Gene Structure of the Human Receptor Tyrosine Kinase *RON* and Mutation Analysis in Lung Cancer Samples

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The human *RON* gene (*MST1R*) maps to 3p21.3, a region frequently altered in lung cancer and other malignancies. It encodes a receptor tyrosine kinase (RTK) closely related to *MET*, whose mutations are associated with neoplasia. We investigated whether *RON* might be involved in the development or progression of lung cancer. We first determined the exon-intron structure of the gene by direct sequencing of *RON* cosmid DNA and PCR products containing intronic sequences, and then developed primers suitable for mutation analysis by the single-strand conformation polymorphism (SSCP) method. Twenty coding exons were characterized, all but the first one small (average size: 170 bp), a feature shared with other RTK genes. We performed SSCP analysis of *RON* in small and non-small cell lung cancer samples, upon detection of its expression in a sample of lung cancer cell lines. A mutation (T915C: L296P) was found in an adenocarcinoma specimen. Several single nucleotide polymorphisms were also found. The panel of intron-anchored primers developed in this work will be useful for mutation analysis of the *RON* gene in different types of human tumors. © 2000 Wiley-Liss, Inc.

INTRODUCTION

The human *RON* gene (*MST1R*, according to the official nomenclature, see http://www.ncbi.nlm.nih.gov/LocusLink/list.cgi) encodes a receptor tyrosine kinase (RTK) of 1400 amino acids that belongs to the *MET* gene family (Ronsin et al., 1993). It is synthesized as a single-chain precursor that undergoes proteolytic cleavage into two single polypeptide chains linked by disulfide bonds. The mature heterodimeric protein is composed of one α- (35 kD) and one β-chain (150 kD). The α-chain is located extracellularly, whereas the β-chain contains an extracellular portion, a one-pass transmembrane helix, and an intracellular portion that harbors the tyrosine kinase domain. The number and the location of the disulfide bonds are still unknown. RON and its ligand, macrophage stimulating protein (MSP), are involved in the development of epithelial tissues, bones, and neuroectodermal tissues, driving cellular proliferation (Wang et al., 1994; Gaudino et al., 1995; Quantin et al., 1995). The genes coding for RON and MSP both map at 3p21.3 (http://www.ncbi.nlm.nih.gov/genemap/map.cgi?CHR=3, Human Genome Map 1998), a region frequently altered in lung, kidney, and other malignancies (Brauch et al., 1987; Zbar et al., 1987; Ronsin et al., 1993; Kok et al., 1997). The predicted amino acid sequences of *RON* and *MET* have overall 33% identity, which increases to 64% in the kinase domain.

Missense mutations located in the tyrosine kinase domain of the *MET* gene have been identified in the constitutional DNA of affected members of families with hereditary papillary renal cell carcinoma and in a subset of sporadic renal cell carcinomas of the same histology (Schmidt et al.,1997, 1998; Zbar and Lerman, 1998). These are likely to be gain/change-of-function mutations that lead to constitutive activation of the MET protein and tumorigenesis in the renal tissue (Schmidt et al., 1997, 1998). Similar mutations occurring in other RTKs are associated with different human malignancies, namely, mastocytomas and gastrointestinal tumors in the case of KIT (Nagata et al., 1995; Piao and Bernstein, 1996; Hirota et al., 1998), multiple endocrine neoplasia types 2A and 2B, and familial medullary thyroid carcinomas in the case of RET (Mulligan et al., 1993; Hofstra et al., 1994). Muta-

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tions (D1232V and M1254T) introduced into the RON kinase domain induce oncogenic and metastatic properties (Santoro et al., 1998). These mutant RON receptors show an increased kinase catalytic efficiency. Additional findings, both in vivo and in vitro, suggest that *RON* could be an oncogene. In liver progenitor cells, RON activation has a strong cell dissociation, motogenic and mitogenic activity (Medico et al., 1996). In a breast cancer cell line (ZR75.1), RON activation led to more rapid proliferation, migration, and invasion (Maggiora et al., 1998). Rearrangements occurring in the extracellular region of the protein result in altered kinase activity. For example, a *RON* splicing variant discovered in a gastric carcinoma cell line (KATO-III) was found to be responsible for invasive properties acquired in vitro by these cells (Collesi et al., 1996). The uncleaved single-chain RON protein undergoes aberrant intermolecular disulfide dimerization and intracellular oligomerization, and shows constitutive activation along with the potential of eliciting a motile-invasive phenotype. The effects on cell motility shown by the RON/MSP system were intriguing in view of the high metastatic activity of SCLC cells.

To investigate whether the putative proto-oncogene *RON* could contribute to the development or progression of lung cancer, we first showed that the gene is expressed in lung cancer cell lines. Next, we performed mutation analysis of the gene in 55 paired lung cancer samples (Johnson et al., 1988). *RON* cDNA was previously cloned (Ronsin et al., 1993), but the gene exon-intron structure was not elucidated. To perform a detailed mutation analysis of the *RON* gene in the lung cancer samples, it was necessary to define the exon-intron structure and develop intron-based primers suitable for exonic and splice-site sequence study. We solved the gene structure through a PCR/sequencing strategy, and designed intronic primers to apply the single-strand conformation polymorphism (SSCP) method to do mutation analysis. We analyzed for mutations in the kinase-domain exons and the region containing the protein cleavage site in exon 1.

MATERIALS AND METHODS

Northern Blot Analysis

Filters carrying polyA+ mRNA from several lung cancer cell lines were prepared according to Sambrook et al. (1989). Radioactive DNA probes were prepared by random priming (Rediprime II, Amersham, Arlington Heights, IL). Hybridization was performed in ExpressHyb hybridization solution according to the manufacturer's instructions (Clontech Laboratories, Palo Alto, CA).

Determination of the Exon-Intron Structure

To define the exon-intron boundaries of *RON*, we designed several PCR primers on the cDNA sequence (GenBank accession number X70040). These primers were used on *RON* cosmid clones and *RON* cDNA. Comparing the PCR products by size allowed us to determine whether an intronic sequence was present. The sequencing of the PCR products to identify intronic sequence led to the characterization of the exon-intron boundaries. When this approach did not work, because of the presence of a large intron, we performed direct primer-walking sequencing on the cosmid DNA.

PCR Reactions

Each PCR primer pair (GIBCO-BRL, Gaithersburg, MD; BioServe Ltd, Laurel, MD) was tested by running the product on a 4% 3:1 Nu-Sieve agarose gel (FMC, Rockland, ME). All pairs were used under the following cycling conditions: 1 min at 95°C, 30 sec at 64°C, 30 sec at 72°C, for 35 cycles. PCR products were cloned using a TA Cloning Kit (Invitrogen, Carlsbad, CA).

Sequencing

Sequencing reactions were done either manually (T7 Sequence Kit, Amersham, Arlington Heights, IL) or automatically (ABI 373 Stretch Automated DNA Sequencer, Applied Biosystems, Foster City, CA).

Web-Based Sequence Analysis Servers

The web-based sequence analysis servers used were: http://gnomic.stanford.edu/GENSCAN.html and http://www.sanger.ac.uk/Pfam/search.shtml.

Single-Strand Conformation Polymorphism Analysis (SSCP)

Each exon of the kinase domain, plus a region containing the proteolytic cleavage site in exon 1, was examined for mutation using a specific PCR primer pair (Table 1). The radioactive reaction was performed in a total reaction volume of 12.5 μl, containing 100 ng of genomic DNA, 12.5 pmol of each primer, 200 μM dNTPs, 1.5 mM $MgCl_2$, 1.25 nCi α^{35}S-dATP, and 0.5 U AmpliTaq DNA polymerase (Perkin-Elmer). After heat denaturation (8 min at 90°C) in formamide buffer (Stop Solution, Amersham, Arlington Heights, IL), PCR products (ranging in size from 88 to 298 bp) were run overnight in a 0.5× MDE gel (FMC Bioproducts,

TABLE 1. PCR Primers Used for SSCP Analysis of the *RON* Gene Kinase Domain-Coding Exons (exons 14–20) and Cleavage Site (exon 1)

PCR product	Size in bp	Forward primer	Reverse primer
876F-971R	115	5′TTAGCGCCACTGAGCCAGAGTTGG	5′AGCACAGGGTAGGGCTGTCC
921F-1011R	112	5′TCGACTGCAGATTTGCTCCA	5′GTGGCAAGTTGGGCACCCACTG
EXON 14	159	5′GCGGAAAGAGTCCATCCAG	5′CTTGAGACTCCATCTCTGC
EXON 15	113	5′ATTATGCACCTCACACCAGGCCAC	5′CTGCCCCACTTACGACTTAGTGAC
EXON 16	206	5′TCGCTCTGCAGGCATCACAGAGATG	5′GATGAACACTGACCCGCTGAGGTGA
EXON 17	162	5′TTGCCCACCAACCCACCTGTG	5′CACCCCAGCTACTCTGGACTC
EXON 18	223	5′AGTCCTAAGTGTGATCCTCTCCCTAC	5′GCCTCACCACATCAGACTTGGTGGT
EXON 19	163	5′CCACAGTGGTCATTTGGTGTGCTGC	5′CCACCTCCACATACTCACAGAGAATCAG
EXON 20	108	5′CAGGTACCAAGTGATGCAGCAATGCTG	5′TCCCCAAGCAGTGCAGACACTATCTG

Rockland, ME), 0.6× TBE, at room temperature, 8 W constant power, transferred to 3 MM paper, dried, and exposed for autoradiography on film (X-OMAT AR, Kodak, Rochester, NY).

Patient DNA and Cell Lines

Fifty-five normal/tumor paired samples were analyzed, including thirty-four small cell lung carcinomas (SCLCs) and twenty-one non-small cell lung carcinomas (N-SCLCs). Tumor and lymphoblastoid cell lines were established from patients with lung cancer according to techniques previously described (Phelps et al., 1996). Tumor and normal tissue were obtained from patients at surgical resection or post-mortem examination (Johnson et al., 1988). The following DNA samples from CEPH individuals were used as negative controls: 1334-01, 1334-02, 66-01, 66-02, 45-01, 45-02, 1331-01, 1331-02, 1345-01, 1345-02, 28-01, 28-02, 21-01, 21-02, 1347-01, 1347-02, 1332-12, 1332-13, 1349-01, 1349-02, 1350-10, 1350-11, 1421-10, 1421-11, 1416-10, 1416-11, 1346-01, 1346-02, 1400-01, 1400-02, 104-01, 104-02, 1355-01, 1355-02, 1427-01, 1427-02, 12-10, 12-11, 17-01, 17-02, 884-01, 884-02, 1420-10, 1420-11.

RON Cosmids

RON cosmid clones (designated numbers 5, 6, 11, 12, 19, and 20) were isolated by screening of a human fetal brain genomic library in the SuperCos 1 vector using the *RON* cDNA as a probe under conditions specified by the vendor (Stratagene, La Jolla, CA). The average insert size of these clones was estimated to be between 35 and 45 kb.

Site-Directed Mutagenesis

T915C base change was introduced into *RON* cDNA using the GeneEditor In Vitro Site-directed Mutagenesis System (Promega, Madison, WI), according to the manufacturer's instructions. The mutagenesis primer sequence was 5′ P-CTA TCG GGA GCC GGT CCT CG (GIBCO-BRL, Gaithersburg, MD).

Cell Lines and Culture Conditions

MDCK cells (ATCC, Manassas, VA) were stably transfected with wild-type or L296P *RON* cDNA in pCl-neo vector (Promega, Madison, WI) or with empty pCI-neo vector (MOCK) by superfect reagent (Quiagen, Santa Clara, CA), and placed in medium with 500 µg/ml geneticin (GIBCO-BRL, Gaithersburg, MD). After two weeks, the geneticin-resistant cells were picked up and expanded into cell lines. Expression of recombinant RON was tested by Western blotting with anti-RON antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Transfected MDCK cell lines were cultured in DMEM (GIBCO-BRL) with 10% FCS (HyClone Lab, Logan, UT).

Cell Stimulation, Lysis, and Western Blotting

After overnight starvation in DMEM without FCS, cells were collected from dishes and stimulated in suspension with 5 nM MSP (Toyobo, Osaka, Japan) for 15 min. After stimulation, cells were lysed in lysis buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 10% glycerol, 1 mM EDTA, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 100 mM NaF, 1% Triton X-100, 10 µg/ml leupeptin, 10 U/ml aprotinin, 1 mM PMSF). Insoluble material was removed by centrifugation. RON receptor was immunoprecipitated from supernatants using rabbit polyclonal antibodies designed on the RON receptor C-terminus (Santa Cruz Biotechnology). RON tyrosine phosphorylation was detected by Western blotting using anti-phosphotyrosine antibodies (anti-PY, clone 4G10, UBI, Lake Placid, NY). Rabbit anti-RON antibodies were used for detection of RON receptor on the membranes.

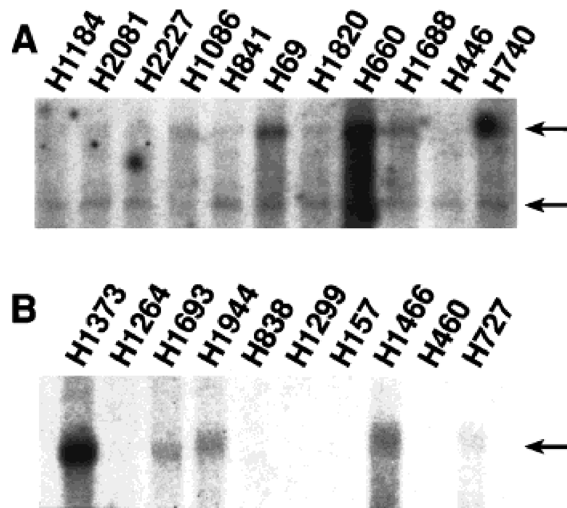


Figure 1. Northern blot analysis. *RON* complete cDNA was used as probe in Northern blot experiments. (**A**) poly-A+ mRNA from small-cell lung cancer cell lines. (**B**) poly-A+ mRNA from non-small cell lung cancer cell lines. The arrow in B and the upper arrow in A point to a 4.5 kb transcript. A smaller transcript was found in small-cell lung cancer cell lines (2 kb, lower arrow in blot A).

RESULTS

RON Gene is Not Homozygously Deleted in Lung Cancer Cell Lines

To investigate the retention of *RON* in the DNA of lung cancer cell lines known to have homozygous deletions in 3p21.3 (Wei et al., 1996), we performed PCR with primers for exon 16 (Table 1). We analyzed the lung cancer cell lines GLC 20, NCI-H740, and NCI-H1450 that harbor the deletions (Wei et al., 1996). The gene is not deleted in any of the three tested cell lines (data not shown).

Expression Study in Lung Cancer Cell Lines

RON cDNA was used as a probe in Northern blotting experiments. A panel of small and non-small lung cancer cell lines showed high to moderate expression of the 4.5 kb species in the majority of samples (Fig. 1A upper arrow; 1B). In addition, a signal of approximately 2 kb was detected in small, but not in non-small cell lines (Fig. 1A, lower arrow).

Characterization of the Exon-Intron Structure

Several PCR primer pairs were designed to cover the whole *RON* cDNA sequence (GenBank accession number X70040). Comparison of PCR products generated from genomic DNA and *RON* cDNA allowed detection of the position of intronic sequences. In the case of large introns, we performed direct primer-walking sequencing using cosmid DNA. We identified a total of twenty exons (Fig. 2). The first exon is also the largest, spanning 1229 bp. The last exon extends from nucleotide 3976 to 4532, where the polyA tail is inserted. Our results confirm the existence of two previously reported exons at bases 2678 and 2825 (Collesi et al., 1996). With the exception of the first one, the coding exons are small, ranging in size from 93 bp to 253 bp (Table 2). The first, 14th, and 19th introns were not completely sequenced. Intron 19 was estimated to be about 2.5 kb in size. The whole gene cannot span more than about 35–45 kb, because it is contained in a single cosmid clone (data not shown). The intronic sequences were deposited with the GenBank (accession numbers: AF164633, AF164634, AF164635, AF164636, AF164637, AF164638, AF164639, AF164640, AF164641, AF164642, AF164643, AF164644, AF164645, AF164646, AF164647, AF164648, AF164649, AF164650, AF164651, AF164652, AF164653, AF164654).

By using the GENSCAN gene prediction program (Burge and Karlin, 1997; http://gnomic.stanford.edu/GENSCAN.html), we checked whether any alternative splicing could be detected. We found that exon 10 could begin with a CAG triplet (coding for a Gln residue) upstream of the canonical TGT (coding for the canonical Cys) without modifying the consensus splicing site sequence 5′…nCAG3′. Another interesting possibility was found at residue 1091, where, after the canonical VIGKG sequence, the theoretical alternative splicing inserts a stretch of bases encoding 6 more amino acids (namely, VGARPG). This last possibility occurs at the very beginning of the kinase domain. A PCR experiment was performed with cDNAs from human fetal and adult lung (Clontech, Palo Alto, CA) to search for the predicted alternatively spliced forms of *RON*. This experiment gave negative results (data not shown). It is worth mentioning, however, that inactivating mutations responsible for disease states caused by the Wilms' tumor suppressor gene (*WT1*) occur in amino acid residues subjected to alternative splicing (Hewitt et al., 1996).

Mutation Analysis

With a panel of primers (Table 1), we performed SSCP analysis of a stretch of bases in exon 1, encoding the putative cleavage site (KRRRR) in the RON protein, to verify whether any mutation at this site could interfere with correct maturation of the protein. We also analyzed exons 14, 15, 16, 17, 18, 19, and part of 20, that encode the tyrosine

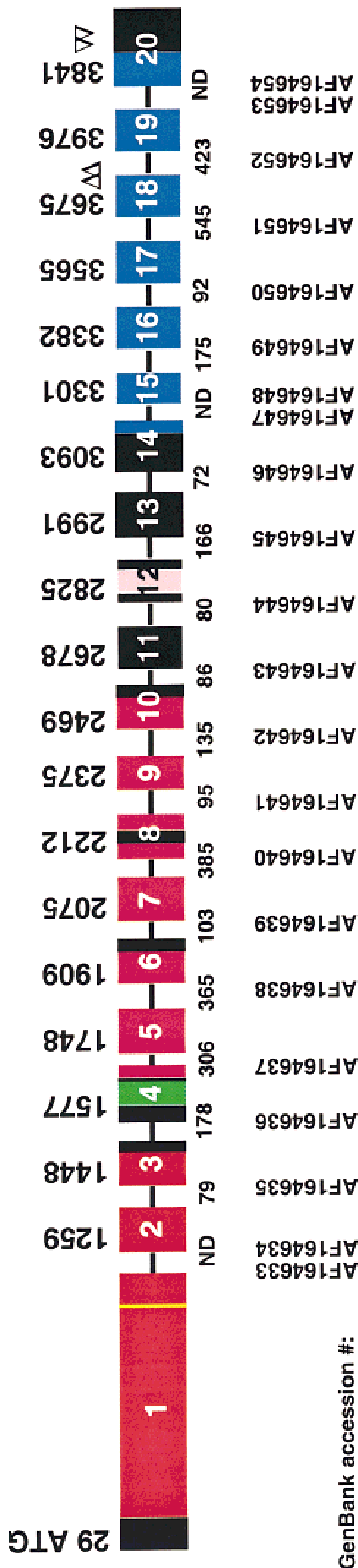


Figure 2. Gene structure of the human receptor tyrosine kinase *RON*. Four-digit numbers refer to splice site location, based on *RON* cDNA sequence (GenBank accession number X70040). Lower numbers refer to the intron length in bp. Red boxes: SEMA domain (amino acids 57–507, http://www.sanger.ac.uk/Pfam/search.shtml). Yellow stripe: putative KRRRR cleavage site. Green box: plexin domain (amino acids 526–568). Fuchsia boxes: IPT domains (amino acids 569–671, 684–767, 770–855; Bork et al., 1999) Pink box: putative transmembrane domain (amino acids 958–982). Blue boxes: kinase domain (amino acids 1073–1335). (△) Tyrosine residues, major putative autophosphorylation sites (Y1238, Y1239). (▽) SH2 domain binding site (Y1353, Y1360). GenBank numbers provide accession to intronic sequences.

kinase domain (PFAM: Bateman et al., 1999; http://www.sanger.ac.uk/Pfam/search.shtml).

A T-to-C transition was found at base 915 (tat cgg gag ct/cg gtc ctc) in the tumor DNA of an adenocarcinoma patient (Patient 122-208). This base change causes an L-to-P change at residue 296 (Fig. 3A) and is present neither in the blood-derived DNA of the patient (Fig. 3A) nor in 44 control individuals (CEPH individuals' DNA; Dausset et al., 1990). The mutation eliminates an *Alu*I site and allows the formation of a *Cfr*10I restriction site, which cuts the PCR product amplified by primers 876F-917R (Table 1) in two fragments of 38 and 77 bp. A change was found at base 993: an A-to-G transition leads to a Q322R amino-acid change (Fig. 3B). This is a single nucleotide polymorphism (SNP), already observed by Collesi et al., (1996). It is present in the normal population with an index of heterozygosity of 0.28 ($P = 0.17$, $q = 0.83$). This SNP cancels a *Bsp*MI restriction site in the PCR product of primers 921F-1011R (Table 1), otherwise cut into two fragments of 31 and 71 bp. In exon 20, two SNPs were found (Fig. 3C, 3D): a C-to-T transition of base 4024 (gtg cga ccc/t acc ttc aga; same-sense variant P1341P) and an A-to-G transition of base 4031 (ccc acc ttc a/gga gta: R1344G). Both SNPs were found in CEPH individuals' DNA (Dausset et al., 1990), with indices of heterozygosity, respectively, of 0.03 and 0.46. These SNPs do not produce any change in the restriction map.

Protein Analysis

Because the T915C mutation was found only in a tumor sample, we investigated the kinase activity of the L296P RON mutant. To compare the mutant protein with wild-type RON, we introduced the T915C base change into *RON* cDNA, through a site-specific mutagenesis procedure. MDCK cells stably transfected with C915T-mutagenized *RON* cDNA express RON protein that shows the same electrophoretic profile as the wild-type protein (Fig. 4A). Therefore, the mutation, although close to the cleavage site, does not block the precursor cleavage and protein maturation. The mutated protein is responsive to MSP, and the kinase activity, elicited upon MSP stimulation, is equivalent to the wild-type activity (Fig. 4B).

DISCUSSION

We established the exon/intron structure of the human *RON* gene. The coding sequence is represented by twenty exons. Ranging in size from 93–253 bp, they all are quite small, a feature shared

TABLE 2. Data on Exons and Introns of the Human *RON* Gene

Exon no. (length in bp)	Ends[a]	Intron no.	Type[b]	Intron 5′ end[c]	Length in bp	Intron 3′ end[c]
1 (1229)	ATG 29–1258	1	0	CCCAACCCG(1258)/gtaagctga	ND	ctttcccag/(1259)CCTGGCCTG
2 (188)	1259–1447	2	0	TATCCTGCAG/gtgggtcctc	79	cctgtcctag/GTGGAGCTGG
3 (128)	1448–1576	3	0	TGGGGACCAG/gtgaggtggg	178	tcacccacag/GTTTTCCAGG
4 (170)	1577–1747	4	0	GCTTACTGAG/gtatggcttc	306	cccatttcag/TTCCACCCCC
5 (160)	1748–1908	5	II	CAAAACTCAG/gtacaatctg	365	tggtgccccag/ACCAGTGCCC
6 (165)	1909–2074	6	0	CTCTTTCATG/gtgaggctac	103	gtcctcttag/GAGCCAGTGC
7 (136)	2075–2211	7	II	TGCTAGCACG/gtaagtacca	385	gttcctgtag/GGTCAGTGAG
8 (162)	2212–2374	8	II	GTGGCTACAT/gtaagcactg	95	tcttccacag/CAACTCCCAC
9 (93)	2375–2468	9	0	GGAAAGCAGG/gtgagtgagt	135	gtcccagcag/TGTGAGAGGC
10 (208)	2469–2677	10	0	TAAGTTTGAG/gtaagtgtaa	86	ctctctgcag/TATATTGGGC
11 (146)	2678–2824	11	0	CCCATTGCAG/gtaggcagcc	80	ctgcccccag/GTCTGCGTAG
12 (165)	2825–2990	12	I	AAGCAGCTAG/gtgagttctc	166	ctttccacag/TTCTTCCTCC
13 (101)	2991–3092	13	I	AGTGGCCTTG/gtgagatagt	72	ttccccacag/CACTCCCTGC
14 (207)	3093–3300	14	I	ATTGGCAAAG/gtgtgggggc	ND	ctcagaccag/GCCACTTTGG
15 (80)	3301–3381	15	I	TCACTAAGTC/gtaagtgggg	175	cgctctgcag/GCATCACAGA
16 (182)	3382–3564	16	0	ACCTCAGCGG/gtcagtgttc	92	gtgcccccag/AACCCCACCG
17 (109)	3565–3674	17	II	GGAACTGCAT/gtgagagtcc	545	taccccctaag/GCTGGACGAG
18 (165)	3675–3840	18	0	GTCTGATGTG/gtgaggcccc	423	ctttccacag/TGGTCATTTG
19 (136)	3841–3977	19	II	CTGATTCTCT/gtgagtatgt	ND	cccccaacag/GTACCAAGTG
20 (556)	3978–4534					

[a]Four-digit numbers refer to *RON* cDNA sequence (GenBank acc. No. X70040).
[b]As classified in Sharp (1981).
[c]Capital letters represent exonic sequence.

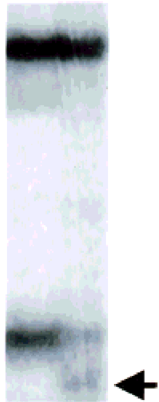
with other receptor tyrosine kinases (Andre et al., 1992; Kwok et al., 1993; Duh et al., 1997). All intronic splice junction sites show the consensus sequence 5′ GT (x)n AG 3′ (Mount, 1982). Excluding the three largest ones, the intronic sequences have an average size of 205 bp, ranging from 79–545 bp in length.

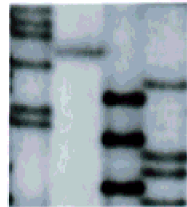
There is quite a remarkable conservation of the exon-intron organization of the human *RON* gene and its mouse ortholog, *Stk* (Waltz et al., 1998). The intervening sequences are of the same type (as classified by Sharp, 1981) and in the same place, with the exception of the human exon 13, which, along with the adjacent intron 13, is not present in the mouse gene. Similarly to the mouse *Stk* gene (Waltz et al., 1998), the first exon is the largest and codes for the putative proteolytic cleavage site KRRRR. The PFAM (http://www.sanger.ac.uk/Pfam/search.shtml) protein domains alignment program detected a SEMA domain, located in the first, second, and third exons of the *RON* gene (corresponding to amino acid residues 58–507). The SEMA domain consists of a highly conserved stretch of about 500 amino acids (Kolodkin et al., 1993), initially identified in the Semaphorin family of proteins. It is characterized by fifteen conserved cysteines, one conserved potential *N*-linked glycosylation site, and several blocks of conserved residues throughout the domain (Kolodkin et al., 1993). Besides the RON and MET receptor tyrosine kinases (human and orthologs), PFAM retrieves the SEMA domain in Semaphorins, Neuropilins, and the orphan receptors of the *SEX* family (Maestrini et al., 1996), expressed in neural and epithelial cells. Unlike the RON/MET family, the SEX family proteins do not have any kinase activity.

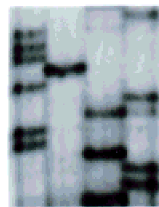
Although the role of the SEMA domain is not clarified, it was shown that it could function in homophilic binding in the presence of calcium ions (Ohta et al., 1995). We propose that, owing to its location in RON and MET, the SEMA domain could be involved in ligand binding and receptor homodimerization upon ligand stimulation. Studies are ongoing to investigate the functional role of the SEMA domain in RON. Other structural features are located as follows (Fig. 2): a plexin domain (http://www.sanger.ac.uk/Pfam/search.shtml) in exon 4; three IPT domains (Bork et al., 1999)

Figure 3. Mutation analysis. The SSCP profile of each base change is shown, along with the sequence. A dot marks the mutated base. (**A**) T915C: L296P mutation. T: DNA from a lung adenocarcinoma sample. The arrow points to the mutated base found in this sample. The mutation affects the SEMA domain. N: blood-derived DNA from the same patient. (**B**) A993G; Q322R SNP. This polymorphism occurs in the SEMA domain. SNPs occurring in exon 20: (**C**) C4024T:P1341P, (**D**) A4031G:R1344G.

A







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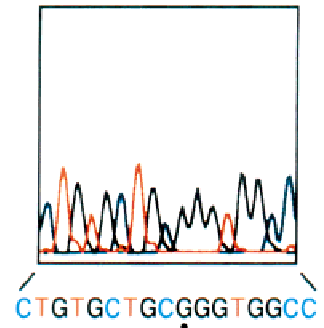

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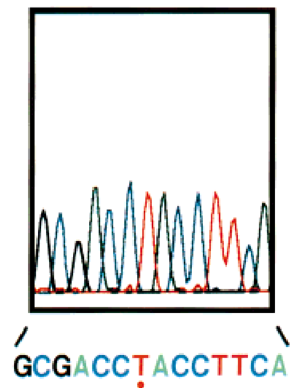

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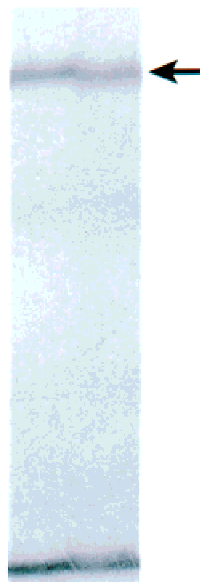

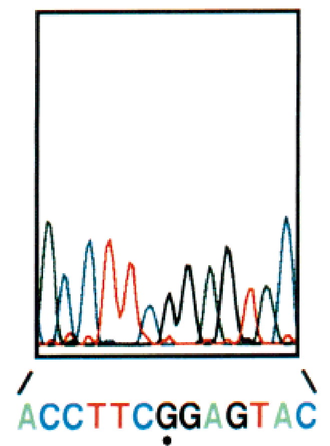

Figure 3.

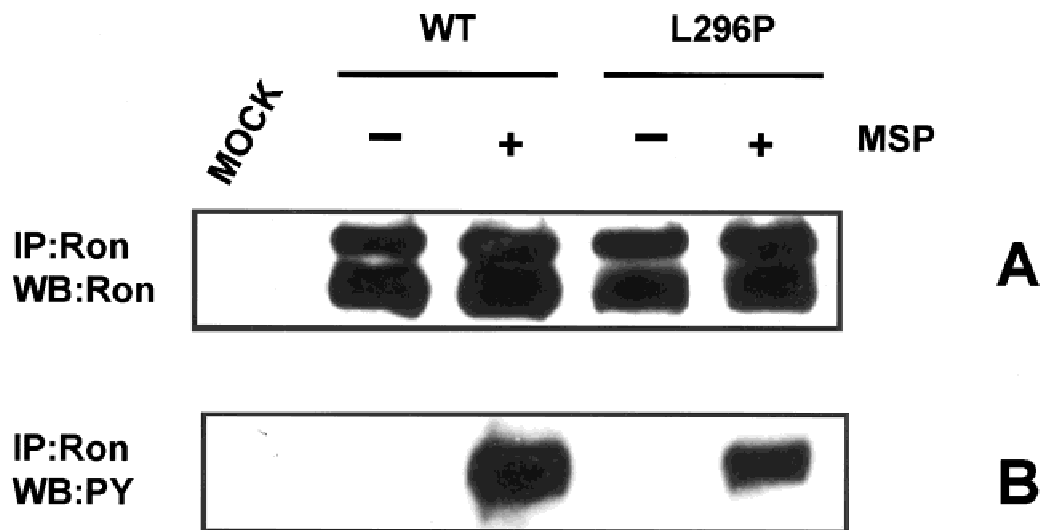

Figure 4. Analysis of the mutant RON protein. MDCK cells stably expressing wild-type RON (WT) or L296P mutant were stimulated with MSP (see Materials and Methods), and then lysed. RON was immunoprecipitated from cell lysates. Parental MDCK cells transfected with empty vector (MOCK) were used as a negative control. (**A**) Immature (upper band) and mature (lower band) forms of RON. This blot, developed with anti-RON antibodies, serves also as a control for the amount of RON in precipitates. (**B**) RON tyrosine phosphorylation was detected by Western blotting with anti-phosphotyrosine (PY) antibodies.

distributed in exons 4–10; a putative transmembrane domain in exon 12; the tyrosine kinase domain in exons 14–20; two conserved, adjacent tyrosine residues, that should serve as putative major autophosphorylation sites in exon 18; and the SH2 binding site (Ponzetto et al., 1994; Zhen et al., 1994) in exon 20.

The hypothesis of RON involvement in lung cancer was tested by mutation analysis of the kinase domain (exons 14–20), because evidence exists that oncogenic mutations found in the MET kinase domain, when introduced into RON, are able to activate its oncogenic potential (Santoro et al., 1998). We also analyzed the region containing the cleavage site in exon 1, to check for mutation that could possibly interfere with the cleavage/maturation process. Using the SSCP protocol followed by sequencing, we identified a C-to-T transition at base 915 (leading to L296P mutation) in the tumor DNA of an adenocarcinoma patient. Leu296 is conserved among human, mouse, and Xenopus *RON* orthologues. It is substituted by Ile, a very similar amino acid, in the chicken homologue, c-SEA. Despite this conservation, however, the mutated amino acid does not seem to play a fundamental role, because the substitution L296P does not change the protein cleavage and maturation (as deduced by the electrophoretic profile) and the ligand-induced phosphorylation status, and therefore, most likely, the overall protein function.

Thus, from our mutation analysis in lung cancer samples, we can rule out the possibility that RON plays a causative role in lung cancer initiation, at least with a mechanism involving mutations of the kinase domain or the cleavage site in exon 1. Several polymorphisms were found: A993G:Q322R (confirming the observation of Collesi et al., 1996); C4024T:same-sense variant; and A4031G:R1344G.

While this manuscript was in preparation, Persons et al. (1999) reported that the FV2 locus, which confers susceptibility to the Friend erythroleukemia virus in mice, encodes a truncated form of Stk, the mouse ortholog of RON (Waltz et al., 1998). Noticeably, the Northern analysis of small-cell lung cancer cell lines (Fig. 1A) showed a smaller (about 2 kb) transcript of *RON*, present in all samples, whereas the normal-size transcript was absent in some. This circa 2 kb transcript was not present at all in the non-small cell lines (Fig. 1 B), that expressed only the normal (4.5 kb) transcript. We are in the process of studying the nature and possible role of this smaller transcript in small cell lung cancer. In fact, a transcript approximately 2 kb in size was reported by others in some normal tissues (Ronsin et al., 1993; Gaudino et al., 1994), but the nature, function, and possible significance of this molecule in human physiology and pathology remain unknown at present.

In conclusion, further research is required to discover human tumors in which *RON* mutations

may play a causative role. The panel of primers developed in this study is a useful tool for further mutation analyses in human malignancies.

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