Research article

Computational analysis and in vivo validation of a microRNA encoded by the IBTK gene, a regulator of B-lymphocytes differentiation and survival

Giuseppe Fiume\textsuperscript{a,*},1, Annalisa Rossi\textsuperscript{b,1} Emanuela Di Salle\textsuperscript{a}, Carmen Spatuzza\textsuperscript{b}, Massimo Mallardo\textsuperscript{b}, Giuseppe Scala\textsuperscript{a,**}, Ileana Quinto\textsuperscript{a,**,**}

\textsuperscript{a} Department of Experimental and Clinical Medicine, University of Catanzaro “Magna Graecia”, Catanzaro, Italy
\textsuperscript{b} Department of Biochemistry and Medical Biotechnology, University of Naples “Federico II”, Naples, Italy

\begin{abstract}
MicroRNAs (miRNAs) are small single-stranded RNA molecules that play an essential role in the regulation of gene expression and cell physiology. Gene rearrangements occurring in the miRNA sequence are associated with cancer. The IBTK genetic locus is located in the genomic sequence 6q14.1 that undergoes chromosomal aberration in lymphoproliferative disorders. The IBTK gene encodes the proteins IBTK-α, β and γ that regulate the B cell receptor signalling through Bruton’s tyrosine kinase, which promotes B cell survival and differentiation. Pro-MiRll-based analysis predicted four precursors of microRNAs (pre-miR) encoded by introns 17, 21, 26 and the 3’ un-translated region of the IBTK gene. Pre-miR-IBTK3, which was encoded by intron 26, was the effective substrate of RNase III Dicer in vitro as well as the precursor of an IBtk miRNA generated in vivo. By CLUSTALW-based analysis, pre-miR-IBTK3 homologues were found in Pan troglodytes, Pongo pygmaeus and Macaca mulatta, suggesting an evolutionary conserved function in primates.

© 2009 Elsevier Ltd. All rights reserved.
\end{abstract}

1. Introduction

MicroRNAs (miRNAs) are single-stranded RNA molecules of 21–23 nucleotides in length that act through base pairing to partially complementary sequences of the 3’ un-translated region (3’UTR) of targeted mRNAs. This event destabilizes the target transcript or inhibits the translation of the related proteins (Ambros, 2001). Similarly to transcription factors, miRNAs play an essential role in the regulation of gene expression (Bartel, 2009).

MicroRNAs result from the processing of the primary transcript (pri-miR) generated by RNA polymerase II (Lee et al., 2004) or RNA polymerase III (Pfeffer et al., 2005). Once capped and polyadenylated (Cai et al., 2004), the pri-miR enters a microprocessor complex (500–650 kDa) consisting of Drosha, an RNase III endonuclease, and an essential cofactor DGR8/Pasha protein containing two double-stranded RNA binding domains (Denli et al., 2004; Gregory et al., 2004). The pri-miR is cleaved by Drosha to generate the precursor of miRNA (pre-miR) consisting of a 60–100 nucleotides stem–loop sequence with a 5’ phosphate and two nucleotides 3’ overhangs. The pre-miR is transported to the cytoplasm by Exportin-5, a member of the Ran transport receptor family (Yi et al., 2003). Finally, the cytosolic RNase III Dicer cleaves the pre-miR stem–loop structure to generate the mature single-stranded miRNA (Grishok et al., 2001). The miRNA is incorporated in the cytosolic effector complex, named RNA-Induced Silencing Complex (RISC), where it anneals to the 3’UTR of targeted transcripts by complementary base pairs causing the destabilization or translational inhibition of the target mRNA (Gregory et al., 2005).

MicroRNAs play an essential role in the regulation of cell survival and differentiation (Schickel et al., 2008). Indeed, gene rearrangements causing miRNA mutations result in deregulated gene expression that has been frequently associated with human cancers (Calin and Croce, 2006, 2007a,b; Li et al., 2008). This evidence suggests that tumor suppressors and oncogenes may harbour miRNAs that target specific mRNAs and thus deregulate cell proliferation and survival.

We have recently characterized the Inhibitor of Bruton’s tyrosine kinase (IBtk) gene (accession number AL050333) that encodes three adaptor proteins in cell signalling (Spatuzza et al., 2008). The IBTK gene is 77.58 kb and includes 29 exons with two promoters and transcriptional start sites that result in the expression of three IBTK transcripts, named IBTK\textalpha, IBTK\textbeta and IBTK\gamma (Spatuzza et al., 2008).

Abbreviations: UTR, un-translated region; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; TBE, Tris–borate–EDTA buffer; PBMC, peripheral blood mononuclear cell.

* Corresponding author. Tel.: +39 0961 3694057; fax: +39 0961 3694090.
** Corresponding author. Tel.: +39 0961 3694093/4057; fax: +39 0961 3694090.
*** Corresponding author. Tel.: +39 0961 3694058/4057; fax: +39 0961 3694090.
E-mail addresses: fiume@unicz.it (G. Fiume), scala@unicz.it (G. Scala),
quinto@unicz.it (I. Quinto).

1 These authors equally contributed to this work.
et al., 2008). IBtkγ has been characterized as a ligand inhibitor of Tec kinases, such as Btk, Itk, and Akt, which regulate signal transduction upon specific stimuli (Spatuzza et al., 2008; Liu et al., 2001). As inhibitor of proliferation, IBTK is a candidate tumor suppressor gene. Consistently, the human IBTK gene is located in the genomic sequence 6q14.1, which is a hot spot of chromosomal rearrangements in lymphoproliferative disorders (Mitelman et al., 1997).

In this study, we have addressed whether the IBTK gene might play a role in transcription regulation as source of miRNAs. By bioinformatics analysis, we identified four putative precursors of miRNA (pre-miR) encoded by three distinct introns and the 3′ un-translated region (3′UTR) of the IBTK gene. Of them, only the pre-miR-IBTK3 encoded by intron 26 occurred in vivo, and was the effective substrate of RNase III Dicer. The presence of homologous in other primates suggests an evolutionary conserved role of pre-miR-IBTK3. Based on this evidence, IBTK miRNA is a novel member of the wide miRNAs genomic network.

2. Materials and methods

2.1. Prediction of potential miRNAs

Search for putative miRNAs generated by the genetic locus IBTK was performed by Pro-MirII software (http://cbit.snu.ac.kr/~ProMiR2/index.php). The pre-miR-IBTK nucleotide sequences were predicted according to the following cut-off values: window size: 100 base pairs; shift size: 10 nucleotides; Pro-Mir values: 0.017; conservation score ≥ 0; free energy (ΔG) ≤−20; G–C ratio = 0.3–0.7; entropy ≥ 1.8.

2.2. DNA plasmids

To generate T7 expression plasmids of pre-miR-IBTK1, pre-miR-IBTK2, pre-miR-IBTK3 and pre-miR-IBTK4, the genomic DNA from MC3 cells was amplified by PCR with appropriate primers and cloned in the HindIII/XhoI-digested pcDNA3 vector (Invitrogen) under the T7 promoter. The regions of the IBTK gene encompassed the nucleotides 82971042–82970943 for pre-miR-IBTK1, the nucleotides 82963276–82963177 for pre-miR-IBTK2, the nucleotides 82945044–82944945 for pre-miR-IBTK3 and the nucleotides 82937481–82937382 for pre-miR-IBTK4. Forward (FW) and reverse (RW) primers were as follows: pre-miR-IBTK1 FW: 5′-CCCAAGCTTTGATTGAAATGTCATAAAACC-3′; pre-miR-IBTK1 RW: 5′-GGCCTCGAGGGAAGTTAATTTTAATCAA-3′; pre-miR-IBTK2 FW: 5′-CCCAAGCTTTGATGAAAGCTACCCCCTTGCTTGG-3′; pre-miR-IBTK2 RW: 5′-GGCCTCGAGGAGGAGATAGTTCTTGGGA-3′; pre-miR-IBTK3 FW: 5′-CCCAAGCTTTGATGAAAGCTACCCCCTTGCTTGG-3′; pre-miR-IBTK3 RW: 5′-GGCCTCGAGGGAAGTTAATTTTAATCAA-3′; pre-miR-IBTK4 FW: 5′-CCCAAGCTTTGATGAAAGCTACCCCCTTGCTTGG-3′; pre-miR-IBTK4 RW: 5′-GGCCTCGAGGGAAGTTAATTTTAATCAA-3′.

2.3. Dicer RNase assay

IBTK transcripts corresponding to predicted pre-miR-IBTK were transcribed with T7 RNA polymerase and [32P]α-UTP (3000 C/mm) using the Riboprobe System–SP6/T7 (Promega); ApaI-digested pre-miR-IBTK pcDNA3 plasmids were used as template. RNA samples (10^6 cpm) were incubated with or without the recombinant human Dicer (2 units) (Genlantis) in a reaction buffer (10 μl) containing 20 mM Tris–HCl pH 7.5, 5 mM MgCl2, 1 mM dithiothreitol (DTT), 1 mM ATP, at 37 °C for 2 h. The RNA products were separated by 15% PAGE in 8 M Urea TBE, and analyzed by autoradiography, as described (Ouellet et al., 2008).

2.4. Cells

Human peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll Paque gradient (GE Healthcare Europe, Munich, Germany) from buffy coats. Healthy subjects blood was diluted 1:1 in PBS and stratified on Ficoll solution with a 3:1 (v/v) ratio.
Fig. 2. Pre-miRNA-IBTK3 is the substrate of the human RNAse III Dicer. Pre-miRs-IBTK were in vitro transcribed with [32P]y-UTP- and 2 h-incubated with or without recombinant human Dicer. Products were resolved by 15% PAGE in 8 M Urea TBE followed by autoradiography. The arrow indicates the ∼22 nucleotides products generated by Dicer cleavage. Molecular weight (MW) markers were [32 P]-ATP-labeled oligonucleotides spanning between 100 and 25 base pairs (lane 1).

After 30 min centrifugation at 1200 × g, PBMCs were recovered and re-suspended in RPMI-1640 medium supplemented with 10% FCS.

2.5. Northern blot analysis

Total RNA was extracted from PBMCs (50 × 10⁶ cells) using TRIzol reagent (Invitrogen, Karlsruhe, Germany) according to supplier’s protocol, and analyzed by Northern blot as previously described (Mallardo et al., 1994). Briefly, RNA samples were dissolved in loading buffer (0.025% bromophenol blue, 0.025% cyanol, 0.025% SDS, 0.5 mM EDTA, 95% formamide), boiled for 5 min at 95 °C and loaded on 15% polyacrylamide gel under denaturing conditions [15% acrylamide–bisacrylamide (19:1), 45 mM Tris, 45 mM boric acid, 1 mM EDTA pH 8.0, 7 M urea, 0.01% N,N,N0,N0-tetramethylethylenediamine (TEMED) and 0.1% ammonium persulfate (APS)]. Samples were resolved by electrophoresis for 90 min at 150 V and transferred to nylon membranes (Hybond N+, Amersham/GE Healthcare, Little Chalfont, UK) by capillary blot. The membranes were then equilibrated in 1 M NaCl and pre-hybridized in 6 × SSC, 5 × Denhart’s solution (1 Denhart’s solution = 0.1% N,N,N0,N0-tetramethylethylenediamine and 0.1% ammonium persulfate (APS)). Samples were resolved by electrophoresis for 90 min at 150 V and transferred to nylon membranes (Hybond N+, Amersham/GE Healthcare, Little Chalfont, UK) by capillary blot. The membranes were then equilibrated in 1 M NaCl and pre-hybridized in 6 × SSC, 5 × Denhart’s solution (1 Denhart’s solution = 0.1% Ficoll, 0.1% Poly-vinyl-pyrolidone and 0.1% bovine serum albumin), 5 mg/ml of sheared salmon sperm double-stranded DNA (Ambion, Austin, TX, USA) at 42 °C for 2 h. After pre-hybridization, [32P]y-ATP-labeled oligonucleotide (3000 Ci/mmol) was added and the hybridization was carried out overnight at 42 °C. The membranes were washed twice in 2 × SSC, 1% SDS at 42 °C for 30 min, and analyzed by autoradiography or Phosphorimage screen (Amersham/GE Healthcare). The nucleotide sequence of probes was as follows: IBTK1–25, for the nucleotides 1–25 of predicted pre-miR-IBTK3 5′-TGGTAGTAAATGATGAGCAAAGAC-3′; IBTK26–50, for the nucleotides 26–50 of predicted pre-miR-IBTK3, 5′-ACATGCCAGAAGCTAATATGAACAC-3′; IBTK51–75 for the nucleotides 51–75 of predicted pre-miR-IBTK3 5′-TCACCAGACTCAGACACCCAAT-3′; probes; IBTK76–100, for the nucleotides 76–100 of predicted pre-miR-IBTK3 5′-AATTTCATTAATTTACTCATT-3′.

2.6. Comparative analysis of nucleotide sequence

The nucleotide sequence of Homo sapiens pre-miR-IBTK3 was aligned with the genome of Pan troglodytes, Pongo pygmæus, Macaca mulatta, Bos taurus and Canis familiaris to identify homologous nucleotide sequences by BLAST software (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The pre-miR-IBTK3 homologues of H. sapiens, P. troglodytes, M. mulatta, B. taurus and C. familiaris were analyzed for the identity percentage by CLUSTALW software (http://www.ebi.ac.uk/Tools/clustalw2/index.html) (Waterhouse et al., 2009).

3. Results

To identify putative pre-miRs encoded by the IBTK gene, we performed an in silico analysis of the genomic nucleotide sequence of the IBTK genetic locus by using the Pro-MirII software that allows the probabilistic prediction of clustered, un-clustered, conserved and un-conserved (Nam et al., 2006). We identified four potential miRNAs located in the introns 17, 21, 26 and in the 3′UTR of the

Fig. 3. In vivo detection of pre-miR-IBTK3 in PBMCs. (A) Schematic representation of the pre-miR-IBTK nucleotides probes used in Northern blotting analysis. (B) Northern blotting of total RNA extracted from human PBMCs using the [32P]y-ATP-labeled IBTK probes. Molecular weight (MW) markers were [32P]y-ATP-labeled oligonucleotides spanning between 100 and 20 base pairs.
IBTK gene, named pre-miR-IBTK1, pre-miR-IBTK2, pre-miR-IBTK3, pre-miR-IBTK4, respectively, according to chemical and physical properties, such as free energy, G–C ratio and entropy (Fig. 1).

To assess whether the predicted pre-miRs were substrates of RNase III Dicer, we generated expression vectors of pre-miR-IBTK sequences under the T7 promoter, which were used to produce the relative $[^{32}P]H_9251$-UTP-labeled transcripts. By in vitro assay, pre-miR-IBTK3, and not the other pre-miRs, was cleaved by the human recombinant Dicer to generate 22–25 nucleotides RNA products (Fig. 2).

Then, we performed the Northern blotting analysis of total RNA extracted from human PBMCs to verify the in vivo occurrence of pre-miR-IBTK3 and relative products under physiological conditions. To this end, we used 25 nucleotides probes annealing to four sequential regions of pre-miR-IBTK3 (Fig. 3A). All nucleotide probes identified both the pre-miR-IBTK3 and IBTK miRNA (Fig. 3B),

![Fig. 4. Comparison of pre-miR-IBTK3 nucleotide sequence in mammals. Alignment (A) and identity percentage (B) of pre-miR-IBTK3 homologues of mammalian species according to CLUSTALW-based analysis.](image1)

![Fig. 5. Prediction of the human pre-miR-IBTK3 homologues in primate species. (A) Chemical and physical properties of predicted pre-miR-IBTK3 homologues in Pan troglodytes, Pongo pygmaeus, Macaca mulatta, as predicted by Pro-MirII software. (B) Secondary structure of the pre-miR-IBTK in mammals.](image2)
indicating that both arms of pre-miR-IBTK3 generated the RNA products in vivo.

To evaluate the grade of evolutionary conservation of pre-miR-IBTK3, we performed a CLUSTALW-based multiple sequence alignment of the nucleotide sequence of pre-miR-IBTK3 in different vertebrates. The pre-miR-IBTK3 of *H. sapiens* showed a high identity degree with pre-miR-IBTK3 of *P. troglodytes, P. pygmaeus* and *M. mulatta*, and a lower identity degree with pre-miR-IBTK3 of *B. taurus* and *C. familiaris* (Fig. 4). In fact, the identity percentage of *H. sapiens* pre-miR-IBTK3 as compared to the pre-miR-IBTK3 of *P. troglodytes, P. pygmaeus* and *M. mulatta* was 95%, 90.4% and 87.5%, respectively. Differently, the identity percentage compared with the pre-miR-IBTK3 of *B. taurus* and *C. familiaris* was 70% and 69%, respectively. Accordingly, the Pro-MirI-based analysis of pre-miR-IBTK3 nucleotide sequence of *P. troglodytes, P. pygmaeus* and *M. mulatta* predicted a miRNA secondary structure (Fig. 5), which was not predicted for the homologue IBTK nucleotide sequence of *B. taurus* and *C. familiaris*.

4. Discussion

The IBTK genetic locus encodes the IBtk proteins α, β, γ (Spatuzza et al., 2008). We previously characterized IBtk as a repressor of Btk (Spatuzza et al. 2008; Liu et al., 2001). Btk is a Tec tyrosine kinase required for B-lymphocytes development and differentiation (Halcomb et al., 2007). Mutations of the BTK gene inactivating the Btk activity caused X-linked agammaglobulinemia in humans (Tsukada et al., 1993) and X-linked immunodeficiency in mice (Vinuesa et al., 2001). In mature B-lymphocytes, Btk is required for calcium signalling and activation of the anti-apoptotic transacting factor NF-κB in response to the triggering of the B cell receptor for the antigen (Spatuzza et al., 2008; Liu et al., 2001); in this context, Btk promotes the antibody production, B cell differentiation and cell cycle progression. IBtky binds the pleckstrin homology (PH) domain of Btk and represses the Btk-mediated signalling leading to B cell activation (Spatuzza et al., 2008; Liu et al., 2001). Based on this evidence, IBTK exerts a prevalent anti-proliferative action. Moreover, IBTK might play a more extended role in cell signalling since the IBtkα and IBtkβ proteins are expressed in distinct cell types and bind the PH domain of distinct Tec kinases, such as Btk, Itk and Akt (Spatuzza et al., 2008). Two promoters and several cis regulatory sequences were identified in the IBTK gene (Spatuzza et al., 2008); however, little is known on the IBTK transcription regulation.

Recent studies have shown that miRNAs regulate cell proliferation and apoptosis and are involved in cancer development (Schickel et al., 2008). Indeed, more than 50% of miRNAs genes are located either in cancer-associated genomic region, or in fragile sites, suggesting that miRNAs rearrangements may play a major role in the pathogenesis of human cancers (Calin and Croce, 2006, 2007a,b). In particular, B and T cell neoplasia showed a high incidence of miRNAs rearrangements (Calin and Croce, 2007a,b; Li et al., 2008), indicating that specific miRNAs mutations may deregulate cell functions that play a role in B and T lymphocyte proliferation.

In this study, we have addressed whether the IBTK gene gen- erates miRNAs in human PBMCs. We found that intron 26 of the human IBTK gene encodes the pre-miR-IBTK3, which is cleaved by Dicer generating ~22-nucleotide-long products. This finding correlated with the occurrence of pre-miR-IBTK3 and the relative miRNA products in human PBMCs. We also found that pre-miR-IBTK3 is an evolutionary conserved function in primates since it is highly conserved in *P. troglodytes, P. pygmaeus* and *M. mulatta*. The evidence of miRNA IBTK occurrence in vivo supports an expanded role of the IBTK gene as regulator of gene transcription through miRNA generation. As the human IBTK gene is located in the genomic sequence 6q14.1, which is a region of recurrent chromosomal aberration in lymphoproliferative disorders (Mitelman et al., 1997), our findings suggest that aberrant production of IBTK miRNA might be involved in tumorigenesis. Identification of the primary structure of the IBtk mature miRNA in vivo will be required to identify its miRNA targets and specific function in gene regulation.

Acknowledgements

We thank Dr. M. Sardiello and Dr. V.A. Gennarino for helpful suggestions. This work was supported by grants from the Associazione Italiana per la Ricerca sul Cancro (AIRC), MIUR-PRIN, MIUR-FIRB and ISS to G.S. and LQ.

References


Tsukada, S., Safran, D.C., Rawlings, D.J., Parolini, O., Allen, R.C., Klisak, I., Sparkes, R.S., Kubagawa, H., Mohandas, T., Quan, S., Belmont, J.W., Cooper, M.D., Conley, M.E.,

