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Long noncoding RNA *HOTAIR* promotes invasion of breast cancer cells through chondroitin sulfotransferase CHST15

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Abstract

The long noncoding RNA HOTAIR plays significant roles in promoting cancer metastasis. However, how it conveys an invasive advantage in cancer cells is not clear. Here we identify the chondroitin sulfotransferase CHST15 (GalNAc4S-6ST) as a novel HOX transcript antisense intergenic RNA (HOTAIR) target gene using RNA profiling and show that CHST15 is required for HOTAIR-mediated invasiveness in breast cancer cells. CHST15 catalyzes sulfation of the C6 hydroxyl group of the N-acetyl galactosamine 4-sulfate moiety in chondroitin sulfate to form the 4,6-disulfated chondroitin sulfate variant known as the CS-E isoform. We show that HOTAIR is necessary and sufficient for CHST15 transcript expression. Inhibition of CHST15 by RNA interference abolished cell invasion promoted by HOTAIR but not on HOTAIR-mediated

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migratory activity. Conversely, reconstitution of CHST15 expression rescued the invasive activity of HOTAIR-depleted cells. In corroboration with this mechanism, blocking cell surface chondroitin sulfate using a pan-CS antibody or an antibody specifically recognizes the CS-E isoform significantly suppressed HOTAIR-induced invasion. Inhibition of CHST15 compromised tumorigenesis and metastasis in orthotopic breast cancer xenograft models. Furthermore, the expression of HOTAIR closely correlated with the level of CHST15 protein in primary as well as metastatic tumor lesions. Our results demonstrate a novel mechanism underlying the function of HOTAIR in tumor progression through programming the context of cell surface glycosaminoglycans. Our results further establish that the invasive and migratory activities downstream of HOTAIR are distinctly regulated, whereby CHST15 preferentially controls the arm of invasiveness. Thus, the HOTAIR-CHST15 axis may provide a new avenue toward novel therapeutic strategies and prognosis biomarkers for advanced breast cancer.

Keywords

HOTAIR; CHST15; long noncoding RNA; breast cancer; invasion; chondroitin sulfate; glycosaminoglycans; RNA-seq

Introduction

Long noncoding RNAs (lncRNAs) have been widely recognized as an important means evolved in mammalian cells to regulate multiple cellular activities without inherently encoding proteins.[1] This class of RNAs, with arbitrarily set lengths of longer than 200 nucleotides, are known to serve as scaffolding plat-forms for multiple proteins or as a molecular sponge of other small RNAs, and thus are able to regulate gene expression.[2] With an increasing number of lncRNAs discovered, an understanding of their biological functions and the underlying cellular mechanisms is urgently needed.

HOX transcript antisense intergenic RNA (HOTAIR) was originally identified by a tiling array of the HOXC locus as an antisense transcript derived from the intergenic region of the locus.[3] In cancer cells, HOTAIR partners with the polycomb repressing complex 2 (PRC2) and the histone demethylation enzyme lysine-specific demethylase 1 (LSD1) to regulate epigenetic programming which leads to enhanced tumor growth and metastasis.[4–6] This is achieved at least in part by the ability of HOTAIR to globally redirect the EZH2-EED-SUZ12 complexes to the target genes and regulate their expression through modulation of histone H3 K27 trimethylation. Recently, a PRC2-independent epigenetic function of HOTAIR was also reported, demonstrating functional versatility of the lncRNA.[7]

Besides developmental biology, HOTAIR is one of the model lncRNAs that plays an important role in tumor biology of multiple cancer types. Expression of HOTAIR is associated with increased malignancy and is closely correlated with poor prognosis and decreased progression-free survival.[4,8–15] In breast cancer, increased HOTAIR expression is frequently observed in metastatic tumor tissues in contrast to the normal tissues.[4,9,16] However, how HOTAIR promotes cancer metastasis and the essential target genes involved in HOTAIR function in cancer invasion have not been fully understood.

To further understand the cellular function of HOTAIR, we have profiled RNA expression in breast cancer cell lines harboring tetracycline-inducible shRNA of HOTAIR (tet-shHOTAIR) or the control shRNA of scrambled sequence (tet-shCtrl).[12] Our study suggests that HOTAIR promotes invasiveness by programming genes involved in conditioning the extracellular environment. Here we demonstrate that HOTAIR preferentially upregulates the expression of chondroitin sulfotransferase CHST15 (carbohydrate N-acetylgalactosamine 4-sulfate 6-O-sulfotransferase 15; GalNAc4S-6ST), an enzyme catalyzing sulfation at position 6 of N-acetylgalactosamine 4-sulfate to form glycosaminoglycans constituted by disaccharide units of glucuronic acid (GlcA) and N-acetylgalactosamine 4,6-bisulfate (4S,6SGalNAc; hereafter referred to CS-E). Previous studies indicated that chondroitin sulfates play an important role in breast cancer metastasis.[17] Using both loss- and gain-of-function approaches, our study demonstrates that CHST15 is required for the proinvasion function of HOTAIR.

Methods and Reagents

Cells, plasmids and qRT-PCR primers

MDA-MB-231 and MDA-MB-468 human breast cancer cell lines were obtained from ATCC. The following antibodies were purchased: CHST15 (Novus Biologicals, Centennial, CO), actin (Santa Cruz Biotechnology, Santa Cruz, CA), chondroitin sulfate (Abcam, Cambridge, UK). The anti-CS-E antibody was developed as described by the Hsieh–Wilson laboratory.[18] The plasmids pInducer-tet-shHOTAIR and pInducer-tet-shCtrl were constructed as described previously. LZRS-HOTAIR was a gift from Howard Chang (Addgene plasmid # 26110).

qRT-PCR primers of human genes: HOTAIR—forward, GGTAGAAAAAGCAACCACGAAGC; HOTAIR—reverse, ACATAAACCTCTGTCTGTGAGTGCC; CHST15—set #1 forward, AAGAGCTTCTGATCTCATCACCT; CHST15—set #1 reverse, CTTGGGTTTTCGCTGTCCATC; CHST15—set #2 forward, CAGCGAAAACCCAAGTGACAC; CHST15—set #2 reverse, CTCAATCCTAGTTGTGATGCTGT, 18S—forward, AGGATCCATTGGAGGGCAAGT; 18S—reverse, TCCAACTA CGAGCTTTTTAACTGCA; Actin—forward, CTTCCCCTCCATC GTGGG; Actin—reverse, GTGGTACGGCCAGAGGCG; GAPDH—forward, CGGAGTCAACGGATTTGGTCGTA; GAPDH—reverse, AGCCTTCTCCATGGTGGTGAAGAC.

RNA-seq and bioinformatics analysis

Poly(A) RNA was purified by NEBNext Poly(A) mRNA Magnetic Isolation Module (New England Biolabs, Ipswich, MA) from a total of 1 μg good quality TRIzol-isolated total RNA. Library preparation and deep sequencing were conducted at the Genomics, Epigenomics and Sequencing Core of the University of Cincinnati. RNA-seq library was prepared using PrepX mRNA Library Preparation kit (WaferGen, Fremont, CA) combined Apollo 324 NGS automated library prep system. Briefly, the isolated poly(A) RNA was fragmented, ligated to adaptors, followed by cDNA conversion. Next, the sample-specific index was added to each

adaptor-ligated cDNA fragments via 12 cycles of PCR. To check the quality and yield of the purified library, the library was analyzed by Bioanalyzer (Agilent, Santa Clara, CA) using DNA high sensitivity chip. To accurately quantify the library concentration for clustering, qPCR was performed by using Kapa Library Quantification kit (Kapabiosystem, Woburn, MA) with ABI's 9700HT real-time PCR system (Thermo Fisher Scientific, Waltham, MA).

High quality individually indexed and compatible libraries were then proportionally pooled (~25 million reads per sample) for clustering in cBot system (Illumina, San Diego, CA). Libraries at the final concentration of 15 pM were clustered onto a single read (SR) flow cell using Illumina TruSeq SR Cluster kit v3, and sequenced to 50 bp using TruSeq SBS v3 kit on Illumina HiSeq system. The reads were aligned with TopHat 2.0.13 to GRCh38 with default parameters and then were assembled by Cufflink 2.2.1, using Ensembl v79 annotations. Transcript abundance was measured in fragments per kb of exon per million fragments mapped. The differentially expressed genes (DEGs) regulated with HOTAIR were identified by DESeq, a bioconductor package of R language, with the criterion $q < 0.05$. We also performed functional enrichment analysis, as described in our previous studies,[19,20] for DEGs to interpret their biological functions. In brief, we used the topGO and GeneAnswers packages of Bioconductor to calculate the topology of the GO graph.

Immunohistochemical staining of CHST15

Formaldehyde-fixed paraffin-embedded (FFPE) tissue sections were dewaxed by baking at 65° for 1 hr. Antigen retrieval was performed by heating with a steamer in 10 mM citrate (pH 6.0). The slides were then incubated with antibodies recognizing CHST15 for overnight at 4°C. Detection was performed using the Novalink Polymer detection system following the manufacturer's protocol (Leica Biosystems, Nußloch, Germany).

Immunofluorescence staining for chondroitin sulfate

MDA-MB-231/tet-shHOTAIR cells were treated with and without doxycycline, then trypsinized and transferred to slides by Cytospin (Thermo Fisher Scientific). The slides were fixed by ice-cold 100% MeOH, followed by blocking in 5% goat-serum in TBST at room temperature for 1 h. Anti-chondroitin sulfate antibody was then applied to the slides and incubated for overnight at 4°C, followed by secondary antibody conjugated with Alexa Fluor 555 (Thermo Fisher Scientific).

In situ RNA hybridization

To design probes, predicted secondary structures of HOTAIR were generated based on thermodynamically favorable models.[21] Probe sequences were chosen by the assumption that sequences in single-stranded regions have the highest potential for target hybridization. The selected region, which is located in the D4 domain of the HOTAIR transcript,[22] were PCR-amplified from HOTAIR cDNA with T7 promoter sequence incorporated in the reverse primer: HOTAIR forward 5′ -GCA AAC GGG ACT TTG CAC TCT-3′, HOTAIR reverse 5′ -CTA ATA CGA CTC ACT ATA GGG CAG TGC ACA GAA AAT GCA TCC-3′. *In vitro* transcription (Ambion, Waltham, MA) and digoxigenin labeling (Roche, Basel, Switzerland) were performed following manufacturers' protocols. Human clinical FFPE tissue sections were treated with proteinase K (20 μg/ml) and rinsed five times in distilled

water after digestion. The slides were immersed in ice-cold acetic acid (20%) for 20 sec and dehydrated by sequential washing in 70, 95, and finally, 100% EtOH, then air-dried. Dried slides were then incubated in hybridization solution containing 50% formamide, 5× salt solution (1 M NaCl, 25 mM EDTA, 50 mM Tris–HCl, pH 7.5, 25 mM phosphate buffer), 5× Denhardt's solution (Alpha Aesar, UK), 10% dextran sulfate, 20 U/ml heparin and 0.1% SDS, at 55°C for 1 hr in a humid chamber. Twenty nanograms of probe RNA was diluted in 30 μl hybridization solution and heated at 95°C for 2 min to denature, then chilled on ice immediately. The tissue slides were then incubated with the probes in the humidified hybridization chamber at 65°C for 19 hr. Hybridized slides were washed three times in 50% formamide in 2× SSC (0.3 M NaCl, 30 mM $Na_3C_6H_5O_7$, pH 5.0) at 37°C for 5 min in each wash, followed by washing in 1× SSC three times at 37°C for 5 min, then in MABT (maleic acid buffer with Tween 20, 150 mM NaCl, 100 mM Maleic acid, 0.1% Tween20), pH 7.0 at room temperature for 30 min. The slides were dried and blocked in MABT with 2% sheep serum for 1 hr. Blocked slides were drained and incubated with anti-DIG antibody in blocking buffer at room temperature for 2 hr. After the incubation, the slides were washed two times in buffer 2 (100 mM Tris–HCl, pH 9.5, 100 mM NaCl, 50 mM MgCl2) at room temperature for 10 min, followed by two times in pres-taining buffer (100 mM Tris, pH 9.5, 100 mM NaCl, 10 mM $MgCl_2$). The slides were then incubated with NBT/BCIP (Roche) solution for about 19 hr. The reaction was stopped by incubating slides in buffer 3 (10 mM Tris–HCl, pH 8.1, 1 mM EDTA). The stained slides were rinsed in deionized water and air-dried for 30 min. Nuclei were stained by 0.1% Nuclear Fast Red (Sigma-Aldrich, St. Louis, MO) for 2 min.

Wound healing cell migration assay

Cells are seeded in 96-well ImageLock plates (Essen Bioscience, Ann Arbor, MI) in a density of 40,000 cells per well. Scratches were made in the cell monolayer with WoundMaker™ (Essen BioScience) by following manufacturer's protocol. After wounding, the media was removed and the culture was gently washed twice with culture media. The culture was supplemented with 100 μl of media and bubbles were carefully removed from the assay plate. Wound healing was monitored by IncuCyte ZOOM™ under scratch wound scanning function and in wide mode of the software. Images of collective cell spreading were recorded every 2 hr for a total duration of 24 hr under 10× objectives. The first scan in the time course was used to generate the initial scratch wound mask, which defined the initial wound region and was used as the base in subsequent quantification processes. Furthermore, scratch wound masks were computed for each subsequent image in the series and were used to calculating the wound width metric. The quantitative results plotted and the statistical significance was determined by linear regression.

Invasion assay

Matrigel (Corning, NY) was applied in transwell (Corning, NY) the day prior to plating cells in it. Cells were suspended in serum-free medium and plated into the upper chamber, while 500 μl complete medium was placed in the lower chamber. Either upper chamber or lower chamber medium contained 1 μg/ml doxycycline. Cells were incubated for another 72 hr, and cell invasion was quantitated by crystal violet staining.

Results

The transcriptomes of MDA-MB-231 cells harboring tet-shHOTAIR (MDA-MB-231/tet-shHOTAIR) or the control shCtrl (MDA-MB-231/tet-shCtrl) upon doxycycline stimulation was assessed by RNA-seq. Unsupervised hierarchical analysis revealed that a distinct group of genes is responding to downregulation of HOTAIR (Supporting Information Fig. S1A). Gene ontology analysis revealed that these genes contribute to a wide range of biological functions, in particular of the categories of vascular development and extracellular regulation (bracketed in Supporting Information Fig. S1B). Focusing on these genes identified the *CHST15* gene, which was the most suppressed gene by ratio in response to HOTAIR depletion (Supporting Information Table S1). Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) using two independent primer sets of CHST15 confirmed that depletion of endogenous HOTAIR of MDA-MB-231 resulted in dramatic reduction of the *CHST15* gene RNA (Fig. 1a). Consistently, protein expression of CHST15 was also diminished upon HOTAIR depletion in MDA-MB-231 cells. To test whether the requirement of HOTAIR for *CHST15* expression was unique to MDA-MB-231, we performed the analysis in another set of cell lines derived from MDA-MB-468, MDA-MB-468/tet-shHOTAIR as well as the control MDA-MB-468/tet-shCtrl, and observed similar results (Fig. 1b). These data show that HOTAIR is required for CHST15 expression. Conversely, ectopic expression of HOTAIR in MDA-MB-231 cells resulted in significant increase of CHST15 expression at RNA and protein levels compared to cells expressing basal level of HOTAIR (Fig. 1c), indicating that HOTAIR is sufficient to promote CHST15 expression. These results together suggest that HOTAIR promotes CHST15 expression.

We next tested whether the enhanced CHST15 expression plays a role in the tumor-promoting functions of HOTAIR. It has been shown that HOTAIR enhances tumor cell invasion.[4] Consistent with the previous reports, MDA-MB-231/tet-shHOTAIR cells treated with doxycycline for HOTAIR downregulation had markedly reduced invasive activity as measured by Boyden chamber assay (Fig. 1d). Data derived from the shRNA inducible system engineered in MDA-MB-468 cells also support an important role of HOTAIR in cell invasiveness (Fig. 1e). To test if this is due to potential effects of HOTAIR in cell proliferation, BrdU incorporation assay was performed which showed that induction of HOTAIR expression has no impact on cell proliferation (Supporting Information Fig. S2A). Conversely, reconstitution of CHST15 in the HOTAIR-depleted cells rescued the invasive activity (Fig. 1f). Ectopic expression of HOTAIR in MDA-MB-231 cells promoted invasive activity (Fig. 1g, comparing the ectopic HOTAIR-expressing Clone #2 and the negative control Clone #6) while depleting CHST15 compromised the gained invasive potential (Fig. 1g). Using the same cell system, we examined the migratory activity in the presence and absence of CHST15 utilizing an image-based real-time wound healing analysis. The result showed that while the levels of HOTAIR expression were correlated with the migratory activity, the migratory activity remained unaffected when *CHST15* was depleted (Supporting Information Fig. S2B). Thus, these results suggest that regulation of cell migration and invasion by HOTAIR is through distinct mechanisms and that CHST15 as a downstream effector of HOTAIR specifically directs cellular invasiveness but not migrating ability (Supporting Information Fig. S2C).

Invasive advantage is known to coevolve with stemness of cancer cell.[23] A previous study has shown that HOTAIR promotes tumor initiation.[24] This can be recapitulated by spheroid formation assays in which ectopic HOTAIR expression in MDA-MB-231/LZRS-HOTAIR cells promoted mammosphere formation compared to the control cells in suspension culture (Supporting Information Fig. S3A). Indeed, ectopic expression of HOTAIR in MDA-MB-231 cells enhanced aldehyde dehydrogenase (ALDH) activity, another independent biomarker of stemness (Supporting Information Fig. S3B). Importantly, depleting *CHST15* abolished the HOTAIR-dependent spheroid-forming activity (Supporting Information Fig. S3C). These results suggest that CHST15 is required for HOTAIR in maintaining stemness of cancer cells. Consistently, gene expression of both HOTAIR and CHST15 increased in the spheroid-forming population compared to the attached monolayer of MDA-MB-231 (Fig. 2a) as well as MDA-MB-468 cells (Fig. 2b).

The HOTAIR-CHST15 axis predicts higher cellular content of chondroitin sulfate in HOTAIR-expressing cells. Indeed, depletion of HOTAIR in MDA-MB-231 cells resulted in downregulation of cell surface chondroitin sulfate as demonstrated by immunofluorescence (Fig. 3a; see Supporting Information Fig. S4A for example images). The cell surface location of the detected chondroitin sulfate is confirmed by confocal microscopy (Supporting Information Fig. S4B). To test whether modulation of cell surface chondroitin sulfate by HOTAIR plays a function in cancer cell invasion, MDA-MB-231 cells expressing ectopic HOTAIR were treated with a pan-chondroitin sulfate-neutralizing antibody and subject to invasion assay. The result showed that blocking chondroitin sulfate resulted in inhibition of cell invasiveness (Fig. 3b). To determine whether CS-E, the specific chondroitin sulfate isoform catalyzed by CHST15, is an important component in the extracellular context of chondroitin sulfate driving cell invasion, MDA-MB-231/LZRS-HOTAIR#2 (with ectopic expression of HOTAIR) and the control MDA-MB-231/LZRS-HOTAIR#6 (with basal expression of HOTAIR) cells were treated by an antibody specifically targeting the CS-E chondroitin sulfate isoform or control IgG while inoculated in the Boyden chamber to measure invasiveness (Fig. 3c; see Supporting Information Fig. S4C for a representative invasion assay).[18] The data showed that CS-E antibody markedly suppressed HOTAIR-mediated invasive activity in MDA-MB-231/LZRS-HOTAIR#2 but not the MDA-MB-231/LZRS-HOTAIR#6 cells, further establishing a causal role of the CHST15-chondroitin sulfate axis in HOTAIR-mediated invasion.

The axis of HOTAIR-dependent CHST15 expression was also observed in the murine mammary cancer cell line 4T1 (Fig. 4a). To further test the physiological function of CHST15 in tumor progression, 4T1 cells stably labeled by luciferase (4T1/Luc) were infected with lentiviral vectors carrying shRNA of CHST15 (4T1/Luc/shCHST15) or control empty shRNA vector (4T1/Luc/shCtrl; Fig. 4b). The cells were then implanted into the mammary gland fat pads of Balb/c syngeneic mice. Assessing tumor progression by the IVIS imaging system showed that the control cells developed prominent primary tumors and distant metastasis to the lung which was significantly compromised by depleting CHST15 (Figs. 4c and 4d; see Supporting Information Fig. S5A for bioluminescence images of end-point tumors). The significant presence of 4T1/Luc/shCtrl but not 4T1/Luc/shCHST15 cells in the lung indicates that the function of CHST15 is important for the development of lung metastasis. The CHST15-dependent tumor progression can be recapitulated in xenografts of

a lymph node-tropic luciferase-labeled subline MDA-MB-231-luc-D3H2LN.[25] MDA-MB-231-luc-D3H2LN cells were infected by lentivirus carrying empty shRNA vector (231LN/shCtrl) or CHST15 (231LN/shCHST15) and then implanted orthotopically to the mammary glands of SCID mice. The results showed that depleting CHST15 nearly diminished tumor development of the aggressive cells as measured by the bioluminescence intensity (Fig. 5a). Tumor xenografts were allowed to grow for 47 days, and then harvested to measure the volumes and weights (Fig. 5b; see Supporting Information Figs. S5B and S5C for end-point images of bioluminescence and tumor tissues).

These results together predict a correlation between the expression of HOTAIR and CHST15 in breast cancer tumor tissues. To test this hypothesis, primary breast cancer tumors of a tissue microarray containing 186 tumors/patients were assessed for expression of HOTAIR RNA by *in situ* hybridization (ISH) and CHST15 expression by immunohistochemical (IHC) staining (Fig. 6). To detect HOTAIR, digoxigenin-labeled antisense RNA probes were produced by T7-driven *in vitro* transcription from the D4 domain of HOTAIR RNA, the largest modular secondary structure of HOTAIR.[22] The specificity of the probe was validated in the MDA-MB-231/Tet-shHOTAIR cell system in the presence and absence of doxycycline in which depletion of HOTAIR by shHOTAIR abolished the ISH signal (Supporting Information Fig. S6). The CHST15 protein was detected in sequential sections of the same cohort by a CHST15 antibody. We found that expression of CHST15 and HOTAIR was highly heterogeneous in breast cancer, ranging from relatively high (Fig. 6a, Case 1) to nearly undetectable (Fig. 6a, Case 2). Consistent with the causal role of HOTAIR in CHST15 expression, tumor expression of HOTAIR and CHST15 was significantly correlated in the examined cohort (Fig. 6b). Analysis of gene expression in a cohort of cancer-normal tissue pairs of breast cancer revealed that CHST15 is preferentially expressed in tumor tissues (Supporting Information Fig. S7). The importance of CHST15 in breast cancer is further supported by the observation that its expression is significantly correlated with poor survival in a cohort of breast cancer patients that received neoadjuvant therapy (Supporting Information Fig. S8). Thus, our previous and the current studies together corroborate a model in which HOTAIR induced by growth signaling pathways transcriptionally upregulates CHST15 which plays a key function in glycosaminoglycan metabolism on the cell surface and subsequently modulates invasive activity (Supporting Information Fig. S9).[12]

Discussion

The current study examines the molecular mechanism of HOTAIR-mediated promotion of invasion in breast cancer cells, which leads to the identification of CHST15 as the key downstream factor of HOTAIR. We previously showed that expression of the HOTAIR lncRNA is coordinately controlled by two independent pathways, EGFR and c-ABL, which promote nuclear function of the oncogenic transcription factor β-catenin to induce gene expression of HOTAIR.[12] Given the dynamic metabolic modifications and versatile biological activities of cell surface glycoproteins, it is conceivable that the HOTAIR-CHST15 axis may regulate multiple cellular functions including tumor progression. For example, the dynamic context of cell surface glycoproteins may regulate the interaction between cancer cells and the microenvironment. Our results also show that CHST15 directs

the maintenance of stem cell properties in breast cancer cells. It is not clear how the glycoprotein modification enzyme regulates cell stemness. One possibility is that the extracellular context of glycosaminoglycans serves as a platform for recruiting cognate cellular and extracellular molecules that subsequently signal intracellular functions for cell differentiation. Alternatively, the context of extracellular matrix composed of the GAGs can complex or form coreceptors of growth factors, cytokines and chemokines, which in turn help shape the microenvironment and tumor progression.[26] The orthotopic tumor model clearly showed that CHST15 is important for tumor progression and metastasis. Our results suggest a new mode of crosstalk between the extracellular context and the intracellular signaling by programming the metabolism of cell surface glycoproteins. Importantly, while HOTAIR enhances migration and invasion, modulating CHST15 affects HOTAIR-dependent invasiveness but has no effect on the migratory ability. Thus, our study identifies a sulfotransferase gene downstream of HOTAIR with the ability of modulating extracellular context and playing a unique role in cell invasion.

The main characteristic of chondroitin sulfates is that they are unbranched polysaccharides consisting of amino sugar–uronic sugar disaccharides carrying different degrees of sulfation modifications and hence different electronic charges, which may regulate the cellular functions of chondroitin sulfate proteoglycans.[27] Two sulfotransferases, CHST11/C4ST1 and CHST13/C4ST3, are known to be able to produce chondroitin-4-sulfate of glucuronic acid-N-acetylgalactosamine disaccharides (the sulfotransferases CHST12/C4ST2 and CHST14/D4ST1 prefer iduronic acid-containing disaccharides of dermatan as substrate).[28] CHST15/GalNAc4S-6ST is the only enzyme subsequently catalyzing the sulfation of chondroitin sulfate 4-sulfate to form the disulfated isoform of chondroitin sulfate 4,6-sulfate (CHST3/C6ST1 and CHST7/C6ST2 mainly catalyze sulfation of the 6-hydroxyl group of the chondroitin sulfate backbone to form the CS-C isoform of chondroitin 6-sulfate).[28,29] Thus, CHST15 may have a unique role in modulating the context of extracellular glycosaminoglycans. Our study provides molecular insights into the regulation of CHST15 expression in breast cancer cells and its functional consequence in tumor progression.

CHST15 was originally identified as the mammalian counter-part of the squid sulfotransferase GalNac4S-6ST,[30] and a gene coexpressed with the RAG1 gene in B cells.[31] The molecular mechanisms of CHST15 and its family members in cancer biology are largely unexplored, but emerging evidence suggests crucial functions of these enzymes in cancer development as well as progression. Elevated CHST15 expression was associated with poor disease-free survival and overall survival in patients with pancreatic adenocarcinoma (PDA).[32] Consistently, downregulation of CHST15 resulted in proliferation inhibition *in vitro* and in xenograft animal models of PDA.[33] While these results indicate that CHST15 plays an important role in cancer cell growth, its function in breast cancer is not clear. Furthermore, it remains to be determined how CHST15 is regulated in cancer cells and how it promotes key events in tumor progression such as metastasis. Our current study demonstrates the molecular mechanisms of the HOTAIR-CHST15 axis taking place in MDA-MB-231 and MDA-MB-468 cell lines, both are EGFR-expressing cells derived from triple-negative breast cancer (TNBC). Our results clearly show that expression of CHST15 is important for the development of lung metastasis at least in the 4T1 mouse TNBC cells. For the MDA-MD-231 system, rudimentary but noticeable lung metastasis was preferentially

detected in the lungs of shCtrl but not shCHST15 mice. We were not able to observe full development of lung metastasis by the time when the sizes of primary tumors reached the limitation of the institutional regulation.

Chondroitin sulfates are known major biological blockades of neuronal regeneration after CNS injury.[34–36] It has been demonstrated that the CS-E isoform exerts axonal suppression activity.[18,37] Besides neuron growth, chondroitin sulfates are also known to play a role in tumor proliferation, migration and angiogenesis.[18,37–44] In direct corroboration with these observations, our studies provide evidence supporting important functions of CHST15 and chondroitin sulfates including the CS-E isoform in cell invasion. Importantly, we show that CHST15 expression is under the positive regulation of the lncRNA HOTAIR and is important for HOTAIR-driven invasiveness. HOTAIR joins the family of lncRNAs which are frequently found playing important roles in cell migration, invasion and cancer metastasis.[45,46] Further insights into the underlying mechanism of the regulation may lead to not only identification of novel biomarkers but also the development of new therapeutic targets to suppress cancer metastasis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Abbreviations:

ALDH	aldehyde dehydrogenase
CHST15	carbohydrate N-acetylgalactosamine 4-sulfate 6-O-sulfotransferase 15; GalNAc4S-6ST
CS	chondroitin sulfate
CS-E	chondroitin sulfate E isoform; 4S,6SGalNAc
EGFR	epidermal growth factor receptor

GAG	glycosaminoglycan
HOTAIR	HOX transcript antisense intergenic RNA
IHC	immunohistochemical staining
ISH	*in situ* RNA hybridization
IVIS	*in vivo* imaging systems
lncRNAs	long noncoding RNAs
LSD1	histone demethylation enzyme lysine-specific demethylase 1
PDA	pancreatic ductal adenocarcinoma
PRC2	polycomb repressive complex 2
qRT-PCR	quantitative reverse transcriptase-polymerase chain reaction
tet-shCtrl	tetracycline-inducible control shRNA of scrambled sequence
tet-shHOTAIR	tetracycline-inducible shRNA of HOTAIR
TNBC	triple-negative breast cancer

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What's new?

The long noncoding RNA HOTAIR is known to influence cancer growth, and now researchers have identified a molecule that mediates the effect. Using both loss-of-function and gain-of-function experiments, these authors identified chondroitin sulfotransferase (CHST15) as a downstream target of HOTAIR required for invasive activity of breast cancer cells. Antibodies against cell surface chondroitin sulfate suppressed HOTAIR-induced invasion in xenograft models. Further, HOTAIR expression correlates with CHST15 protein both in primary tumors and metastases. The identification of HOTAIR-CHST15 regulation of cell surface moieties could lead to new therapeutic targets or diagnostic biomarkers for breast cancer.

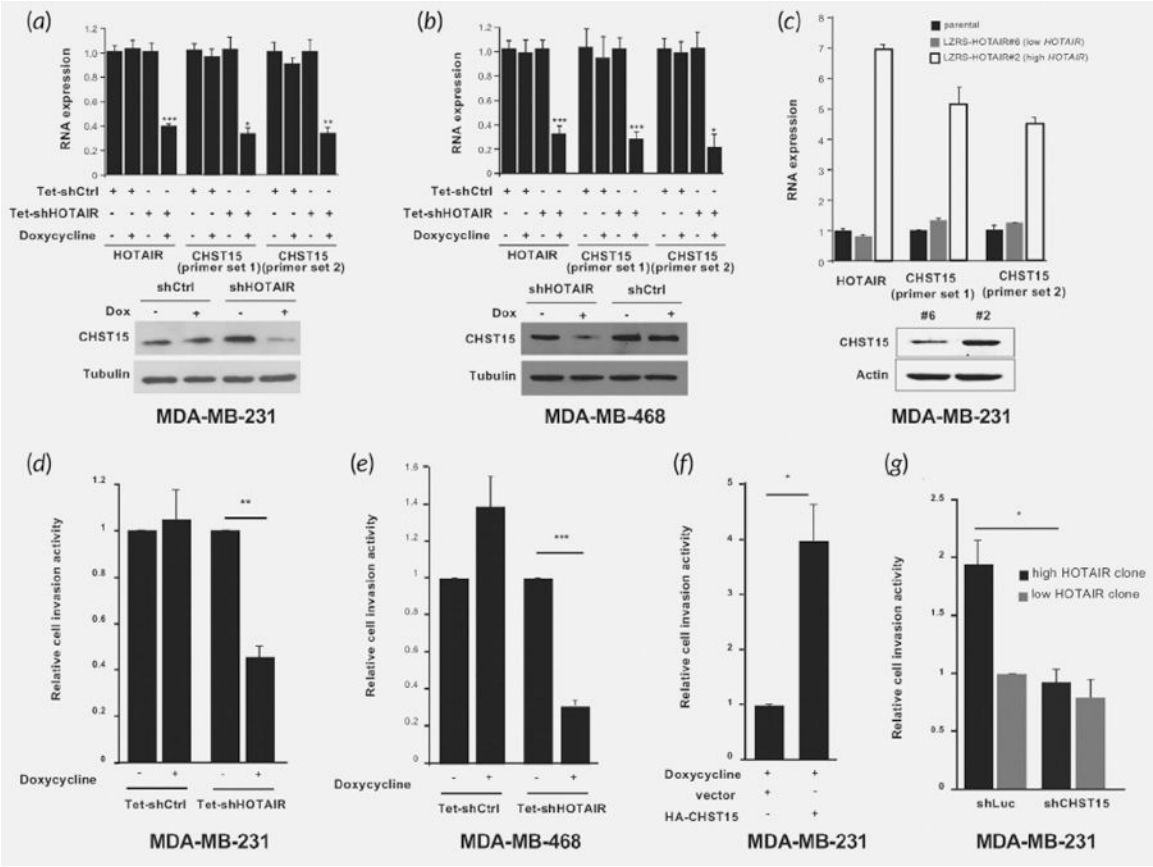


Figure 1.

HOTAIR is required for CHST15 expression and CHST15 is a major downstream factor of HOTAIR-induced invasiveness. (*a*) Top panel, MDA-MB-231 cells stably transfected with a tetracycline-inducible shRNA of HOTAIR (pInducer10/Tet-shHOTAIR) or the control shRNA of random sequence (Tet-shCtrl) are treated with and without doxycycline. RNA was isolated from the treated cells, the level of endogenous HOTAIR and *CHST15* were then assessed by qRT-PCR. Two different sets of *CHST15*-specific primers were tested. The results were normalized to 18S. *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$. Bottom panel, expression of the CHST15 protein was measured by Western blotting analysis. (*b*) Depleting HOTAIR in MDA-MB-468 cells using the same system as described in *a* resulting in downregulation of the endogenous CHST15 gene. (*c*) Ectopic expression of HOTAIR induced CHST15 expression. Top panel, MDA-MB-231 cells were stably transfected by a plasmid encoding HOTAIR cDNA (LZRS-HOTAIR). Stable clones were analyzed for expression levels of HOTAIR and *CHST15* RNA by qRT-PCR. Two different sets of *CHST15*-specific primers were used. Bottom panel, protein expression of CHST15 was measured by Western blotting analysis. (*d*) MDA-MB-231/Tet-shHOTAIR and MDA-MB-231/Tet-shCtrl cells were treated with or without doxycycline and the invasive activities were determined *in vitro* by Boyd chamber assay. **$p < 0.01$. (*e*) MDA-MB-468/Tet-shHOTAIR and MDA-MB-468/Tet-shCtrl cells were treated with or without doxycycline for 48 hr and the invasive activities were determined *in vitro* by Boyd chamber assay. ***$p <$ 0.005. (*f*) Reconstitution of CHST15 in MDA-MB-231/Tet-shHOTAIR by transient

transfection in the presence of doxycycline (HOTAIR-depleted) rescued invasive activity. *$p < 0.05$. (*g*) MDA-MB-231 cells with (Clone #2) and without (Clone #6) ectopic expression of HOTAIR by stable transfection of the LZRS-HOTAIR plasmid followed by infecting with lentivirus carrying shRNA specific for *CHST15* (shCHST115) or the control shRNA targeting *luciferase* (shLuc) were assessed for relative invasiveness by Boyd chamber assay. *$p < 0.05$.

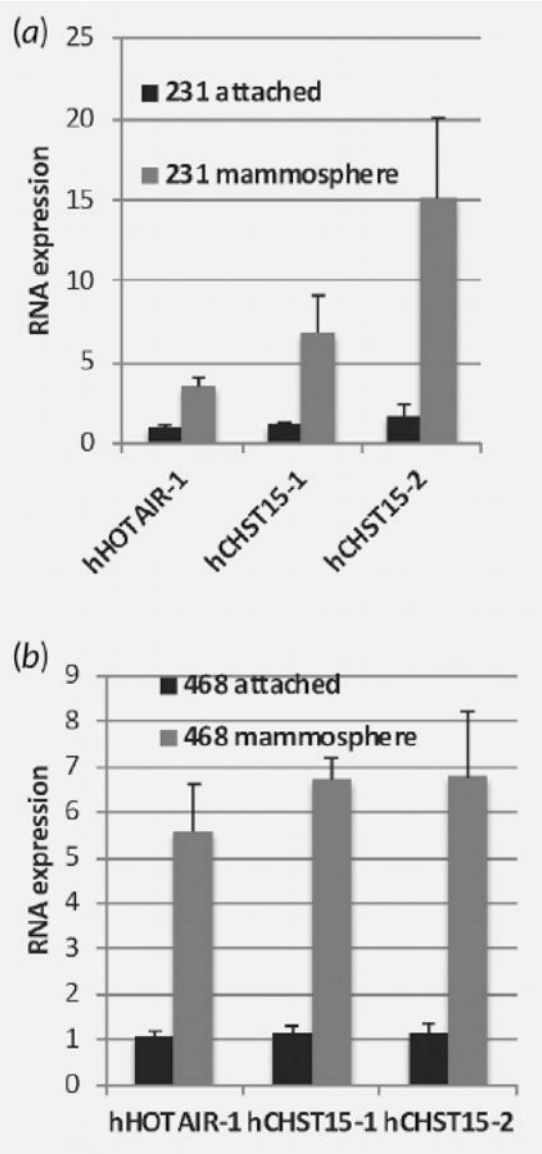


Figure 2.

CHST15 is a key downstream factor of HOTAIR in promoting stemness in cancer cells. (*a*) MDA-MB-231 cells were grown in suspension culture and allowed to form mammospheres. Relative RNA expression of *HOTAIR* and *CHST15* in the spheroid compared to the parallel culture of attached monolayer was assessed by qRT-PCR. (*b*) The same experiment in MDA-MB-468 cells.

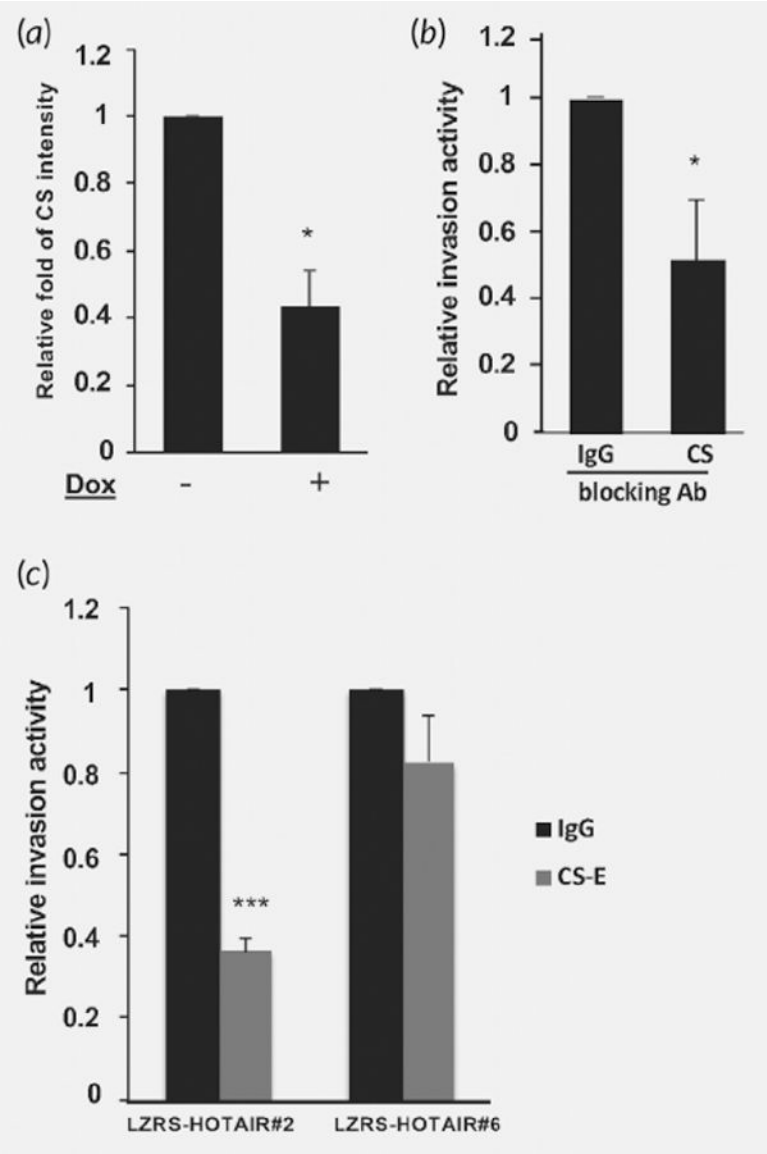


Figure 3.

HOTAIR-regulated chondroitin sulfate plays an important role in invasiveness *in vitro.* (*a*) MDA-MB-231/Tet-shHOTAIR cells were treated with or without doxycycline and the levels of chondroitin sulfate (CS) were detected by immunofluorescent staining using a pan-CS antibody. The results were quantitated by Image J which shows that depleting HOTAIR resulted in CS downregulation. *$p < 0.05$. (*b*) MDA-MB-231/LZRS-HOTAIR#2 cells were treated with IgG or a CS-blocking antibody. Cell invasiveness was assessed in Boyden chamber assays and quantitated based on the absorbance of crystal violet staining of the invaded cells. *$p < 0.05$. (*c*) MDA-MB-231/LZRS-HOTAIR#2 and MDA-MB-231/LZRS-HOTAIR#6 cells were incubated with a CS-E-targeting antibody or control IgG (10 μg/ml) while loaded to the Boyden chamber to assess cell invasiveness. Results of the invasion assay were quantitated based on the absorbance of crystal violet staining of the invaded cells. ***$p < 0.005$.

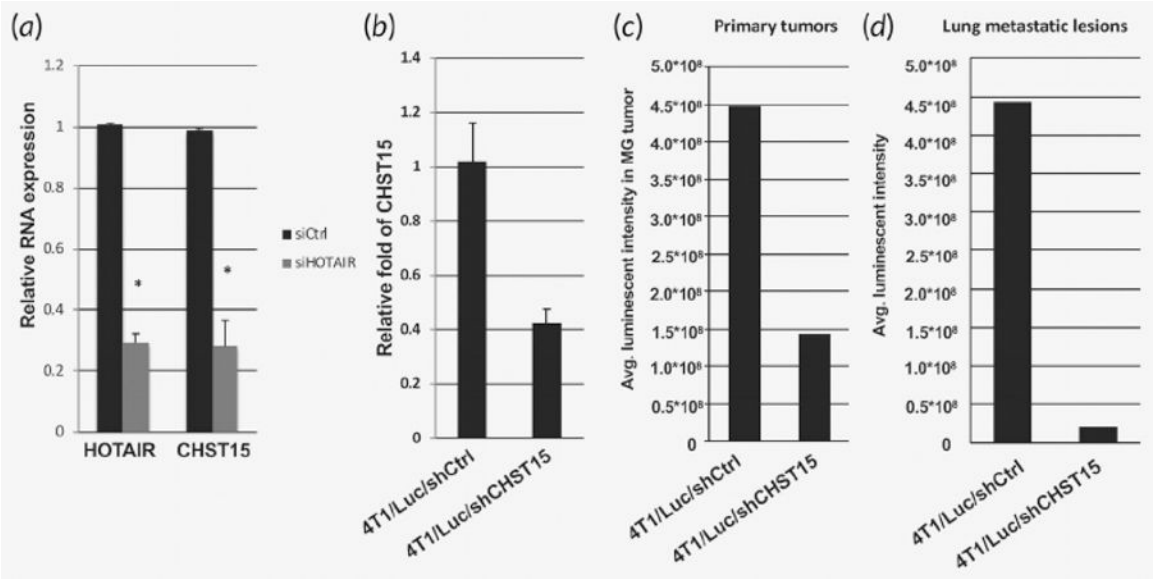


Figure 4.

The HOTAIR-CHST15 axis is effective in murine breast cancer cells. (*a*) Mouse HOTAIR gene was silenced by siRNA of HOTAIR (siHOTAIR) or the scramble control siRNA (siCtrl) in 4T1 cells. RNA expression of HOTAIR and CHST15 was determined by qRT-PCR. (*b*) 4T1 cells stably expressing the luciferase marker gene were infected with lentivirus carrying shRNA of CHST15 (shCHST15) or the control virus harboring empty vector (shCtrl). The expression of CHST15 was confirmed by qRT-PCR. (*c*) The engineered cells were implanted orthotopically to the abdominal mammary glands BALB/c syngeneic mice. Tumor growth and progression were monitored by IVIS imaging system for the luciferase reporter activity using the same exposure intensity for both groups, luminescent signals derived from the primary mammary tumors (*c*) and in the lung metastasis (*d*), were quantitated and plotted.

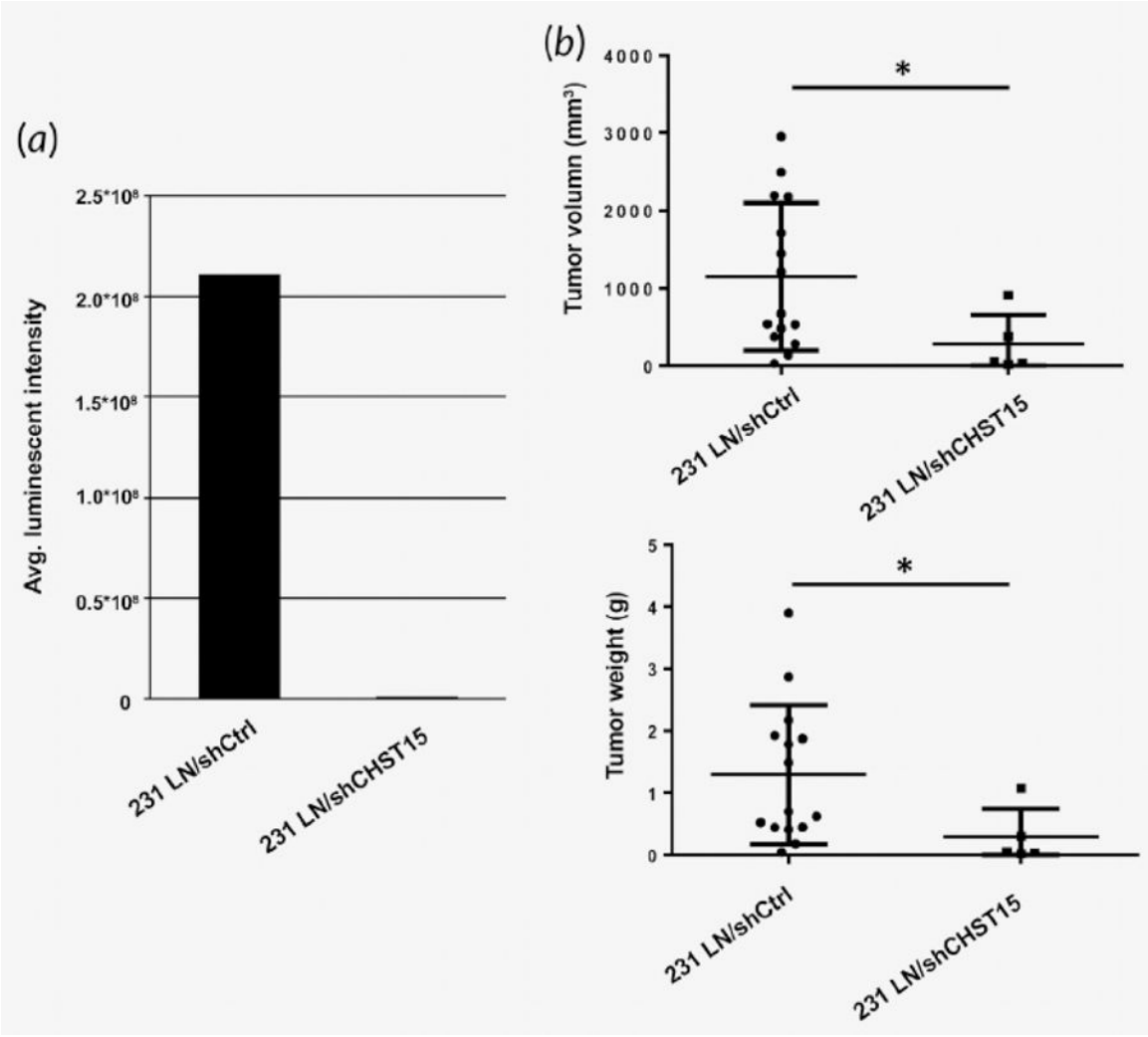
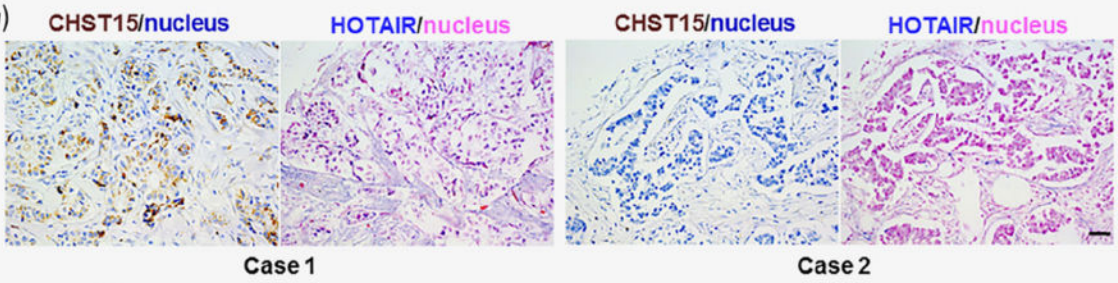


Figure 5.

CHST15 is required for tumor development of MDA-MB-231 breast cancer cells. (*a*) MDA-MB-231-luc-D3H2LN cells stably expressing the luciferase marker and infected by lentivirus carrying shRNA of CHST15 (231LN/shCHST15) or the control empty vector (231LN/shCtrl) were implanted into the abdominal mammary glands of SCID mice. IVIS images were taken 7 weeks after tumor inoculation. Note that the 231LN/shCHST15 group was imaged with prolonged exposure to reveal signals of the residual tumors. (*b*) The volumes and weights of the tumors are plotted. *$p < 0.05$.

(*a*)



(*b*)

IHC \ ISH	Cut-off by median: HOTAIR high	Cut-off by median: HOTAIR low	IHC \ ISH	Cut-off by average: HOTAIR high	Cut-off by average: HOTAIR low
CHST15 high	72	23	CHST15 high	25	23
CHST15 low	37	54	CHST15 low	48	90
Fisher exact test P < 0.00001; Chi square test P = 0.000001			Fisher exact test P = 0.0403; Chi square test P = 0.0344		

Figure 6.

Correlation between HOTAIR and CHST15 in breast cancer tumor tissues. Primary breast cancer tumor tissues ($n = 20$) were analyzed for expression of HOTAIR by FISH and CHST15 by IHC. (*a*) Representative stained tissues with positive (Case 1) and negative (Case 2) expression of HOTAIR and CHST15 are shown. (*b*) Compiled result was analyzed by Fisher's exact test which showed the expression of the two factors is significantly correlated.