REVIEW ARTICLE

Role of Nischarin in the pathology of diseases: a special emphasis on breast cancer

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Nischarin has been demonstrated to have tumor suppressor functions. In this review, we comprehensively discuss up to date information about Nischarin. In addition, this paper aims to report the prognostic value, clinical relevance, and biological significance of the Nischarin gene (NISCH) in breast cancer (BCa) patients using the Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) and The Cancer Genome Atlas (TCGA) datasets. We evaluated NISCH gene expression and its correlation to patient survival, baseline expression, and expression variation based on age groups, tumor stage, tumor size, tumor grade, and lymph node status in different subtypes of BCa. Since NISCH has been extensively reported to inhibit EMT and cancer cell migration, we also checked for the correlation between NISCH and EMT genes in addition to the correlation between NISCH and cell migration genes. Our results indicate that NISCH is a tumor suppressor that plays a critical role in BCa initiation, progression, and tumor development. We find that there is a higher level of NISCH expression in normal breast tissues compared to breast cancer tissues. Also, aggressive subtypes of breast cancers, such as the triple negative/basal category, have decreased levels of NISCH as the disease progresses. Finally, we report that NISCH is inversely correlated with many EMT and cancer cell migration genes in BCa. Interestingly, we identified a significant negative correlation between NISCH expression and its methylation in breast cancer patients. Overall, the goal of this report is to establish a strong clinical basis for further investigation into the cellular, molecular, and physiological roles of NISCH in BCa. Ultimately, NISCH gene expression might be clinically harnessed as a biomarker or predictor of invasiveness and metastasis in BCa.

INTRODUCTION

NISCH is a novel tumor suppressor protein that was identified by our laboratory as an interacting partner of integrin α5 subunit [1]. It is ~200kD in size and is presently being studied as a potent suppressor of migration and metastasis in breast cancer (BCa). The NISCH gene has been successfully mapped to chromosome 3p21, a metastasis-suppressor locus that is frequently lost in breast and several other types of cancer [2]. In accordance with this, NISCH overexpression inhibits growth of breast tumors and cellular events related to BCa cell metastasis [3]. Furthermore, STK11/LKB1 tumor suppressor interacts with the N terminus of NISCH and that the amino acids 416–624 of NISCH are crucial for this interaction [4]. This interaction between both tumor suppressors has been shown to enhance LKB1 kinase activation and regulate cell migration of breast epithelial cells [4]. Furthermore, the correlational status of these essential genes in BCa patients will serve as an important validatory approach. Interestingly, NISCH has been reported to have an anti-neuronal growth effect as well [5, 6]. Furthermore, it is shown that NISCH also regulates exosome secretion, long noncoding RNA expression, apoptosis, mammary gland development, and epithelial to mesenchymal transition (EMT) in BCa [7–15].

BCa is the most common invasive malignant disease that affects women worldwide, with estimates of over 280,000 new cases of invasive disease and over 40,000 deaths just in the United States in 2021 [16]. For more insight on this disease, it should be noted that the American Cancer Society regularly updates its website with vital information regarding the risks, diagnosis, and treatment of BCa and can be found using the link in parenthesis (https://www.cancer.org/cancer/breast-cancer/about/how-common-is-breast-cancer.html). Despite all tumors originating from the breast being referred to as BCa, this disease can be additionally categorized into subtypes based on hormone receptor status of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor type 2 (HER2/neu) amplification [17]. Each BCa subtype has unique attributes such as the age of onset, disease progression, and even therapeutic strategy. For example, tamoxifen, a selective estrogen receptor modulator, would be used to treat ER + BCa, while trastuzumab, an anti-HER2/neu monoclonal antibody, would be used to treat HER2 + tumors [18].

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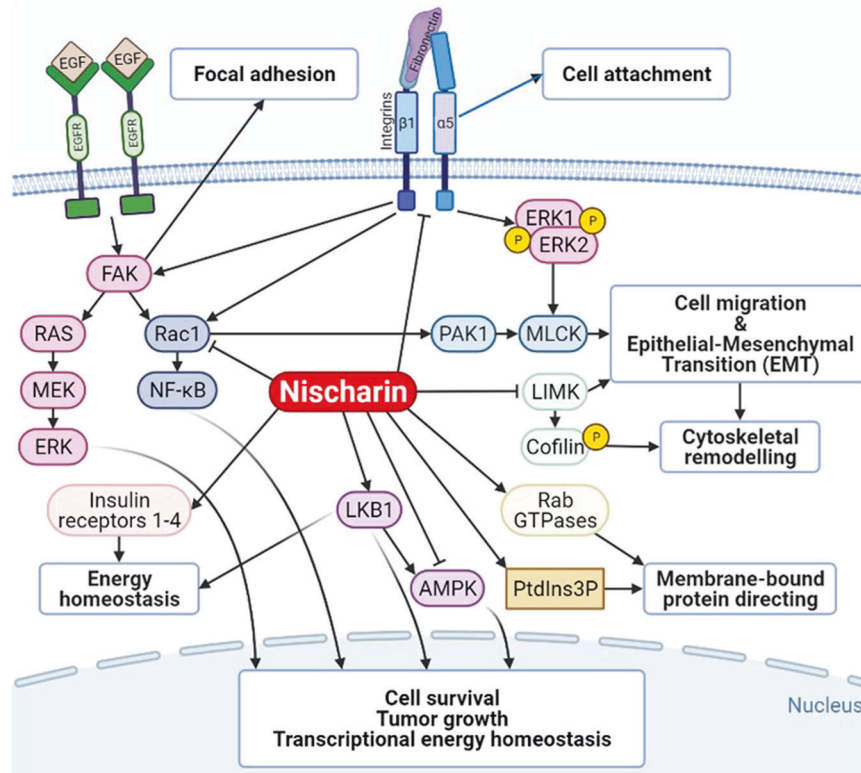
Fig. 1 Signaling pathways regulated by Nischarin. Nischarin regulates α5-integrin expression resulting in reduced FAK activation leading reduced Ras signaling which regulates cell survival. Also, Nischarin affects NFkB signaling through Rac1 that regulates cell survival. Nischarin and LKB1 interact to reduce tumor growth. Interestingly Nischarin also directly affects AMPK, a downstream effector of LKB1. Nischarin directly interacts with PAK1, LIMK1 and affects their kinase activities that regulate cell migration and EMT. In addition, phosphorylation of LIMK through PAK inhibits cofilin, an actin severing protein, thus leading to cytoskeletal remodeling. Nischarin regulates Rab GTPases and PtdIns3P that are known to regulate membrane-bound protein targeting. Nischarin regulates energy homeostasis through LKB1 and AMPK pathways.

HISTORY OF NISCH
We discovered a protein that links the cytoplasmic domains of various α integrin subunits, especially the α5 subunit. Previously it was identified as IRAS [19]. This protein was named Nischarin, which signifies slowness of motion in Sanskrit as we observed reduced cell migration upon expression of Nischarin (NISCH). NISCH associates with proteins Rac1, PAK1, LIMK, and LKB1 to decrease focal adhesions (adhesive contact between cell and extracellular matrix), organization of the cytoskeleton, cell migration, growth of tumors, cell survival, and has been shown to play a role in the phagocytosis of pathogens (Fig. 1) [2]. RNA and protein expression of NISCH is elevated in low-grade human breast cancers but is much lower in high-grade breast cancers [3]. The NISCH promoter is often hypermethylated in breast and lung cancers [20]. NISCH contains a terminal PX domain at the amino terminus, a leucine repeat region, a coiled-coil domain, and a very long carboxy-terminal region. Coiled-coil region helps NISCH to homo-oligomerize [21]. Phosphatidylinositol 3-phosphate binds to NISCH through its PX domain, suggesting that different domains of NISCH have distinct functions. NISCH binds to the GTP-bound Rab14 and PI(3)P to lead the maturation from early endosomes to Rab9/CD63-containing late endosomes [22]. These data indicate that NISCH regulates multiple signaling pathways, and the interaction between NISCH and GTPases controls maturation and acidification of the vacuoles after phagocytosis of pathogens.

ENDOGENOUS EXPRESSION OF NISCH
As depicted on the protein atlas (https://www.proteinatlas.org/ENSG00000010322-NISCH/tissue), NISCH is ubiquitously expressed; highest in cerebellum, adrenal gland, bronchus, rectum, gall bladder, heart muscle, and skin; medium level of expression in cerebral cortex, thyroid gland, nasopharynx, oral mucosa, salivary gland, esophagus, stomach, duodenum, small intestine, colon, kidney, testis, ovary, fallopian tube, endometrium, placenta, breast, smooth muscle, skeletal muscle, soft tissue, adipose tissue, appendix, lymph node, and tonsil; and lowest expression in lung, liver, pancreas, urinary bladder, epididymis, seminal vesicle, prostate, vagina, cervix, and spleen (Supplementary Table 1).

POST-TRANSLATIONAL MODIFICATIONS OF NISCH
NISCH has several potential post-translational modification sites. Ubiquitination is one of the major post-translational events that plays an important role in several diseases such as metabolic syndromes, muscular dystrophy, cancer, neurodegeneration, autoimmune, and inflammatory diseases. NISCH is potentially ubiquitinated at these different lysine sites: K59, K79, K353, K358, K454, K843, K865, K902, K942, K1009, K1015, K1290, K1299, K1303, K1316, and K1335. Acetylation plays an important role in protein that regulates gene transcription and signal transduction. The acetylation of proteins chiefly depends on lysine acetyltrans-ferases and lysine deacetylases. Acetylation is predicted to take place at the K1015 site of NISCH. Protein phosphorylation plays a crucial role in several protein signal transduction pathways. Several proteins get activated/deactivated by phosphorylation and dephosphorylation events. Protein kinases are responsible for phosphorylation, and their activation/expression regulates the progression of several diseases, mostly tumors. The predicted phosphorylation sites for NISCH include S51, S246, S250, T252, S477, S541, S546, S883, S1004, S1022, S1038, S1200, T1282, S1284, Y1293, S1294, Y1307, T1372, and T1376 (Fig. 2). For further details
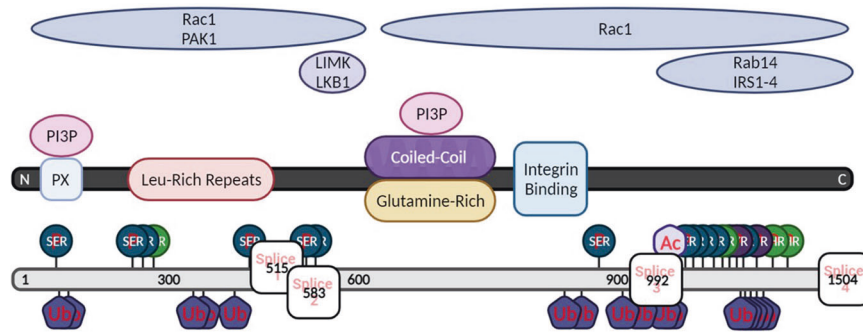
Fig. 2 Mutation diagram for NISCH gene (exons) and protein from The Cancer Genome Atlas (TCGA) extracted from cBioPortal (https://www.cbioportal.org). The schematic diagram shows the NISCH protein domains and the locations of specific mutations. Post translational mutations has also been shown. PX (PX domain); LRR_8: (Leucine rich repeat).

regarding the afore-mentioned post-translational modifications associated with NISCH, please access this link: (https://www.phosphosite.org/proteinAction?id=15675&showAllSites=true)

MUTATIONS OF NISCH

Based on the analysis and results of a high throughput screening, it is believed that NISCH is mutated in several cancers (https://www.phosphosite.org/proteinAction?id=15675&showAllSites=true). According to the publicly available data, gene alterations and mutations associated with NISCH expression varies across different cancers. Notably, colorectal adenocarcinoma has the highest percentage of total alteration frequency, while uterine corpus endometrial carcinoma has the second-highest percentage of total alteration frequency and the highest percentage of mutation frequency (Fig. 2, Supplementary Fig. 1, Supplementary Fig. 2 and Supplementary Table 2).

NON-CODING RNAS AND NISCH

Interestingly, noncoding RNAs modulate the function of NISCH as well. MicroRNAs control and regulate the expression of NISCH as it is enhanced by the knockdown of microRNA-23b and microRNA-27b [23]. Both microRNAs have an inverse correlation with NISCH expression in human breast cancers, which implicates their biological relevance. Surprisingly, NISCH controls the expression of both microRNAs through a feedback loop mechanism by subduing the phosphorylation of NF-KB [23]. Furthermore, NISCH also affects long noncoding RNAs. Specifically, NISCH affects the functions of long noncoding RNA Malat1 [12]. It was demonstrated that the expression of NISCH is needed for Malat1 to function in breast cancer [12].

KNOCK-OUT MODELS OF NISCH

Currently, there are three knock-out mouse models for NISCH. Zhang and colleagues made a knock-out model for NISCH by deleting the fourth exon [24]. These mice were much smaller than the normal mice, suggesting that NISCH plays an important role in embryonic development. Furthermore, the knock-out mice gained a tolerance to morphine and developed a dependence through regulating the expression of the μ opioid receptor [25]. Another mouse model that is used is the Otitis Media model. This was made from a large N-ethyl N-nitrosurea mutagenesis of the mutation L972P in the NISCH gene [26]. Homozygous mice that have this mutation develop a type of conductive hearing loss due to inflammation of the middle ear, which is analogous to otitis media in humans [26]. Our group made NISCH knock-out mice as well by deleting exons 7 thru 10 [27]. The embryos of these mice

showed delayed development and were characterized by their small size and decreased weight gain. One possible reason for this is due to the fact that NISCH links to and inhibits the functions of AMPK, a metabolic regulator. AMPK controls energy homeostasis by obstructing anabolic and promoting catabolic functions [27]. Also, the signaling of insulin was altered in many insulin-sensitive tissues in the female NISCH knock-out mice [28]. These females gained significant insulin resistance and reduced tolerance to glucose. These mice accumulated much more white fat, had bigger adipocytes, and developed hepatic steatosis indicating potential metabolic abnormalities in these mice. Interestingly, NISCH mRNA expression is inversely correlated to obesity, fat distribution, and the metabolism of lipids and glucose in humans [28]. Another study revealed that there was a defect in the development of the mammary gland [10]. Increased tumor growth and metastasis in the NISCH knock-out mice expressing the gene PyMT was observed. Unexpectedly, the NISCH mutation reduced the function of AMPK and its downstream effectors [10]. Thus, the mice were treated with metformin, which is known to improve the activity of AMPK. From this, it was shown that not only does metformin activate AMPK signaling, but it also inhibits tumor growth in PyMT tumors without NISCH. In summary, metformin can potentially be used as a cancer therapeutic, especially in breast cancers that lack NISCH.

ASSOCIATION OF NISCH WITH DISEASES

NISCH is associated with several diseases, including hypertension, xerostomia, morphine dependence, depression, anxiety, ventricular hypertrophy, congestive heart failure, rosacea, and multiple cancers [2]. NISCH interacts with 18 proteins to influence cell adhesion, cell proliferation, cell migration, vesicle trafficking, apoptosis, cancer growth, exosome secretion, glucose metabolism, and cell signaling. The proteins that interact with NISCH include α5 integrin, Rac1, P21 activated kinase, LIM kinase, LKB1, phosphatidylinositol 3-phosphate, Rab14, Rab4a, Rab9a, Rab38, Insulin receptor substrates 1-4, and AMP kinase [2] (Fig. 1).

NISCH is also found to be hypermethylated in advanced cancers, leading to low levels of expression. A recent study revealed that serum NISCH and E-cadherin promoters are hypermethylated in lung cancer, indicating that hypermethylation of NISCH may be used as a prognostic liquid biopsy marker for cancers. Surprisingly, methylation of NISCH is also connected to cigarette smoke and suggests that cigarette smoke induces methylation of the NISCH promoter [20], making NISCH methylation a potential prognostic marker before patients develop lung cancer. Since NISCH expression is regulated by hypermethylation, it is possible to use plasma DNA for early detection of lung cancer by methylation-specific PCR. Abnormalities of lung cancer are usually detected by computed tomography (CT) scan; however,

these CT scans are not reliable, and thus methylation-specific plasma markers can distinguish between noncancerous and cancerous lungs. NISCH inhibits cell migration, invasion, and EMT by increasing E-cadherin expression and inhibiting transcription factors such as Snail, ZEB1, N-cadherin, Slug, Twist-1, and vimentin in breast cancer cells [9]. Interestingly, the expression of NISCH negatively correlates with estrogen receptor in breast cancer cells [15]. Consistent with our own data, a study showed that NISCH expression is low in patients with lymph node metastasis and suggested that expression of NISCH could be used as a marker to predict metastasis of breast cancer [29]. In addition to a decrease in expression levels of NISCH in breast cancer tissues, NISCH expression negatively correlates with estrogen receptor status [15]. NISCH functions as a tumor suppressor in ovarian cancer as well by regulating the FAK/ERK signaling pathway [30]. Thus, NISCH plays an important role in various diseases, and its function is well-studied in cancers.

Genome-wide association studies (GWAS) gave some potential clues of NISCH's function in several psychiatric disorders such as attention deficit hyperactivity disorder (ADHD), bipolar disorder, and schizophrenia [31]. One very interesting finding was reported using an artificial intelligence algorithm, direct casual learner (DCL) [32]. The authors applied DCL to both simulated, clinical, and genome-wide association study (GWAS) datasets. From this analysis, DCL identified rs6784615, located on the NISCH gene, as an important player in developing late-onset Alzheimer's disease [32]. Furthermore, DCL identified previously implicated ER and Her2 genes as predictors of cancer mortality [32]. NISCH has been shown to have an association with otitis media, an inflammatory disease of the middle ear [33]. NISCH has also been depicted to have pharmacological importance, which includes its role in rilmedine evoked hypotension [34], alteration of agmatine effect on calcium signal in morphine dependent cells [35, 36], and increased binding to alpha (2 A)-noradrenegric receptor [37]. NISCH interacts with μ opioid receptor (MOR) and regulates trafficking [38] suggesting that this might be a mechanism by which NISCH modulates opioid tolerance and dependence [39]. Consistent with this, NISCH (IRAS) knockout mice showed aggravated analgesic tolerance and physical dependence morphine [38], and mechanistically it is due to altered MOR expression and phosphorylation of AMPA receptor GluR1-S845. In addition, NISCH plays an important role in the regulation of spinal cord injury [13]. Oxidative stress-induced apoptosis is a mechanism involved in spinal cord injury. NISCH has been suggested to function as pro-apoptotic protein, which regulates oxidative stress coinciding with the function in spinal cord injury [13]. High levels of NISCH are expressed in fat tissues and islets, and eight different splice variants were detected in islets [40]. A positive correlation was detected between GLP1 and GIP receptors. Glucose-stimulated insulin secretion is enhanced upon treatment with imidazoline receptor agonist on non-diabetic and type 2 diabetic islets suggesting that the agonist could be an attractive insulinotropic and beta cell protective agent [40]. Morin is a flavonoid which has been implicated in reducing hyperglycemia in diabetes [41]. Morin promotes glucose-stimulated insulin secretion in MIN cells. Suppression of NISCH expression reduced insulin secretion, suggesting that NISCH may play an important role in diabetes [29, 41]. Visceral fat accumulation (VFA) plays an important role in developing metabolic disorders such as type 2 diabetes, and a single nucleotide polymorphism (SNP) of NISCH (rs6784615) is associated with increased VFA [42]. Agmatine is an endogenous ligand for imidazoline receptor, and NISCH has been implicated in the effect of agmatine on morphine dependence [25]. A meta-analysis of 32 GWAS studies indicated that NISCH-STAB1 locus associated with waist-hip ratio (WHR) suggesting NISCH 's role in overall adiposity [43]. Another GWAS analysis from South African tuberculosis patients detected association between NISCH and tuberculosis susceptibility [44]. A compound

5-isothiocyanato-2-benzofuranyl-2-imidizalonie (BU99006) has been shown to upregulate NISCH expression in the hippocampus and lead to inhibition of MAP kinase signaling pathway [45]. These reports indicate that NISCH is not only important in cancer arena but in other disease processes as well.

INFLUENCE OF NISCHARIN IN THE NERVOUS SYSTEM

Though we have covered some of the roles of NISCH in the nervous system [2], there are newer findings that are worth highlighting more in this review. Researchers are steadily investigating the different roles of NISCH in the proper functioning of the brain and the findings so far have been very significant. A recent report indicated that NISCH expression and associated signals were broadly observed throughout the brainstem of newborn rats within the ages of 3–5 days [46]. These data indicate that NISCH is expressed in the brainstem of rats including the motor nuclei, the rostral and caudal ventrolateral medullar regions [46]. In addition, NISCH has been implicated the PI3K/PKB pathway-dependent neuronal apoptosis [47]. NISCH expression is evident in the hippocampus region in mouse brains [45] and this is associated with the regulation of MAPK signaling pathways [45]. Interestingly, platelet NISCH expression level has been investigated and reported as a potential biomarker important in differentiating between primary and cocaine induced major depressive disorder [48]. Another study found that NISCH is dysregulated in depressed brains, supporting a potential role for NISCH in the neurobiology of major depressive disorder and in the molecular mechanisms of antidepressant drugs [49]. Inhibition of NISCH has also been reported to promote neurite outgrowth and neuronal regeneration in Neuro-2a cells and primary cortical neurons [50]. This inhibition shown to induce the expression of growth-associated protein-43 and accelerate function recovery after spinal cord injury [50]. PC12 cells are commonly used in neuroscience research, including studies on neurotoxicity, neuroprotection, neurosecretion, neuroinflammation, and synaptogenesis [51]. These cells are a type of catecholamine cells that synthesize, store and release norepinephrine and dopamine. In PC12 cells, NISCH downregulation attenuates cell injury induced by oxidative stress via the Wnt signaling pathway [52]. In addition, NISCH attenuates apoptotic events induced by oxidative stress in PC12 cells [13]. Altogether, these studies show that NISCH has rapidly emerged as an important player in the cellular and molecular functioning of the nervous system.

ANALYSIS OF NISCH GENE EXPRESSION IN BREAST CANCER USING BIOINFORMATICS APPROACHES

The role of NISCH in breast cancer has been extensively studied by our group and many other research groups around the world. However, there is an urgent need for the understanding of the clinical relevance of NISCH based on human data. Therefore, we have carefully explored different databases comprised of a large set of patient data in order to extrapolate and better understand the clinical and prognostic value of NISCH. Here, we gathered and analyzed datasets from the molecular taxonomy of breast cancer international consortium (METABRIC) and the cancer genome atlas (TCGA). These are the two of the largest datasets documenting a vast amount of information on BCa patients. In brief, we analyzed datasets and compared large-scale patient sample data of NISCH mRNA expression and methylation status in normal vs cancer tissues, intrinsic subtypes, and hormone receptor subtypes. In addition, we performed further analysis of NISCH mRNA expression in samples of different tumor stages, tumor sizes, tumor grades, lymph node status, and patient age. Lastly, we used gene expression analysis to identify correlations of NISCH mRNA expression with known downstream-target expression as well as EMT markers.
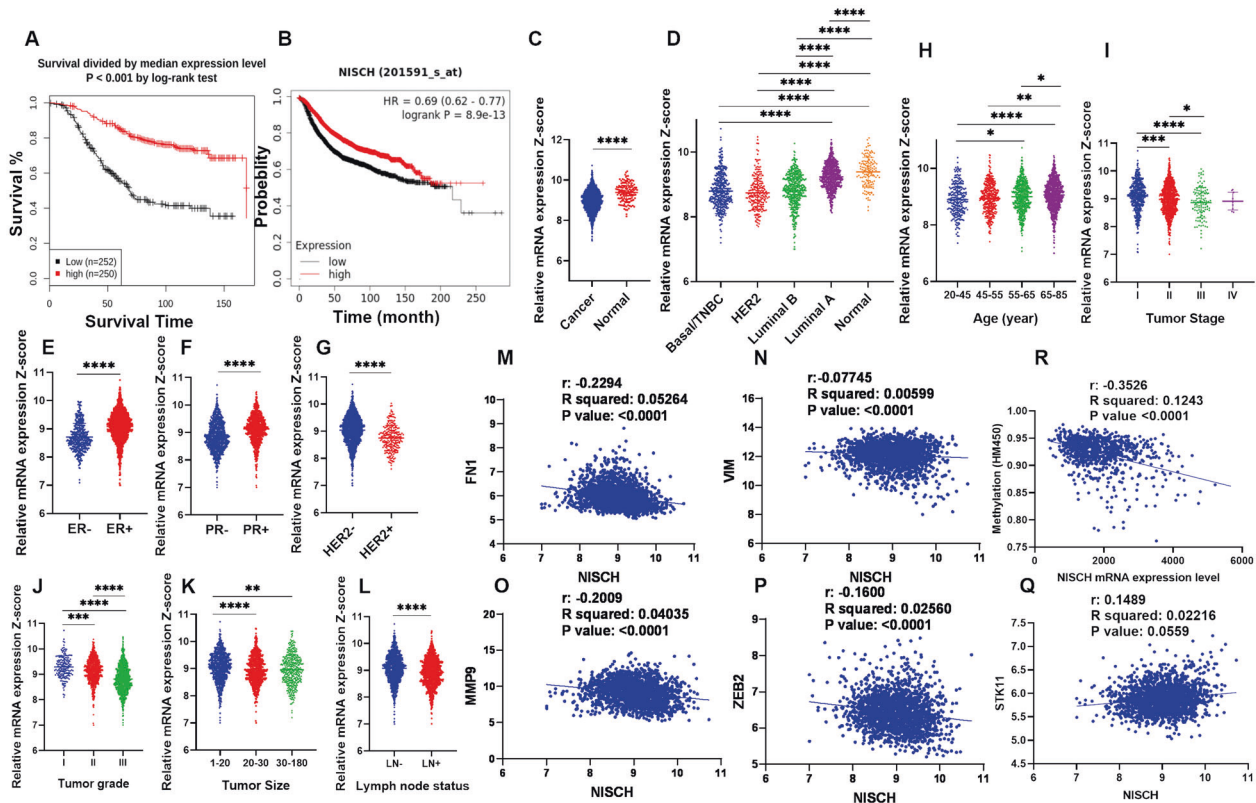
Fig. 3 Nischarin expression is associated with tumor subtype, grade, stage, size and DNA methylation status in breast cancer patients. Kaplan–Meier online platforms validated the association of clinical outcome and Nischarin expression **A** overall survival (OS) and **B** relapse-free survival (RSF). Kaplan–Meier overall survival (OS) curves for patients with breast cancer divided by the median value into low and high Nischarin mRNA expression using the GENT2 data set. Kaplan-Meier analysis (kmplot.com) of relapse-free survival (RFS) was used based on the mean value of Nischarin in breast cancer (*n* = 4929 patients), and probe 201591_s_at (NISCH). Nischarin is differentially expressed across different breast cancer intrinsic subtypes. **C** RNA-Seq mRNA expression data for normal and breast cancer tissue from METABRIC database in normal (*n* = 148) and cancerous (*n* = 1826) breast tissue samples. **D** Gene expression of Nischarin in METABRIC by Pam50 gene expression subtype classification. Scatterplots show that there is a significant association between breast cancer subtypes and the level of Nischarin gene expression in breast cancer patients. Nischarin expression is partially correlating with estrogen receptor breast cancer ER+, PR+ and HER2 subtypes. Relative Nischarin gene expression in different breast cancer tumors (METABRIC) based on **E** ER, **F** PR, and **G** HER2 status examined by IHC. Nischarin expression is differentially modulated by age, tumor stage, tumor size, tumor grade, and lymph node metastasis in breast cancer. Relative expression of Nischarin gene in different breast cancer tumors **H** ages, **I** stages, **J** tumor grade, **K** tumor size, and **L** lymph node metastasis. Nischarin gene expression correlation with EMT markers in breast cancer. Correlation analysis of the METABRIC breast cancer data set comparing the correlation between **M** Nischarin:FN1 expression, **N** Nischarin:VIM expression, **O** Nischarin:MMP9 expression, **P** Nischarin:ZEB2. Analysis was based on the mRNA expression Z-scores ratios. Nischarin directly correlates with STK11 and inversely correlates with its methylation status in breast cancer. **R** Correlation analysis of the TCGA breast cancer data set comparing the correlation between Nischarin gene expression and its DNA methylation status. Gene expression was found to be negatively correlated with DNA methylation. **Q** Correlation analysis of the METABRIC breast cancer data set comparing the correlation between Nischarin:STK11 expression. Analysis was based on the mRNA expression Z-scores ratios. The Pearson correlation coefficients (*r*) and the relative p-values are shown. The association between genes was measured using the Pearson correlation coefficient (*r*) and respective computed *P*-value. Data were analyzed by one-way ANOVA followed by a *t*-test. Statistically significant values of *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$ were determined.

NISCH ENHANCES OVERALL SURVIVAL AND RELAPSE-FREE SURVIVAL IN BCA PATIENTS

Based on our analysis of data from the Kaplan-Meier plotter database, as explained in the approach section in the supplementary methods section, we found that NISCH is critical for overall survival and relapse-free survival of BCa patients (Fig. 3A, B). Overall survival is a relevant parameter that helps to determine the efficacy of specific treatments or the significance of target genes in regulating cancer initiation and progression. Our analysis showed that a low level of NISCH is significantly associated with worse overall survival for BCa patients, and this is observed over a time duration of 150-months (Fig. 3A). As expected, we found that high levels of NISCH is significantly associated with better overall survival (Fig. 3A). On the other hand, low levels of NISCH were found to be significantly associated with poor relapse-free survival for BCa patients, and

this was observed over a time duration of 250-months (Fig. 3B). Accordingly, we also found that high levels of NISCH is significantly associated with better relapse-free survival (Fig. 3B). Altogether, the results of our analysis indicate that NISCH plays a critical regulatory role that is essential for overall and relapse-free survival in BCa patients.

NISCH IS DIFFERENTIALLY EXPRESSED ACROSS DIFFERENT BCA INTRINSIC SUBTYPES

Following our analysis of the METABRIC dataset, we found that the relative mRNA expression of NISCH is significantly higher in normal subjects compared to BCa patients. This was more evident when samples from all different intrinsic subtypes of BCa were pooled together (Fig. 3C). This outcome has been previously reported by our group following a careful examination
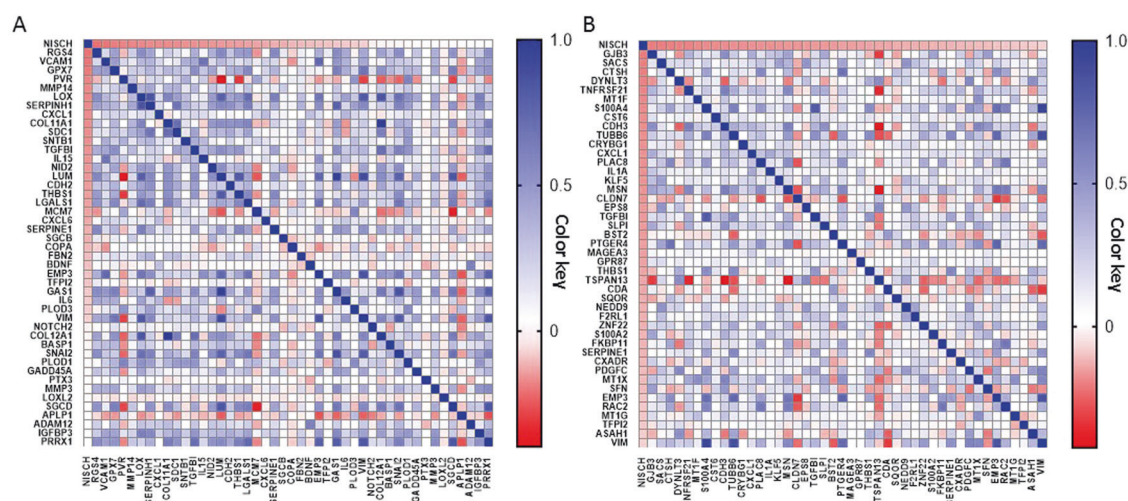
Fig. 4 Correlation analysis between NISCH and other genes. Correlation analysis between Nischarin gene and the (**A**) EMT and (**B**) Migration-related markers using the marker genes from HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION and WU_CELL_MIGRATION gene sets, respectively (https://www.gsea-msigdb.org/gsea/msigdb). Correlation heatmap (Pearson r) of the transcriptomes from METABRIC breast cancer project samples ($n = 1986$). All the EMT and migration markers analyzed here showed a negative correlation (P value < 0.0115 to <0.0001), and (P value < 0.0122 to <0.0001), respectively. Red color refers to negative correlation, and blue color indicates positive correlation.

of NISCH expression in about three hundred (300) human BCa and normal tissues using quantitative polymerase chain reaction (qPCR) and immunohistochemistry [3]. According to Baranwal et al., normal human breast tissue samples had statistically significant higher expression of NISCH mRNA compared to BCa tumor tissue samples [3]. NISCH protein expression levels have been reported to be lower in primary BCa tissues compared to adjacent noncancerous tissues [29]. To further understand the clinical relevance and tumor suppressor role of NISCH, we examined the expression of NISCH between the intrinsic subtypes of BCa versus normal-like control. Our result clearly shows that luminal B and basal-like/triple-negative categories have the least relative mRNA expression of NISCH among all subtypes, while the normal-like category has the highest relative mRNA level of NISCH (Fig. 3D). Overall, the relative mRNA expression of NISCH is low in subtypes with poor or worse prognosis and high in the normal-like category. Altogether, our clinically relevant mRNA expression data show that NISCH tumor suppressor function in BCa is essential and as such, more research might be needed to understand further how NISCH affects the different subtypes of BCa.

NISCH EXPRESSION IS DOWNREGULATED IN HUMAN BCA LACKING ESTROGEN AND PROGESTERONE RECEPTORS BUT NOT HER2/NEU RECEPTORS

Based on our analysis of the METABRIC dataset, we found that the relative mRNA expression of NISCH is significantly down-regulated in ER-negative and PR-negative BCa (Fig. 3E, F). On the contrary, the relative mRNA expression of NISCH was found to be significantly downregulated in HER2 + BCa (Fig. 3G). This is a very interesting clinical finding because this result has been reported earlier by our group in 2013 [23]. Jin et al. reported that NISCH and HER2 status have an inverse relationship [23]. Briefly, HER2 + receptors are one of the factors that promote the expression of oncogenic miR-23b/27b. To this end, when cancer-associated miR-23b/27b are inhibited, NISCH expression is significantly upregulated in BCa [23]. Therefore, it is logical to speculate that NISCH expression will be low in HER2 + BCa and significantly elevated in HER2- BCa (Fig. 3G). This relationship between HER2 and NISCH is worth further investigation as this might harbor some unexplained biological concepts that will add more insight into the existing body of knowledge about

the biology and function of NISCH in BCa. Altogether, these reports show that NISCH gene expression and activity can be impacted by endogenous receptors in BCa.

NISCH EXPRESSION IS DIFFERENTIALLY MODULATED BY AGE, TUMOR STAGE, TUMOR SIZE, TUMOR GRADE, AND LYMPH NODE METASTASIS IN BCA

Analysis of the METABRIC dataset showed that the expression of NISCH is progressively downregulated with an increase in age of BCa patients (Fig. 3H). Remarkably, this finding has been reported by McGuire et al. where they showed that BCa survival is strongly associated with age at diagnosis [53]. They found that lower survival is seen in BCa patients under 50 years, while patients over 70 years have the lowest survival since these patients most likely have age-related co-morbidities [53]. Consistent with this, NISCH expression is also downregulated with an increase in tumor stage (Fig. 3I), tumor grade (Fig. 3J), tumor size (Fig. 3K) and the presence of lymph node metastasis (Fig. 3L). These findings further strengthen the case that NISCH is a potent tumor suppressor in BCa. In our analysis, we found that as BCa stage increases, the relative mRNA expression of NISCH decreases significantly (Fig. 3I). Stage four (4) is the last stage of cancer; hence many patients are expected to be dead; and thus, this clarifies why the sample size is the least among all stages. Accordingly, the tumors with the largest size were found to have the least expression of NISCH mRNA, while tumors with small size have the highest NISCH expression (Fig. 3K). Furthermore, the expression level of NISCH was downregulated with increasing tumor grades (Fig. 3J). At the highest grade (3), tumor cells are completely different from normal cells, and this is where NISCH level is the lowest (Fig. 3J). Migration of cells to the lymph node is a unique feature of advanced stage BCa and other types of cancer. Breast tumor cells are known to travel through the bloodstream in the lymph system; hence, this can be another method of confirming the severity of disease. Our analysis found that NISCH expression is significantly downregulated in lymph node-positive BCa compared to lymph node-negative tumors where NISCH expression is elevated (Fig. 3L). Consistent with this, it has been reported that there is a significant difference in the expression of NISCH between patients with and without lymph node metastasis [29]. Collectively, it is evident that NISCH plays a critical role as a tumor suppressor in BCa; that is why its expression is low in severe cases of disease. Overall, our findings, when put

together, strengthens the fact that NISCH has great clinical relevance as a tumor suppressor in BCa pathology.

NISCH IS NEGATIVELY CORRELATED TO MANY EPITHELIAL-MESENCHYMAL TRANSITION MARKERS IN BCA

Based on the analysis of the METABRIC dataset, we report that NISCH has a significant inverse correlation with many EMT markers (Fig. 4). This is a novel finding because there is still no clinical dataset reporting on the correlation of NISCH with EMT markers in BCa patients. According to our analysis, NISCH is inversely correlated to fibronectin 1 (Fig. 3M), vimentin 1 (Fig. 3N), MMP9 (Fig. 3O), and ZEB2 (Fig. 3P). These are some of the critical markers that drive the EMT process, enhancing the initiation of aggressive mesenchymal features in BCa cells. We also find that there is a significant inverse correlation between NISCH and many cell migration genes in BCa (Fig. 4B). The result of our analysis supports and validates that NISCH has a very significant role in preventing the migration of BCa cells from the breast to secondary organs in patients. These findings show that NISCH plays an active role in inhibiting BCa metastasis. This is of high clinical value, especially in the design of future therapeutics for patients with advanced metastatic BCa.

NISCH DIRECTLY CORRELATES WITH STK11/LKB1 AND INVERSELY CORRELATES WITH ITS METHYLATION STATUS IN BCA

LKB1 acts as a multitasking tumor suppressor, and has a well-established role in the regulation of metabolism, partly through its ability to regulate the mTOR pathway [54]. It has been shown that LKB1 acts as a tumor suppressor and that loss of expression/function is associated with NISCH. Furthermore, AMPK is a downstream effector of LKB1 and is associated with NISCH [4]. Thus, we decided to analyze NISCH and LKB1 connection further. We report here for the first time that there is a significant positive correlation between NISCH and STK11 (LKB1) genes in the pathogenesis of BCa in humans and this is based on the analysis of BCa clinical samples generated from the METABRIC dataset (Fig. 3Q). An increase in the gene expression of NISCH resulted in a corresponding increase in the gene expression of STK11. This is a finding that was reported earlier using biochemical assays [4]. In terms of NISCH DNA methylation, our analysis of the TCGA dataset shows that NISCH expression is inversely correlated with its gene methylation status (Fig. 3R). According to a previous report, NISCH promoter experiences hypermethylation in several cancers, whereas some highly aggressive breast cancer cells exhibit genomic loss of the NISCH locus [2]. Our present result confirms that NISCH is highly methylated in BCa patients at baseline levels, and this might be the reason for the downregulation of the tumor suppressor NISCH in BCa patients (Fig. 3C). Altogether, using both TCGA and METABRIC datasets, we report that NISCH and STK11 are interacting partners that are positively correlated. Also, we showed that the methylation of NISCH might be a plausible reason why its gene expression is low in BCa patients.

Our results show that NISCH levels are generally correlated to positive patient outcomes, and NISCH levels are differentially expressed across different BCa intrinsic subtypes. Interestingly, the more aggressive basal subtypes have lower levels of NISCH compared to the less aggressive luminal A types. Further correlations of NISCH expression with tumor age, stage, size, grade, and metastasis show decreasing levels of NISCH as the tumor severity progresses. Overall, we report that NISCH is a promising tumor suppressor with the potential for impact in clinical translational research applications and therapeutic development.

SUMMARY

Based on the 2021 American Cancer Society statistics, BCa has the highest number of estimated new cases and the second-highest number of deaths for women, after lung cancer. Therefore, it is plausible to state that BCa is having a negative impact and rapidly becoming a huge burden on the growth of the national and global economy. BCa, just like other types of cancer, progressively gets worse with time and with metastasis being the main cause of mortality. Despite available therapies, there are often cases of relapse or drug resistance following completion of treatment. This could be due to the expression or lack of expression of specific genes associated with BCa signaling cascade. Therefore, it is important to emphasize that the time of BCa diagnosis is very critical as early detection will allow for quicker treatment intervention. To date, there is no patient-centered clinical dataset validation of NISCH as a potent tumor suppressor in BCa. Thus, we sought to investigate the role and functionality of NISCH in BCa patients using publicly available databases containing records from BCa large studies. Datasets from both METABRIC and TCGA were analyzed using bioinformatic methods for specific parameters. Interestingly, we found that NISCH expression is progressively downregulated with an increase in age and advances in tumor size, shape, and grade. It is worth mentioning that when the size of a tumor exceeds a certain threshold, cancer cells intravasate into the bloodstream and begin to metastasize to other essential organs, including the lymph node. When this cell migratory event happens, it is often regarded as an indicator of late tumor stage. Therefore, lymph node-positive BCa have a lower level of NISCH expression, while the reverse is the case for lymph node-negative samples. EMT is a cellular migratory process that promotes the initiation of the aggressive mesenchymal phenotype in BCa. Thus, we report that NISCH expression in BCa is critical for disease initiation and progression, making a case for its clinical relevance and therapeutic application. Though we and others have reported several in-vitro and in-vivo anti-tumor effects of NISCH, our present clinical findings further validate the prognostic value of NISCH and make a case for further studies investigating the role of NISCH in the pathogenesis of other cancers and in the development of specific anti-cancer therapeutics.

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AUTHOR CONTRIBUTIONS
SO wrote the first draft, HY did bioinformatic analysis and prepared figures, KN, NA, BB, MB contributed to the text, TC created some figures, and SKA finalized the text and figures.

COMPETING INTERESTS
The authors declare no competing interests.

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