Universiteit
Leiden
The Netherlands

Unraveling multifaceted roles of Grainyhead-like transcription factor-2 in breast cancer

Coban, B.

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Chapter 1

Introduction, aim and scope of the thesis

Breast Cancer

Breast cancer is the leading cause of death among women worldwide.[1] The highly heterogenous and complex nature of the disease, characterized by diverse biological and histological features influence the breast cancer prognosis and response to the treatments. To overcome this heterogeneity and tailor the treatment options, breast cancer has been subcategorized into four groups based on the expression of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2); Luminal A, Luminal B, Human epidermal growth factor receptor 2 enriched ($HER2^+$) and Triple-negative Basal-like.[2]

Luminal A tumors accounts for the most prevalent subtype with ER, PR and low Ki67 expression (a proliferation marker) but not HER2. It shows good prognosis and hormone therapy response. Compared to Luminal A, Luminal B has a higher level of Ki67 expression, causing a worse prognosis. It represents 20% of luminal tumors and expresses ER, PR and Her2. The Her2 enriched subtype is characterized by overexpression of Her2 and absence of ER and PR. HER2+ breast tumors are associated with poor prognosis and require therapies targeting Her2 such as trastuzumab.[3,4] The last subtype Basal-like lacks ER, PR and Her2 expression. The high metastatic potential and the aggressiveness of TNBC tumors result in poor prognosis and survival outcomes.[5,6]

While this categorization based on the molecular markers guides the disease prognosis and treatment strategies, metastasis remains a major challenge for achieving therapeutic success. Metastasis accounts for more than 90% of cancer related deaths. It has been previously reported that the interactions between the components of tumor microenvironment (TME) (extracellular matrix, tumor cells, fibroblasts, blood and lymph vessels, immune cells and cytokines) promotes tumor progression and metastasis.[7–10] Therefore, it is crucial to identify key molecules and processes modulating the TME to channel novel targeted therapies to combat metastasis.

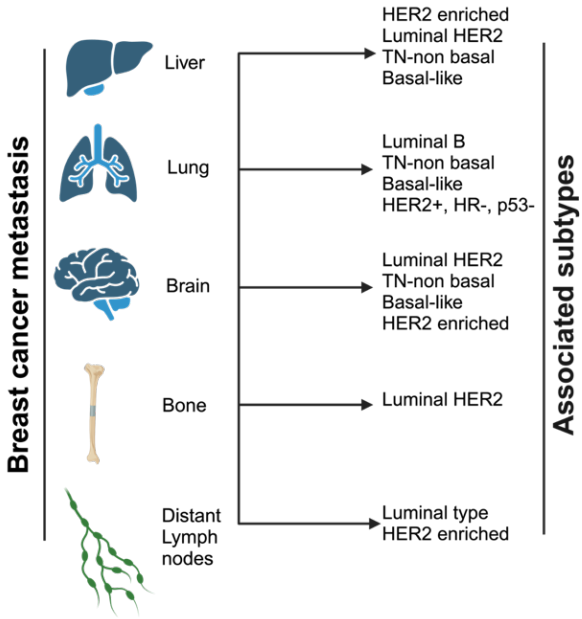

Figure 1: Association of breast cancer molecular subtypes with metastasis. Organ-specific metastasis of breast cancer is governed by the molecular subtypes of breast cancer. The most prevalent metastatic site in breast cancer patients is the bone, followed by the brain as the second most common site, with the liver and lungs being subsequent sites. (Created by Biorender.com)

Grainyhead-like transcription factors

Transcriptional regulatory networks play pivotal roles in embryogenesis, wound healing and disease-onset (e.g., cancer) in humans. The Grainyhead-like (Grhl) family of transcription factors belong to such group of genes. It was initially identified in Drosophila melanogaster which harbor an embryonic mutation, resulting in a unique head defect phenotype characterized by holes in specific large cuticular regions and abnormal cuticular structures.[11] Three orthologues of Grhl; GRHL1-3 are expressed in mammals.[12,13] GRHL transcription factors consist of an N-terminal transactivation domain, a DNA binding domain homologous to tumor suppressor gene p53, a DNA binding domain, and a C-terminal dimerization domain, enabling GRHL proteins to form homo and heterodimers.[14]

GRHL family members exert tissue-specific functions during mammalian development by being involved in the regulation of neural tube closure,[15,16] formation of the lungs,[17,18] skin barrier function,[19,20] and epithelial morphogenesis.[21] They are also involved in the repair of epidermal barrier after tissue damage modulated by receptor tyrosine kinases.[22] These important roles are facilitated in part by the negative regulation of epithelial-mesenchymal transition (EMT); a cellular process where epithelial cells lose apical-basal polarity and cell-cell adhesion through downregulation of epithelial genes[23,24] (i.e. E-cadherin, OVOL2) enabling to gain mesenchymal features such as motility, invasion and plasticity through upregulation of mesenchymal genes (i.e.; ZEB1, Vimentin SNAIL1).[25] EMT in embryonic development also lead to the differentiation of germ layers (ectoderm, endoderm and mesoderm), giving rise to the formation of different organs.[26] The members of GRHL family act as master regulators of epithelial characteristics and drive the establishment and maintenance of tissue integrity and epithelial differentiation in the embryonic development and tissue homeostasis.

In addition to their roles in the developmental processes, GRHL family transcription factors have been linked to various types of cancer including breast cancer,[23,27] lung cancer,[28,29] ovarian cancer,[30] colorectal cancer (CRC),[31,32] skin cancer,[33,34] and neuroblastoma.[35] This association is characterized by the disruption of the epithelial integrity and the dysregulation of growth and survival pathways, exhibiting both tumor suppressive and supportive functions, in part through modulation of EMT.[36–38]

Deregulation of GRHL1 and GRHL3 in cancer

GRHL1 has been associated with squamous cell carcinoma of the skin. Grhl1 deletion in mice resulted in skin barrier defects, accompanied by more chemically-induced skin tumor formations.[33] This implied that GRHL1 functions as a tumor suppressor gene. Another significant role of GRHL1 as tumor suppressor has been reported in neuroblastoma. GRHL1 was identified as an early response gene to the treatment with histone deacetylase inhibitors. Its overexpression in neuroblastoma cell lines inhibited proliferation and hampered anchorage independent growth. Neuroblastoma patients with high

GRHL1 expression had favorable prognosis.[35] In contrast, a tumor promoter function of GRHL1 in cell-cycle regulation via EGFR-ERK axis has been shown in lung cancer.[28]

Similar to GRHL1, GRHL3 also exhibits context-specific activities in multiple tumors types. GRHL3 was found to be expressed mainly in early stage and hormone receptor positive breast cancers and decreased GRHL3 expression was observed during tumor progression. This indicated a possible function of GRHL3 in suppressing the tumor growth.[39] However, an overexpression study of GRHL3 in skin and breast cancer cell lines showed the direct transcriptional repression of E-cadherin by GRHL3, resulting in induction of cell migration and invasion.[40] Furthermore, decreased GRHL3 expression levels promoted EMT and deregulated MAPK/ERK pathway in CRC cells, demonstrating the tumor promoter role of GRHL3.[41] In addition to CRC, reduced levels of GRHL3 in squamous cell carcinoma of the skin also contributed to the tumor progression and upregulated of the oncomir miR-21, an indicator of malignant transformation.[42]

GRHL2 transcription factor in tumorigenesis

GRHL2 is located on the chromosome 8q22 within human genome, a region that is commonly amplified in different tumor types.[43] Dysregulation of GRHL2 expression has been linked to numerous cellular processes, affecting the tumor progression and metastasis. In this regard, GRHL2 confers both tumor suppressive[44,45] and oncogenic roles in cancers.[46,47]

GRHL2 as a tumor suppressor

It has been demonstrated that the overexpression of GRHL2 expression prevented the invasive and migratory capabilities of gastric cancer cell lines and transforming growth factor-β (TGFβ)-induced EMT in both gastric cancer[48] and oral carcinogenesis.[49] Downregulation of GRHL2 expression in lung cancer cell lines induced partial EMT -a hybrid state where the expression of both epithelial and mesenchymal genes coexists- by influencing the cell proliferation and colony formation.[29] Moreover, functional studies on GRHL2 expression revealed that GRHL2 inhibits EMT through its direct interactions with E-

cadherin and ZEB1. Silencing GRHL2 expression in a human mammary epithelial cell line resulted in downregulation of E-cadherin (CDH-1) expression and EMT. [50] ZEB1 was found to interact with the GRHL2 promoter upon activation of an important EMT-supporting pathway, Transforming growth factor-β (TGF-β), thereby suppressing GRHL2 in different cancer types including breast cancer and colorectal cancer.[46,51] In contrast, it has been shown that GRHL2 also inhibits ZEB1 expression by upregulating the miR200b/c, a known EMT suppressor.[52] These results suggested a double negative feedback loop between GRHL2 and ZEB1 in the regulation of EMT and tumor progression. A direct and indirect regulation of epithelial phenotype via GRHL2 is further represented in Figure 2.

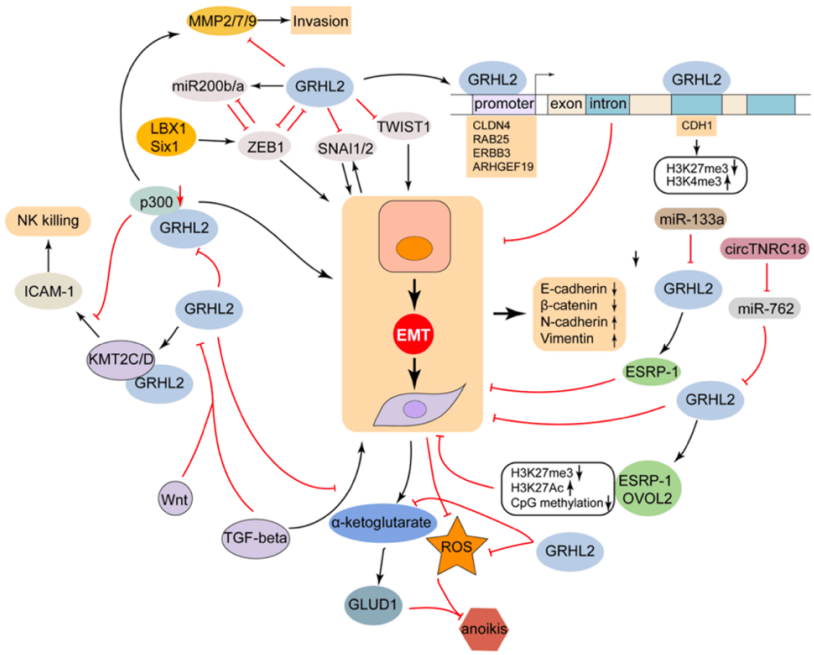

Figure 2: Complex regulatory loops illustrating GRHL2-mediated EMT suppression.

Here, a double-negative feedback loop between GRHL2 and ZEB1 is indicated upon GRHL2 suppression by ZEB1. Additionally, miR-200 is a direct target of GRHL2, thereby allowing the regulation of ZEB1 and EMT by GRHL2. GRHL2 bound

to the promoters of RAB25, CLDN4, ARHGEF19, and ERBB as well as intron of E-cadherin/CDH1, resulting in alterations in histone methylation. GRHL2 also inhibits SNAI2, TWIST1 and TGF-beta/Wnt-induced EMT. Furthermore, it increases ICAM-1 expression and the sensitivity of NK killing via KMT2C/D interactions and inhibition of p300. GRHL2 is downregulated by miR-133a, resulting in decreased ESRP1expression and EMT inhibition. MiR-762 reduction by circTNRC18 increases GRHL2 expression. EMT enhances mitochondrial oxidative phosphorylation accompanied by the overall declined level of ROS and increased GLUD1 expression which is restored by GRHL2. EMT elevates mitochondrial oxidative phosphorylation, leading to a decrease in ROS levels and an increase in GLUD1 expression. This effect is reversed by GRHL2. (Adopted from He J. et.al.; 2020)

GRHL2 as an oncoprotein

Several studies have also attributed a tumor supportive role for GRHL2. A study with colorectal cancer cell lines identified GRHL2 as an oncoprotein due to its ability for enhancing cell viability and decreasing apoptosis through PI3K/Akt pathway.[31] Increased GRHL2 expression has been associated with poor prognosis and resistance to cisplatin, regulated by via ERK/MAPK signaling in ovarian cancer.[53] GRHL2 also employed oncogenic roles in lung cancer by stabilizing partial EMT; a hybrid epithelial/mesenchymal phenotype that holds a higher metastatic potential than the mesenchymal state.[24] Lastly, it has been found that GRHL2 functions as a key player for telomerase activity by controlling hTERT expression, thereby enhancing the viability of tumor cells.[54]

Role of GRHL2 in stemness and epigenetic regulation

A mechanism based mathematical modeling analyzed the tumor initiating potential (stemness) of GRHL2 in stemness through EMT. GRHL2-centered positive and negative feedback loops including ZEB1 and SNAIL constituted a regulatory network between GRHL2 and stemness associated genes such as Oct4 and LIN28.[24] Direct regulation of Oct4 by GRHL2 modulated the stemness and plasticity in oral cancer cells.[47] Notably, decreased GRHL2 expression enabled pancreatic cancer cells to retain their stem-like characteristics, including self-renewal capacity and resistance to anoikis.[45]

Multiple studies have examined the interaction between GRHL2 and epigenetic modifiers, particularly Histone deacetylases (HDACs). HDACs are enzymes that induce loss of gene expression through chromatin condensation, impacting cellular processes such as cancer stemness, cell proliferation, and EMT.[55] The induction of GRHL2 suppresses the expression of mesenchymal proteins like ZEB1 and SNAI2 and augments apoptosis mediated by HDAC inhibitors in glioblastoma.[56] In a study on Basal-like breast cancer, the mechanisms behind cell survival upon treatment with conventional therapy were linked to the loss of histone acetylation by H3K27ac, a known transcription enhancer, at regulatory regions of epithelial master regulators like GRHL2.[57]

GRHL2 in breast cancer

GRHL2 exhibits context-specific and subtype-specific functions in breast cancer, similar to its behavior in other cancer types. The role of GRHL2 in maintaining epithelial phenotype of breast cancer cells was highlighted above. This was further supported by Werner et al., who demonstrated that GRHL2 knockdown led to the downregulation of its target gene Erbb3, resulting in phenotypic and genotypic changes related to EMT in Basal subtype of breast cancer cells.[58] In contrast, elevated expression of GRHL2 has been associated with increased metastatic potential and poor relapse-free survival in breast cancer patients.[59]

Downregulated GRHL2 expression was observed in the Basal-B subtype.[46] Accumulating evidence suggested a specific role for GRHL2 in modulating transcriptional activity of ER in hormone receptor positive breast cancer. A virtual ChIP-seq based network analysis revealed that GRHL2 restricts estrogen transcriptional activity by downregulating the estrogen responsive enhancer RNAs.[60] In Tamoxifen-resistant breast tumors, FOXA1, a pioneer factor for ER transcriptional activity, has been discovered to interact with GRHL2. This interaction further regulated the LYPD3/AGR2 complex, promoting therapy resistance.[61] It was further shown that GRHL2 not only controls the ER activity but also prevents estrogen-induced migration of ER-positive breast cancer cells.[62] Altogether, these studies manifest a multifaceted role for GRHL2 function across multiple breast cancer subtypes.

Role of GRHL2 in immune modulation

The immune landscape of the TME is heterogenous and determines tumor progression. Therefore, it is important to understand the dynamic interplay between tumor and immune cells.[63] The immune cells exert functional adaptations to the local TME and perform dual roles. These can be tumor growth promoting: Regulatory T cells (Treg), M2-like Tumor-associated Macrophages, Mast cells, Innate Lymphoid Cells type 2 and 3 (ILC2/3). Or they can be tumor growth inhibiting: Natural killer cells (NK) and Tumor infiltrating CD4+ and CD8+ Lymphocytes (TILs), Dendritic cells (DC).[64] During tumor progression, tumor cells adopt several immune evasive mechanisms to limit infiltration of anti-tumor immune cells and impair their cytotoxic or effector functions.[65] An overview of immune evasive mechanisms in breast cancer is shown in Figure 3.

Besides enabling the tumor cells to gain the cellular plasticity, EMT also confer immune evasion in different cancer types including breast cancer.[66] It has been previously reported that diverse epithelial and mesenchymal phenotypes modulate the sensitivity to the immune cells, thereby determining the therapy response in metastatic melanoma patients.[67] A comprehensive study on immune infiltration in breast cancer patient tumors revealed a correlation between EMT signature and the immune response by using the expression of epithelial markers associated with EMT. It has found out that in the triple-negative tumors and poorly differentiated tumors where E-cadherin and GRHL2 expression was downregulated, the inflammatory infiltrate was more prominent.[68] This was further supported by Song S. et.al, demonstrating the inverse correlation between GRHL2 expression and CD8+T cell infiltration in breast tumors.[69] Moreover, type I interferon (IRF1) has been identified as a direct target of GRHL2 and GRHL2 upregulated the expressions of both IRF1 and IRF3; stimulants of immune responses. Therefore, high GRHL2 protein expression was found to be associated with suppression of tumor recurrence.[27] Regulation of interferon response genes by GRHL2 has been also demonstrated in another study. Upon GRHL2 overexpression, an interaction of GRHL2 with two the histone methyltransferase genes was formed and this further induced mesenchymal-epithelial transition and sensitized the cells

NK cell mediated killing.[70] Lastly, in gastric cancer, GRHL2/ZEB1 axis was shown to regulate PD-L1 downregulation -a critical factor for T-cell mediated cell killing- via a direct target of GRHL2, miR-1290, resulting in immune evasion.[71]

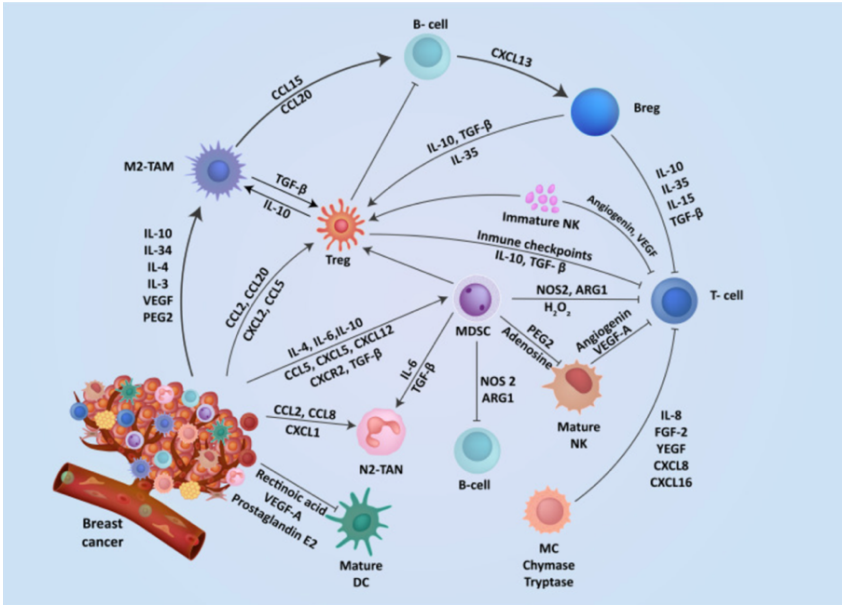

Figure 3: The mechanisms of immunosuppression in breast TME.
The tumor microenvironment (TME) harbors various resident cells, pivotal for tumor progression and metastasis. These cells, along with their secretory elements and receptors such as cytokines, chemokines, and growth factors, form a complex network. This diverse network actively promotes an immunosuppressive TME. (Adopted from Akinsipe T. et.al., Frontiers in Immunology, 2024)

Aim and scope of the thesis

This thesis aimed to decipher GRHL2-mediated signaling networks, cellular processes and targetable vulnerabilities in multiple breast cancer subtypes to identify novel therapeutic avenues for breast cancer. We discuss how the interplay between cellular plasticity and mechanical cues in the TME enable tumor cells to navigate during dissemination, providing an overview of therapeutic opportunities to prevent metastasis in chapter 2. We explore DNA

binding sites as well as direct and indirect targets of GRHL2 in luminal breast cancer in chapter 3. ChIP-seq analysis revealed few overlapping binding sites of GRHL2 with ERα in luminal breast cancer cell lines. We found the direct and indirect targets of GRHL2 in response to GRHL2 loss in luminal breast cancer using Bru-seq. An integrative analysis of ChIP-seq and Bru-seq data identified distinct gene regulatory networks controlled by GRHL2 in luminal breast cancer, differ from those in other tissues. Chapter 4 demonstrates the shared and distinct roles for GRHL2 in growth and motility of luminal and basal breast cancers. A common outcome of GRHL2 deletion was characterized by cell cycle arrest. We observed a reduction in epithelial markers particularly in the luminal line while mesenchymal markers were induced only in basal cells alongside enhanced migration in response to GRHL2 loss. This is further confirmed by an in vivo model with silenced GRHL2 which demonstrated reduced primary tumor growth and fewer lung colonies, indicating that growth suppression predominated upon GRHL2 deletion. We focus on delineating GRHL2-mediated drug resistance/sensitivities in the Basal B subtype of breast cancer in Chapter 5. GRHL2 overexpression did not result in any MET-like changes in Basal B cell line in contrast to the literature previously reported. We also didn't find an effect of elevated GRHL2 expression on the cellular proliferation. A kinase inhibitor approach was implemented on GRHL2 overexpressing Basal B cells that four kinase candidates have been associated with the presence GRHL2. However, this needs further validations to confirm GRHL2 mediated responses. Chapter 6 focuses on the impact of GRHL2 deletion in immune modulation via NT5E/CD73 in luminal breast cancer. Loss of GRHL2 increased the expression of NT5E/CD73, resulting in elevated levels of adenosine production in the TME. This further enhanced the CD8+ T cell migration. In chapter 7, we provide an overview of the research presented in this thesis, accompanied by the discussions and future perspectives. Overall, this thesis unveils the novel regulatory roles of GRHL2 across breast cancer subtypes and highlighting novel GRHL2-mediated pathways for potential therapeutic interventions in breast cancer.

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