LYMPHATIC RESEARCH AND BIOLOGY
Volume 6, Number 2, 2008
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DOI: 10.1089/lrb.2008.1524

HGF and MET Mutations in Primary and Secondary Lymphedema

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Abstract

Background: Lymphedema is the abnormal accumulation of protein-rich fluid in the interstitial space. Primary lymphedema is a rare genetic condition with both autosomal dominant and autosomal recessive modes of inheritance. Three genes, FLT4 (VEGFR3), FOXC2, and SOX18 cause varying forms of primary lymphedema. In industrialized countries, secondary lymphedema is usually associated with cancer therapy and/or trauma. Recent observations suggested that hepatocyte growth factor/high affinity hepatocyte growth factor receptor (HGF/MET) were new candidate lymphedema genes.
Methods and Results: The coding exons and flanking regions of HGF and MET were directly sequenced in 145 lymphedema probands, 59 unrelated women with secondary lymphedema following treatment for breast cancer, 21 individual patients with lymphedema and intestinal lymphangiectasia, and at least 159 unrelated ethnic matched control individuals. Mutations leading to truncation or missense changes in evolutionarily conserved residues of HGF and MET were identified. These mutations were not polymorphic in control individuals.
Conclusions: The identification of HGF/MET mutations in primary lymphedema, lymphedema/lymphangiectasia, and breast cancer-associated secondary lymphedema suggests that the HGF/MET pathway is causal or alters susceptibility for a broad range of lymphedema phenotypes. The HGF/MET pathway provides a new target for the prevention and/or treatment of lymphedema.

Introduction

LYMPHEDEMA, THE ABNORMAL ACCUMULATION OF PROTEIN-RICH FLUID in the interstitial space, is a chronic disabling condition. Patients with lymphedema suffer from recurrent local infections, physical impairment, cosmetic and psychosocial stigmatization, and may be at increased risk for developing cancers such as lymphangiosarcomas.[1] Lymphedema is a poorly treated condition requiring lifelong attention. Despite the fact that the lymphatic vessels were identified hundreds of years ago, limited understanding exists of lymphatic development, function, and disease. Primary, or inherited, lymphedema is a rare genetic condition with both autosomal dominant and autosomal recessive modes of inheritance, and is due to mutations in specific genes important to lymphatic development or function. The phenotype, even within families, is variable in age at onset, expressivity, and penetrance. In industrialized countries, secondary lymphedema is more commonly seen following trauma or following breast cancer therapy, post surgery, and/or radiation to the axilla.

Many genes that play a role in lymphatic development have been identified.[2,3] Only three genes, FLT4, FOXC2, and SOX18 have been proven to cause varying forms of primary lymphedema. The report by Kajiya *et al*. that cultured lymphatic endothelial cells (LEC), but not blood endothelial cells, significantly expressed the hepatocyte growth factor (HGF) receptor MET, that treatment of LEC with recombinant HGF promoted the proliferation, migration and tube formation of LEC, and that the subcutaneous or transgenic delivery of

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Supported in part by a grant from the American Cancer Society, NIH Grant HD 37243, and Deutsche Forschungsgemeinschaft, SFB-TR23.

HGF promoted lymphatic vessel formation in the mouse, suggested HGF/MET as viable candidate genes for hereditary lymphedema.[4] This was further supported by the observation by Saito *et al*. that the expression of HGF by plasmid transfer promotes lymphangiogenesis and ameliorates secondary lymphedema in the rat tail injury model.[5] Using a mouse corneal lymphangiogenesis model, Cao *et al*. also demonstrated that HGF could be lymphangiogenic.[6] These observations supported our hypothesis that HGF and its receptor, MET, previously unrecognized lymphedema genes, were attractive causal candidate genes for lymphedema.

Materials and Methods

We sequenced the coding exons and flanking regions of HGF and MET in 154 lymphedema probands identified in the Lymphedema Family Study (http://www.pitt.edu/AFShome/g/e/genetics/public/html/lymph/), and 59 women with secondary lymphedema following treatment for breast cancer, all of whom were already screened for mutations in FLT4 (VEGFR3), FOXC2, and SOX18. We also sequenced 21 patients ascertained with intestinal lymphangiectasia and lymphedema (Hinterzarten, Germany). Informed consent was obtained from all subjects. Controls were 159 unrelated ethnicity-matched individuals.

The sequences of human HGF and MET were downloaded from GenBank (accession numbers NM_000601 and NM_000245). Unique primers flanking each exon and at least 500 bp of the 5′ and 3′ untranslated regions (UTR) were designed and used to amplify the target sequence. Each resulting amplimer was treated with shrimp alkaline phosphatase, and sequenced in both directions using ABI Big Dye 3.1 chemistry (Applied Biosystems, Foster City, California 94404). The fragments were resolved on an ABI 3730 DNA analyzer, and the sequences aligned and curated using Sequencher V5.0 software (Gene Codes Corp., Ann Arbor, Michigan 48108).

Results

Table 1 shows six individual mutations (Nos. 1–6) in HGF and MET. Each mutation is considered to be causal because:

1. They are found only in lymphedema probands and their families.
2. They are not present in over 300 chromosomes from ethnically matched controls.
3. They cause a truncation or missense mutation in evolutionarily conserved residues of the proteins.
4. They are not present in the public databases of population polymorphisms (HapMap, dbSNP).

Three of four HGF mutations occur at exon 17 of the HGF gene. Two of these, (Nos. 3 and 4) were found in at least one affected first-degree relative (parent, child) of the proband. The other (No. 2) was seen in a patient with lymphedema and lymphangiectasia. These three mutations occur in a domain of HGF which is predicted to strongly interact with its high affinity receptor, MET. We hypothesize that these three mutations would likely have a similar effect on downstream signaling, particularly that they may perturb the normal phosphorylation of tyrosine residues involved in signal transduction in the HGF/MET system. The HGF mutation (No. 1) in exon 7 results in a premature termination occurring in the α-chain of the N1 domain of HGF, which is also a region essential to HGF/MET binding.[7] This abnormal HGF protein might compete with normal HGF for binding and activation of MET.

A number of additional nucleotide changes in HGF were observed (data not shown), but they appeared in the normal populations, and the more frequent ones (minor allele frequency >5%) were found in dbSNP (NCBI single nucleotide polymorphism data base). These were not further characterized.

Two mutations were identified in exon 2 of the MET gene. One of these mutations (No. 5) was found in a lymphedema family and the other (No. 6) was found in a patient with lymphedema and intestinal lymphangiectasia. Mutations in exon 2 are located in the Sema domain of the MET gene which contains the HGF and heparin binding domain.[8,9] The crystal structure of the Sema domain in MET in complex with the α chain of HGF has more recently confirmed the seven blade β propeller structure binding HGF.[10] Moreover, this extracellular Sema domain has recently been shown to be necessary for MET receptor dimerization and activation *in vitro* using several human cell lines.[11]

The shared mutation (Nos. 7 and 8) in Table 1 shows a nonconservative arginine to cysteine change in exon 14 of the MET gene. This mutation was observed in an isolated patient with lymphedema and protein losing enteropathy secondary to intestinal lymphangiectasia, and also observed in an unrelated patient with secondary lymphedema following breast cancer treatment. This nonconservative amino

Table 1. HGF and MET Mutations Found in Lymphedema Patients

Patient	*Sequence*	*Amino acid change*	*Location*	*Population frequency*
HGF				
1	TTAAAACATG(c/a)GGTAAGTGA	C → Term	exon 7	0/344
2	GGCCTCTTA(c/t)GAGTGGCA	R → Term	exon 17	0/360
3	TATTACGA(g/a)TGGCACATCT	V → M	exon 17	0/360
4	AGTGGCACAT(c/t)TCTATATAA	L → F	exon 17	0/360
MET				
5	AGGAGCAATG(g/a)GGAGTGTA	G → E	exon 2	0/362
6	GTGTGGTGAGC(g/a)CCCTGGGA	A → T	exon 2	0/354
7, 8	TGAATTAGTT(c/t)GCTACGATG	R → C	exon 14	1/318

acid substitution is listed in dbSNP as a polymorphism, rs3458946 (http://ncbi.nlm.nih.gov/sites/entrez). However, this putative SNP has no information on population frequency, and was reported in an individual from the Burroughs Wellcome Sanger Center Cancer Genome project. It was observed in one chromosome of 359 from normal ethnic matched controls from Pittsburgh. This nonconservative change is in the juxtamembrane domain of MET[9] in an autophosphorylation domain of several protein tyrosine kinases.[12,13]

Conclusions

Both HGF and its high affinity receptor MET are expressed in numerous tissues, although their expression is predominantly in cells of mesenchymal and epithelial origin, respectively.[14,15] Both are expressed in embryonic and adult cells. HGF/MET signaling affects a broad range of biological activities including morphogenic differentiation,[16,17] cell motility,[14] growth,[18,19] intercellular junctions,[20] and survival.[21]

Initially, HGF was independently identified for its potent effects on cell motility in epithelial cells (scatter factor[14]) and cell proliferation in hepatocytes (hepatocyte growth factor/hepatopoietin A[22–24]). HGF is a large multidomain polypeptide with similarities to plasminogen, and as such is synthesized as a single long polypeptide which requires proteolytic conversion to its active form as a heterodimer. So far as is known, HGF's biologic activity is dependent on its binding to the MET receptor. MET is a prototypical receptor tyrosine kinase, but was first recognized by Cooper *et al*.[25] as a protein product of an oncogene, TPR-MET. It was not until 1991 that MET was identified by Bottaro *et al*.[26] to be the HGF receptor. To accomplish the breadth of biologic activities, MET signals are channeled by an unconventional multidocking site that consists of two tyrosines that, when phosphorylated, recruit a wide spectrum of transducers and adaptors (e.g., PI3K, Src, Grb2, Shp2 Gab1, and STAT3). Whether mutations in any of these downstream genes acting in the HGF/MET signaling pathway are responsible for lymphatic phenotypes has not been studied.

In the Lymphedema Family Study, we ascertained 239 individuals with physician-diagnosed lymphedema and a positive family history of lymphedema. Among these, 5% (12/239) have mutations in the VEGFC/VEGFR3 pathway, 7% (17/239) in FOXC2, and none (0/239) in SOX18. This is similar to the distribution seen by other investigators. Mutations in the HGF/MET pathway appear to account for another equally small proportion of lymphedema.

The identification of a MET mutation shared in patients with both secondary lymphedema following breast cancer treatment and peripheral lymphedema and intestinal lymphangectasia suggests that HGF/MET mutation confers susceptibility to secondary lymphedema and other syndromic lymphatic variation. Pain *et al*.[27,28] found evidence suggesting that underlying genetic susceptibility may influence the risk of secondary lymphedema. They studied 18 women with breast cancer-related lymphedema using dual isotope lymphoscintigraphy, and demonstrated significant differences in lymphatic function in the swollen treated and contralateral supposedly unaffected arms. Subgroup analysis of their patients demonstrated significant abnormalities in lymphatic function in the contralateral unaffected arm when compared to affected women whose hands in the affected limb had been spared lymphedema. The observation that mutation in the VEGFC/VEGFR3 pathway was a cause of primary lymphedema provided the framework linking clinical lymphedema with basic research on lymphatic development and biology, and suggested a pathway for therapy of lymphedema by direct or transgenic delivery of VEGFC.[29] The identification of the HGF/MET pathway as a cause of lymphatic phenotypes provides another pathway that may be exploited in the prevention and treatment of lymphedema.

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