Identification of novel splicing variants from RON proto-oncogene pre-mRNA

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Abstract. RON is a proto-oncogene that induces cell dissociation, migration and matrix invasion. RONΔ160, a splicing variant of RON, is a natural splicing product in colon cancers that is produced through skipping of exons 5 and 6 in alternative splicing process. RONΔ160 promotes cellular transformation *in vitro* and tumor formation *in vivo*. We present, here, two novel splicing variants of RON in the partial splicing events that involve exons 5 and 6. The common facts of these two novel splicing variants are that exons 4-7 are included. In addition, intron 4 is spliced whereas intron 5 is included in both variants. The difference of these two isoforms is the inclusion or skipping of intron 6. In one variant intron 6 is included, but intron 6 is skipped in another variant. These two variants should be truncated but these proteins have not yet been detected.

Introduction

RON receptor tyrosine kinase is a member of the MET proto-oncogene that induces cell dissociation, migration, and matrix invasion (1-3). Receptor tyrosine kinases consist of a large group of cell surface proteins with unique structure and biological activities (4,5). Abnormal accumulation and activation of receptor tyrosine kinases causes the initiation and progression of a variety of malignancies including tumors derived from breast and kidneys (6,7). The invasive growth features are controlled by a genetic program which is conserved in MET family members (8,9). Mature RON is a 180-kDa heterodimeric protein that is composed of a 40-kDa extracellular α chain and 150 kDa transmembrane β chain with intrinsic protein tyrosine kinase activity (10,11). α and β chains are proteolytic products of a 180-kDa RON precursor (12). RON is activated by macrophage-stimulating protein (MSP), a serum protein that is constitutively expressed by liver cells as an inactive form and requires proteolytis conversion for receptor binding (13-15). The binding of MSP to RON results in receptor autophosphorylation and upregulation of RON kinase activity, which in turn stimulates a number of intracellular pathways mediating MSP effects (16). In normal cells MSP-induced activation is a transient event, whereas in tumor cells RON activity is often constitutively upregulated (17). RON activation causes invasive growth and motility of certain epithelial tumor cells (18,19). A number of facts suggest that RON might be involved in the progression of certain epithelial malignancies, particularly at the stage of tumor metastasis (20).

It has been shown that receptor tyrosine kinases can be activated by a variety of mechanisms, including mutation, deletion, gene rearrangement and alternative splicing of pre-mRNA (21). Various RON protein isoforms are produced through alternative splicing of pre-mRNA (12). One of the protein variants is RonΔ160, a naturally occurring oncogenic form of RON identified in human colon cancers, which is produced from a splicing mRNA transcript by exons 5 and 6. RonΔ160 causes structural changes that lead to cellular transformation *in vitro* and tumor growth *in vivo* (22,23). Another variant RonΔ170 is a 170-kDa variant is generated by skipping of exon 19 in alternative splicing (24). RonΔ170 is kinase defective and acts as dominant negative agent that inhibits tumorigenic activities mediated by oncogenic variant RONΔ160 in colon cancer cells (24). RonΔ165 is produced by an in-frame deletion of exon 11 that affects the proteolytic process which results in the accumulation of single-chain pro-RonΔ165 in the cytoplasm (25). RonΔ155 is a derived from a combined deletion of exons 5, 6 and 11, the function of RonΔ155 is similar to RonΔ160 (25,26).

Here we show two novel splicing variants of RON proto-oncogene. From the sequence analysis it is proved they are partially spliced during the splicing events of exons 5 and 6. These two variants contain either intron 5, or introns 5 and 6, in addition to exons 4-7. The proteins encoded by these variants are not identified yet.

Materials and methods

Cell culture. HeLa and C33A cells were maintained in Dulbecco's modified Eagle's medium (DMEM) and HCT116 and HT-29 cells were maintained in RPMI-1640 medium supplemented

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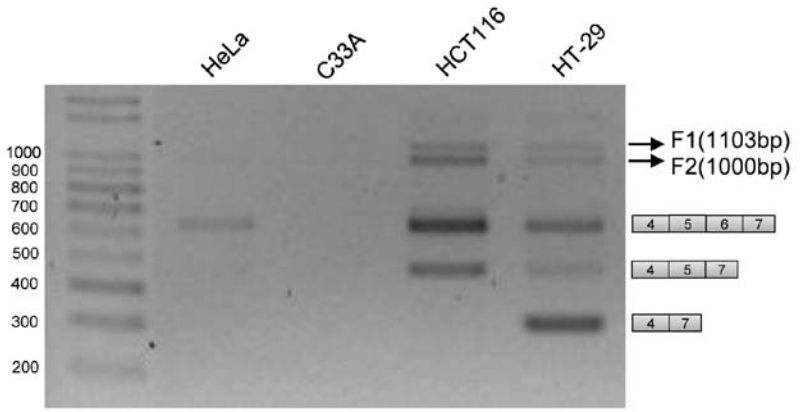

Figure 1. Alternative splicing of endogenous RON pre-mRNA. RT-PCR products of RON mRNA using a RON exon 4-specific forward primer and an exon 7-specific reverse primer are shown.

A.

<F1 sequence> - 1103bp

GTTTTCCAGGTACCTATCCAAGGCCCTGGCTGCCGCCACTTCCTGACCTGTGGGCGTT
GCCTAAGGGCATGGCATTTCATGGGCTGTGGCTGGTGTGGGAACATGTGCGGCCAGC
AGAAGGAGTGTCCTGGCTCCTGGCAACAGGACCACTGCCCACCTAAGCTTACTGAGTT Exon 4,5
CCACCCCCACAGTGGACCTCTAAGGGGCAGTACAAGGCTGACCCTGTGTGGCTCCAA
CTTCTACCTTCACCCTTCTGGTCTGGTGCCTGAGGGAACCCATCAGGTCACTGTGGGC
CAAAGTCCCTGCCGGCCACTGCCCAAGGACAGCTCAAAACTCAGgtacaatctggtccctcccct
ccctttccctgaaggggggaaaccaagcagccccttcccca**tga**gaccctgttctctgcttatcagaggcaaggggggatggggg
aagctgcagtggttctgactgctttttgagagtcaaaaaggtctctgtcccttttgagctttcatgtgcccttcctgtctgttcactcatgg Intron5
accagccaaggttaatctctgccccaccagaaccttccttccatggagggaggcatgggtggagaaatgccattctctggctcag
aggagccctgtggcttgtggcaggcatgcatctaggcctgtgtaattcctggctgacctcaggggttccctggtgccccagACC
AGTGCCCCGGAAAGACTTTGTAGAGGAGTTTGAGTGTGAACTGGAGCCCTTGGGCAC Exon6
CCAGGCAGTGGGGCCTACCAACGTCAGCCTCACCGTGACTAACATGCCACCGGGCAA
GCACTTCCGGGTAGACGGCACCTCCGTGCTGAGAGGCTTCTCTTTCATGgtgaggctacctt Intron6
gccctgtctgtgcccttggccagtgcatggtacgggaagggaggggcttggagtggaggacctgcctaagccacctctatgtcct
cttagGAGCCAGTGCTGATAGCAGTGCAACCCCTCTTTGGCCCACGGGCAGGAGGCACC Exon7
TGTCTCACTCTTGAAGGCCAGAGTCTGTCTGTAGGCACCAGCCGGGCTGTGCTGGTCA
ATGGGACTGAGTGTCTGCTAGCACG

B.

<F2 sequence> - 1000bp

GTTTTCCAGGTACCTATCCAAGGCCCTGGCTGCCGCCACTTCCTGACCTGTGGGCGTT
GCCTAAGGGCATGGCATTTCATGGGCTGTGGCTGGTGTGGGAACATGTGCGGCCAGC
AGAAGGAGTGTCCTGGCTCCTGGCAACAGGACCACTGCCCACCTAAGCTTACTGAGTT Exon 4,5
CCACCCCCACAGTGGACCTCTAAGGGGCAGTACAAGGCTGACCCTGTGTGGCTCCAA
CTTCTACCTTCACCCTTCTGGTCTGGTGCCTGAGGGAACCCATCAGGTCACTGTGGGC
CAAAGTCCCTGCCGGCCACTGCCCAAGGACAGCTCAAAACTCAGgtacaatctggtccctcccct
ccctttccctgaaggggggaaaccaagcagccccttcccca**tga**gaccctgttctctgcttatcagaggcaaggggggatggggg
aagctgcagtggttctgactgctttttgagagtcaaaaaggtctctgtcccttttgagctttcatgtgcccttcctgtctgttcactcatgg Intron5
accagccaaggttaatctctgccccaccagaaccttccttccatggagggaggcatgggtggagaaatgccattctctggctcag
aggagccctgtggcttgtggcaggcatgcatctaggcctgtgtaattcctggctgacctcaggggttccctggtgccccagACC
AGTGCCCCGGAAAGACTTTGTAGAGGAGTTTGAGTGTGAACTGGAGCCCTTGGGCAC Exon6
CCAGGCAGTGGGGCCTACCAACGTCAGCCTCACCGTGACTAACATGCCACCGGGCAA
GCACTTCCGGGTAGACGGCACCTCCGTGCTGAGAGGCTTCTCTTTCATGGAGCCAGT
GCTGATAGCAGTGCAACCCCTCTTTGGCCCACGGGCAGGAGGCACCTGTCTCACTCTT Exon7
GAAGGCCAGAGTCTGTCTGTAGGCACCAGCCGGGCTGTGCTGGTCAATGGGACTGAG
TGTCTGCTAGCACG

Figure 2. Sequence of the F1 and F2 fragment. (A) Partial sequences of the F1 fragment. F1 fragment contains exons 4 and 5, intron 5, exon 6, intron 6 and exon 7. (B) Partial sequences of the F2 fragment. F2 fragment contains exons 4 and 5, intron 5, exons 6 and 7. The stop codon in intron 5 in frame with exons 1-4 is shown in bold.

with 10% of Fetal Bovine Serum (FBS) at 37˚C in a humidified 5% CO_2 condition.

RT-PCR. Total RNA was extracted using RiboEx (GeneAll) following the manufacturer's instructions. Total RNA (1 μg) was reverse transcribed with a RON exon 8 specific primer (5'-TGGCACATAAAAGCTG-3') using ImProm-II™ reverse transcriptase (Promega) following the manufacturer's instructions. RON cDNA was amplified by PCR using RON exon 4-specific primer (5'-GTTTTCCAGGTACCTATCCAAG-3')

Figure 3. The schematic diagrams of the RON pre-mRNA, F1 andF2 fragments. Intron 4 is spliced in the F1 fragment while introns 4 and 6, but not intron 5, are spliced in F2 fragment through the alternative splicing. A premature stop codon in frame with exons 1 to exon 4 is located in intron 5 at 63 nt downstream from the 5' splice site of exon 5.

and RON exon 7 specific primer 5'-CGTGCTAGCAGACACT CAGTC-3'). The PCR products were loaded onto 2% agarose gel and visualized by staining with ethidium bromide solution. RT-PCR bands were purified and sequenced.

Results

Novel RT-PCR products are produced from RNA fragment covering exons 4-7. In the pre-mRNA splicing of RON gene, RonΔ160 is produced due to skipping of exons 5 and 6. In order to compare the skipping/inclusion of exons 5 and 6 in different cell lines, we performed RT-PCR reaction using primers from exons 4 and 7. As expected we observed several splicing variants that had been previously described to include or exclude exons 5 and 6 in human colon cancer HT-29 and HCT116 cells (23). In addition to these two isoforms, a variant which includes exon 6, but not exon 5, is also produced in these two cell lines, consistent with previous results (27). To our surprise, we also found that two other PCR products which we name as F1 and F2, are also produced, as shown in Fig. 1. As compared with size markers on the agarose gel, the length of F1 is ~1,100 bp; the length of F2 is ~1,000 bp. On the other hand, in cervix cancer cell C33A, none of any RT-PCR product RON RNA is produced; consistent with the fact that RON is not expressed in cervix cells. In HeLa cells, the only RT-PCR products we have observed are variants that have exon 5 and 6 inclusion. F1 and F2 products are not found in HeLa and C33A cells.

F1 and F2 fragments are partially spliced product of RON pre-mRNA. In order to characterize two unknown pre-mRNA splicing products from exons 4 and 7 of RON, we performed sequence analysis. As shown in Fig. 2, we found that the RNA fragment, which corresponds to F1 RT-PCR product, contains exons 4 and 5, intron 5, exon 6, intron 6 and exon 7 (1103Base). Another RNA fragment, which corresponds to F2 RT-PCR product, contains exons 4 and 5, intron 5, exons 6 and 7 (1000Base). Therefore F1 is a partial splicing product in which only intron 4 is spliced while F2 is a product in which both introns 4 and 6 are spliced as shown in Fig. 3. F1 and F2 intron 5 are not spliced in either F1 or F2 products. We conclude that F1 and F2 are partially spliced product of RON pre-mRNA.

Intron 5 includes a stop codon. Both novel RNA variants which correspond to F1 and F2 contain intron 5. We analyzed intron 5 sequence. We found that intron 5 contains a stop codon at the position 63-nt from 5' splice site of exon 5 in frame with exons 1-4. Therefore it is likely that F1 and F2 will encode truncated RON isoforms from exons 1-4, but not beyond exon 4.

Discussion

The full length RON precursor is 180 kDa. But it has been well-documented that RON has several isoforms that are produced from alternative splicing or protein truncation. RonΔ160 without exons 5 and 6 is oncogenic while RonΔ170 is a dominant negative isoform of RON protein. The different effects of these two isoforms suggest that it is important to investigate how many isoforms and what isoforms are present in particular cells. In this study, we used RT-PCR and identified two additional RON RNA variants both of which include intron 5. The intron 5 sequence contains stop codons that prevent these two RON RNA variants to translate proteins beyond exon 4, leading to potential truncated isoforms of RON protein (from exons 1-4). The existence and possible functions of these isoforms should be investigated in the future.

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