5 and exon6 were joint head to tail. Therefore, we inferred its full length is length of exon5 plus that of exon6, that is 297 bp. The novel transcript was identified in different cells or cell lines of human and rat cells we selected, but not in lower organisms. The circular RNA transcripts may suggest a novel RNA species, which may help to integrate the genomic context. But this still leads to the debate about their unknown biological role. They should attract our attention, just like the non-coding RNA. We believe there is likely to be circular RNA transcripts of other genes, though there is yet no direct method to detect them. Moreover, the specific mechanism of their formation also needs to be elucidated.

MATERIALS AND METHODS

Sample preparation

Bone marrow samples were from healthy individuals and AML patients, who were diagnosed using the MICM classification standard. Oral mucosa and nucleus pulposus cells of an intervertebral disc were also obtained from healthy individuals. All samples were collected with informed consent from the subjects. The patients' IRB number is NO. 2012057 from Shanxi Medical University Medical Ethics Committee.

U266, K562, SMMC-7721, AGS and NRK52E cells were deposited in our laboratory, and cDNAs of tetrahymena, Euplotes elegans and Tartary buckwheat were given by Dr. Shen Quan.

Preparation of total RNA, poly(A)+ and poly(A)-RNA

Total RNA was extracted using RNAiso Plus (Takara, Catalog#-D9108A) according to the instruction of manufacturer. The mRNA was isolated from total RNA using PolyATtract® mRNA Isolation Systems III (Promega, Cat#Z5300), and the poly(A)-RNA was recycled from the supernatant by ethanol precipitation. Purity and concentration were detected by NanoDrop 2000 (Thermo, USA) before being stored at −80°C.

Reverse transcription-polymerase chain reaction (RT-PCR)

RNA (1 μg) was *in vitro* reverse-transcribed into cDNA with reverse transcriptase M-MLV (RNase H-) (Takara, Tokyo, Japan, Cat#D2640A) using random primers (Promega, Madison, MI, USA) and an oligo(dT) primer (Takara, Cat#D511), respectively. As a negative control, cDNA prepared without reverse transcriptase was employed.

The PCR was performed with a set of outward-facing primers designed to bind in exon 5, oF and oR. Amplification conditions were as follows: denaturation at 94°C for 3 min; 35 cycles of denaturation at 94°C for 30 s, annealing at 53°C for 30 s, and extension at 72°C for 30 s; plus a final extension step at 72°C for 10 min. PCR was also employed with other sets of primers, using 60°C as the annealing temperature. The primers were designed by Primer 5.0 (Table 1).

Each 25 μl PCR reaction contained 1 μl cDNA template, 2.5 μl of 10× Taq buffer, 1.5 μl of 25 mM $MgCl_2$, 0.5 μl of 10 mM dNTPs, 1.5 U of Taq DNA polymerase (Fermentas, Cat#EP0282) and 1 μmol/L of forward and reverse primers. Aliquots (10 μl) of the amplified PCR products were loaded onto 2% agarose gels for electrophoresis and visualization with ethidium bromide. The band was cut from the gel, purified using the Gel Extraction Kit (Omega, Cat#D2500-01), and sequenced by the Beijing Aucg Biotechnology Company.

RNase R digestion

RNA (20 μg) was incubated with or without 40 U of RNase R (Epicentre Biotechnologies, Cat# RNR07250) for 2 h at 37°C. The resulting RNA was purified by ethanol precipitation and quantified. Equal amounts of RNA with or without RNase R treatment were subjected to reverse transcription using random primers. Expression of the novel transcript was detected using SYBR Green-based real-time PCR, which was performed using the 7300 Real-Time PCR System (Applied Biosystems, USA) with SYBR Green I detection and Tm analysis. SYBR® Premix Ex Taq™ II (perfect real time) kit (Cat#DRR081A) was purchased from Takara. The optimized reaction was performed in a 25 μl final reaction volume containing 12.5 μl of kit-supplied SYBR® Premix Ex Taq™ II, 1 μl ROX Reference Dye (50×), 0.5 μl of both the forward and reverse primers (each 10 μM), 2 μl cDNA template, and 8.5 μl distilled water giving a final volume of 25 μl. Amplification conditions were as follows: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, 60°C for 31 s with a dissociation step. The equation $2^{-\Delta\Omega}$ was used to measure the change of the novel transcript expression after RNase R digestion.

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REFERENCES

1. De Braekeleer, E., Douet-Guilbert, N., Morel, F., Bris, M., Ferec, C. and De Braekeleer, M. (2011) RUNX1 translocations and fusion genes in malignant hemopathies. *Future Oncology*. **7**, 77-91.
2. Burd, C. E., Jeck, W. R., Liu, Y., Sanoff, H. K., Wang, Z. and Sharpless, N. E. (2010) Expression of linear and novel circular forms of an INK4/ARF-Associated Non-Coding RNA correlates with atherosclerosis risk. *PLoS Genet*. **6**, e1001233.
3. Suzuki, H., Zuo, Y., Wang, J,, Zhang, M. Q., Malhotra, A. and Mayeda, A. (2006) Characterization of RNase R-digested cellular RNA source that consists of lariat and circular RNAs from pre-mRNA splicing. *Nucleic Acids Res*. **34**, e63.
4. Miyoshi, H., Ohira, M., Shimizu, K., Mitanil, K., Hirai, H., and Imai, T. (1995) Alternative splicing and genomic structure of the AML1 gene involved in acute myeloid leukemia. *Nucleic Acids Res*. **23**, 2762-2769.
5. Nigro, J., Cho, K., Fearon, E., Kern, S., Ruppert, J. and Oliner, J. (1991) Scrambled exons. *Cell* **64**, 607-613.
6. Cocquerelle, C., Daubersies, P., Majerus, M. A., Kerckaert, J. P. and Bailleul, B. (1992) Splicing with inverted order of exons occurs proximal to large introns. *EMBO J*. **11**, 1095-1098.
7. Cocquerelle, C., Mascrez, B., Hetuin, D. and Bailleul, B. (1993) Mis-splicing yields circular RNA molecules. *FASEB J*. **7**, 155-160.
8. Capel, B., Swain, A., Nicolis, S., Hacker, A., Walter, M. and Koopman, P. (1993) Circular transcripts of the testis-determining gene Sry in adult mouse testis. *Cell* **73**, 1019-1030.
9. Zaphiropoulos, P. G. (1996) Circular RNAs from transcripts of the rat cytochrome P450 2C24 gene: correlation with exon skipping. Proc. *Natl. Acad. Sci*. **93**, 6536-6541.
10. Zaphiropoulos, P. G. (1997) Exon skipping and circular RNA formation in transcripts of the human cytochrome P-450 2C18 gene in epidermis and of the rat androgen binding protein gene in testis. *Mol. Cell. Biol*. **17**, 2985-2993.
11. Surono, A., Takeshima, Y., Wibawa, T., Ikezawa, M., Nonaka, I. and Matsuo, M. (1999) Circular dystrophin RNAs consisting of exons that were skipped by alternative splicing. *Hum. Mol. Genet*. **8**, 493-500.
12. Gualandi, F., Trabanelli, C., Rimessi, P., Calzolari, E., Toffolatti, L., Patarnello, T., Kunz, G., Muntoni, F. and Ferlini, A. (2003) Multiple exon skipping and RNA circularisation contribute to the severe phenotypic expression of exon 5 dystrophin deletion. *J. Med. Genet*. **40**, e100.
13. Li, X. F. and Lytton, J. (1999) A circularized sodium-calcium exchanger exon 2 transcript. *J. Biol. Chem*. **274**, 8153-8160.
14. Sänger, H. L., Klotz, G., Riesner, D., Gross, H. J. and Kleinschmidt, A. K. (1976) Viroids are single-stranded covalently closed circular RNA molecules existing as highly base-paired rod-like structures. *Proc. Natl. Acad. Sci*. **73**, 3852-3856.
15. Salzman, J., Gawad, C., Wang, P. L., Lacayo, N. and Brown, P. O., (2012) Circular RNAs are the predominant transcript isoform from hundreds of human genes in diverse cell types. *PloS ONE* **7**, e30733.
16. Takahara, T., Kanazu, S., Yanagisawa, S. and Akanuma, H. (2000) Heterogeneous Sp1 mRNAs in human HepG2 cells include a product of homotypic trans-splicing. *J. Biol. Chem*. **275**, 38067-38072.
17. Flouriot, G., Brand, H., Seraphin, B. and Gannon, F. (2002) Natural trans-spliced mRNAs are generated from the human estrogen receptor-α (hERα) gene. *J. Biol. Chem*. **8**, 218-222.
18. Axtell, M. J., Westholm, J. O. and Lai, E. C. (2011) Vive la différence: biogenesis and evolution of microRNAs in plants and animals. *Genome Biol*. **12**, 221.
19. Chen, C. Y. and Sarnow, P. (1995) Initiation of protein synthesis by the eukaryotic translational apparatus on circular RNAs. *Science* **268**, 415-417.
20. Kozak, M. (1989) The scanning model for translation: an update. *J. Cell Biol*. **108**, 229-241.
21. Sachs, A. B. and Davis, R. W. (1989) The poly(A) binding protein is required for poly(A) shortening and 60s ribosomal subunit-dependent translation initiation. *Cell* **58**, 857-867.
22. Storzm, G. (2002) An expanding universe of noncoding RNAs. *Science* **296**, 1260-1263.