Integrative genomic analyses of recepteur d'origine nantais and its prognostic value in cancer

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Abstract. Recepteur d'origine nantais (RON) is a receptor tyrosine kinase (RTK) normally expressed at low levels in epithelial cells. RON is a 180-kDa heterodimeric protein composed of a 40-kDa α-chain and a 150-kDa transmembrane β-chain with intrinsic tyrosine kinase activity. The extracellular sequences of RON contain several domains including an N-terminal semaphorin (sema) domain, followed by the plexin, semaphorin, integrin (PSI) domain, and four immunoglobulin, plexin, transcription factor (IPT) domains. Here, we identified RON genes from 14 vertebrate genomes and found that RON exists in all types of vertebrates including fish, amphibians, birds and mammals. We found that the human RON gene showed predominant expression in the liver, lymph node, thymus, intestine, lung, mammary gland, bone marrow, brain, heart, placenta, bladder, cortex, cervix, skin, kidney and prostate. When searched in the PrognoScan database, human RON was also found to be expressed in bladder, blood, breast, glioma, esophageal, colorectal, head and neck, ovarian, lung and skin cancer. The relationship between the expression of RON and prognosis was found to vary in different cancer types, even in the same cancer from different databases. This suggests that the function of RON in these tumors may be multidimensional, not just as a tumor suppressor or oncogene. Six available single-nucleotide polymorphisms (SNPs) disrupting existing exonic splicing enhancers were identified in RON. This may contribute to the generation of active RON variants by alternative splicing, which is frequently observed in primary tumors.

Introduction

Recepteur d'origine nantais (RON) is a receptor tyrosine kinase (RTK) normally expressed at low levels mostly in epithelial cells (1,2). The human RON gene was originally cloned from keratinocytes (1). It contains 20 exons and 19 introns and is located on chromosome 3p21 (1-3), a region frequently altered in certain cancer types (4). The RON cDNA encodes 1,400 amino acids, which are synthesized first as a single-chain precursor (pro-RON) (1). Maturation occurs in the cell membrane resulting in a 180-kDa heterodimeric protein composed of a 40-kDa α-chain and a 150-kDa transmembrane β-chain with intrinsic tyrosine kinase activity (5,6). The extracellular sequences of RON contain several domains including an N-terminal semaphorin (sema) domain, followed by the plexin, semaphorin, integrin (PSI) domain, and four immunoglobulin, plexin, transcription factor (IPT) domains (7).

RON is activated in response to macrophage-stimulating protein (MSP), and then induces an invasive program (8) consisting of cell proliferation, migration, and invasion, all of which are important at multiple points during tumorigenesis. RON gene transcripts are present in the liver, lung, brain, kidney, bone, adrenal glands, testis and digestive tract (2). RON was found to be primarily expressed in cells of epithelial origin such as colon, breast and skin (9). Constitutively active RON variants may be generated by alternative splicing (RONΔ165, RONΔ160, and RONΔ155) or by methylation-dependent promoter usage [short form RON (sfRON)] (10,11). Among these RON variants, RONΔ160 is located at the plasma membrane, whereas RONΔ165 and RONΔ155 are retained in the cytoplasm. sfRON lacks almost all of the extracellular domain and is incapable of ligand binding. Recent studies have indicated that altered RON expression contributes significantly to cancer progression and malignancy. In primary tumors, such as colon and breast cancers, overexpression of RON exists in a large number of cases and is often accompanied by the generation of different splicing variants (12-14). However, a comprehensive investigation of whether RON is involved in the formation of various types of tumors has not been adequately carried out.

In the present study, we identified RON genes in the human, chimpanzee, macaque, orangutan, dog, cow, horse, mouse, rat, opossum, chicken, *Xenopus tropicalis*, zebrafish, and fugu by comparative genomic analyses. Conserved transcription factor-binding sites within promoter regions of human RON genes were then searched. The expression data, functional relevant single-nucleotide polymorphisms

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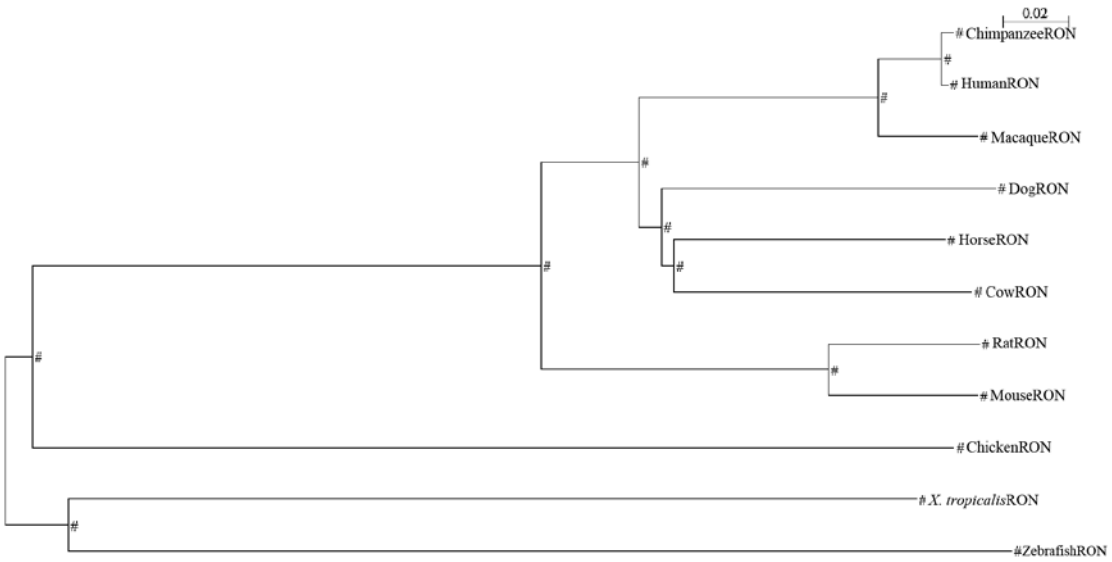

Figure 1. Phylogenetic analysis of RON. RON genes were identified in the genome sequences of the human, chimpanzee, macaque, orangutan, dog, cow, horse, mouse, rat, opossum, chicken, *Xenopus tropicalis*, zebrafish and fugu. The phylogenetic tree of the RON gene was obtained using maximum likelihood and neighbor-joining methods. It appeared that primate RON was clustered into one group, different from other RON genes.

(SNPs) and comparative proteomic analyses were conducted. Furthermore, meta-analysis of the prognostic value of RON genes in various cancers was also performed.

Materials and methods

Identification of novel RON genes in vertebrate genomes and integrative genomic analyses. RON genes were searched in the genome sequences of the human (*Homo sapiens)*, chimpanzee (*Pan troglodytes*), macaque (*Macaca mulatta*), orangutan (*Pongo pygmaeus)*, dog (*Canis familiaris*), cow (*Bos taurus*), horse (*Equus caballus*), mouse (*Mus musculus*), rat (*Rattus norvegicus*), opossum (*Monodelphis domestica*), chicken (*Gallus gallus*), *Xenopus tropicalis*, zebrafish (*Danio rerio*), and fugu (*Takifugu rubripes*) by standard methods using the human RON gene (NM_002447) as a query. The assemblies used were human NCBI 36, chimpanzee CHIMP2.1, macaque MMUL 1.0, orangutan PPYG2, dog CanFam 2.0, cow Btau 4.0, horse Equ Cab 2, mouse NCBI m37, rat RGSC 3.4, opossum monDom5, chicken WASHUC2, *X. tropicalis* JGI 4.1, zebrafish Zv8 and fugu FUGU 4.0. The identified putative Ikaros genes were BLASTed against the nr database of GenBank to confirm that the best hits were RON genes (15-17). Conserved transcription factor-binding sites within the promoter region of the human RON gene was obtained from SABiosciences proprietary database which combines Text Mining Application and data from the UCSC Genome Browser.

Comparative proteomic analyses of RON proteins. The amino acid sequences of RON were deduced from the identified RON genes and aligned using Clustal X 1.8 software (18). The phylogenetic tree of RON was obtained by using maximum likelihood (ML) (PHYML v2.4.4) (19) and neighbor-joining (NJ) (MEGA 3.0) (20) methods, and the reliability of the tree was evaluated by the bootstrap method with 1,000 replications. The program Codeml implemented in the PAML 3.14 b software package was used to investigate whether Ikaros proteins are under positive selection (21). Six models of codon substitution, M0 (one-ratio), M1a (nearly neutral), M2a (positive selection), M3 (discrete), M7 (β), and M8 (β and ω) were used in the analysis (22).

Functional relevant SNP evaluation of the human RON gene. Functional relevant SNPs of the human RON gene were identified as previously described (15). The SNPs were extracted from Ensembl (http://www.ensembl.org) and NCBI's SNPdb (http://www.ncbi.nlm.nih.gov). The SNPs that could disrupt exonic splicing enhancer/exonic splicing silencer (ESE/ESS) motifs and cause a missense mutation were also identified.

In silico expression analyses of the human RON gene. Expressed sequence tags (ESTs) derived from the human RON gene were searched using the BLAST programs as previously described (23-28). Human RON gene (NM_002447) was used as query sequences for the BLAST programs. The expression profiles for normal human tissues were obtained from GeneAnnot (29) and ArrayExpress (30). Northern analysis of NCBI's uniGene dataset was also extracted (15).

Meta-analysis of the prognostic value of the RON gene in cancer. A database named 'PrognoScan' has been developed (31). This is i) a large collection of publicly available cancer microarray datasets with clinical annotations, as well as ii) a tool for assessing the biological relationship between gene expression and prognosis. PrognoScan employs the minimum P-value approach for grouping patients for survival analysis. PrognoScan provides a powerful platform for evaluating potential tumor markers and therapeutic targets and is publicly accessible at http://gibk21.bio.kyutech.ac.jp/PrognoScan/index.html. Human RON (MST1R) gene was input as a query and the data were collected for analysis.

Table I. Evaluation of the functionally relevant SNPs of the human RON gene.

SNP ID	Chr 3 position	Sequence	Type
rs1062633	49924940(-)	CCTTC**A**/**G**GAGTA	Missense
rs2230590	49936102(-)	TATCC**A**/**C**/**G**/**T**AGGCC	Missense
rs2230593	49940078(-)	GCTGC**A**/**C**/**G**/**T**GGTGG	Missense
rs13078735	49933274(+)	CCGCA**C**/**T**CACTC	Missense
rs2230591	49936626(-)	CTTCT**C**/**T**ACGTG	Missense
rs2230592	49936608(-)	ATTCA**A**/**C**/**G**/**T**TGGGC	Missense
rs34350470	49936338(+)	AAGAC**A**/**G**ACTGA	Missense
rs7433231	49928691(+)	CATGC**C**/**T**GCGGG	Missense
rs35986685	49935526(+)	GACTT**G**/**T**GGCCC	Missense
rs34564898	49936533(+)	TTGTG**C**/**T**CCATG	Missense
rs35887539	49940820(+)	ATTGC**G**/**T**TATGG	Missense
rs56330223	49924864(+)	TCATG**C**/**T**AGGTT	Missense
rs56091918	49933492(+)	ACCCA**C**/**T**GGTCA	Missense
rs55633379	49940490(+)	TACAC**A**/**G**GGTGC	Missense
rs3733136	49940706(+)	AGGGC**C**/**T**GTGGG	Missense
rs61729096	49933469(+)	AACTC**C**/**T**TGCTG	Missense
rs55908300	49940760(+)	AGCAG**G**/**T**GCCCG	Missense
rs56066753	49924837(+)	TCTCA**C**/**T**GCGAG	Missense
rs35924402	49939976(+)	GACCA**C**/**T**CATCC	Missense
rs34740617	49928852(+)	ATGAA**-**/**A**CTGGA	Frameshift
rs34211295	49936120(+)	GAAAA**-**/**T**CCTGT	Frameshift
rs66570013	49933436(+)	GGGGC**-**/**G**AGGGG	Frameshift
rs66589847	49933500(+)	TCACG**-**/**T**TGATA	Frameshift
rs67360293	49934262(+)	CTAAG**-**/**G**GGGGA	Frameshift
rs67811243	49932778(+)	GTGGT**-**/**G**GAATC	Frameshift
rs1062633	49924940(-)	CCTTCA/GGAGTA	Exonic splicing enhancer
rs2230591	49936626(-)	CTTCTC/TACGTG	Exonic splicing enhancer
rs2230592	49936608(-)	ATTCAA/GTGGGC	Exonic splicing enhancer
rs2230593	49940078(-)	GCTGCG/AGGTGG	Exonic splicing enhancer
rs3733136	49940706(+)	AGGGCC/TGTGGG	Exonic splicing enhancer
rs13318943	49940499(+)	GCCCAA/GTGGGC	Exonic splicing enhancer

A total of 380 available SNPs were identified in the human RON gene. Among these, 33 SNPs were functionally relevant, including 5 available alleles disrupting an existing exonic splicing enhancer, 21 SNPs causing a missense mutation and 6 frame shift SNPs.

Results

Comparative proteomics of RON proteins identified in vertebrate genomes. RON genes were identified in the genome sequences of the human, chimpanzee, macaque, orangutan, dog, cow, horse, mouse, rat, opossum, chicken, *Xenopus tropicalis*, zebrafish and fugu. Refined phylogenetic trees using the identified RON protein amino acid sequences by ML and NJ methods were almost identical (Fig. 1). We were unable to identify any site under positive selection with any of the 6 models in RON proteins. Instead, the RON proteins were under purifying selection (data not shown).

Expression profile of the human RON gene. By EST sequence searching, the human RON gene was expressed in prostate, colon, stomach, an adenocarcinoma cell line, kidney, B-cells of chronic lymphatic leukemia, squamous cell carcinoma, testis, thalamus, thymus, uterus, transitional cell papilloma, brain, liver, coronary artery smooth cells, human retinal pigment epithelium and epithelial. The investigation of available microarray experiments and 'virtual northern blotting' showed a predominant expression of RON in the liver, lymph node, thymus, intestine, lung, mammary gland, bone marrow, brain, heart, placenta, bladder, cortex, cervix, skin, kidney and prostate. When searched in PrognoScan database, human RON was also found to be expressed in bladder cancer, blood cancer, breast cancer, glioma, esophageal cancer, colorectal cancer, head and neck cancer, ovarian cancer, lung cancer and skin cancer tissues.

Comparative genomics of the human RON gene. NF-κB, signal transducer and activator of transcription 5A (STAT5A), signal transducer and activator of transcription 3 (STAT3), C/EBPα, ZID, peroxisome proliferator-activated receptor (PPAR)-γ, serum response factor (SRF), POU domain, class 2, transcription factor 1 (POU2F1) regulatory transcription factor

Table II. Dataset content from PrognoScan showing an association between microarray expression in RON and cancer prognosis.

Database	Case type	Subtype	No. of patients	Endpoint	Cut off point	P-value	Prognosis	Refs.
GSE13507	Bladder cancer		165	Overall survival	0.53	0.04	1	(60)
GSE13507	Bladder cancer	Transitional cell carcinoma	165	Disease-specific survival	0.53	0.046	1	(61)
MGH-glioma	Brain cancer	Glioma	50	Overall survival	0.12	0.015	1	(62)
GSE9195	Breast cancer		77	Relapse-free survival	0.79	0.017	2	(63)
GSE12093	Breast cancer		136	Distant metastasis-free survival	0.73	0.008	2	(64)
GSE9893	Breast cancer		155	Overall survival	0.79	0.006	2	(65)
GSE4922-GPL96	Breast cancer		249	Disease-free survival	0.9	0.01	2	(66)
GSE7390	Breast cancer		198	Recurrence-free survival	0.74	0.036	1	(67)
GSE22138	Eye cancer	Uveal melanoma	63	Distant metastasis-free survival	0.22	0.038	1	(68)
MICHIGAN-LC	Lung cancer	Adenocarcinoma	86	Overall survival	0.85	0.045	2	(69)
jacob-00182-UM	Lung cancer	Adenocarcinoma	178	Overall survival	0.89	0.004	2	
GSE14814	Lung cancer	NSCLC	90	Overall survival	0.7	0.001	2	(70)
GSE14814	Lung cancer	NSCLC	90	Disease-specific survival	0.86	0.004	2	(70)
GSE17260	Ovarian cancer		110	Overall survival	0.11	0.013	1	(71)

Fourteen tests showed an association between microarray expression in RON and cancer prognosis (bladder cancer 2/3, blood cancer 0/9, breast cancer 5/31, colorectal cancer 0/9, esophageal cancer 0/1, eye cancer 1/1, brain cancer 1/5, head and neck cancer 0/2, lung cancer 4/21, ovarian cancer 1/10, prostate cancer 0/1, skin cancer 0/1 and soft tissue cancer 0/1) with a 5% significance level. NSCLC, non-small cell lung cancer. RON, recepteur d'origine nantais.

binding sites were identified in the MST1R gene upstream (promoter) region.

Functional relevant SNP evaluation of the human RON gene. Three hundred and eighty available SNPs were identified in human RON gene. Among these, 33 SNPs were functionally relevant, including five available alleles disrupted an existing exonic splicing enhancer and 21 SNPs causing missense mutation. Moreover, six frame shift SNPs were also found (Table I).

Meta-analysis of the prognostic value of the human RON gene in cancer. PrognoScan displays a summary in table format of tests for the specific gene with columns for dataset, cancer type, subtype, endpoint, cohort, contributor, array type, probe ID, number of patients, optimal cut-off point, Pmin and Pcor. Among the databases which detected the expression of the RON gene, 14 out of 97 tests showed an association between microarray expression in the RON gene and cancer prognosis (bladder cancer 2/3, blood cancer 0/9, breast cancer 5/31, colorectal cancer 0/9, esophageal cancer 0/1, eye cancer 1/1, brain cancer 1/5, head and neck cancer 0/2, lung cancer 4/21, ovarian cancer 1/10, prostate cancer 0/1, skin cancer 0/1 and soft tissue cancer 0/1) with a 5% significance level (Table II). Of the 6 breast cancer cases, higher expression of the RON gene was found to relate to poor survival in 4 cases (GSE9195, GSE12093, GSE9893 and GSE4922). However, low expression of the RON gene was related to poor survival in 2 cases of breast cancer (GSE7390). Regarding lung cases, we found a higher expression of the RON gene to be associated with poor survival in all 4 lung cancer cases including adenocarcinoma and NSCLC. In the other cancer cases, a low expression of the RON gene was associated with poor survival in 2 cases of bladder cancer, 1 case of brain cancer, eye cancer and ovarian cancer.

Discussion

RON is a RTK containing 20 exons and 19 introns. It is located on chromosome 3p21 (1-3) in the human genome, a region frequently altered in certain cancers (4). In the present study, we identified other RON genes from an additional 13 vertebrate genomes and found that RON exists in all types of vertebrates including fish, amphibians, birds and mammals. Moreover, all identified RON proteins contain the semaphorin (sema) domain, followed by the PSI domain, and four IPT domains. The phylogenetic tree showed that RON is separated according to the order fish, amphibians, birds and mammals. Primate RONs are almost identical and clustered together. From the alignment and phylogenetic tree, mammalian RONs are conserved among vertebrate genomes, suggesting that the function of RON is essential for all vertebrates during the long evolutionary process. Moreover, this process was under purifying selection.

In adult tissues, RON expression has been found in brain, adrenal glands, epithelium of the gastrointestinal tract, testis, kidneys and ovaries (2). Systematic analysis of RON expression in normal tissues and cancer samples has not been adequately carried out. In general, RON has been detected in certain types of normal cells such as epithelial cells and

tissue macrophages. We found that the human RON gene was expressed in many tissue or organs. It showed predominant expression in the liver, lymph node, thymus, intestine, lung, mammary gland, bone marrow, brain, heart, placenta, bladder, cortex, cervix, skin, kidney and prostate.

Ligand-dependent or -independent activation of RON results in cell proliferation, migration, and matrix invasion, collectively known as invasive growth (32,33). These activities facilitate epithelial cell transformation and malignant progression. Elevated RON expression has been found in breast, colon, lung, bladder and ovarian cancer (34-40). In the present study, we found predominant expression of RON in the liver, lymph node, thymus, intestine, lung, mammary gland, bone marrow, brain, heart, placenta, bladder, cortex, cervix, skin, kidney and prostate by the investigation of available microarray experiments and 'virtual northern blotting' as shown. When searched in the PrognoScan database, human RON was also found to be expressed in bladder cancer, blood cancer, breast cancer, glioma, esophageal cancer, colorectal cancer, head and neck cancer, ovarian cancer, lung cancer and skin cancer tissues.

Wang *et al* (41) reported that the roles of RON in cancer pathogenesis are as follows. First, aberrant splicing, resulting in the formation of oncogenic RON variants, is frequently observed in primary tumors such as colon and breast cancers. Second, RON overexpression exists in various types of primary and metastatic tumors, indicating that RON overexpression is involved in tumorigenic progression. Third, RON activation promotes a malignant phenotype in cancer cells. Fourth, altered RON expression results in increased survival and pro-apoptotic activity of tumor cells, which sustains tumor growth under hostile conditions such as hypoxia. Fifth, abnormality in RON expression contributes to the acquired resistance to conventional chemoagents. Therefore, various strategies have been reported to block the c-MET/RON pathways for targeted cancer treatment (42-45). Aberrant RON expression is featured by generation of biologically active RON variants (46). Currently, seven RON variants including RONΔ170, RONΔ165, RONΔ160, RONΔ155, RONΔp110, RONΔ85 and RONΔ52 have been identified in primary cancer samples and in established cell lines (46). The switch from constitutive to alternative pre-mRNA splicing is the major event in producing RON variants in cancer cells. These RON variants have the ability to activate multiple signaling cascades, and consequently regulate cell migration, invasion and proliferation, which contribute to the invasive phenotype and promote malignant progression (12). We identified 5 available SNPs disrupting an existing exonic splicing enhancer, which may affect the alternative splicing of the RON gene. The effects of these SNPs on RON physiological and pathological function warrant further investigation. We also identified 21 SNPs causing a missense mutation and 6 frame shift SNPs. Although RON gene mutations were not found in primary cancer samples, these mutations of RON warrant further observation.

In the present study, in regards to the 5 breast cancers, high expression of the RON gene was associated with poor survival in 4 cases. However, low expression of the RON gene was related to poor survival in 1 cases of breast cancer. Concerning lung case, high expression of the RON gene was associated with poor survival in all 4 lung cancer cases including adenocarcinoma and NSCLC. In the other cancer cases, low expression of the RON gene was associated with poor survival in 2 cases of bladder cancer, 1 case of brain cancer, eye cancer and ovarian cancer. This suggests that the expression of RON is related to the prognosis of many solid tumors. The mechanism of RON involved in the process of these tumors requires further investigation. It is important to note that the relationship between the expression of RON and prognosis varied in different types of cancers, even in the same cancer from different databases. This implies that the function of RON in these tumors may be multidimensional, not just as a tumor suppressor or oncogene.

NF-κB, STAT5A, STAT3, PPAR-γ, SRF, POU2F1 regulatory transcription factor binding sites were identified in the MST1R gene upstream (promoter) region. NF-κB is widely used by eukaryotic cells as a regulator of genes that control cell proliferation and cell survival. As such, many different types of human tumors have misregulated NF-κB: that is, NF-κB is constitutively active. Active NF-κB turns on the expression of genes that maintains cell proliferation and protects the cell from conditions that would otherwise cause it to die via apoptosis (47-50). Constitutively activated STAT proteins, in particular STAT3 and STAT5, have been demonstrated to directly contribute to oncogenesis, in part, by stimulating proliferation and preventing apoptosis in various types of tumor cells (51-53). SRF is a member of the MADS box family of transcription factors. It was recently shown that SRF is involved in the epithelial-mesenchymal transition (EMT) of various types of cancer and it regulates migration and invasion of these cells with subsequent acquisition of the mesenchymal phenotype (54-56). PPAR-γ belongs to the family of nuclear hormone receptors (NHRs), which directly regulate transcription of target genes. PPAR-γ activation by specific agonists leads to growth inhibition, apoptosis and differentiation of tumor cells (57-59). These two tumor-related transcriptional factors may be involved in the effect of RON on tumors.

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