EBioMedicine xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

EBioMedicine

journal homepage: www.ebiomedicine.com

Research Paper

The Protein Encoded by the *CCDC170* Breast Cancer Gene Functions to Organize the Golgi-Microtubule Network

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ARTICLE INFO

Article history:
Received 2 May 2017
Received in revised form 23 June 2017
Accepted 23 June 2017
Available online xxxx

Keywords:
CCDC170/C6ORF97
Golgi-associated microtubules
Tubulin acetylation
Polarized cell migration
Genome-wide association studies (GWAS)
Differential allele specific expression (DASE)

ABSTRACT

Genome-Wide Association Studies (GWAS) and subsequent fine-mapping studies (>50) have implicated single nucleotide polymorphisms (SNPs) located at the *CCDC170/C6ORF97-ESR1* locus (6q25.1) as being associated with the risk of breast cancer. Surprisingly, our analysis using genome-wide differential allele-specific expression (DASE), an indicator for breast cancer susceptibility, suggested that the genetic alterations of *CCDC170*, but not *ESR1*, account for GWAS-associated breast cancer risk at this locus. Breast cancer-associated *CCDC170* nonsense mutations and rearrangements have also been detected, with the latter being specifically implicated in driving breast cancer. Here we report that the wild type CCDC170 protein localizes to the region of the Golgi apparatus and binds Golgi-associated microtubules (MTs), and that breast cancer-linked truncations of CCDC170 result in loss of Golgi localization. Overexpression of wild type CCDC170 triggers Golgi reorganization, and enhances Golgi-associated MT stabilization and acetyltransferase ATAT1-dependent α-tubulin acetylation. Golgi-derived MTs regulate cellular polarity and motility, and we provide evidence that dysregulation of CCDC170 affects polarized cell migration. Taken together, our findings demonstrate that CCDC170 plays an essential role in Golgi-associated MT organization and stabilization, and implicate a mechanism for how perturbations in the *CCDC170* gene may contribute to the hallmark changes in cell polarity and motility seen in breast cancer.

1. Introduction

Advances in single nucleotide polymorphism (SNP) array technologies and Next Generation Sequencing have made it practical to perform Genome-Wide Association Studies (GWAS), an unbiased genomic approach to identify genetic factors that account for cancer susceptibility. Following initial GWAS (Zheng et al., 2009; Turnbull et al., 2010), subsequent fine-mapping studies (>50) have implicated genetic variants located at the *CCDC170/C6ORF97-ESR1* locus (6q25.1) as being associated with the risk of breast cancer. Estrogen receptor α (ERα), the protein encoded by the *ESR1* gene, binds to estrogen, and the estrogen-ERα axis promotes the growth of breast epithelial cells and thereby contributes to breast cancer risk (Ali and Coombes, 2000). It is therefore logical to hypothesize that the breast cancer-associated SNPs at the *CCDC170/C6ORF97-ESR1* locus impact function of the *ESR1* gene (Hein et al., 2012; Koller et al., 2013; Paternoster et al., 2013; Yang et al., 2013). However, thus far, few studies have identified any strong causal variants regulating *ESR1* function or expression (Cai et al., 2011; Stacey et al., 2010). Interestingly, the *CCDC170-ESR1* intergenic *rs2146210 SNP* was found to have stronger risk-association in ER- breast tumors than those in ER+ breast tumors, which suggests that this risk variant is likely *ESR1*-independent (Hein et al., 2012; Mulligan et al., 2011; Stacey et al., 2010; Zheng et al., 2009).

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http://dx.doi.org/10.1016/j.ebiom.2017.06.024

Nonsense mutations (e.g. p.E48* and p.Q405*) in the *CCDC170* gene have been reported in sporadic breast cancer and other cancers by both the Cancer Genome Atlas (TCGA) and Cancer Genome Project. Importantly, several tumor-specific gene rearrangements from the second noncoding exon of *ESR1* to the sixth and/or seventh coding exon(s) of *CCDC170* were also reported by several studies using high-throughput RNA-seq (Robinson et al., 2011; Sakarya et al., 2012; Veeraraghavan et al., 2014). This *ESR1-CCDC170* gene arrangement presents in ~14% of ER + breast cancer and could be one of the most important recurrent gene fusions in breast cancer (Veeraraghavan et al., 2014). This recent study by Veeraghavan et al. demonstrated that N-terminally truncated CCDC170 proteins were produced as a result of this *ESR1-CCDC170* rearrangement (Veeraraghavan et al., 2014). Ectopic expression of these truncated proteins increased breast cancer cell motility and enhanced the transformation of normal mammary epithelial cells (MECs) (Veeraraghavan et al., 2014), indicating the important role of *CCDC170* gene abnormalities in breast cancer initiation and/or progression. Taken together, the findings from GWAS, TCGA, cell culture, and mouse xenograft studies strongly indicate that a variety of perturbations of the CCDC170 protein are capable of driving breast cancer. Despite the wealth of genetic information relating to the *CCDC170* gene, nothing was known about the encoded protein.

Here, we initially show that the *CCDC170* locus is associated with significant Differential Allele Specific Expression (DASE), which supports specifically a link to breast cancer risk. As nothing was known about the molecular function of the CCDC170 protein, the present work focused largely on identifying a potential molecular mechanism for *CCDC170*-associated breast cancer risk and progression. We demonstrate that the CCDC170 protein, a predicted coiled-coil domain containing (CCDC) protein, associates with the Golgi apparatus, stabilizes perinuclear microtubules (MTs), and plays an essential role in the known process of MT-dependent Golgi organization. Distinct Golgi-derived MTs that extend into the cytoplasm are now understood to contribute to cell polarity and directional migration. We hypothesized that breast cancer-related perturbations of the CCDC170 Golgi-MT network could lead to altered cell polarity and migration, and thereby drive breast cancer initiation and progression. We provide evidence that dysregulation of CCDC170 indeed affects polarized cell migration.

2. Materials and Methods

2.1. Cell Lines

Thirty human mammary epithelial cell (HMEC) lines were utilized as starting materials for DASE analysis at the *CCDC170-ESR1* locus. Under an approved protocol by the Institutional of Review Board (IRB) at Fox Chase Cancer Center, we derived primary HMEC lines from adjacent or contralateral normal mammary tissue of breast cancer patients as described previously (Gao et al., 2012). Non-tumorigenic MEC lines, MCF-10A and -10F, and human breast cancer cell lines, MCF-7, T-47D, ZR-75-1, MDA-MB-231, HCC-1937, and SK-BR-3, were purchased from American Type Culture Collection (ATCC). Cell lines were maintained in medium recommended by ATCC at 37 °C in the presence of 5% CO_2. MCF10ADCIS·COM cells were a gift from Dr. Fariba Behbod (University of Kansas Medical Center) and were maintained as previously described (Behbod et al., 2009). MCF-7 Tet-On® cells were purchased from Clontech and were maintained according to the manufacture's guidelines. U2OS cells were obtained from Dr. Sanjeevani Arora, Fox Chase Cancer Center. U2OS cell clones stably expressing WT GFP-CCDC170 were created by transfection, followed by single cell sorting. Single cell clones displayed heterogeneous CCDC170 levels and localization.

2.2. DASE Analysis at CCDC170-ESR1 Locus

Genomic DNA (gDNA), RNA and double-stranded cDNA (ds-cDNA) from primary HMEC lines were prepared as previously described (Gao et al., 2012). gDNA (quantified by PicoGreen assay) and ds-cDNA samples were subjected to whole genome application and fragmentation prior to Illumina HumanOmni5-quad BeadChip hybridization. To obtain the array probes that fall within the *CCDC170* and *ESR1* exons, SNP information from the HumanOmni5 BeadChip was retrieved and SNP coordinates were used. For each probe marker with the heterozygous genotype, scanned raw signal intensities were processed by GenomeStudio Software (Illumina) to generate X and Y intensity values for allelic expression at each marker position. Raw expression data for each SNP site were filtered (X + Y value >1000). A total of 20 and 10 SNP probes mapping to *CCDC170* or *ESR1* exons, respectively, were obtained and used for final DASE analysis.

2.3. CCDC170 Expression Plasmids

The CCDC170 orf (RefSeq accession NM_025059) was obtained from OriGene Technologies in the pCMV6-AC-GFP C-terminal turboGFP fusion vector. The CCDC170 orf was transferred to the pCMV6-AN-mGFP N-terminal monomeric GFP fusion vector using the OriGene Precision Shuttle cloning sites Sgf I and Mlu I. The *CCDC170* wild-type gene was subsequently subcloned into a pTRE-Tight (Clontech) at Kpn I and Not I sites. Fragments of CCDC170 1-48, 1-405, 1-591, 1-649, 1-689, 355-591, 355-689, 355-715, 593-715 were generated in the N-terminal GFP CCDC170 fusions by either gene synthesis (GenScript) or using the Agilent QuickChange II site-directed mutagenesis kit. Non-tag wild-type CCDC170 was also subcloned in the pRetroX-IRES-ZsGreen1 vector at the Not I site.

2.4. CRISPR/Cas9 Mediated CCDC170 Editing

CCDC170/C6orf97 knockout MCF-7 cells were generated using CRISPR/Cas9 technology. A guide RNA (gRNA-GTTCGGAAGTCCCGGTCACG) with predicted highest target specificity was selected using the CRISPR design tool from MIT (http://crispr.mit.edu). Guide RNA synthetic DNA fragments were ordered from Integrated DNA Technologies (IDT). The gRNA sequence was cloned into the px459 vector (Addgene plasmid #62988) (Ran et al., 2013). CCDC170$^{-/-}$ or CCDC170$^{+/-}$ clones were generated by single cell expansion. To detect the presence of out-of-frame insertions/deletions (indels) in all CCDC170 alleles, the genotypes of knock-out clones were screened and verified by Sanger DNA sequencing. CCDC170 protein levels were further examined by SDS PAGE/immunoblot analysis.

2.5. Immunofluorescence

Cells were plated on 4-well coverslips and transfected after 24 h with the indicated vectors using Lipofectamine® 3000 (Thermo Fisher Scientific) according to the manufacturer's recommendations. After 2 days of transfection, the cells were washed three times with PBS and fixed in 4% paraformaldehyde for 15 min at RT. Fixed cells were then washed with PBS three times for 5 min and permeabilized with 0.5% Triton X-100 for 15 min at RT. After three 5-minute washes, cells were incubated in 3% BSA in PBS containing 0.1% Tween 20 (PBS-T) for 30 min and washed twice in 1% BSA in PBS-T. Cells were incubated with primary antibodies at optimized dilutions for 1 h at RT if not specifically mentioned, washed four time for 5 min with 1% BSA in PBS-T and incubated with secondary Alexa 488/555/647 antibodies (1:100 Molecular Probes) for 1 h. The coverslips were washed four times for 5 min with PBS and Vectashield Mounting Medium with DAPI (Vector laboratories) was added. The cells were than visualized by confocal microscopy (Leica TCS SP8). The following primary antibodies were used for IF: AKAP9 rabbit antibody (1:100, #HPA008548, Sigma-Aldrich), CCDC170 rabbit antibody (1:100, HPA027114, Sigma-Aldrich). MAP4 rabbit antibody (1:139, #HPA038149, Sigma-Aldrich), TGN46 Rabbit antibody (1:100, #ab50595, Abcam), Mannosidase II rabbit antibody (1:100, #ab12277, Abcam), chicken GFP antibody (1:400, #A10262, Life Technologies),

Please cite this article as: Jiang, P., et al., The Protein Encoded by the *CCDC170* Breast Cancer Gene Functions to Organize the Golgi-Microtubule Network, EBioMedicine (2017), http://dx.doi.org/10.1016/j.ebiom.2017.06.024