Predictive Biomarkers and Personalized Medicine

Connexin 47 Mutations Increase Risk for Secondary Lymphedema Following Breast Cancer Treatment

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Abstract

Purpose: Secondary lymphedema is a frequent complication of breast cancer associated with surgery, chemotherapy, or radiation following breast cancer treatment. The potential contribution of genetic susceptibility to risk of developing secondary lymphedema following surgical trauma, radiation, and other tissue insults has not been studied.

Experimental Design: To determine whether women with breast cancer and secondary lymphedema had mutations in candidate lymphedema genes, we undertook a case–control study of 188 women diagnosed with breast cancer recruited from the University of Pittsburgh Breast Cancer Program (http://www.upmccancercenter.com/breast/index.cfm) between 2000 and 2010.

Candidate lymphedema genes, *GJC2* (encoding connexin 47 [Cx47]), *FOXC2*, *HGF*, *MET*, and *FLT4* (encoding VEGFR3), were sequenced for mutation. Bioinformatics analysis and *in vitro* functional assays were used to confirm significance of novel mutations.

Results: Cx47 mutations were identified in individuals having secondary lymphedema following breast cancer treatment but not in breast cancer controls or normal women without breast cancer. These novel mutations are dysfunctional as assessed through *in vitro* assays and bioinformatics analysis and provide evidence that altered gap junction function leads to lymphedema.

Conclusions: Our findings challenge the view that secondary lymphedema is solely due to mechanical trauma and support the hypothesis that genetic susceptibility is an important risk factor for secondary lymphedema. A priori recognition of genetic risk (i) raises the potential for early detection and intervention for a high-risk group and (ii) allows the possibility of altering surgical approach and/or chemo- and radiation therapy, or direct medical treatment of secondary lymphedema with novel connexin-modifying drugs. *Clin Cancer Res; 18(8); 2382–90. ©2012 AACR.*

Introduction

Secondary lymphedema is a frequent serious chronic complication of breast cancer treatment. The staging and treatment of other cancers involving removal and/or radiation of lymph nodes may also precede secondary lymphedema. Secondary lymphedema following breast cancer therapy occurs in approximately 30% of patients, but estimates range from 2% to 80%, depending on the timing and method of ascertainment of lymphedema (1). As many as 600,000 women may suffer from secondary lymphedema following breast cancer treatment (2). Risk factors for secondary lymphedema, although not found to be the same in all studies, include treatment related factors: extent of surgery, radiation, and chemotherapy; disease related factors: stage at diagnosis, pathologic nodal status, and number of dissected lymph nodes; and patient related factors: age at diagnosis, body mass index, and presence of a sedentary lifestyle (2, 3). As shown by the nature of these risk factors, secondary lymphedema is viewed as the consequence of a traumatic event. This contrasts with familial or primary lymphedema which has a genetic etiology and is viewed as a developmental abnormality often segregating within families and having mutations in one of several causal genes (4–7). The contribution of genetic susceptibility to the subsequent risk of developing secondary lymphedema following surgical trauma, radiation, and other tissue insults has not been evaluated.

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Translational Relevance

Secondary lymphedema is a frequent and serious chronic complication of breast cancer treatment. Our finding of four independent mutations in Cx47, including one shared mutation previously reported in primary lymphedema, not only supports these mutations as a genetic risk to the development of secondary lymphedema but raises the likelihood that other genes may contribute to such a genetic risk to secondary lymphedema as well.

Finegold and colleagues (6) reported a shared, rare mutation in the high-affinity receptor for hepatocyte growth factor, MET, between a patient with primary lymphedema and an unrelated patient with breast cancer and secondary lymphedema. This observation supported our hypothesis that some cases of secondary lymphedema are conditioned by mutation in genes causing primary lymphedema influencing lymphatic development or function. This hypothesis is further supported by quantitative lymphoscintigraphy in women with breast cancer secondary lymphedema showing abnormalities in the unaffected contralateral normal arm (8). The presymptomatic identification of individuals susceptible to secondary lymphedema would identify a subset of patients for preventive intervention or early therapy, with the potential of ameliorating the negative effects of secondary lymphedema (9). We studied women with breast cancer, posttreatment, with and without secondary lymphedema to determine whether they carried mutations in known causal genes for primary lymphedema.

Materials and Methods

Recruitment

This study was approved by the Institutional Review Board of the University of Pittsburgh, and written informed consent was obtained from all participants. We studied 188 breast cancer patients recruited from the University of Pittsburgh Breast Cancer Program (http://www.upmccancercenter.com/breast/index.cfm) between 2000 and 2010: 80 cases with secondary lymphedema and 108 breast cancer controls. Subjects were recruited through an initial mailing as well as during prospective follow-up. Subjects were eligible if they had a defined diagnosis of breast cancer, were willing to participate, and were willing to provide a DNA sample. Population controls were derived from a western Pennsylvania sampling of healthy women.

Blood was obtained for DNA isolation and analysis. We classified participants as cases if diagnosed with secondary lymphedema by a physician, physical therapist, or had received therapeutic treatment for lymphedema. Those without lymphedema were treated as breast cancer controls. All participants returned a questionnaire to assess risk factors for secondary lymphedema (Supplementary Material). Comparisons of nongenetic risk factors between cases and controls were carried out with a t test for continuous measures and with the Fisher exact test for binary measures. Genotype frequency differences were also tested with the Fisher exact test. $P < 0.05$ was considered statistically significant.

Sequencing

Each participant was sequenced for the candidate lymphedema genes FLT4 (encoding VEGFR3), FOXC2, HGF, MET, GJC2 (connexin 47 [Cx47]) as previously described (10). We previously reported numbering for amino acid sequence based on the first ATG start site for human GJC2 as originally published by Uhlenberg and colleagues (11). There is now sufficient evidence supporting the second ATG site for initiation of translation for human GJC2 (12–15), and we use this site for initiation of numbering the amino acid sequence. This results in the amino acid substitutions in this report being numbered 3 amino acids less than our original report of GJC2 mutation in primary lymphedema (16). Statistical comparisons of mutation frequencies in case and control groups were carried out with the Fisher exact test.

Functional assays in HeLa cells

The Cx47 mutations were transfected (transient and stable) into communication deficient HeLa cells (17) to determine functional changes in gap junction intercellular communication (GJIC) or connexin function. The 4 mutations were introduced by site-directed mutagenesis into a vector containing wild-type human Cx47 pIRES2-EGFP (WT-hCx47-EGFP), a gift from Dr. S. Scherer (Department of Neurology, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA) and the fidelity of the wild type and all mutant constructs confirmed by bidirectional sequence analysis.

Immunofluorescence microscopy was used to determine the presence or absence of Cx47 gap junction plaques when the constructs are transiently expressed in HeLa cells. A human Cx47 antibody was obtained from the Scherer laboratory (18). We used human central nervous system (CNS) tissue and positive oligodendrocyte staining as a positive control and primary antibody delete as a negative control (data not shown). Cultured HeLa cells were routinely fixed and stained with the primary antibodies against Cx47, along with a nuclear marker, and transfected cells were identified by their enhanced green fluorescent protein (EGFP) signal. Plaques were imaged using an Olympus Fluoview 1000 confocal microscope, ×100 oil objective.

Electrophysiologic characteristics of GJIC were measured between HeLa cell pairs transfected with the mutant constructs (as indicated by EGFP expression) by dual whole cell patch clamp recording. Pipette series resistance averaged 7.2 + 0.5 MΩ (mean ± SEM, $n = 50$). All experiments were carried out in a blinded manner. Coupling current was quantified by measuring peak current recorded in the pair when the neighboring cell received a 100 mV step membrane potential change (in both positive and negative

Table 1. Characteristics of secondary lymphedema cases and controls

	Cases	Controls	*P*
N	80	108	
Age, y (current)	60 (37–93)	54 (22–78)	<0.01
Age at diagnosis (BC)	54 (30–77)	51 (20–74)	0.015
Age at diagnosis (LE)	56 (37–82)	—	
Body mass index	28.6 (19.6–48.4)	27.5 (19.2–43.9)	0.23
Mastectomy	33 (41%)	51 (47%)	0.46
Radiation	68 (85%)	63 (58%)	<0.01
Risk factors			
Blood draw	7 (9%)	21 (19%)	0.06
Blood pressure	9 (11%)	18 (17%)	0.40
Cat scratch	7 (9%)	20 (18%)	0.09
Cut	24 (30%)	47 (44%)	0.07
Insect bite	22 (28%)	24 (22%)	0.49
Manicure	18 (22%)	31 (29%)	0.40
Sun pain	9 (11%)	16 (15%)	0.52

NOTE: Values in table expressed as either mean (%) or mean (range).

Abbreviations: BC, breast cancer; LE, lymphedema.

directions). Step changes in membrane potential were delivered to each cell in the pair in sequence and the average current recorded in the neighboring cell, determined and divided by 100 to generate coupling conductance (nS). Untransfected HeLa cells and cells transfected with empty vector (i.e., no hCx47) were used as additional controls.

The wound assay measure of proliferation/migration used differential interference contrast (DIC) time lapse imaging of over 24 hours with confluent transfected HeLa cells. Analysis was done using TScratch (19), mean ± SEM of at least 10 positions along the wound; the scrape width was normalized to the first image for each position. In all cases, we tested for statistical significance with a 2-tailed Student *t* test.

Functional assay in human lymphatic endothelial cells

Adult human dermal lymphatic microvascular endothelial cells (LEC) were cultured in EGM-2 MV (both from Lonza). Cells were electroporated with 2 μg cDNA of the EGFP-tagged mutant constructs and then selected with G418 as described above. Cells were microinjected with a combination (1:4 ratio) of 70 kd Texas Red dextran (Invitrogen) to mark the injected cell for reference, and Lucifer yellow, a known gap junction permeable dye (mw, 443; −2 charge), to assess change in extent of spread (20). All cells were injected using constant conditions and scored for dye spread in tiers from a reference cell, using constant exposure time and thresholding, and imaged using a Nikon TE2000 with a temperature controlled motorized stage and QImaging Retiga CCD camera. Images were obtained using DIC and standard filters for EGFP (identify expression of mutation), DAPI (for Lucifer yellow), and dsRed (for dextran) preinjection, immediately postinjection and 2 minutes after injection. Results were calculated as mean ± SEM. Statistical significance was determined in compar-

ison with WT-hCx47-EGFP–expressing cells by a Mann–Whitney test.

Results

Patient characteristics and mutation analysis

The characteristics of the study subjects derived from the questionnaire responses are shown in Table 1. Few significant differences were seen in demographic, clinical, or treatment variables between women who developed secondary lymphedema and controls that did not. Women with secondary lymphedema were older, diagnosed at a slightly later age, and were more likely to have had radiation therapy as part of their treatment. No differences were seen between cases and controls in other variables associated with increased risk for secondary lymphedema. None of the cases or controls had amino acid substitutions in the lymphedema genes FLT4 (VEGFR3), FOXC2, or HGF. A single case had a mutation in *MET* previously reported (6).

Among the 80 sequenced breast cancer patients with secondary lymphedema, we observed Cx47 mutations in 4 patients and no mutations among 108 sequenced breast cancer controls that did not develop secondary lymphedema (Table 2; *P* = 0.03). Comparable Cx47 mutations were not seen among at least 298 population controls (596 alleles; *P* = 0.002). One secondary lymphedema patient had the same mutation (G146S) seen in a family with primary lymphedema recently reported by our group (16). The other 3 mutations (*G183C*, *P381S*, and *H409Y*) are unique. The Cx47 mutations found in these secondary lymphedema patients all met the following criteria for relevance of mutation status (similar to the Cx47 mutations observed in our previously reported primary lymphedema patients). Each mutation causes a change in the amino acid sequence of Cx47, is not present in at least 298 sequenced,

Table 2. Connexin 47 variation seen in secondary lymphedema

cDNA	Δnt	Amino acid	Protein location	Comment
bp 436	G→A	G 146 S	Cytoplasmic loop	Identified in primary lymphedema also reported in PMLD
bp 547	G→T	G 183 C	Cytoplasmic loop	
bp 1141	C→T	P 381 S	C terminal	
bp1225	C→T	H 409 Y	C terminal	Sister with secondary lymphedema following breast cancer
bp 585	C→T	H195H	Cytoplasmic loop	Polymorphism

ethnically matched controls (0 of 596 alleles), and is well conserved in mammalian evolution. The second allele in each of these mutation patients was wild type.

None of the cases with these mutations reported a personal or family history of primary lymphedema. Two cases (P381S and H409Y) had sisters with breast cancer and one case (H409Y) reported her sister also having secondary lymphedema following breast cancer treatment. All 4 women with mutations were receiving therapy for the lymphedema including bandaging, compression garments, and in one case, exercise. None of them reported metastatic disease. Of note, all 4 women had prior surgeries including hysterectomy, cholecystectomy, knee surgery, and other procedures. They did not report lymphedema following these surgical procedures.

We identified a fifth synonymous Cx47 polymorphic mutation, H195H, occurring in secondary lymphedema patients, breast cancer controls, and Western Pennsylvania nonbreast cancer population controls with essentially equal frequency (5 of 80 secondary lymphedema patients, 8 of 108 breast cancer controls, and 27 of 298 population controls).

The 3 mutations found in probands with breast cancer and secondary lymphedema, the shared G146S mutation (by probands with primary and secondary lymphedema), and our previously identified mutations in families with primary lymphedema are distributed throughout the Cx47 monomer (although no mutations have been found in the transmembrane domains; Fig. 1, Supplementary Fig. S2). Mutations G146S and G183C are located within the

Figure 1. Schematic drawing of connexin 47 protein locating secondary lymphedema (blue), shared primary and secondary (blue/red), and primary lymphedema-associated amino acid substitutions (red). Domain break points and sequence are as described by Orthmann-Murphy and colleagues (18). Locations are representative.
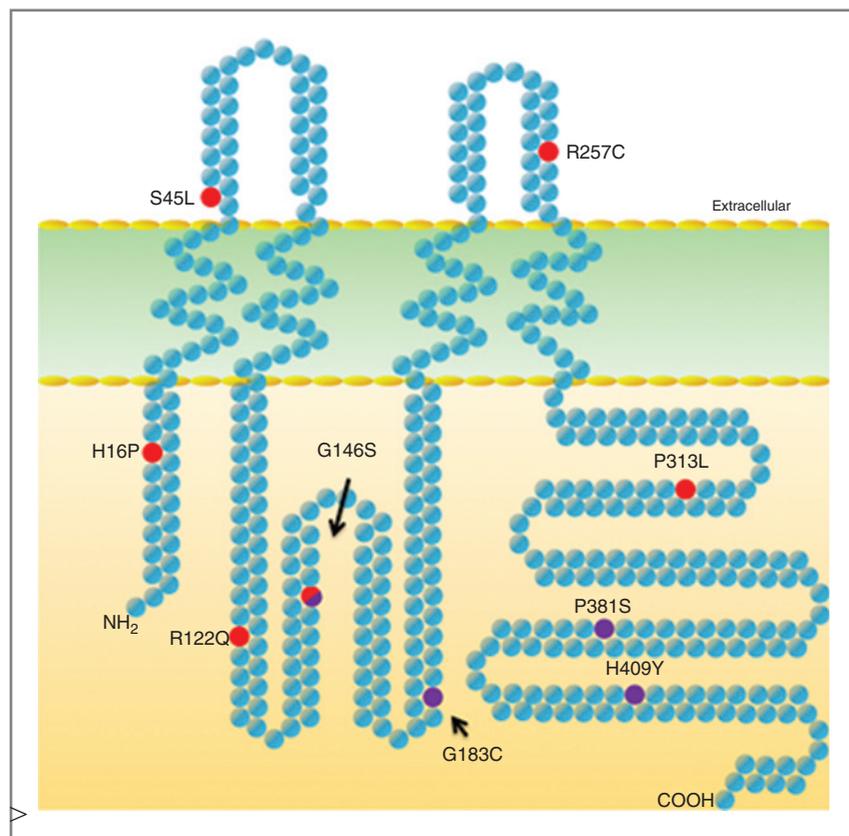
cytoplasmic loop domain while P381S and H409Y are located in the C-terminal domain.

No single functional assay is adequate to assess the complex spectrum of connexin physiology and the effect of connexin (Cx) mutations. We used a combination of frequently used assays in HeLa cells and another assay done in human dermal LECs, the cell type most likely to manifest the dysfunction causing clinical lymphedema. Each of the 4 mutations found in patients with secondary lymphedema show a phenotype different from that found in cells (HeLas and/or LECs) expressing WT-hCx47-EGFP.

Multiple assays for Cx function were conducted in HeLa cells because they have little endogenous Cx expression, allowing the role of the specific Cx of interest to be isolated, and because of the ease of manipulation of these cells. The most common functional assays use immunofluorescent microscopy to show the presence or absence of Cx plaques along the cell membrane between adjoining cells, dye transfer studies to determine transport of gap junction permeable dyes between cells, and dual whole cell patch clamp recordings to measure electrical coupling between paired cells. Because there is increasing evidence that Cxs function independent of gap junctional communication (21–23), we also conducted a wound healing assay to

quantify the mutations' effects on cell migration/proliferation.

Mutant-transfected HeLa cells showed no significant change in plaque formation: by immunofluorescent confocal microscopy intercellular plaques were indistinguishable from those detected in WT-hCx7–transfected cells (Fig. 2). However, both mutations in the cytoplasmic loop domain showed significant functional differences as compared with WT-hCx47-EGFP–transfected cells. G146S-transfected cells showed faster wound closure in a conventional cell scratch assay than the WT- hCx47-EGFP–transfected cells (Table 3). G183C-transfected cells showed increased electrical coupling as compared with the WT-hCx47–transfected cells (this effect was statistically significant, but we recognize there are limitations in interpretation due to potential voltage errors in the whole cell patch clamp method when coupling currents are large, see Fig. 2 and Table 3). Hela cells transfected with the mutations located in the C-terminal domain (P381S and H409Y) were not functionally distinct from WT-hCx47-EGFP–transfected HeLa cells.

When dye spread was evaluated in LECs expressing the human mutations, C-terminal domain mutations were phenotypically distinguished from WT-hCx47-EGFP–
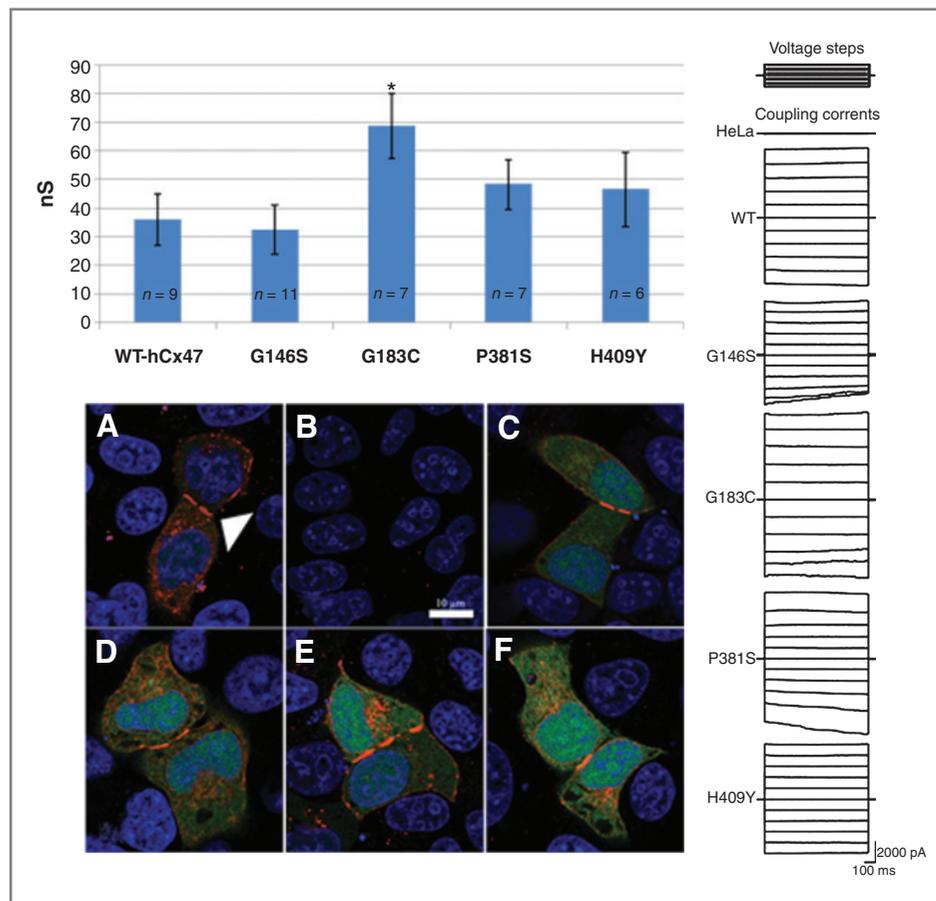

Figure 2. Top, right, representative transjunctional currents and average peak coupling current in HeLa cell pairs measured by dual whole cell patch clamping in response to a voltage step protocol, top right (−100 to +100 in 20 mV steps). *, $P < 0.05$; 2-tailed Student t test. Bottom, immunofluorescent confocal microscopy of Cx47 intercellular plaques in near confluent HeLa cells transfected with WT-hCx47-EGFP (A), and mutants: G146S (C), G183C (D), P381S (E), and H409Y (F). Untransfected (B). White arrow head indicates sample plaques in WT-hCx47-EGFP–expressing cells (A). Green indicative of EGFP and transfection, red Cx47, and blue nuclei. Scale bar, 10 μm.

Table 3. Summary of functional assessment of mutations

	WT-Cx47	G146S[a]	G183C	P381S	H409Y
LEC spread[b,c]	1.4 ± 0.14	2.0 ± 0.13	1.4 ± 0.18	2.26 ± 0.17[d]	0.07 ± 0.07[d]
Electrical coupling[b,f]	35.9 ± 8.9	32.4 ± 8.6	68.6 ± 11.3[e]	48.2 ± 8.6	46.4 ± 12.9
Wound assay[b,g]	0.72 ± 0.01	0.62 ± 0.03[e]	0.68 ± 0.16	0.70 ± 0.03	0.73 ± 0.02

[a]G146S mutation also found in patients with primary lymphedema (16).
[b]Mean ± SEM.
[c]Dye spread to tiers of LECs after 2 minutes.
[d]Indicates significance determined by Mann–Whitney test.
[e]Indicates significantly different than WT-hCx47 at $P < 0.05$, 2-tailed Student t test.
[f]In nS.
[g]Expressed as fraction of original wound in HeLa cell monolayer after 24 hours.

expressing LECs (Table 3, Supplementary Fig. S1). The H409Y mutation showed dramatically impaired dye transfer of Lucifer yellow after microinjection. In contrast, the P381S mutation showed significantly enhanced dye transfer.

Discussion

Secondary lymphedema is a serious chronic complication of breast cancer treatment. Detection of increased risk of lymphedema is particularly important given the value of preoperative assessment and early postoperative intervention in reducing the impact of secondary lymphedema (9). Typically, studies of secondary lymphedema use patient-specific information, like age and body mass index, in evaluating the risk of secondary lymphedema, but family history for lymphedema and genotype is not usually considered. A decision to intervene with treatment is usually based on the clinical burden of secondary lymphedema.

Recently, Ferrell and colleagues (16) identified mutations in Cx47 encoded by *GJC2* as a frequent cause of primary lymphedema. This finding was confirmed by Ostergaard and colleagues (24). Cxs are the major constituents of gap junctions which mediate intercellular communication. Increasingly, there is evidence of Cxs' role in a large signaling complex of associated proteins which serve to regulate coordination of conventional cell–cell communication in adhesion and motility, but also other basic cell processes including proliferation (22, 25). Gap junctions form as 2 apposing hexamers of Cx in adjoining cells. Gap junction communication mediates the propagation of spontaneous contractions in mesenteric lymphatics (26, 27).

Mutations leading to secondary lymphedema might be expected to result in fairly subtle dysfunction *in vitro* as, clinically, no lymphedema is observed until after some other significant insult *in vivo*, for example, breast cancer treatment. This is consistent with our observation that all 4 of the mutations show normal plaque formation when expressed in HeLa cells. The detection of plaques indicates fairly normal trafficking of the Cx proteins to the cell membrane and subsequent organization into clusters of gap junctions, that is, plaques. Of relevance, one of these 4 Cx47 mutations associated with secondary lymphedema, that is, G146S, causes primary lymphedema inherited as an autosomal dominant mutation with reduced penetrance. As yet unknown, modifying genetic or environmental factors must influence the expression of clinically detectable lymphedema. Variation in penetrance and expression has been shown for other lymphedema genes such as *FLT4* and *FOXC2* (5, 28).

In 2 mutations, including *G146S*, we detected abnormal gap junction or Cx function using *in vitro* assays in HeLa cells. We documented autosomal dominant inheritance in 2 primary lymphedema families with Cx47 mutations and thus might expect a dominant negative effect of Cx47 mutations. However, these *in vitro* assays in HeLa cells are likely independent of such an effect because HeLa cells show little Cx expression (17; and data not shown).

Functional data that support the significance of these mutations in the development of secondary lymphedema is also shown in human dermal LECs. We confirmed Cx47 expression in LECs (data not shown) along with other Cx species. Little is known about Cx expression and gap junction function in lymphatics, but gap junctions are important to the propagation of spontaneous contractions through mesenteric lymphatics in animal models (26, 27). It was recently reported that Cx47 had very limited expression in LECs in lymphatic valves in the thoracic duct and mesentery of developing mice. (29) We postulate that Cx47 mutations cause or contribute to the development of dermal lymphedema by impaired gap junction function causing impaired conduction of lymph from the periphery to more central lymphatic trunks. This is also supported through the identification of 2 Cx47 mutations in families with 4 limb lymphedema, one novel and one previously reported by us, in which lymphoscintigraphy showed normal anatomy in distal lymphatics, but impaired uptake (24).

The specific mechanisms through which Cx47 mutations result in increased susceptibility to secondary lymphedema

remain to be determined. There is increasing evidence of the sensitivity of gap junction function in various connexin species to physiologic changes to ambient conditions such as pH and oxygenation (30–33). We suspect that these secondary mutations have preexisting impairment of connexins' ability to adapt to such changes in milieu that do not result in overt lymphedema until they are exacerbated by local changes in tissue posttreatment. Further experimentation will be required before these mechanisms are clarified. Our findings of significant changes in gap junction function in LECs expressing the 4 mutations found in patients with secondary lymphedema: (i) confirms the significance of these mutations in patients with secondary lymphedema and (ii) suggests impaired gap junction function as a novel mechanism for the development of lymphedema.

Identification of Cx47 mutations in secondary lymphedema, and previously in primary lymphedema, expands the clinical pathology of Cx47 in human disease. Until recently, Cx47 was only considered important for CNS myelination (34) because Cx47 mutations are causal for Pelizaeus-Merzbacher-like disease (PMLD; refs. 11, 18, 35–38) and a milder phenotype of spastic paraplegia (39). These were all reported to be recessive mutations but recently dominant mutations in Cx47 were also identified as causing PMLD, among them a G146S mutation (identified as G149S; ref. 12). In contrast to disease caused by recessive mutations, autosomal dominant mutations in Cxs are more likely to cause syndromes in a similar fashion to the Cx43 mutations causing oculodentodigital dysplasia and the Cx26 mutations causing hearing loss and a variety of skin diseases (21). Thus, the recent identification of dominant mutations in Cx47 causing PMLD coupled with our findings of Cx47 mutations causing and/or predisposing to lymphedema suggests some patients may manifest both neurologic and lymphatic deficits.

Our finding of 4 independent mutations in Cx47, including one shared mutation previously reported in primary lymphedema (16), not only supports these mutations as a genetic risk to the development of secondary lymphedema but raises the likelihood that other genes may contribute to such a genetic risk to secondary lymphedema as well. Gap junctions participate in a multiprotein complex, and our observations implicate any of these proteins as potential candidates for risk mutations for secondary lymphedema and targets for novel drug therapy. A patient's family history of lymphedema would be useful in identifying women at higher than normal risk of developing secondary lymphedema, and sequencing genes such as Cx47 and other genes known to cause primary lymphedema may prospectively identify a group of women who would benefit from early, aggressive surveillance, and therapy before the clinical onset of lymphedema. Our findings challenge the commonly held view that secondary lymphedema is solely due to mechanical trauma (40, 41). Genetic susceptibility is an important risk factor which must be included with mechanical trauma, radiation, and/or chemical insult. *A priori* recognition of such a genetic susceptibility (i) raises the potential for early detection of a group at high risk, and (ii) allows the possibility of altering surgical approach and/or chemotherapy radiation therapy or direct medical treatment of the lymphedema.

The prospect of preventive intervention or pharmacologic treatment in secondary lymphedema is especially attractive given the estimated prevalence of up to 600,000 (2) women who suffer from secondary lymphedema following treatment for breast cancer. The limited treatment options currently available to these patients include early education and physiotherapy (42, 43). With regard to the Cx47 mutations specifically, there is potential for rapid translational progress given the preexisting effort to develop Cx modifying drugs for application to cardiovascular disease (44). Our findings offer the possibility that early detection and intervention may be possible before breast cancer treatment is complete, and also offers the chance to ameliorate the severity of secondary lymphedema in a subset of breast cancer patients.

Disclosure of Potential Conflicts of Interest

Authors' Contributions

Acknowledgments

Grant Support

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