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Identification of the splice variants of Recepteur d'Origine nantais (RON) in lung cancer cell lines

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1   **Identification of the Splice Variants of Recepteur d'Origine nantais (RON) in Lung Cancer**
2   **Cell Lines**
3

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Abstract

30

31 RON receptor tyrosine kinase is a transmembrane protein directly involved in suppression of

32 inflammation and its aberrant expression linked to cancers and metastasis. Efforts to block

33 deregulated RON signaling in tumors using small molecule kinase inhibitors or antibodies have

34 been complicated by the presence of unknown number/types of isoforms of RON, which, despite

35 being structurally similar, localize differently and mediate varied functions. Current study was

36 designed to identify the splice variants of RON transcripts formed by skipping of sequences

37 between exons 9 and 14 for better understanding of isoform specific RON signaling in cancers.

38 PCR amplification and bi-directional sequencing of a 901bp cDNA sequence located between

39 exons 9 to 14 of RON from lung cancer cell lines revealed the presence of two splicing variants

40 formed by skipping of exons 11 and 11-13. Each of these transcripts was found in more than one

41 cell line. Expressed sequence tag (EST) database search indicated that the splicing variant lacking

42 exons 11-13 was a novel one. Here we conclude that the splice variants of RON lacking exon 11

43 and exons 11-13 were detected in several lung cancer cell lines. Novel variant formed by skipping

44 exons 11-13, the sequence of which code for transmembrane region, is predicted to code for a

45 truncated isoform that may be secreted out. Tumors may antagonize the ligand dependent anti-

46 inflammatory function of wild-type RON by secreting out the ligand binding isoforms.

47 **Keywords:** alternative splicing; lung cancer; macrophage stimulating protein (MST1R); receptor

48 tyrosine kinase; RON; RON isoform

49

50

Introduction

RON, also known as MST1R (macrophage-stimulating 1 receptor), is a member of the MET family of receptor tyrosine kinases (RTKs). MSP (macrophage stimulating protein, the ligand for RON) driven RON signaling has been shown to be anti-inflammatory via many models (Correll et al., 1997; Liu et al., 1999; Waltz et al., 2001). MSP/RON signaling is activated in macrophages following acute inflammation and serves to suppress synthesis of pro-inflammatory mediators like nitric oxide (NO), prostaglandins and several other pro-inflammatory cytokines and upregulate anti-inflammatory cytokines like IL-10 (Gunella et al., 2006). RON activation also blocks NF-kappaB activation (Zhou et al., 2002), and further is a critical determinant of macrophage activation states (M1/M2) (Sharda et al., 2011).

Ample evidence indicate deregulated expression and functioning of RON in a number of cancers (Gaudino et al., 1995; Wang et al., 1996; Sakamoto et al., 1997; Maggiora et al., 1998; Wang et al., 2000; Camp et al., 2007; Zhou et al., 2008; Kretschmann et al., 2010). While RON overexpression is associated with tumor aggressiveness and metastasis (Thomas et al., 2007), knockdown of RON expression in different cancer cell lines using siRNA/shRNA showed suppression of tumorigenic properties (Xu et al., 2004; Wang et al., 2009; Logan-Collins et al., 2010). Validation of overexpressed RON as a therapeutic target in tumors has been hampered due to the simultaneous production of its several isoforms. Previously, protein expression analyses indicated the absence of wild type RON but its isoforms present in lung cancer cell lines could not be detected with Western blotting (Kanteti et al., 2012). Several variant transcripts and their protein products have been described for RON in various cancer cell lines as well as solid and pleural tumors (Lu et al., 2007). Despite exhibiting diverse functions in cancers, the isoforms show considerable sequence similarity and to the wild type RON. The isoforms include both active

74 (Wang et al., 2000; Angeloni et al., 2007; Zhou et al., 2008) as well as various N-terminally

75 truncated dominant negative variants (Lu et al., 2007; Jin et al., 2008; Eckerich et al., 2009; Ma et

76 al., 2010). Ectopic expression of some RON splice variants in NIH3T3 cells induced tumor

77 formation *in vivo* (Zhou et al., 2003).

78 We hypothesize that aberrant expression of RON via alternative splicing in cancers may alter its anti-

79 inflammatory role and hence structural and functional characterization of the individual isoforms may

80 elucidate their role in cancer development. Recently, we reported the presence of several, frequently

81 occurring novel transcript variants affecting the intracellular region of RON in lung cancer cell lines

82 (Krishnaswamy et al., 2015; Krishnaswamy et al., 2016). In this study, we focused on RON mRNA

83 sequence between exons 9 and 14 to identify any novel splicing variants, using various lung cancer

84 cell lines, by PCR amplification and sequencing.

85 **Materials and Methods**

86 **Cell lines**

87 SCLC cell lines H526, H446, H249, H69, H2171, H345, H82, H146, H889 and H524 and

88 NSCLC cell lines SW1573, H358, A549, H1838, H661, H522, H1437, H2170, SW900, H1993,

89 SKLU-1, H1703 and SKMES were obtained from ATCC (Manassas, VA) and were cultured in

90 RPMI 1640 medium (Gibco/BRL) supplemented with 10% (v/v) fetal bovine serum supplemented

91 with L-glutamine and 1% (v/v) penicillin/streptomycin at 37°C with 5% CO_2.

92 **cDNA preparation, PCR and sequencing**

93 Total RNA from the cell lines was isolated using TRIZol reagent (Invitrogen, Carlsbad, CA, USA)

94 following manufacturer's instructions. cDNA was generated using 1μg of total RNA and oligo dT

95 primer by using Single Strand cDNA Synthesis Kit (Clontech, Palo Alto, CA, USA). cDNAs of

96 RON were amplified thermal cycler using specifically designed primers covering 901 bps of RON

4

97 reference mRNA sequence (NM_000247) using forward primer (located in exon 9) 5′-

98 CAGCATCTAACTTCAGCATGGCACTTAG - 3′ and reverse primer (located in exon 14) 5′-

99 CAGTGACCGAGTCATTGGCAAAG - 3′. Sequencing was performed in final volume of 10µl.

100 BigDye® Terminator (V 3.1) Sequencing reactions were carried out by using 0.4 pmole of either

101 forward or reverse primers. The 10µl reaction consisted of 0.5-1µl of BigDye mix, 1.5µl of 5X

102 sequencing buffer (Tris HCl 400 mM pH 9, MgCl2 10 mM) and 5-6µl purified PCR product.

103 Sequencing PCR reaction mixes were initially denatured at 96C for 1 minute and 30 seconds,

104 followed by 35 cycles of denaturation at 96C for 45 seconds and annealing at 50C for 30 seconds,

105 and extension at 60C for 4 minutes. Sequencing PCR products ere purified and dissolved in 12µl

106 Hi-DiTM formamide before loading on a genetic analyzer (Applied Biosystems PRISM 310,

107 Foster City, CA). Sequence variations in the PCR products were identified by aligning sequencing

108 chromatograms with reference RON sequence using Mutation Surveyor version 3.1 software

109 (SoftGenetics, State College, PA). The numbering of nucleotide positions is relative to the first

110 base of the translational initiation codon of the full-length RON coding sequence (CCDS 2807.1).

111 **Results**

112 A number of alternatively spliced forms of RON mRNA and their protein products have previously

113 been reported. However, amplifying the entire coding sequence of RON (4200 bps) as a single

114 amplicon and sequencing would have failed to provide a complete picture of all the alternative

115 splicing events due to the formation of multiple products possessing largely similar sequences.

116 Hence, we amplified a short section of RON mRNA, covering region between exons 9 and 14, by

117 converting to cDNA and using forward and reverse PCR primer sequences located in exons 9 and

118 14, respectively (Figure 1). Sequence chromatograms were obtained using the forward

119 amplification primer for NSCLC cell line H661 indicated the presence of a predominant variant

120 lacking exon 11 (Figure 2). This splicing variant was also found in A549, SKLU1, A249, H69,

121 H82, H345 and H526 cell lines.

122 Chromatograms of cell line H249 were obtained by sequencing from 5′ end indicated the co-

123 occurrence of two splice variants, one formed by skipping of exon 11 and the other formed by

124 skipping of exons 11-13. Further, exon 10 of the major variant (based on peak heights) ended with

125 TTTTAG sequence, while it ended with TTT in the minor variant due to the deletion of the last

126 three (TAG) nucleotides. Two additional overlapping new sequences starting at nucleotide 2650

127 (of RON reference sequence) were also identified; these sequences corresponded to starting

128 nucleotides of exons 12 and 14, identifying them as distinct splicing variants arising from the loss

129 of exon 11 and exons 11-13, respectively. Exon 11 containing wild type/reference RON transcript

130 sequence was completely missing from H249 PCR products. Skipping of exon 11 results in an in-

131 frame deletion of 147 nucleotides, corresponding to 49 amino acids, and skipping of exons 11-13

132 leads to loss of 415 nucleotides and consequently a frame-shift leading to the appearance of

133 premature termination codon. RON splicing variant lacking exons 11-13 was also found in H358,

134 H146, H524, A549, SKLU1, SKMES, H69, H1703, H1993, H82, and H889 cell lines (Figure 3).

135 The overlapping of sequences started at nucleotide 3066 of RON reference sequence and the two

136 overlapping sequences matched with exons 10 and 13 of the reference sequence in H1993. This

137 confirmed the presence of the splicing variant formed by loss of exons 11-13, which occurred

138 along with the wild-type transcript. Furthermore, from the size of the peaks, alternatively spliced

139 transcript variant was identified at a higher level than the wild-type transcript. In this cell line, only

140 the splicing variant whose exon 10 sequence ends with bases TTTGAG was found (Figure 4).

141 Intron sequence located between exons 9 and 10 was spliced out in two different ways in H1993

142 (Figure 5): the last three nucleotides of this intron (CAG) were retained in the minor (based on

6

143   peak heights) splicing variant, while these nucleotides were not included in the major splicing

144   variant. The splicing variant that retained CAG at the beginning of exon 10 ended up losing the

145   last three nucleotides, GAG, of exon 10, as shown in H249 cell line (Figure 3).

146   **Discussion**

147   Aberrant expression of RON in tumors is accompanied by alternative splicing of mRNA transcripts

148   leading to expression of an array of isoform products having varying functions. However, high

149   level of sequence similarities among the transcript variants and their protein isoforms pose

150   problems in specific target discovery and validation. In this study, we screened lung cancer cell

151   lines for splicing variants between exons 9 and 14 of RON transcripts through partial cDNA

152   sequencing. Results revealed the presence of a novel alternatively spliced variant lacking exons

153   11-13 and a previously known variant formed by skipping of exon 11. Both these variants occurred

154   together in several cell lines. Both variants were found with or without deletion of the last three

155   nucleotides of exon 10, GAG, which codes for glutamic acid. This single codon difference between

156   transcript variants was created by differential splicing of exon 10. Exon 12 contains sequence

157   coding for transmembrane (TM) domain, and skipping of exons 11-13 leads to frame-shift and

158   appearance of premature termination codon. Translation product of transcript variant lacking

159   exons 11-13 is expected to be secreted and the produced isoform may block MSP/RON signaling

160   by binding to MSP or by dimerizing with normal RON, by making N-terminally truncated isoforms

161   of RON. Thus, tumors may nullify the anti-inflammatory/anti-carcinogenic role of MSP/RON

162   signaling via altering the splicing pattern of RON RTK.

163   Two RON transcripts involving differential splicing of exon 11 have been reported previously;

164   one of these lacked exons 5, 6 and 11 and the other lacked only exon 11. Skipping of exon 11

165   caused an in-frame deletion of 147 nucleotides, corresponding to 49 amino acids of the

166 extracellular region of RON beta chain, resulting in RONdelta165, a constitutionally active

167 cytoplasmic isoform (Zhou et al., 2003; Lu et al., 2007). Transcript lacking exons 5, 6 and 11 was

168 translated into RONdelta155, a cytoplasmic isoform of RON that was also constitutively active

169 (Zhou et al., 2003; Lu et al., 2007). Partial splicing of exons 5 and 6 (P5P6) produces a RON

170 isoform that lacks the first extracellular immunoglobulin-plexin-transcription domain which

171 express in human pancreatic cancer (Chakedis et al, 2016). The expression of RON wildtype,

172 p165, p160, and p155 transcripts were studied in different cancer tissues. The higher expression

173 of RON transcripts was noted in lung, gastroesophageal, and colon tissues (both normal and

174 cancerous) than breast, prostate, and ovarian tissues (both normal and cancerous)

175 (Wortinger and Liu, 2008). We used an antibody specific for amino acids 531-690 of the

176 extracellular region of beta RON for Western blot analysis in NSCLC and SCLC cell lines but no

177 expression of RON was found.

178 RON splicing variant lacking exons 11-13 is the novel finding of this study. Defective splicing

179 reactions causing large deletions and appearance of early termination codons in mRNAs have been

180 reported to be degraded via non-sense mediated decay (NMD). Even though the deleted sequence

181 is large (415 nucleotides) and deletion of exons 11-13 result in appearance of early termination

182 codon, this transcript is not expected to undergo NMD; this is because alternatively spliced RON

183 transcript lacking exon 6, which acquires a premature early termination codon caused by frame-

184 shift, was shown to yield a viable isoform, RONdelta90 (Eckerich et al., 2009). Also, RONdelta85

185 was shown to be an N-terminally truncated isoform of RON formed due to retention of 49 bases

186 of intron 5 (lying between exons 5 and 6) and consequently undergoing a reading-frameshift (Ma

187 et al., 2010). We expect that the translation product of transcript variant lacking exons 11-13 may

188 be secreted extracellularly in a fashion similar to the two isoforms, RONdelta90 and RONdelta85.

8

189   The isoform product coded by transcript variant lacking exons 11-13 is predicted to act in a

190   dominant negative fashion and block MSP stimulated RON signaling, as in the case RONdelta85

191   and RONdelta90 (Eckerich et al., 2009; Ma et al., 2010). We speculate that the constitutively active

192   isoform, encoded by transcript lacking exon 11, and the dominant negative isoform, which may

193   serve to block ligand dependent RON signaling, together may enable tumors acquire ligand (MSP)

194   independent RON signaling.

195   One of the primary hallmarks of cancer is growth factor independent signaling. However, how

196   cancer cells achieve this is not yet understood and more research on dominant negative isoforms

197   produced by cancer cells may shed light on this aspect. Ubiquitous presence of RON isoforms

198   exhibiting dominant negative functions, such as the secreted ones capable of nullifying the effect

199   of ligand (MSP), truncated transmembrane or cytoplasmic isoforms capable of dimerizing with

200   wild type RON, in cancer also raises important questions regarding the appropriateness of targeting

201   wild type RON, which in fact may lead to tumors.

202   Identification and characterization of all the transcript variants and their protein products is

203   essential for RON target validation in cancer therapeutic development. Presence of alternatively

204   spliced transcripts lacking different coding regions in tumor cells is expected to interfere with

205   estimation of wild type RON expression, either by immunological or PCR methods, leading to

206   exaggerated values. Further, application of siRNAs, which usually lack transcript variant

207   specificity, may knockdown different transcripts affecting results of correlational studies (Celotto

208   and Graveley, 2002). In this context, several genes and their protein products have been found to

209   exert dual roles as tumor suppressors and stimulators, but the specific underlying mechanisms are

210   yet to be determined (Perkins, 2004; Krisenko and Geahlen, 2015). A complete understanding, at

211   a structural and functional level, of the various transcription products of RON is expected to lead

212 to resolving the mechanism underlying their specific roles in cell signaling regulation and cancer

213 development and eventually help us to target its cancer specific signaling.

214 **Conclusion**

215 Tumors are normally screened for RON expression in target identification and validation studies.

216 Even though the functions of many of its isoforms may be different and even oppose each other in

217 some cases, current methods for quantification and functional analysis of RON cannot distinguish

218 between its isoforms. This study describes the identification of a novel alternatively splice site

219 sequence variant of RON that may affect its transmembrane localization. Sequence

220 characterizations presented here together with knowledge of previously identified isoforms point

221 to the need for design and application of isoform specific primers, siRNAs and antibodies for more

222 accurate isoform – functional correlational studies and the therapeutic development. Further,

223 inflammation is expected drive tumor development as well as metastasis and aberrant expression

224 of RON by tumors - via alternative splicing of transcripts - may attenuate its anti-inflammatory

225 functions.

226 **Availability of data and material**

227 The sequence reported in this paper has been deposited with the National Center for Biotechnology

228 Information (NCBI) Sequence Read Archive (SRA) (accession no. SRS354082).

229 **Competing interests**

230 All authors declare no competing interest.

231 **Authors' contributions**

232 SK and ND have made substantial contributions towards design, conceptualization, execution,

233 drafting and revision of the manuscript. AKM and OEA have helped with experimental part. IB

234 and GT have thoroughly revised the MS. MSA has participated in analysis of data. All authors

235 have read and approved the final version of the manuscript for publication.

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348 **<u>Figure legends</u>**

349 **Figure 1. Schematic diagram showing structural features of RON.** A: various domains of RON

350 protein and two juxtaposed tyrosine residues at position 1238 and 1239 respectively (Y-Y) in the

351 kinase domain; carboxy-terminal docking site for multiple substrates with *src* homology 2 (SH2)

352 domain contains two phosphorylation sites for tyrosine at amino acid positions 1353 and 1360; B:

353 20 coding exons of RON with exons are shown in proportion to length; C: PCR amplified and

354 sequenced segment of RON coding sequence, lying between exons 9 and 14; starting nucleotide

13

355 numbers are given for each exon. D: cDNA obtained from different cell lines were PCR amplified

356 using pairs of primers covering increasing lengths RON cDNA sequence. The agarose gel

357 showing fragment sizes above the wells. Results indicate the presence of increasing number of

358 transcripts with increasing amplicon size for each of the cell lines.

359

360 **Figure 2. RON splicing variant lacking exon 11 from cell line H661.** PCR amplification product

361 of RON cDNA from cell line H661 sequenced from 3′ end showing deletion of exon 11. The only

362 splicing variant in the sequencing chromatogram, as seen from peak heights, lacked 147

363 nucleotides, from 2650 to 2796, corresponding to exon 11 (minor peaks in this sequencing

364 chromatogram were not analyzed) of RON reference cDNA sequence.

365 **Figure 3. Splicing variants lacking exon 11 and exons 11-13 from cell line H249.** PCR

366 amplification product of RON cDNA from cell line H249 sequenced from 5′ end showing the

367 presence of two splicing variants; one caused by loss of exon 11 and the other caused by loss of

368 exons 11-13. Exon 10 of the major variant (based on peak heights) ends with TTTGAG sequence,

369 while the same exon ends with TTT in the minor variant due to the skipping of nucleotides GAG.

370 **Figure 4. RON splicing variant lacking exons 11-13 from cell line H1993.** RON cDNA PCR

371 product of cell line H1993 sequenced from 3′ end showing the presence of alternatively spliced

372 transcript variant formed due to deletion of exons 11-13.

373 **Figure 5. Differential splicing of exon 10 in RON transcripts of H1993.** RON cDNA PCR

374 product of cell line H1993 sequenced from 5′ end showing inclusion of nucleotides CAG at the

375 beginning of exon 10 when compared to reference RON sequence. During the formation of

376 reference RON transcript, CAG, which was part of the intron was spliced out.

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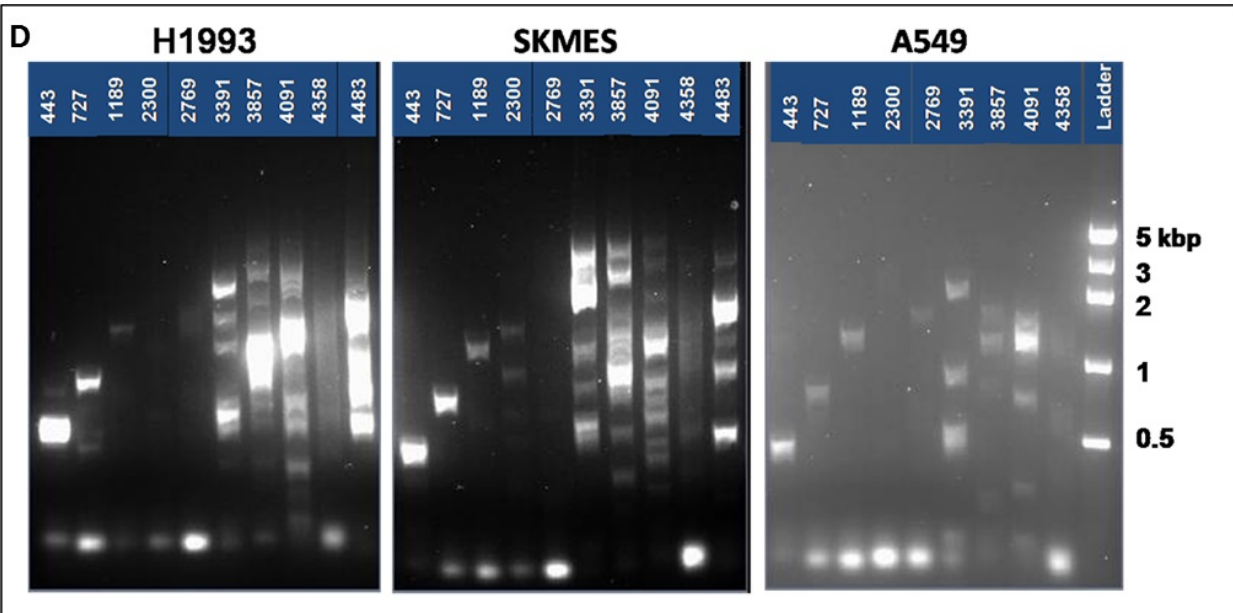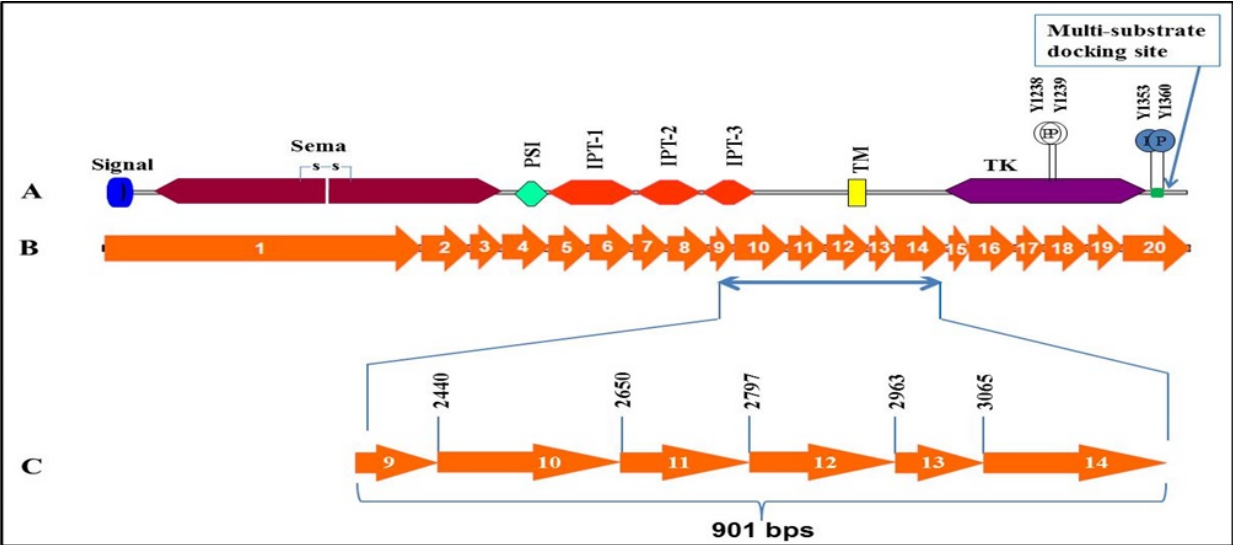384

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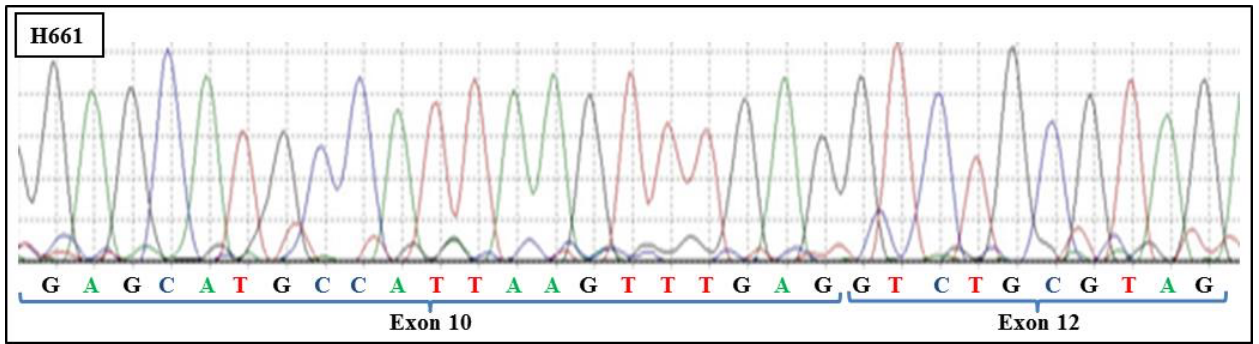390

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398   Figure 1

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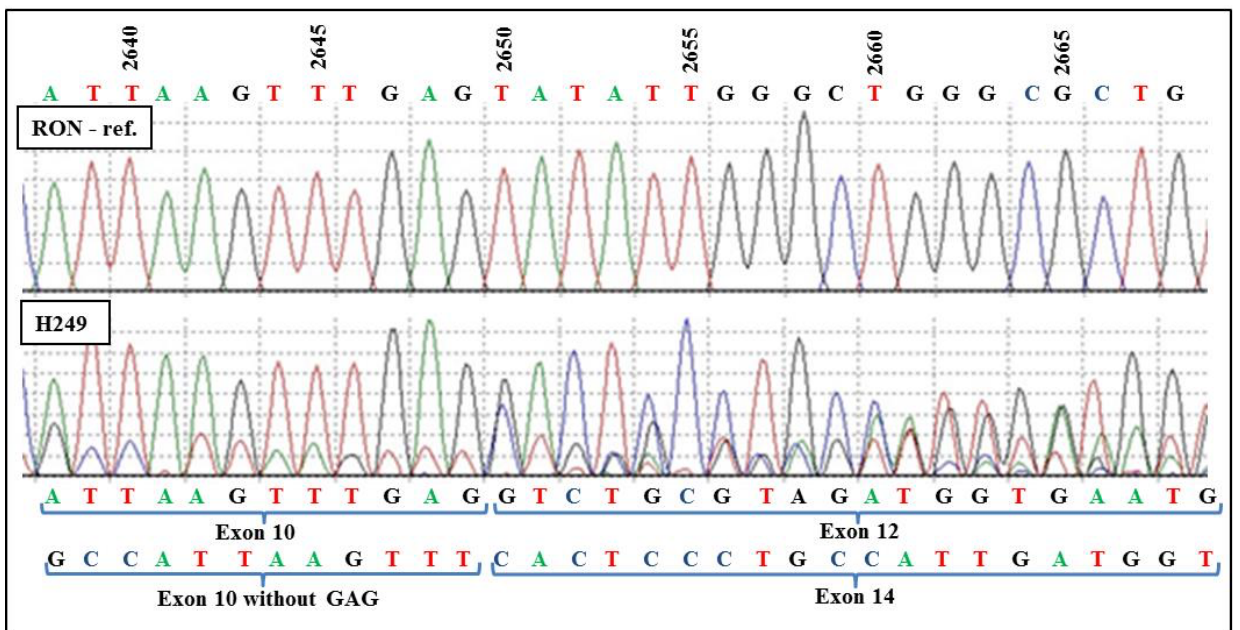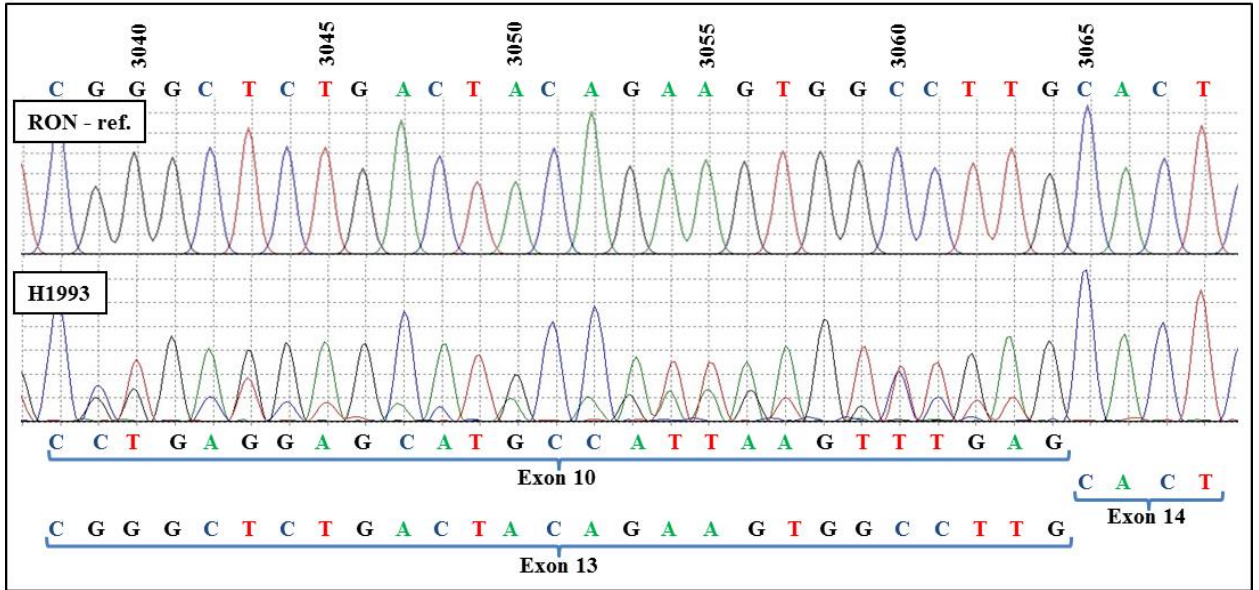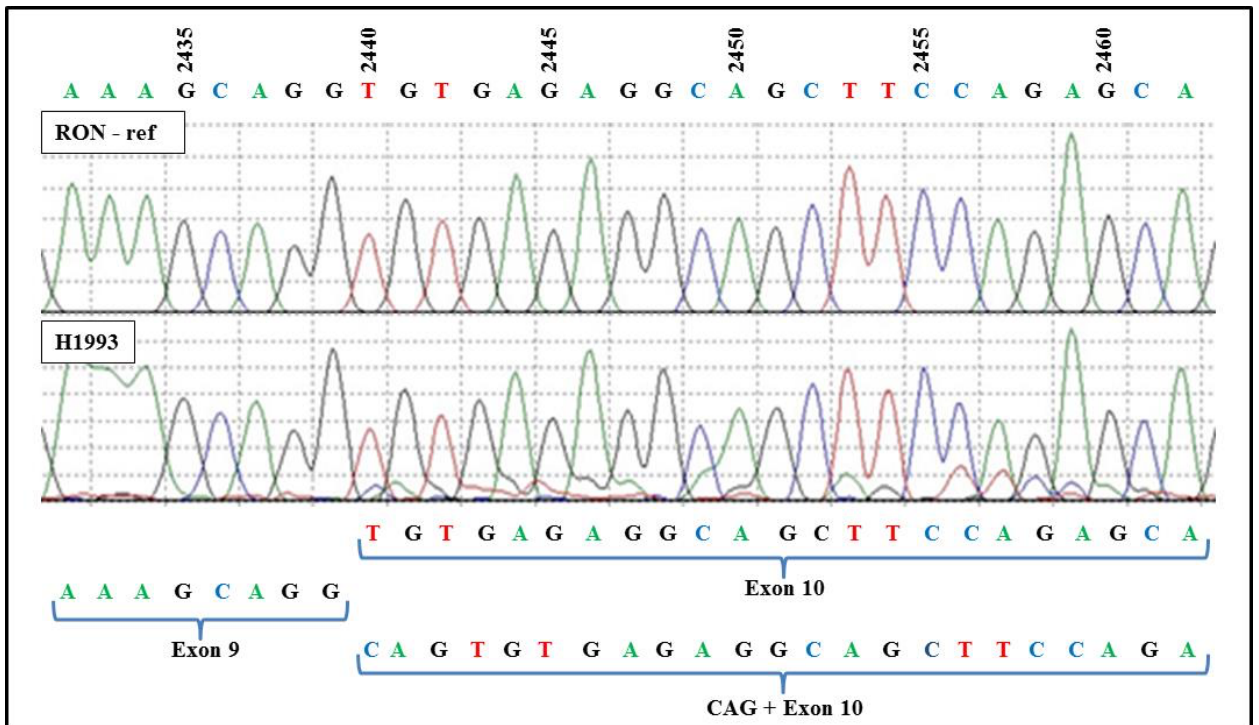405 Figure 2

Figure 3

Figure 4

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417   Figure 5

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