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Role of Biglycan on the immunogenicity of tumor cells

Dissertation obtaining the academic degree
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“Wherever the art of medicine is loved there is also a love of humanity”.

- Hippocrates

“I’m a big believer in what’s called personalized medicine, which refers to customizing your health care to your specific needs based on your physiology, genetics, value system and unique conditions”.

- David B. Agus

Abstract

Biglycan (BGN), a class I small leucine-rich proteoglycans (SLRPs), is a vital component of the extracellular matrix (ECM) that participates in scaffolding the collagen fibrils and mediates cell signaling. BGN underexpression was found in tumors of distinct origin and evidence suggests an association with tumorigenicity. The incapacitated immune system is a characteristic hallmark of solid tumors and the oncogene HER-2/neu is an essential target for cancer immunotherapy.

To establish a mechanistic link between HER-2/neu-mediated immune escape and BGN reduction, *in vitro* models of HER-2/neu-overexpressing murine fibroblasts and human mammary and melanoma carcinoma lesions with defined HER-2/neu status were used.

Upon BGN restoration, BGN^{high} $HER\text{-}2/neu^{+}$ fibroblasts were less tumorigenic in immune-competent mice when compared to $BGN^{low/neg}$ $HER\text{-}2/neu^{+}$ cells, which was associated with enhanced immune cell responses and higher frequencies of immune effector cells in tumors and peripheral blood. The increased immunogenicity of BGN^{high} $HER\text{-}2/neu^{+}$ fibroblasts appears to be due to upregulated major histocompatibility complex (MHC) class I and reduced expression levels of the oncomir, miR-21-3p and transforming growth factor (TGF)-β suggesting a network among BGN, miR-21-3p, TGF-β pathway and HER-2/neu-mediated downregulation of MHC class I antigens. High miR-21-3p levels due to HER-2/neu transformation were inversely correlated to the expression of SMAD family member 2 (SMAD2), BGN and MHC class I. These effects were due to the binding of miR-21-3p to the 3' untranslated region of murine SMAD2 or the human peptide transporter TAP1, accompanied by an impaired MHC class I expression and increased natural killer (NK) cell recognition. This was in accordance with *in silico* analyses of mRNA datasets obtained from breast cancer (BC) patients. The BC datasets confirmed the positive correlation between miR-21-3p and HER-2/neu expression and its inverse correlation to BGN, MHC class I and TAP1, which was associated with a reduced patients' overall survival. It is noteworthy that, BGN has no mutation which is associated with increased survival in BC.

Thus, this study reports for the first time the role of BGN in the HER-2/neu-mediated immune escape and identified the miR-21-TGF-β-BGN-MHC class I axis as a novel, innovative therapeutic concept for $HER\text{-}2/neu^{+}$ cancers.

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Referat

Biglykan (BGN) gehört zur Familie der kleinen, Leucin-reichen Proteoglykane, *small leucine-rich proteoglycans* (SLRPs), und stellt eine wichtige strukturelle und stabilisierende Komponente der extrazellulären Matrix dar, die vielfältige physiologische Prozesse beeinflusst und am Aufbau von Kollagenfibrillen sowie der Signalübertragung beteiligt ist. Eine verminderte BGN-Expression wurde in verschiedenen Tumoren gefunden und es gibt Hinweise, dass auch eine Assoziation mit Tumorigenität besteht. Das geschwächte Immunsystem ist ein charakteristisches Kennzeichen solider Tumore und das Onkogen HER-2/neu ist ein wesentliches Ziel für die Krebsimmuntherapie.

Um eine mechanistische Verbindung zwischen den HER-2/neu-vermittelten *immune escape* und der Herunterregulation von BGN- herzustellen, wurden *in vitro*-Modelle von HER-2/neu-überexprimierenden murinen Fibroblasten und humanen Mamma- und Melanomkarzinomzellen mit definiertem HER-2/neu-Status verwendet.

Nach BGN-Rekonstituierung waren BGN^{high} $HER\text{-}2/neu^{+}$ Fibroblasten in immunkompetenten Mäusen im Vergleich zu $BGN^{low/neg}$ $HER\text{-}2/neu^{+}$ Zellen weniger tumorig, was mit einer Zunahme von Immuneffektorzellen in Tumoren und peripheren Blut einherging. Die erhöhte Immunogenität von BGN^{high} $HER\text{-}2/neu^{+}$ Fibroblasten war mit einer Hochregulation von Haupthistokompatibilitätskomplex (MHC)-Klasse-I-Antigenen und einer reduzierten Expression der OncomiRs, miR-21-3p und dem transformierenden Wachstumsfaktor (TGF)-β zurückzuführen, was auf ein Netzwerk zwischen BGN, miR-21-3p, TGF-β und HER-2/neu-vermittelter Herunterregulation von MHC-Klasse-I- Antigenen hinweist. Die starke Expression von miR-21-3p nach der HER-2/neu-Transformation von Zellen war invers mit der Expression von *SMAD family member 2* (SMAD2), BGN und MHC Klasse I korreliert. Diese Effekte waren auf die Bindung von miR-21-3p an die 3'-untranslatierte Region von murinem SMAD2 oder dem humanen Peptidtransporter TAP1 zurückzuführen, was zu einer beeinträchtigten MHC-Klasse-I-Expression und einer erhöhten natürliche Killer (NK)-Zellerkennung führt. Diese Resultate waren vergleichbar mit *in silico* Analysen von mRNA-Datensätzen von Brustkrebspatienten. Dabei wurde die positive Korrelation zwischen miR-21-3p- und HER-2/neu-Expression und eine Assoziation mit BGN, MHC-Klasse I und TAP1 beschrieben, die mit einem reduzierten Gesamtüberleben der Patientinnen einherging. Bemerkenswert ist, dass BGN keine Mutation innerhalb der mRNA-Datensätzen von Brustkrebspatienten aufweist, die mit einer erhöhten Überlebenszeit verbunden ist.

Zusammenfassend zeigen die Untersuchungen zum ersten Mal eine Rolle von BGN beim HER-2/neu-vermittelten *immune escape* und identifiziert die miR-21-TGF-β-BGN-MHC-Klasse-I-Achse als neuartiges, innovatives Therapiekonzept.

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List of abbreviations

APM, antigen processing machinery
BC, breast cancer
BGN, biglycan
CALR, calreticulin
CHST, carbohydrate sulfotransferase
CHSY, chondroitin sulfate synthase
CNA, copy number alterations
CNX, calnexin
CPTAC, Clinical Proteomic Tumor Analysis Consortium
CS, chondroitin sulfate
DC, dendritic cells
DCN, decorin
DS, dermatan sulfate
EGFR, epidermal growth factor receptor
EMT, epithelial–mesenchymal transition
ER, endoplasmic reticulum
FMOD, fibromodulin
GAG, glycosaminoglycan
HA, hyaluronan
HC, heavy chain
HLA, human leukocyte antigen
HS, heparan sulfate
IFN, interferon
IL, interleukin
KS, keratan sulfate
LRR, leucine rich repeats
MAPK, mitogen-activated protein kinase
MDSC, myeloid-derived suppressor cells
MHC, major histocompatibility complex
miRNA, microRNA
NK, natural killer
OS, overall survival
PDIA3, protein disulfide-isomerase A3
PG, proteoglycan
PLC, peptide loading complex
PRELP, prolargin

SLRP, small leucine rich proteoglycan

SMAD, small mothers against decapentaplegic

SMAD2, SMAD family member 2

TA, tumor antigen

TACE, tumor necrosis factor-α-converting enzyme

TAP, transporter associated with antigen processing

TAPBP, tapasin

TCGA, The Cancer Genome Atlas

TCR, T cell receptor

UCEC, uterine corpus endometrial carcinoma

TGF, transforming growth factor

TLR, toll-like receptor

TME, tumor microenvironment

TNF, tumor necrosis factor

Treg, regulatory T cells

UTR, untranslated region

WHO, World Health Organization

1 Introduction and Objectives

1.1 Cancer, a global threat

Cancer has always afflicted humans, even though its relative impact was overshadowed by an early death from infectious diseases for centuries. Cancer ranks as a leading cause of death and a critical barrier to increasing life expectancy in every country of the world (1).

1.1.1 Global cancer statistics

Globally, an estimated 19.3 million new cancer cases and almost 10 million cancer deaths occurred in 2020 (2). Cancer is the first or second leading cause of premature death (ages 30-69 years) in 134 of 183 countries. According to the statistics, one in every eight men and one in every ten women are likely to develop the disease during their lifetimes (3). The predicted global cancer burden exceeds 27 million new cancer cases per year by 2040, a 50 % increase on the estimated 18.1 million cancers in 2018 (4).

1.1.2 Cancer burden statistics in Europe

With more than 3.7 million new cases and 1.9 million deaths each year, cancer represents the second most important cause of death and morbidity in Europe. Europe comprises only one-eighth of the total world population but has around one-quarter of the global total cancer cases with some 3.7 million new patients per year (5).

1.1.3 Breast cancer (BC) statistics

BC has become the leading cause of death from malignant tumors among women worldwide in the past few decades (6). BC has become a key research focus because of its high susceptibility and malignancy in women. According to World Health Organization (WHO) fact sheets 2020, there were 2.3 million women diagnosed with BC and 685,000 deaths globally. As of the end of 2020, there were 7.8 million women alive who were diagnosed with BC in the past 5 years, making it the world's most prevalent cancer (7). The best survival pattern was observed among women with $HR^+/HER2^-$ subtype (survival rate of 92.5 % at 4 years), followed by $HR^+/HER2^+$ (90.3 %), $HR^-/HER2^+$ (82.7 %), and finally worst survival for triple-negative subtype (77.0 %) (8).

1.2 Immunobiology of cancer

Over the past 150 years, researchers have made significant accomplishments against cancer. Cancer survival rates are increasing, and cancer patients' quality of life is improving. Recently, cancer research focuses more on targeted and precision medicine, adapted to individual patients and their tumors (9). Cancer will remain an urgent priority on the global public health agenda. Immunotherapy has now firmly established itself as a novel pillar of cancer care in numerous cancer types (10). Since cancer immunotherapy was prized as a

breakthrough (11), immune escape has become one of the most important topics to be investigated. Indeed, the ability of the tumor cells to escape impairment by the immune system has been recognized as a novel “hallmark of cancer” (12).

1.2.1 Different immune escape strategies of tumors

In general, there exists interplay between immune and tumor cells, which profoundly influences each other. An association between host immune responses and prognosis has been described in a variety of tumor types, which is also influenced by the tumor microenvironment (TME), consisting of a heterogeneous mix of cellular and non-cellular components. The immune cells can recognize tumor cells, thereby leading to their apoptosis and tumor rejection. This activity is mainly mediated by T cells, NK cells as well as macrophages. During disease progression, host immune responses have been biased against antitumor immune responses. Tumors could not be recognized by immune cells resulting in tumor cell proliferation and in the induction of an immune suppressive microenvironment, which negatively interferes with the systemic and local adaptive immune responses.

There exist different strategies how tumors evade the immune system. These include defects in the elucidation or maintenance of an effective antitumor responses such as insufficient antigen processing by dendritic cells (DC), poor recruitment of or impaired activation of effector cells, the secretion of immune suppressive factors, like cytokines, prostaglandine and growth factors, the expression of molecules of negative regulatory pathways, such as CD274, CD276 and VTCN1 and the non-classical HLA-G antigen, increased frequency of regulatory T cells (Treg) and myeloid-derived suppressor cells (MDSC), altered metabolite expression, modulation of the pH as well as downregulation or lack of the expression of classical HLA class I molecules and signal transduction molecules. Although there exists evidence for improved responses at earlier disease stages, not only activated effector $CD8^+$ T cells, but also tumor antigen (TA)-specific T cells could be induced. For the antitumoral T cell responses, the interaction of the T cell receptors with their specific HLA class I complex is required. Therefore, the HLA class I antigen processing machinery (APM) plays a crucial role in mediating immune responses by the generation and expression of the trimeric HLA class I, β2-microglobulin (B2M) and peptide complex.

1.2.2 Cancers often lose expression of MHC class I

Prof. Dr. Barbara Seliger was one of the first to establish a connection between oncogene expression and an MHC class I-mediated immune escape phenotype. With more than 25 years of experience in the field of immune-oncology, Prof. Dr. Barbara Seliger pioneering her research in MHC class I abnormalities and APM components in tumors (13–23). Currently there is abundant evidence that loss of MHC I antigen presentation is a frequent event in cancers including non-small-cell lung carcinoma, breast, prostate, colorectal, head and neck

squamous cell carcinoma, hepatocellular carcinoma, and melanoma (24–26) that results in immune evasion (12).

1.2.3 MHC class I presentation

The processes relying on MHC class I surface expression such as T cell development, DC-mediated cross presentation and NK cell responses are pursued by the complex MHC class I antigen processing and presentation machinery, which has been well characterized during the last three decades (Figure 1). It consists of four major steps: (1) peptide generation/trimming; (2) peptide transport; (3) MHC class I assembly and (4) antigen presentation (27,28). MHC class I molecules are assembled in the endoplasmic reticulum (ER) and consist of two types of chain – a polymorphic heavy chain (HC) and B2M. The HC is stabilized by the chaperone calnexin (CNX), prior to association with the B2M. Without peptides, these molecules are stabilized by chaperone proteins: calreticulin (CALR), Protein disulfide-isomerase A3 (PDIA3) and tapasin (TAPBP) (18). TAPBP interacts with the transport protein TAP (transporter associated with antigen presentation) which translocate peptides an ATP-dependent manner from the cytosol into the ER. Prior to entering the ER, peptides are derived from the degradation of proteins, which can be of viral- or self-origin. Degradation of proteins is mediated by proteasomes, and the resulting peptides are translocated into the ER by means of TAP (Figure 1). TAP translocates peptides of 8 –16 amino acids and they may require additional trimming in the ER before binding to MHC class I molecules (29). The complex of TAP, TAPBP, MHC class I, PDIA3 and CALR is called the peptide loading complex (PLC) (17).

When peptides bind to MHC class I molecules, the chaperones are released and peptide–MHC class I complexes leave the ER for presentation at the cell surface (Figure 1). In some cases, peptides fail to associate with MHC class I and they must be returned to the cytosol for degradation (17). There are different proteasomes that generate peptides for MHC class I presentation: 26S proteasome, which is expressed by most cells; the immunoproteasome, which is expressed by many immune cells; and the thymic-specific proteasome expressed by thymic epithelial cells (26).

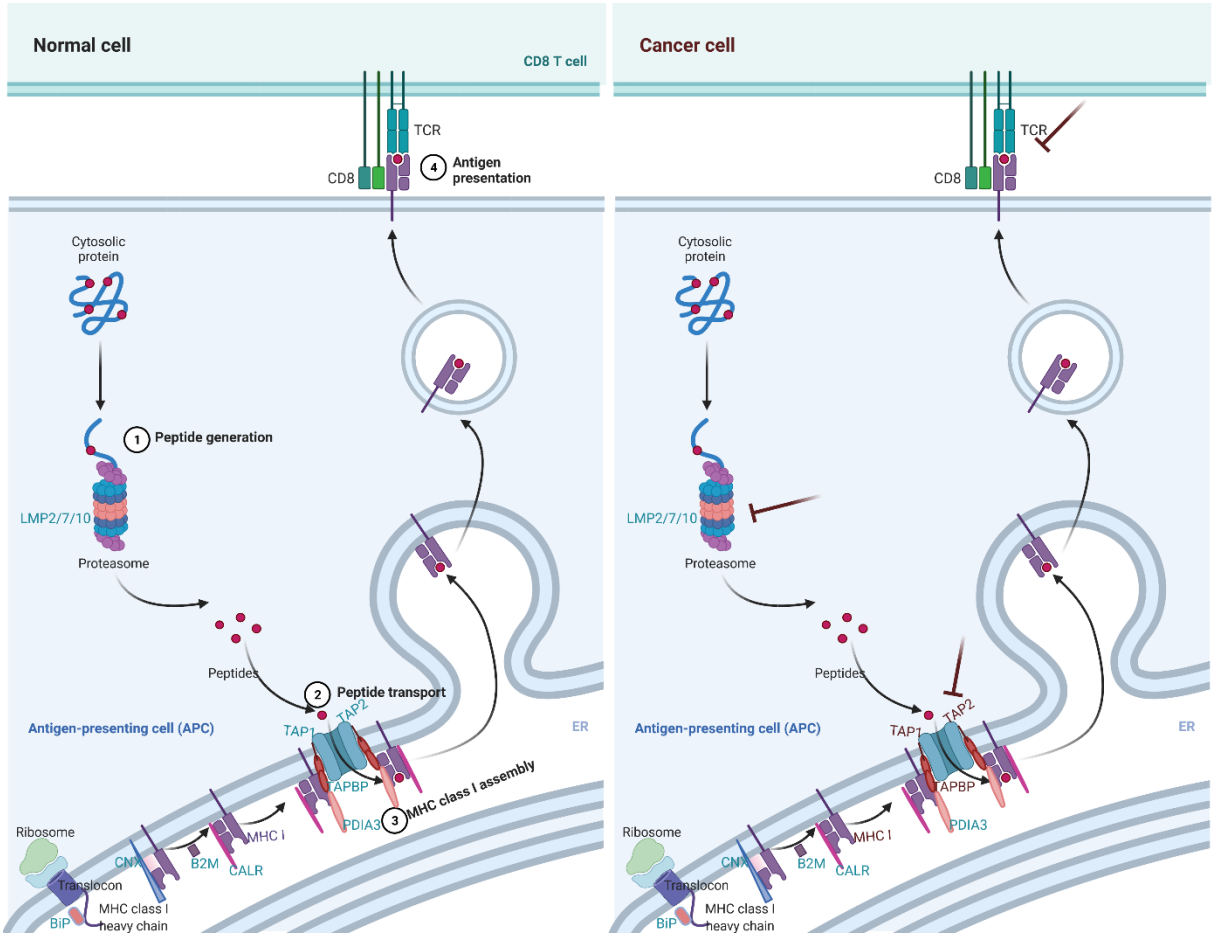


Figure 1. *Schematic diagram of MHC class I APM and steps deficient in tumors. Cellular proteins are hydrolyzed by the ubiquitin-proteosome pathway into oligopeptides, which are subsequently transported into the ER through the TAP transporter. In the ER, MHC class I HC and B2M, assisted by the chaperones CNX and CALR, are stabilized and associate. A multimeric PLC consisting of the TAP subunits, MHC class I HC, B2M, TAPBP, CALR and PDIA3 is yielded. Upon peptide loading, the PLC dissociates and the peptide/MHC class I/B2M complex is transported via the trans-Golgi to the cell surface. The red-highlighted the MHC class I APM components, which have been found to be dysregulated in tumor cells. Figure was prepared with Biorender.*

1.3 Immune escape and oncogenic transformation

In murine *in vitro* models of oncogenic transformation, a reduced surface expression of MHC class I antigens was demonstrated, which is based on a transcriptional downregulation of components of the MHC class I-APM (17–19,22,23,30–32). This was associated with reduced T-cell recognition, a poor prognosis, and lower patient survival rates, as well as the development of resistance to T-cell-based immunotherapies (33–37). The reduced expression of MHC class I APM components is rarely due to structural changes, but is often due to complex regulatory mechanisms, which include transcriptional, epigenetic, post-transcriptional and / or post-translational control (15,38,39).

1.3.1 The proto-oncogene, HER-2/neu and its clinical significance

ERBB2 (HER-2/neu), a known proto-oncogene, is located at human chromosome 17 (17q12). The ErbB family consists of four plasma membrane-bound receptor tyrosine kinases. One is ERBB2, and the other members are epidermal growth factor receptor (EGFR), ERBB3 (HER-3, which is neuregulin-binding and lacks kinase domain), and ERBB4 (HER-4). When the human epidermal growth factor receptor 2 (HER-2) is activated, the cells to grow and reproduce. Normal breast cells have two copies of the gene that makes HER-2. Overexpression of HER-2 leads to increased breast cell production or cancer. Molecular cloning of the gene showed that HER-2, Neu, and ERBB2 are all encoded by the same orthologs (40).

Uncontrolled growth is a common feature of tumors and is often based on amplification, overexpression, or increased stimulation of growth factor receptors. Amplification, also known as the overexpression of the ERBB2 gene, occurs in approximately 15-30 % of BC (41,42). It is strongly associated with increased disease recurrence and a poor prognosis; however, drugs targeting HER-2 in BC have significantly improved (43). Overexpression is also known to occur in ovarian, stomach, adenocarcinoma of the lung (44) and aggressive forms of uterine cancer, such as uterine serous endometrial carcinoma (45,46), e.g., HER-2 is over-expressed in approximately 7-34 % of patients with gastric cancer (47,48) and in 30 % of salivary duct carcinomas (49). HER-2 proteins have been shown to form clusters in cell membranes that may play a role in tumorigenesis (50).

1.3.2 HER-2/neu-mediated signal transduction and TGF-β

The truncated epidermal growth factor HER-2/neu induces various oncogenic signaling pathways like TGF-β signaling. Increased expression of TGF-β receptors and TGF-β, as well as secretion of TGF-β by HER-2/neu overexpressing tumor cells, is associated with increased migration, proliferation, invasion, metastasis and epithelial–mesenchymal transition (EMT) as well as decreased survival of patients with HER-2/neu-overexpressing tumors associated (51). TGF-β also suppresses the activation, maturation and differentiation of immune cells and inhibits the anti-tumor function of $CD8^+$ T cells by inducing Treg (52,53). Synergistic effects of HER-2/neu and TGF-β on tumor progression have been described and are based on functional alterations of anti-mitogenic effects of SMAD (small mothers against decapentaplegic)-mediated transcription and induction of anti-apoptotic and pro-migratory functions by HER-2/neu-dependent mechanisms (54), while the influence on the anti-tumor immune response was not investigated.

1.4 Extracellular Matrix (ECM) in tumor microenvironment (TME)

Indeed, all tumors independently of etiology are distinguished by an early inflammatory milieu and characterized by discrete interactions with the immune system at all stages of disease

progression (55). During malignant transformation, cells obtain complex biological characteristics correlated with more efficient survival, invasion, metastasis, and evading the immune response. The transformed cells, through continuously evolving interactions, communicate with and alter the surrounding microenvironment consisting of ECM components, cytokines embedded in the ECM, and the stromal cells (e.g., fibroblasts, endothelial cells, adipocytes, and immune cells) (56,57). The resulting ECM remodeling crucially contributes to the abnormal tumor inflammatory pattern (12,57).

1.4.1 Proteoglycans (PGs) and glycosaminoglycans (GAGs)

The ECM is a complex network of macromolecules comprised of PGs and fibrous proteins. The composition is tissue-specific and plays a critical role in modulating cellular functions (58,59). Because multicellularity evolved independently in different multicellular lineages, the composition of ECM varies between multicellular structures; however, cell adhesion, cell-to-cell communication and differentiation are common functions of the ECM (60).

The ECM is composed of an interlocking mesh of fibrous proteins and GAGs (61). GAGs are carbohydrate polymers and mostly attached to ECM proteins to form PGs (hyaluronic acid is a notable exception). PGs have a net negative charge that attracts positively charged sodium ions (Na^+), which attracts water molecules via osmosis, keeping the ECM and resident cells hydrated (62).

PGs are complex molecules consisting of a protein core into which one or more GAG chains are covalently tethered. The bound GAGs can be heparan sulfate (HS), chondroitin sulfate (CS), dermatan sulfate (DS), or keratan sulfate (KS) type. In mammalian cells, PGs are associated with the plasma membranes, released into the ECM, or intracellularly localized. Presently, 45 PGs have been identified with each member characterized by alterability attributed to the protein core modifications and the type and different stoichiometry of the GAG chain substitutions (63). Thus, PGs have particular and multifaceted biological roles, including: (i) contributing to ECM superstructure (64); (ii) defining biochemical and physicochemical properties (64); (iii) acting as receptors of diverse responsiveness as well as a pool of various biologic effectors such as growth factors (65,66).

1.4.2 Small Leucine Rich Proteoglycans (SLRPs)

SLRPs, a diverse sub-group of PGs, are involved in matrix organization and regulation of cell growth and signaling. SLRPs are ubiquitously distributed in the ECM. It consists of 17 members (Table 1) which are further categorized into five distinct classes based on their evolutionary protein conservation, leucine rich repeats (LRR), N-terminal cysteine rich clusters and chromosomal organization (67,68). The N-terminal Cysteine rich cluster consists of four characteristic cysteine residues with intervening short amino acid consensus sequences (67) (Table 1). SLRPs are synthesised and secreted into the pericellular spaces which then gets incorporated into the tissue ECM (69). A characteristic feature of SLRPs is

their ability to interact with extracellular receptors, such as tyrosine kinase receptors and toll-like receptors (TLR), facilitated by the bare β-sheets present on the concave surface of their LRR.

Table 1. *Classification of SLRP with respective gene symbol. This classification is adapted from Schaefer et al.* (67) *and Appunni et al.* (70)*.*

Class	Cys-rich cluster consensus	SLRP	Chromosome	Binding partners
I	CX3CXCX6C	Decorin (DCN)	12	MET (71), EGFR (72), VEGFR2 (73), TGF-β1 (74), TLR2/TLR4 (75,76)
		BGN	X	TLR2/TLR4 (77,78), P2X4/P2X7 (79), TGF-β1 (21,80), LRP6 (81)
		Asporin (ASPN)	9	EGFR (82), CD44 (83), TGF-β1 (84–86)
		ECM2	9	
II	CX3CXCX9C	Fibromodulin (FMOD)	1	EGFR (87), CD44, TGF-β1 (88)
		Lumican (LUM)	12	ITGB1/ITGB2 (89), MMP14 (90), EGFR (91)
		PRELP	1	
		Keratocan (KERA)	12	
		Osteomodulin (OMD)	9	BMP2 (92)
III	CX2CXCX6C	Epiphycan (EPYC)	12	
		Opticin (OPTC)	1	
		Osteoglycin (OGN)	9	
IV	CX3CXCX6-17C	Chondroadherin (CHAD)	17	
		Nyctalopin (NYX)	X	
		Tsukushi (TSKU)	11	BMP4, BMP7 (93), CHRD (94)
V	CX3-4CXCX9C	Podocan (PODN)	1	CDKN1A (95)
		Podocan-like protein 1 (PODNL1)	19	

The gene expression of all these SLRPs were studied in HER-2/neu transformed NIH3T3 cells and their expression profile is displayed in Figure 2.

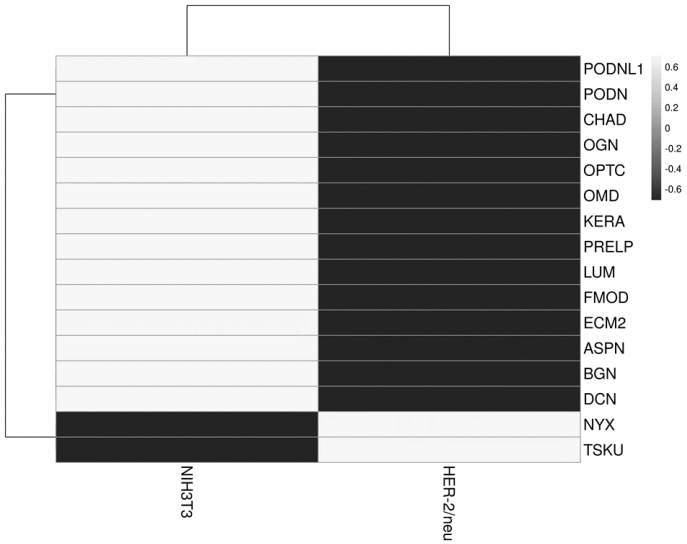


Figure 2: *SLRP transcriptomic data of RNA-seq analysis from HER-2/neu overexpression in NIH3T3 mouse fibroblast cells. The heatmap projected that 14 were downregulated out of 16 listed SLRPs upon the HER-2/neu overexpression.*

Among the downregulated SLRPs in the HER-2/neu transformed NIH3T3 cells, BGN expression was the lowest (log fold change of -15.89; unpublished data). Similar data were reported using a comparative proteomic approach of Ki-ras-transformed mouse fibroblasts and respective controls: BGN expression was downregulated when compared to parental NIH3T3 cells or mock transfectants (96).

1.4.3 Structure and function of BGN

BGN, a member of the SLRPs, is differentially expressed in tumors (70) and modulates molecular events relevant to either tumor progression (97–99) or suppression depending on the cancer type analyzed (21,77,100). The ubiquitously expressed BGN is one important structural and stabilizing component of the ECM. BGN is located on the X chromosome that is found in a variety of ECM tissues, including bone, cartilage and tendon (101).

BGN consists of a protein core containing leucine-rich repeat regions and two GAG chains consisting of either CS or DS, with DS being more abundant in most connective tissues (Figure 3). The CS/DS chains are attached at amino acids 5 and 10 in human BGN (102). The composition of the GAG chains has been reported as varying according to the tissue of origin (103).

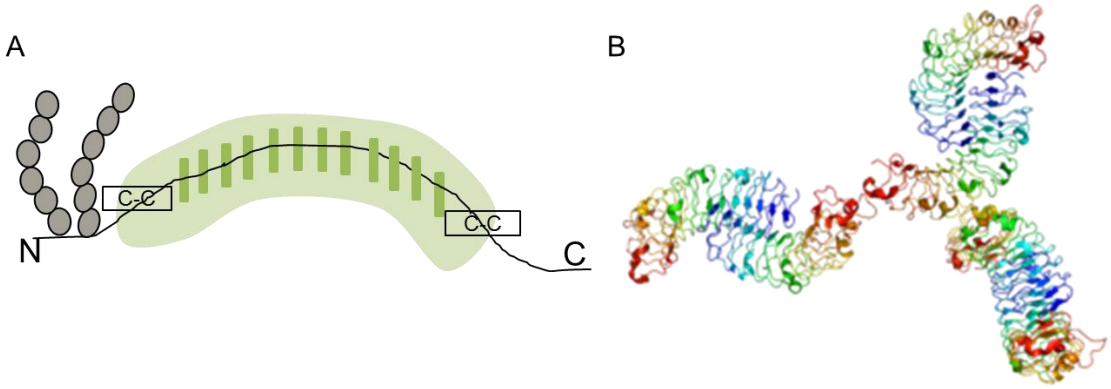


Figure 3: *Basic structure of BGN* (70) *(A) and PyMOL rendering of PDB 2ft3 (B). BGN possess a curved solenoid shaped core protein comprising of 10–12 LRR motifs that are flanked on either side by cysteine rich clusters. BGN consist of two CS/DS residues along the N-terminal domains (N: Amino terminal; C: Carboxyl terminal).*

The structure of the BGN core protein is highly conserved across species; over 90% homology has been reported for rat, mouse, bovine and human BGN core proteins (104). DCN is a small cellular or pericellular matrix PG and shares homology (55 % of amino acids are identical) with BGN in mouse and human (105).

BGN is believed to play a role in the mineralization of bone. Knock-out mice that have had the gene for BGN suppressed ($Bgn^{-/-}$) have an osteoporosis-like phenotype with reduced growth rate and lower bone mass than mice that can express BGN (106). By interacting with different types of collagens and elastin, BGN is involved in the assembly of collagen fibrils and the bone matrix. BGN regulates complex cellular processes such as growth, differentiation, adhesion, migration, inflammatory reactions and angiogenesis. Soluble BGN can interact with the TLR2 and TLR4 on macrophages and thus for the activation of NF-κB, mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinases (ERK), stress-activated protein kinase (SAPK) and p38 signaling pathways and the synthesis and secretion of proinflammatory cytokines such as IL-1β, CCL2, CXCL1, CCL5, TNF-α and IL12B (78,107). BGN core protein binds to the growth factors BMP4 and influences its bioactivity (108). It has also been reported that the presence of BGN is necessary for BMP4 to exert its effects on osteoblasts. Like DCN, there is also evidence that BGN binds to TGFB1 (109).

1.4.4 Differential expression and genetic alterations of BGN in tumors

BGN expression was reduced or lost in breast, colon, ovarian and uterine corpus endometrial carcinoma (UCEC) compared to their normal counterparts (Figure 4) (110). In contrast, literature reports overexpression of BGN compared to corresponding normal tissue has been

demonstrated in pancreatic, colon, ovarian, cholangial, prostate, esophageal and endometrial carcinomas and melanoma (111).

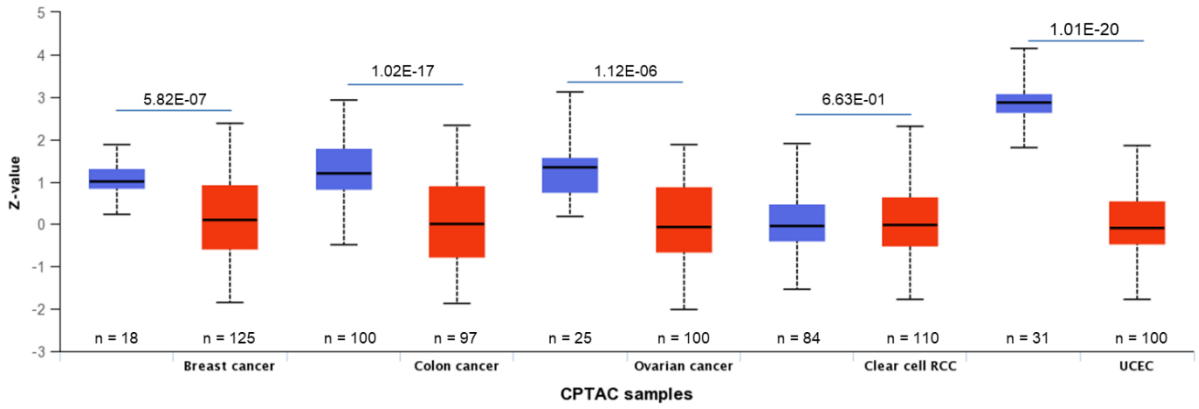


Figure 4: Expression of BGN across cancers (with tumor and normal samples)
UALCAN (110)*-based proteogenomic analysis for BGN expression in different cancer samples from the CPTAC (Clinical Proteomic Tumor Analysis Consortium) dataset was performed. The protein expression of BGN from normal and primary tumors was compared. The z-values represent standard deviations from the median across the samples. Log2 spectral count ratio values from CPTAC were first normalized within each sample profile followed by normalization across samples.*

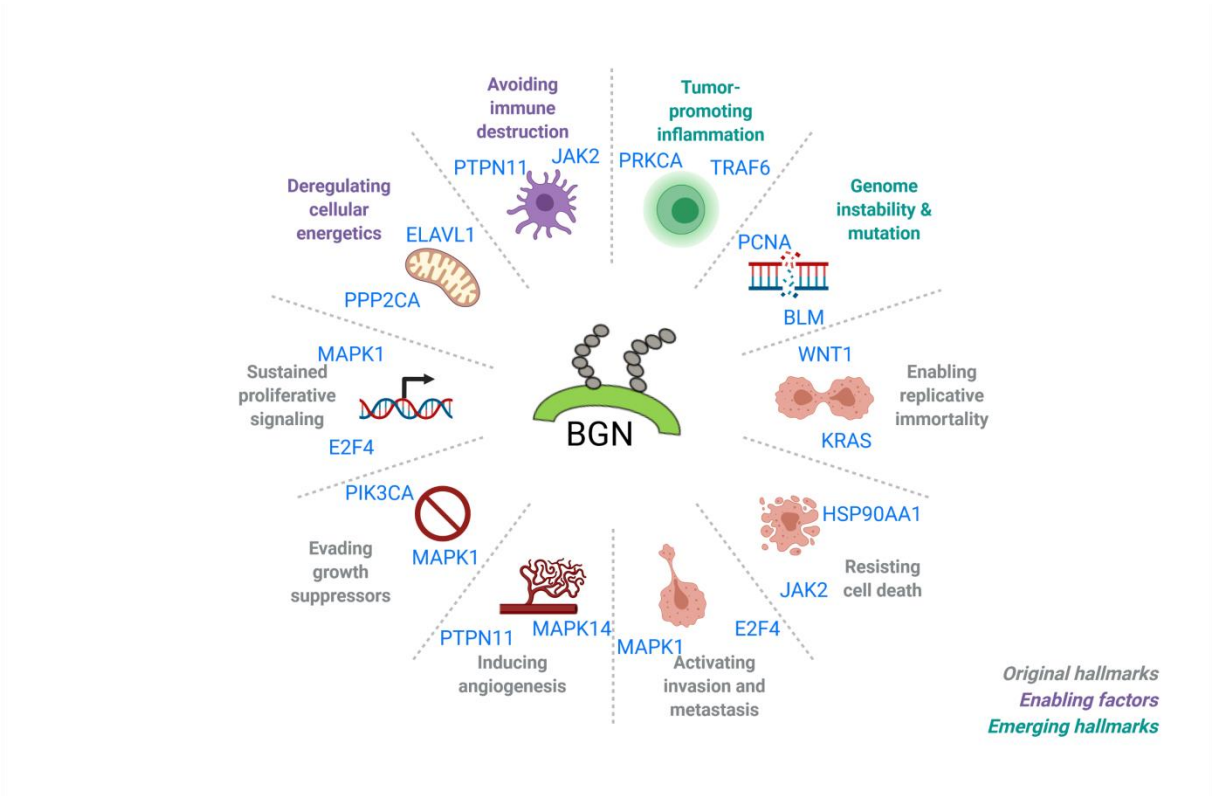


Figure 5: *Cancer hallmarks and BGN. The therapeutic connection between BGN and key cancer hallmark genes. Based on TCGA data sets (https://www.cancer.gov/tcga;*

http://r2.amc.nl) of 11003 mixed cancer samples, the expression data of the BGN and key cancer hallmark genes that were negatively correlated were plotted.

As per the TCGA data sets of 11003 mixed cancer samples, BGN play a crucial role in suppressing tumorigenesis, displayed a wide range of interactions with different key cancer hallmark genes (Figure 5). BGN controls tumorigenicity directly via the TLR2/4-NF-κB cascade or the P2X7-NLRP3-caspase-1 signaling pathway or indirectly via downstream mediators (77,78). This process is coupled with apoptosis resistance and increased production of growth factors and proinflammatory cytokines, which influences the TME and facilitates the migration of leukocytes into the peritumoral zone. The interaction of BGN with TLR2 stabilizes and increases HIF-2α (112), which influences tumor progression. In gastrointestinal tumors, BGN increases the migration and invasion of tumor cells by activating the focal adhesion kinase and inhibits apoptosis by regulating the p38 MAPK signaling pathway (97), while the depletion of BGN in endometrial and colorectal tumor cells resulted in reduced growth and a p21-mediated cell cycle arrest as well as reduced migration and invasion (113). The upregulation of BGN induced by TGF-β via p38 MAPK, indicating a connection between BGN and metastasis (114). In addition to altering signaling pathways, BGN also influences the biophysical properties of the matrix. Furthermore, BGN overexpression increases the rigidity of the matrix, which facilitates the migration of tumor cells and thus metastasis. Thus, at least in these tumor entities, BGN is involved in tumor progression, a prognostic marker as well as a potential therapeutic target. Due to the induction of a proinflammatory microenvironment and the interference with central signaling pathways in tumor cells, BGN was assigned a regulatory role in tumorigenesis (115).

However, BGN has not only pro-tumorigenic functions but also a tumor-suppressive potential (Figure 6). Lack of BGN expression or blocking of BGN expression leads to increased proliferation and/or tumor progression in pancreatic carcinomas (113), while BGN expression in the bladder and pancreatic carcinoma cells is anti-proliferative works. Although high BGN expression levels in bladder cancer correlate with increased tumor grading and invasiveness, BGN expression is associated with better survival (100). In diffuse large B-cell lymphomas, BGN induces an intra-tumor inflammatory reaction and an increased autologous tumor response by establishing a TLR2/4-mediated, proinflammatory microenvironment, which is associated with a better prognosis and treatment response (116).

Based on the discrepant results described a tumor cell type-specific and differentiation stage-dependent role of BGN is postulated, caused by alterations of various signaling pathways. A BGN-mediated chronic inflammatory microenvironment can promote tumor growth. At the same time, acute inflammation inhibits malignant growth by administering BGN in established tumors, analogous to the effects of the DCN on TLR2/4 signal transduction

(117). Although BGN expression has essentially been detected in tumors, BGN can be secreted by tumor and stromal cells or released proteolytically from the host and tumor-derived ECM (118).

Cancer development and progression is associated with genetic alterations. Although many genomic events with direct phenotypic impact have been identified (119), the role of well-known glycosylation-associated genes, like PG and GAG has not yet been studied in detail in cancers. On the other hand, the discrepant function of BGN in tumor development and progression depends on the tumor entity and is caused by alterations in the activity of various signaling pathways and immunogenicity (Figure 6). A multitude of mechanisms can contribute to the aberrant expression of PGs including oncogenic microRNA (miRNA) signatures (120).

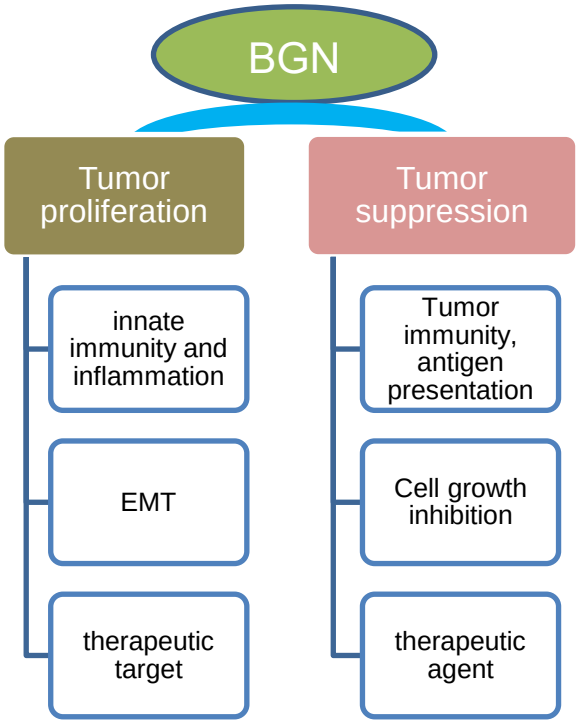


Figure 6: *Dual function of BGN on tumorigenesis.*

1.5 miRNA mediated post-transcriptional regulation

Post-transcriptional regulation controls are critical for the regulation of numerous genes in human tissues (121). It also plays a big role in cell physiology, being implicated in pathologies such as cancer and neurodegenerative diseases (122). miRNAs appear to regulate the expression of more than 60% of protein coding genes of the human genome (123). If a miRNA is abundant it can behave as a "switch", turning certain genes on or off (124). A miRNA is a small single-stranded non-coding RNA molecule (containing about 22 nucleotides) found in plants, animals and some viruses (125). Several hundred target genes

are often repressed by a single miRNA (123,126). Repression usually occurs either through translational silencing of the mRNA or through degradation of the mRNA, via complementary binding, mostly to specific sequences in the 3' UTR of the target gene's mRNA (127).

1.5.1 Role of the oncomir, miR-21 in TME

The dysregulation of certain miRNAs has been associated with oncogenic events. Oncomir is a portmanteau, derived from "oncogenic" + "miRNA", coined by Scott M. Hammond in a 2006 publication characterizing OncomiR-1 (128). Many different oncomirs have been identified in many types of human cancers (128). Oncomirs are associated with carcinogenesis, malignant transformation, and metastasis (128,129). In 2002, a down-regulation of miR-15a and miR-16-1 in B-cell chronic lymphocytic leukemia patients was reported as the first link between miRNA and cancer progression (130).

The miRNA miR-21, the prototype of oncogenic miRNAs, is encoded on chromosome 17q23.2, where it overlaps with the protein-coding gene VMP1 (131,132). It is involved in mammary gland development and epithelial cell proliferation (133), but also in the initiation and progression of numerous tumor types (134). Significantly increased miR-21 expression levels were found in e.g., cervical cancer, laryngeal squamous cell carcinoma, colorectal cancer (CRC) and BC when compared to adjacent normal tissues (135–137), which was associated with an increased proliferation, migration, invasiveness and cisplatin resistance by targeting RBPMS, RCBTB1, ZNF608, and NAV3 genes (138,139).

1.6 Declaration on the contribution to the publications

I, Karthikeyan Subbarayan, was bringing the data sets together, planning the evaluations and performing all statistical analyses. In addition, I collaborated with Dr. Sandra Leisz, Dr. Chiara Massa and Dr. André Steven to plan basic molecular research and animal studies. Under Prof. Dr. Barbara Seliger’s correspondence and supervision, I contributed as a ‘First author’ to all three manuscripts.

1.7 Aims and objectives

A relationship between the overexpression of miR-21-3p, impaired BGN and MHC class I expression and increased TGF-β signaling in HER-2/neu$^+$ cells has not yet been analyzed. This dissertation discusses for the important role of BGN and miR-21-3p in the immune escape of murine and human HER-2/neu-overexpressing cells by modulating the MHC class I and TGF-β network.

1. Investigating BGN-mediated modulation of MHC class I expression in murine and human HER-2/neu-overexpressing cells.
2. Studying a link between the lack of BGN expression and impaired MHC class I expression in HER-2/neu$^+$ cells.
3. Identification of miR-21-3p/TGF-β signaling-driven immune escape via the MHC class I/BGN axis in tumor cells.
4. Exploring the machinery behind the dual role of BGN by analyzing the influence of genetic alterations of BGN and its association with GAG synthesizing enzymes using computational models.

2 Discussion

2.1. BGN-mediated upregulation of MHC class I expression in HER-2/neu cells

HER-2/neu amplification and/or overexpression has been shown to be associated with altered growth properties and a reduced immunogenicity of tumors, which might be at least partially mediated by a HER-2/neu-induced downregulation of MHC class I surface expression due to transcriptional suppression of major APM components leading to escape from immune surveillance (140,141). Furthermore, the TME is accompanied by a reduced activation, migration and cytotoxic activity of T cells, while the frequency of immune suppressive cells, e.g., Treg, M2 macrophages and myeloid suppressor cells, is increased (142). Thus, there is an urgent need to recover the immune escape phenotype of tumors to enhance the efficacy of T cell-based immunotherapies (143).

The SLRP BGN has a broad range of functions. It links soluble matrix with innate immune responses via TLR 2 and 4 thereby inducing “danger” signals (77,144). Furthermore, BGN has been shown to promote angiogenesis via VEGF signaling (145) and tumorigenesis via the wnt/β-catenin pathway (146). In contrast, HER-2/neu-transformed cells with high angiogenic activity expressed decreased levels of BGN in a PKI/CREB-dependent manner (147). This dissertation demonstrated that the lack of BGN expression in HER-2/neu transformants was associated with reduced MHC class I surface expression, which could be reverted by BGN overexpression or by the addition of exogenous BGN. *In vivo*, BGN overexpression in HER-2/neu^{+} cells resulted in reduced tumorigenicity of these cells in immune competent mice when compared to BGN$^{low/neg}$ HER-2/neu^{+} cells suggesting that BGN acts as a tumor suppressor and enhances immunogenicity. This might be explained by a stronger immune cell infiltration as shown by an increased frequency of CD3^{+} cells and higher mRNA expression levels of CD3 and CD8 in BGN^{high} vs BGN$^{low/neg}$ HER-2/neu^{+} tumors. Particularly the presence of T cells (CD3^{+}) and T cell subpopulations (CD8^{+}) are indicators for a better prognosis (148), strongly suggesting that the anti-tumoral immune responses could be exploited as a therapeutic option. It is noteworthy that CD4 transcription was low in BGN^{high} HER-2/neu^{+} cells. These data suggest that the reduced frequency and size of BGN^{high} HER-2/neu^{+} tumors might be due to an increased immunogenicity of these cells accompanied by a strong infiltration of effector T cells when compared to BGN$^{low/neg}$ HER-2/neu^{+} tumors. Since CD4 transcription was reduced in BGN^{high} HER-2/neu^{+} tumors, one might speculate that BGN restoration downregulates the frequency of immune suppressive CD4^{+} FoxP3^{+} Treg. In addition, overexpression of BGN in HER-2/neu^{+} cells was accompanied by an upregulation of DCN expression suggesting a link between BGN and DCN expression. Treatment of BGN$^{low/neg}$ HER-2/neu^{+} cells with recombinant BGN or DCN resulted in an upregulation of MHC class I surface antigens due to increased expression of

major APM components including TAP1 and TAP2 demonstrating that both SLRPs have an immune modulatory potential.

Neoplastic malignancies often overexpress TGF-β and its receptor (149). In a murine neu-driven BC model, TGF-β can accelerate metastasis formation possibly through the synergistic activation of PI3K/AKT and Ras/MAPK pathways with neu-dependent signaling (150). Furthermore, TGF-β signaling is activated in HER-2/neu-overexpressing BC cells (151), which is accompanied by increased tumor cell motility and metastatic progression. The crosstalk between HER-2 and TGF-β not only alters intracellular signaling in cancer cells, but also influences components of the TME through the induction of several pro-invasive growth factors. In $BGN^{low/neg}$ $HER\text{-}2/neu^{+}$ cells high transcript levels of TGF-β and of the TGF-β receptor were detected, while BGN expression in $HER\text{-}2/neu^{+}$ cells reduced their expression. This is in line with the regulation of BGN by the ALK5-Smad2/3 TGF-β1 signaling pathway (152) and its function as a TGF-β repressor (153). Thus, BGN expression could be linked to changes in the TGF-β pathway known to negatively interfere with MHC class I surface expression (154) and anti-tumoral immune responses (155). These data confirm the TGF-β-mediated escape from immune surveillance due to the downregulation of MHC class I expression (156) and an induction of the epithelial mesenchymal transition (156) as demonstrated by increased SNAIL expression and activation of MMP9 (157). Interestingly, TGF-β inhibition induced MHC class I expression in $BGN^{low/neg}$ $HER\text{-}2/neu^{+}$ cells. Such data highlight the important the role of microenvironmental TGF-β signaling on escape of tumor cells from immune surveillance leading to progression.

On the other hand, DCN has also been shown to block TGF-β transcription and protein expression in glioblastoma cells, which was accompanied by a strong inhibition of tumor formation *in vivo* (158). DCN-expressing glioblastoma showed an altered TME characterized by an increased frequency of infiltrating T and B cells. Furthermore, the DCN-induced inhibition of TGF-β was accompanied by significantly enhanced anti-glioblastoma immune responses in tumor necrosis factor-α-converting enzyme signaling (158). These data are in line with our BGN^{high} HER-2/neu model demonstrating an increased DCN expression in these cells. Thus, a better understanding of the contextual networks of BGN and DCN in tumors is required to modulate immunogenicity by targeting the MHC class I surface expression.

TGF-β1 binds to its receptors (TGF-βRI and TGF-βR2) and induces the phosphorylation of the tumor necrosis factor-α-converting enzyme (TACE) (Figure 7), which resulted in its translocation to the cell surface, where TACE induces integrins and cleaves the EGFR pro-ligands (159). EGFR ligands will initiate an autocrine and paracrine EGFR signaling, which is amplified in HER-2/neu-overexpressing cells ($BGN^{low/neg}$ $HER\text{-}2/neu^{+}$ cells). In BGN^{high} $HER\text{-}2/neu^{+}$ cells, BGN and DCN bind to TGF-β1 and restrict HER-2/neu signaling, which might induce tumor suppression (Figure 7). It can be suggested that TGF-β by signaling via the

TGF-β receptor enhances the HER-2/neu-initiated signal transduction by increasing HER-2/neu ligand shedding, HER-2/neu-containing heterodimers, and their cross talk with integrins (151).

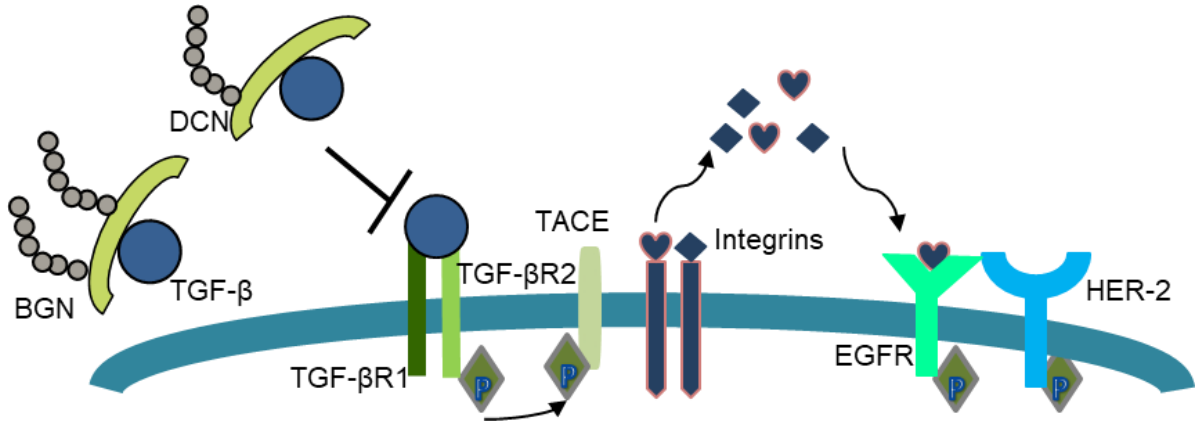


Figure 7. *Schematic representation of BGN- and DCN-mediated inhibition of the TGF-β pathway and restriction of HER-2/neu signaling. TGF-β1 binds to its receptors (TGF-βRI and TGF-βR2) and induces phosphorylation of TACE, resulting in its translocation to the cell surface, where TACE induces integrins and cleaves EGFR pro-ligands. EGFR ligands will initiate autocrine and paracrine EGFR signaling, which is amplified in HER2-overexpressing cells (BGN$^{low/neg}$ HER-2/neu^{+} cells). In BGN^{high} HER-2/neu^{+} cells, BGN and DCN bind to TGF-β1 and restricts HER-2/neu signaling, thus allowing tumor suppression to occur.*

In our study, an enhanced expression of TGF-β1 and its receptor TGF-βR1 was found in BGN^{low} HER-2/neu, which could be reverted to normal levels by BGN overexpression. Both BGN and DCN regulate the TGF-β availability in BGN^{high} HER-2/neu cells. In BGN^{high} HER-2/neu^{+} cells, the amount of BGN and DCN in the ECM increases and both PGs bind to TGF-β and sequester it to the ECM. In this way both DCN and BGN translocate TGF-β from the membrane thereby reducing the binding to its receptor resulting in a decreased TGF-β signaling and restricting HER-2/neu-mediated carcinogenic effects.

2.2 miR-21-3p/TGF-β signaling-driven immune escape via the MHC class I/BGN axis

The oncogenic miR-21 is overexpressed in many cancers, including BCs (160) and has often a prognostic value (139,161–169). It plays an important role by affecting the expression of cancer relevant genes involved in proliferation, migration and cell death (169–172) as well as in angiogenic activity, inflammatory responses and T cell activation (173–178). For example, miR-21 can be dynamically regulated after T cell activation by controlling the sustained activation of the MAPK and AKT-mTOR signaling pathways (179) and the PTEN/Akt pathway in response to T cell stimulation (180). However, miR-21 expression can be also increased in tumor suppressive immune cells, like tumor associated macrophages, known to be partially

responsible for promoting tumor growth, while its absence is accompanied by a decreased tumor growth (177). In addition, miR-21 is involved in the COX-2 inflammatory pathway, which promotes CRC progression and downregulates the tumor suppressor gene PDCD4 (181). Thus, miR-21 expression and activity have a dual role and affects multiple physiologic and pathophysiologic functions in a context-dependent manner (178). This dissertation postulates that miR-21 induced by HER-2/neu transformation might emerge as a key player in immune evasion as postulated for other miRs (182–184).

Therefore, the link between the immune modulatory role of BGN in HER-2/neu^{+} cells and miR-21-3p was explored *in vitro* and on tumor samples *in vivo* based on our previous work demonstrating a reduced tumorigenicity of BGN^{high} HER-2/neu^{+} cells in DBA-1 mice (21). A downregulated miR-21-3p expression and a reduced TGF-β signaling was not only found *in vitro,* but also in tumor lesions of BGN^{high} HER-2/neu^{+} cells suggesting that the enhanced immunogenicity of these cells might be mediated by a decline of miR-21-3p expression, which was further strengthened by a strong tumor infiltration of effector CD8^{+} T cells when compared to BGN$^{low/neg}$ HER-2/neu^{+} tumors (21). This study therefore extended the oncogenic function of miR-21-3p to immune modulatory properties and showed for the first time a miR-21-3p-mediated downregulation of MHC class I surface antigens in different model systems, which is linked to an altered BGN expression and TGF-β signaling in HER-2/neu^{+} cells. Our results suggest that HER-2/neu transformation induces the expression of miR-21-3p, which interferes with the expression of immune modulatory molecules thereby accelerating immune suppression and reducing tumor immunogenicity (Figure 8).

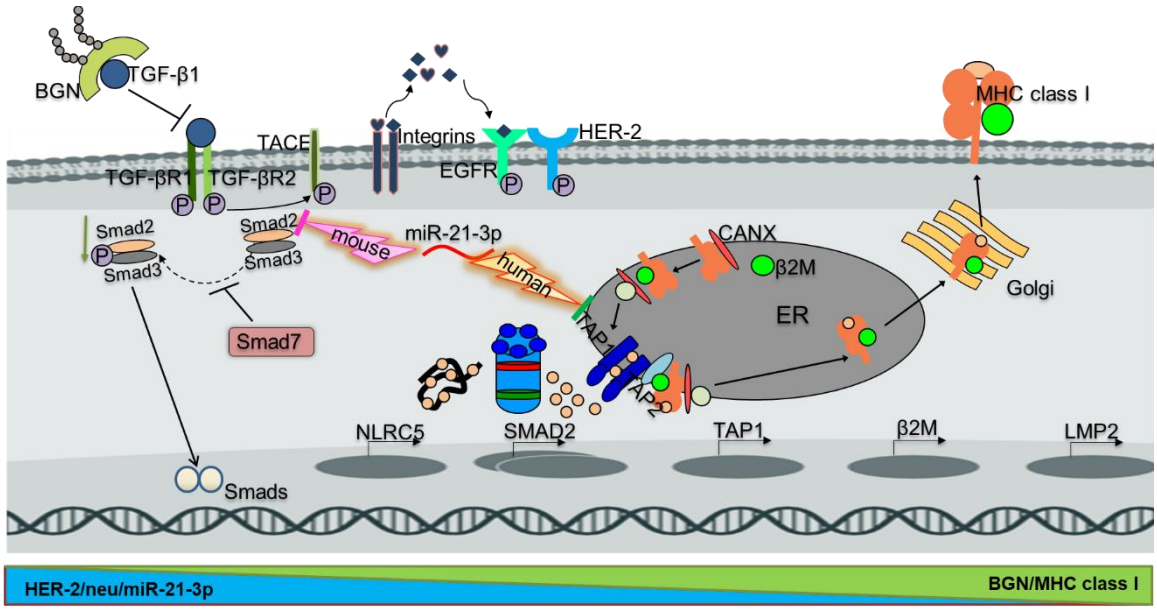


Figure 8: miR-21-3p/TGF-β signaling-driven immune escape via the MHC class I/BGN axis

MiR-21 targets important tumor suppressor genes as well as genes involved in carcinogenesis, such as SPRY2 (185), PTEN (186), RECK (187), TIMP3 (188), BCL2 (189)

and PDCD4 (190). For example, the knock down of miR-21-3p overexpression caused an increased PTEN expression associated with an increased apoptosis (191), while miR-21-3p inhibited the tumor suppressive function of the p53 dimer (192). Moreover, an immune suppressive effect of miR-21 was described by inhibiting e.g., the IFN-γ-induced STAT1 signaling pathway (183,193). This dissertation reports for the first time that miR-21-3p targets a MHC class I APM component and an inhibitor of the TGF-β pathway thereby interfering with the MHC class I antigen expression (21): Targeting of the 3' UTR of SMAD2 by murine miR-21-3p enhances the TGF-β signaling, which is accompanied by a reduced MHC class I surface expression (Figure 8). In human cells, hsa-miR-21-3p binds to the 3'UTR of the TAP1 subunit thereby restricting the peptide transport into the ER leading to an impaired peptide loading of MHC class I antigens. The oncogenic miR-21-3p is increased by HER-2/neu transformation and linked to altered growth properties and a reduced immunogenicity of HER-2/neu^{+} tumors (140). Interestingly, the BGN-mediated overexpression in BGN$^{low/neg}$ HER-2/neu^{+} cells induced MHC class I expression and reduced miR-21-3p thereby highlighting the critical role of miR-21-3p on the immune escape of tumor cells (Figure 8). These results were confirmed by TCGA data analysis demonstrating a significant correlation of miR-21-3p with HER-2/neu (ERBB2) expression with a reduced patients' survival. The crosstalk between HER-2/neu and miR-21-3p alters the intracellular signaling in cancer cells by enhancing pro-invasive growth factors, like TGF-β, or inhibiting immune stimulatory factors, like BGN. In BGN$^{low/neg}$ HER-2/neu^{+} cells high miR-21-3p transcript levels were detected, while BGN transfection into BGN$^{low/neg}$ HER-2/neu^{+} cells reduced miR-21-3p expression. This is in line with the reduced expression of SMAD2 upon miR-21-3p overexpression and the promotion of the cell viability and proliferation of bovine mammary gland epithelial cells (133). Despite further *in vitro* and *in vivo* studies are required to elucidate the precise underlying molecular mechanisms of miR-21-3p in the tumor development, pharmacological targeting of the miRNA-PG-MHC class I axis as a novel innovative therapeutic concept for HER-2/neu^{+} cancers is suggested.

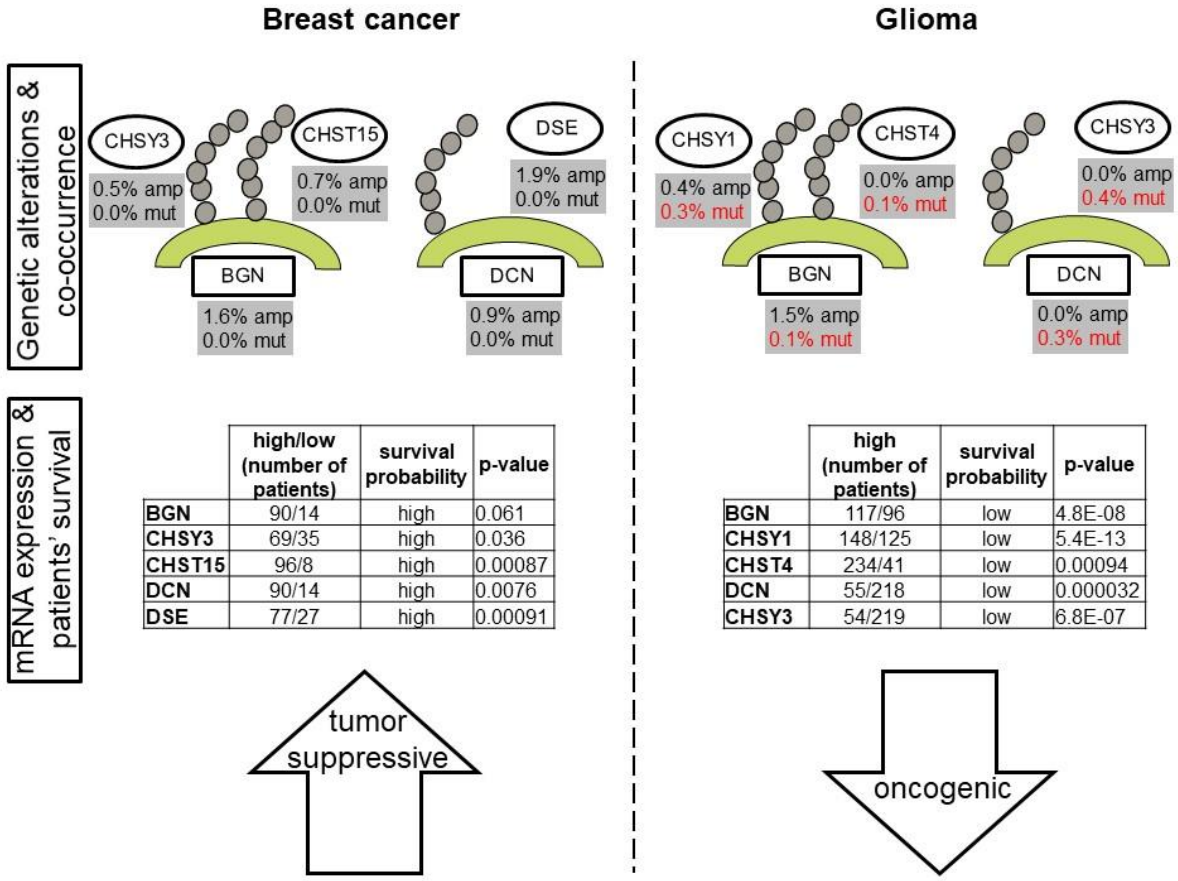
In cancer, contradicting data exist regarding the clinical significance of BGN expression. In some tumors an increased BGN expression was linked to poor prognosis (194), whereas in others its overexpression was associated with inhibition of cancer cell growth and a good prognosis (113,195). The expression of BGN was increased in liver, ovarian, endometrial, pancreatic and gastric cancer (196) suggesting an important role of BGN in the pathogenesis of these malignancies. In contrast, several other studies demonstrated an anti-tumoral activity of BGN associated with anti-proliferative capacity. Similar results were obtained in our HER-2/neu model system suggesting that BGN has tumor suppressive activity, which might be associated with an increased immunogenicity. *In silico* analysis of TCGA data from BC patients demonstrated a prognostic value of BGN and DCN, which is in line with our *in*

vitro results. Based on these reports BGN displays very contradicting roles, which might depend on the cellular context.

2.3 Prognostic associations of mutations in BGN and co-occurred GAG genes

In the era of individualized therapies, grouping patients based on their co-association pattern of genetic alterations is the first step towards the implementation of genomics-based personalized medicine. Our report describes how the genetic alterations co-occur between PGs and CS/DS modification enzymes in every individual BC patient.

Tumor cells display a wide range of glycosylation alterations compared to their non-transformed counterparts and have already been described (197,198). Changes in the connective tissue within and around tumors are increasingly recognized as important contributors to tumor development and progression (199). A central role for PGs, containing at least one GAG chain, such as HS, DS, CS, or KS covalently attached to the protein core, has been described in tumors (200). Among PG family members, the expression profiles of DCN and/or BGN are well-studied in different cancers and have been associated with the clinical outcome of tumor patients (99,201,202). However, their tumor-suppressive or tumor-promoting potential is controversially discussed.



	high/low (number of patients)	survival probability	p-value
BGN	90/14	high	0.061
CHSY3	69/35	high	0.036
CHST15	96/8	high	0.00087
DCN	90/14	high	0.0076
DSE	77/27	high	0.00091

	high (number of patients)	survival probability	p-value
BGN	117/96	low	4.8E-08
CHSY1	148/125	low	5.4E-13
CHST4	234/41	low	0.00094
DCN	55/218	low	0.000032
CHSY3	54/219	low	6.8E-07

Figure 9: Using computational tools, this dissertation demonstrates a distinct association of BGN and DCN with sulfate modification enzymes in BC and glioma. The unique pattern of structural alterations and expression with clinical relevance was found for PGs and sulfate

modification enzymes in BC and glioma, which might help to identify high risk patients and to develop personalized therapeutics (203).

In this study, the structural alterations of the ECM components BGN and DCN in BC with special emphasis on their GAG chains focusing on CHST and CS/DS modification enzymes were investigated. It is well-known that mutations and aberrant glycosylation occur in all major human cancers (198–200). BC was selected to unravel the distinct roles of BGN, DCN, CHST and CS/DS modification enzymes as the cancer entities have already been shown for the RNA-binding protein Musashi-1 (204) and STAT3 inhibitors (205). We have addressed the frequency of glycosylation modifications and structural abnormalities of CS/DS chains, BGN and DCN and their associations between genomic, transcriptomic and clinical features. Computational approaches allow to correlate gene expression with mutation rates in order to identify frequently mutated genes while eliminating many false-positive cells made in several single-tumor-type projects (206). A better understanding of the relevant alterations will help to identify glycan-based biomarkers with prognostic and diagnostic value.

Using network analyses, the mechanisms of interaction among the different genes could be determined. For example, mutual exclusivity analyses have been published for K-RAS and p53 and co-occurred in pancreatic adenocarcinoma (log odds ratio = 1.599, p=0.006), where K-RAS and p53 were not neighboring genes (207). In our investigation, BGN and DCN were not neighboring genes; but many CS, DS and carbohydrate sulfotransferases enzymes were reported as neighboring genes of BGN and DCN. BGN was associated with CS modifying enzymes, CHSY3 and carbohydrate sulfotransferases, CHST15. Except for CHST4, all these CHST and CS/DS modification enzymes were directly interacting with BGN and DCN suggesting that the CHST and CS/DS modification enzymes influence and control the expression of BGN and DCN. BGN and DCN showed differential co-occurrence, while DCN co-occurs with DSE (Figure 9).

Since we hypothesized that the prognostic effects may be influenced by mutations, the mutation rates of BGN, DCN and modification enzyme genes and their survival analyses were correlated with the patients' survival by R2 Genomics. There is an association between the occurrence of mutations and poor patients' outcome. No mutations were found in BGN/DCN and their co-occurred CHST and CS/DS modification enzymes in BC, which were linked with good clinical outcome of patients (Figure 9). Germ-line mutations in DCN and p53 have been reported to cooperate in the transformation of lymphocytes and ultimately lead to a more aggressive phenotype by shortening the latency of lymphoma (208). BGN and their associated CHSY1 and CHST4 were also mutated in glioma (203), which was also in line with a statistically significant increase in variant allele frequency in melanoma and bladder cancer (209,210). The specificities of glycosylation and mutation depend on various intrinsic

factors of the glycosylation process within a given cell or tissue type (211). Cancer drivers identified only by integrating less frequent events across tumor types with mutations and copy number alterations (CNA) and aggregating genes using gene network and pathway analyses (212).

BGN and their associated CHST and CS/DS modification enzymes were over-expressed in BC. However, overexpression of BGN in BC was correlated with higher patients' survival. Many studies have already shown an association of BGN and DCN with cancer in the context of oncogenic (113,213) and anti-tumor properties (100,118,214). The changes in the glycosylation pattern of BGN and DCN will increase the molecular heterogeneity as well as the functional diversity (203,215).

Targeting altered glycosylation as an immunotherapeutic strategy provides an appealing option for cancer treatment (216,217). While the HA-CD44 was the most studied GAG associations in cancer leading to an altered signal transduction (218,219), this dissertation reports here the first evidence of BGN and DCN interacting with different CHST and CS/DS modification enzymes. Understanding of the structure and specific biological roles of GAGs might lead to the development of novel therapeutic approaches, including the development of mimics as well as delivery systems for anti-cancer drugs targeting over-expressed BGN/DCN and their associated enzymes in the TME. Therefore, an increased knowledge of BGN/DCN and GAG chains in tumors of distinct origin is important for diagnosis, prognosis, drug delivery and design of novel treatment of tumor patients.

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4 Theses

1. The declining BGN expression in HER-2/neu transformants was associated with reduced MHC class I surface expression, which could be reverted by BGN overexpression or by the addition of exogenous BGN.
2. The reduced frequency of tumor formation and tumor size of BGN^{high} HER-2/neu^{+} tumors might be due to increased immunogenicity of these cells accompanied by a strong infiltration of effector T cells compared to $BGN^{low/neg}$ HER-2/neu^{+} tumors.
3. Overexpression of BGN in HER-2/neu^{+} cells was accompanied by an upregulation of the PG DCN expression suggesting a link between BGN and DCN expression. Since recombinant DCN also elevated MHC class I surface expression in $BGN^{low/neg}$ HER-2/neu^{+} cells, both PGs might act synergistically.
4. TGF-β inhibition induced MHC class I expression in $BGN^{low/neg}$ HER-2/neu^{+} cells. Like other neoplastic malignancies, $BGN^{low/neg}$ HER-2/neu^{+} cells expressed high transcript levels of TGF-β, while BGN expression in HER-2/neu^{+} cells reduced their expression.
5. The miR-21-3p enhances TGF-β signaling by binding to the 3′UTR of SMAD2, resulting in a reduced MHC class I surface expression, while hsa-miR-21-3p binds to the human TAP1 3′UTR, thereby restricting the peptide transport and loading of MHC class I antigens. Hence, the miR-21-3p targets a MHC class I APM component and an inhibitor of the TGF-β pathway, thus interfering with the MHC class I antigen expression. Interestingly, the BGN-mediated overexpression in $BGN^{low/neg}$ HER-2/neu^{+} cells reduced miR-21-3p.
6. The miRNA-BGN-MHC class I axis along with the unique pattern of structural alterations and expression of BGN, which has clinical relevance in BC dataset, which might help to identify high-risk patients and to develop personalized therapeutics.
7. BGN exhibits tumor-suppressive or oncogenic potentials depending on the cellular context, mutation status and association with GAGs. The lack of mutations and CNA in BGN, and their associated CS polymerizing enzyme, CHSY3 and carbohydrate sulfotransferase, CHST15 accompanied with increased survival in the BC patients' dataset. Therefore, further experimental and clinical studies are warranted.

5 Publications

List of publications included in this dissertation

1. **Subbarayan K,** Leisz S, Wickenhauser C, Bethmann D, Massa C, Steven A, Seliger B. Biglycan-mediated upregulation of MHC class I expression in HER-2/neu-transformed cells. Oncoimmunology. 2018 Apr 3;7(4):e1373233. Available from: https://pubmed.ncbi.nlm.nih.gov/29632715/
2. **Subbarayan K,** Massa C, Lazaridou MF, Ulagappan K, Seliger B. Identification of a novel miR-21-3p/TGF-β signaling-driven immune escape via the MHC class I/biglycan axis in tumor cells. Clinical and translational medicine. 2021 Mar;11(3). Available from: https://pubmed.ncbi.nlm.nih.gov/33783999/
3. **Subbarayan K,** Seliger B. Tumor-dependent effects of proteoglycans and various glycosaminoglycan synthesizing enzymes and sulfotransferases on patients' outcome. Current cancer drug targets. 2019 Mar 1;19(3):210-21. Available from: https://pubmed.ncbi.nlm.nih.gov/29984655/

5.1. Publication - One

1. **Subbarayan K,** Leisz S, Wickenhauser C, Bethmann D, Massa C, Steven A, Seliger B. Biglycan-mediated upregulation of MHC class I expression in HER-2/neu-transformed cells. Oncoimmunology. 2018 Apr 3;7(4):e1373233. Available from: https://pubmed.ncbi.nlm.nih.gov/29632715/

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ORIGINAL RESEARCH

Biglycan-mediated upregulation of MHC class I expression in HER-2/neu-transformed cells

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ABSTRACT

The extracellular matrix protein biglycan (BGN) has oncogenic or tumor suppressive potential depending on the cellular origin. HER-2/neu overexpression in murine fibroblasts and human model systems is inversely correlated with BGN expression. Upon its restoration BGN^{high} $HER\text{-}2/neu^{+}$ fibroblasts were less tumorigenic in immune competent mice when compared to $BGN^{low/neg}$ $HER\text{-}2/neu^{+}$ cells, which was associated with enhanced immune cell responses and higher frequencies of immune effector cells in tumors and peripheral blood. The increased immunogenicity of BGN^{high} $HER\text{-}2/neu^{+}$ fibroblasts appears to be due to upregulated MHC class I surface antigens and reduced expression levels of transforming growth factor (TGF)-β isoforms and the TGF-β receptor 1 suggesting a link between BGN, TGF-β pathway and HER-2/neu-mediated downregulation of MHC class I antigens. Treatment of $BGN^{low/neg}$ $HER\text{-}2/neu^{+}$ cells with recombinant BGN or an inhibitor of TGF-β enhanced MHC class I surface antigens in $BGN^{low/neg}$ HER-2/neu-overexpressing murine fibroblasts, which was mediated by a transcriptional upregulation of major MHC class I antigen processing components. Furthermore, BGN expression in $HER\text{-}2/neu^{+}$ cells was accompanied by an increased expression of the proteoglycan decorin (DCN). Since recombinant DCN also elevated MHC class I surface expression in $BGN^{low/neg}$ $HER\text{-}2/neu^{+}$ cells, both proteoglycans might act synergistically. This was in accordance with *in silico* analyses of mRNA data obtained from The Cancer Genome Atlas (TCGA) dataset available for breast cancer (BC) patients. Thus, our data provide for the first time evidence that proteoglycan signatures are modulated by HER-2/neu and linked to MHC class I-mediated immune escape associated with an altered TGF-β pathway.

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Introduction

The biglycan (BGN) gene localized on chromosome X in humans and mice[1] encodes an extracellular matrix (ECM) protein of the small leucine-rich proteoglycan (SLRP) family characterized by cysteine residues in the N-terminus and a protein core with side chains containing chondroitin and/or dermatan sulfate. It is expressed in most tissues in particular in the ECM of epithelial cells. Major functions of BGN include modulation of matrix assembly, cell migration, adhesion, bone mineralization, inflammation, cell growth, autophagy regulation as well as apoptosis.[2] Consequently BGN is involved in several physiologic and pathophysiologic processes such as tumorigenesis.[3] SLRPs including BGN interact with a number of receptors that regulate growth, motility and immune responses. Consequently, proteoglycans can induce a cross talk among various families of receptors and interact with natural receptor ligands.[4] In their soluble form, proteoglycans can become stress signals and may e.g. act as ligands for toll-like receptors (TLR) thereby regulating innate immunity. Furthermore, BGN has been shown to enhance antigen-specific T cell activation thereby triggering autoimmune peri-myocarditis.[5,6]

The role of BGN in tumorigenesis is currently controversially discussed. In some tumor types, higher levels of BGN expression have been detected when compared to normal counterparts[7-9], which was associated with enhanced migration and invasion *in vitro* and *in vivo*. Overexpression of BGN in e.g. colorectal cancer (CRC) cells possesses pro-angiogenic properties by binding to vascular endothelial growth factor (VEGF)-A leading to an activation of vascular endothelial growth factor receptor (VEGF-R) signaling and the extracellular signal-regulated kinase (ERK) pathway.[4, 10] Furthermore, BGN can promote tumorigenesis via enhanced Wnt/β-catenin signaling.[11] Thus, BGN plays an important role in cancer progression and metastasis.[12]

In contrast, in bladder cancer silencing of BGN resulted in enhanced tumor cell proliferation indicating that BGN acts as a growth suppressor in this disease.[13] *In vivo* BGN expression might inhibit tumor growth of established tumors by creating a TLR2/4-mediated pro-inflammatory microenvironment.[14] Decorin (DCN) is another member of the SLRP family and was first described in dcn/p53 double knock out mice, which developed tumors faster than wild type (wt) counterparts.[3] BGN

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Subbarayan K, Leisz S, Wickenhauser C, Bethmann D, Massa C, Steven A, Seliger B. Biglycan-mediated upregulation of MHC class I expression in HER-2/neu-transformed cells. Oncoimmunology. 2018 Apr 3;7(4):e1373233. Available from: https://pubmed.ncbi.nlm.nih.gov/29632715/

shares > 65% homology with DCN in mouse and human.[15] Like BGN, DCN exert pro-angiogenic activities[16]. In addition, impaired DCN expression was found in many solid tumor entities including CRC[17-20] and breast cancer (BC).[21] Due to the inhibitory properties against receptor tyrosine kinases (RTK) and cancer growth pathways other SLRP members have been shown to have tumor suppressive effects *in vivo* and *in vitro*.[16,22-25]

These data were further confirmed using a comparative proteomic approach of Ki-ras-transformed mouse fibroblasts and respective controls: BGN expression was downregulated when compared to parental NIH3T3 cells or mock transfectants.[26] Similar results were also obtained upon HER-2/neu transformation of fibroblasts.[27] Restoration of BGN expression in HER-2/neu$^+$ cells reduced their growth and migration capacity[27] when compared to BGN$^{low/neg}$ HER-2/neu$^+$ cells, which was due to the enhanced expression and function of the cAMP response element binding protein (CREB).[28] HER-2/neu amplification and/or overexpression were found in a variety of human epithelial tumors of distinct origin including breast and lung cancer, which was in the majority of studies associated with a more aggressive disease and poor patients' outcome.[29] Poor survival patients' outcome was also correlated with an overexpression of the transforming growth factor (TGF)-β in breast cancer,[30] which is required for the activation of the epidermal growth factor receptor (EGF-R).[31] In *in vitro* models high levels of HER-2/neu expression in murine fibroblasts and human HER-2/neu-overexpressing tumor cells caused a downregulation of MHC class I surface expression leading to a reduced T cell recognition.[32-35] This inverse expression of HER-2/neu and MHC class I surface antigens was caused by a transcriptional downregulation of major components of the MHC class I antigen processing machinery (APM) and controlled by activation of the mitogen-activated protein kinase (MAPK) pathway.[32, 36, 37]

Since a link between the lack of BGN expression and impaired MHC class I expression in HER-2/neu$^+$ cells has not yet been described, the role of BGN in murine and human HER-2/neu-overexpressing cells on MHC class I APM components *in vitro* and/or *in vivo* was determined by overexpressing BGN in BGN$^{low/neg}$ HER-2/neu$^+$ cells or by their treatment with recombinant BGN.

Results

BGN-mediated induction of MHC class I expression in HER-2/neu overexpressing cells

In order to determine the effect of BGN on the MHC class I surface expression of HER-2/neu$^+$ cells, both a BGN expression vector and a vector control were stably transfected into BGN$^{low/neg}$ HER-2/neu$^+$ cells followed by analyses of the BGN and MHC class I mRNA and/or protein expression. The BGN$^{low/neg}$ HER-2/neu$^+$ cells showed low transcription levels of MHC class I antigens (Fig. 1A), whereas BGN overexpression in HER-2/neu$^+$ cells resulted in an upregulation of MHC class I heavy chain transcription (Fig. 1A) as well as MHC class I surface expression (Fig. 1B, C). In addition, treatment of BGN$^{low/neg}$ HER-2/neu$^+$ cells with exogenous recombinant BGN significantly increased MHC class I surface expression (Fig. 1D, S1A). Vice versa, silencing of BGN expression in BGN^{high} NIH3T3 fibroblasts by shRNA resulted in a downregulation of MHC class I surface expression in these cells (Fig. 1E).

BGN-mediated upregulation of MHC class I antigens due to transcriptional increase of APM components

To test whether the BGN-mediated increase of MHC class I surface antigens was due to a transcriptional upregulation of MHC class I APM components, mRNA expression and/or promoter activity of selected APM components were determined by qPCR and promoter reporter assays, respectively[37]. As shown in Fig. 2A and 2B, the transcript and protein expression levels of TAP1 and TAP2 were increased in BGN transfectants when compared to BGN$^{low/neg}$ HER-2/neu$^+$ cells, but to a different extent. This was further confirmed by enhanced TAP1 and TAP2 promoter activities in BGN transfectants using luciferase (luc) reporter assays (Fig. 2C), which was more pronounced for TAP2. In contrast, LMP2 transcription (Fig. 2A) and promoter activity (Fig. 2C) were reduced in BGN transfectants, while the expression of TPN (data not shown) and other APM components (Figure S2) were comparable in BGN transfectants and BGN$^{low/neg}$ HER-2/neu$^+$ cells.

Inverse correlation of BGN expression with HER-2/neu expression in human tumor cells

To confirm the inverse correlation of BGN and HER-/neu expressions in the murine model system, human HER-2/neu-overexpressing breast cancer cells were selected for further experiments. As expected, an inverse correlation of BGN and HER-2/neu expression was detected in wt HER-2/neu (HTB122 E2), but not in mut HER-2/neu (HTB122 E2A)-transfected HTB122 cells (Fig. 3). In addition, qPCR analysis of a large panel of melanoma cells with known HER-2/neu status demonstrated an inverse correlation of HER-2/neu and BGN expression levels (Figure S3).

Role of BGN expression in vivo

In order to assess whether BGN overexpression in BGN$^{low/neg}$ HER-2/neu$^+$ cells also affects the *in vivo* tumorgenicity, BGN$^{low/neg}$ HER-2/neu$^+$ cells, mock and BGN transfectants were injected into both immune competent (DBA-1) and immune deficient mice (Fox 1 nude), respectively, and tumor growth regarding incidence and tumor diameter was monitored over time. BGN^{high} HER-2/neu$^+$ cells exhibited a reduced frequency of tumor formation and tumor diameter in immune competent mice, which was associated with an increased survival of mice (Fig. 4A) when compared to BGN$^{low/neg}$ HER-2/neu$^+$ cells. Despite the tumor formation in immune deficient mice was an early event in mice injected with BGN$^{low/neg}$ HER-2/neu$^+$ cells compared to those injected with BGN^{high} HER-2/neu$^+$ cells, all mice injected with either BGN$^{low/neg}$ or BGN^{high} HER-2/neu$^+$ cells, respectively, developed tumors at later stage without a significant difference in tumor diameter. The expression of HER-2/neu and BGN in tumors was determined by qPCR, western blot (Fig. 4B) and/or immunohistochemistry (IHC) demonstrating high levels of HER-2/neu expression in all tumor lesions analyzed independent of the presence of BGN, while BGN expression was only detected in the

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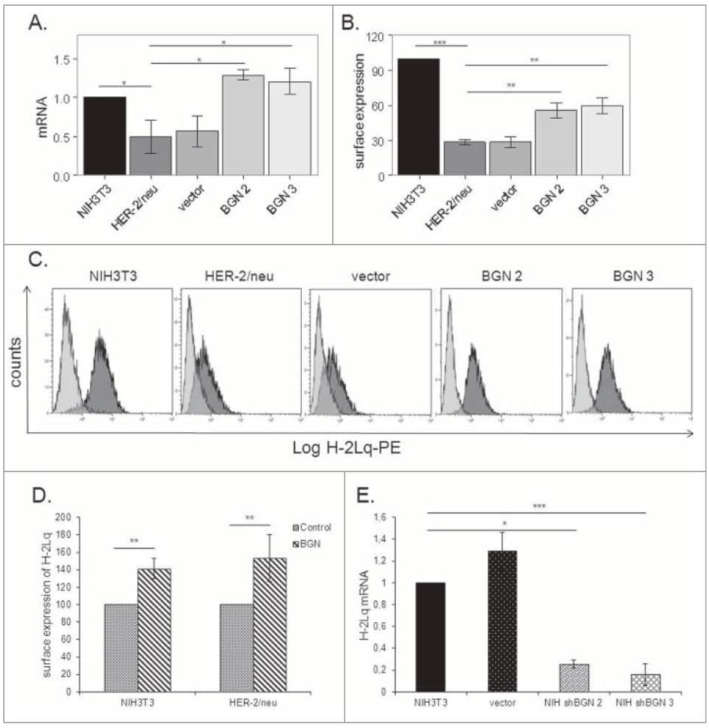

Figure 1. Induction of MHC class I expression upon BGN overexpression in BGN$^{low/neg}$ HER-2/neu^{+} cells. A. mRNA expression levels of the MHC class I heavy chain in BGN$^{low/neg}$ vs. BGN^{high} HER-2/neu^{+} cells. Transcription of H-2L^{q} was analysed by qPCR as described in Materials and Methods. B. BGN-mediated regulation of MHC class I surface antigen expression MHC class I surface expression was assessed by flow cytometry as described in Materials and Methods. C. A representative histogram of MHC class I surface expression. D. Influence of recombinant BGN on MHC class I surface antigens. Cells were left untreated or treated with recombinant BGN (1 μg/mL) for 24 h before MHC class I expression was assessed by flow cytometry using an anti-H-2L^{q} mAb as described in Materials and Methods. E. Downregulation of MHC class I surface expression upon silencing of BGN. BGN expression was silenced in parental BGN^{high} HER-2/neu$^{low/neg}$ NIH3T3 cells using shBGN and transfectants were analysed by qPCR for the expression of H-2L^{q} as described in Materials and Methods. The results were displayed as bar diagrams and represent the mean ± SE format of three independent experiments.

BGN-transfected HER-2/neu^{+} cells. Along with BGN expression, IHC analysis displayed also a higher frequency of CD3^{+} cells in BGN^{high} HER-2/neu^{+} cells when compared to BGN$^{low/neg}$ or BGN^{high} HER-2/neu^{+} cells (Fig. 4C, 4D). Analysis of the immune cell infiltration in tumor tissues determined by qPCR revealed higher transcript levels of CD3 and CD8, but reduced CD4 mRNA levels in BGN^{high} HER-2/neu^{+} cells, which were accompanied by high MHC class I heavy chain mRNA expression in these tumors (Fig. 4E). This was further supported by the fact that the mice bearing tumors induced by BGN^{high} HER-2/neu^{+} cells showed an enhanced CD8^{+} T cell frequency and slight reduction of CD4^{+}/CD25high cells in peripheral blood (Fig. 4F).

Correlation of BGN expression with TGF-β-mediated MHC class I downregulation

BGN$^{low/neg}$ HER-2/neu^{+} cells expressed significantly higher mRNA levels of TGF-β1, TGF-β3 and TGF-βR1 than parental NIH3T3 cells and BGN^{high} HER-2/neu^{+} cells suggesting that BGN overexpression inhibits the HER-2/neu-mediated TGF-β signaling (Fig. 5A). These *in vitro* data were further confirmed *in vivo* demonstrating a significant downregulation of molecules of the TGF-β pathway in the BGN^{high} HER-2/neu^{+} tumor lesions (Fig. 5B).

In order to study a possible link between BGN and MHC class I expression with the TGF-β pathway BGN^{high} cells were treated with recombinant TGF-β1, while BGN^{low} HER-2/neu^{+} cells were treated with the TGF-β1 inhibitor SB431542. IFN-γ treatment of the cells served as positive control. As expected IFN-γ significantly enhanced the expression of MHC class I surface antigens (Fig. 5C, S1B) in these cells, while it was downregulated in the presence of TGF-β1 (Fig. 5D) and slightly increased in the presence of SB431542 (Fig. 5E, S1C). The combination of TGF-β1 and IFN-γ treatment demonstrated that IFN-γ counteracted the TGF-β1-mediated inhibition of MHC class I surface antigens (Figure S4H) suggesting that IFN-γ overcomes the TGF-β-mediated downregulation of MHC class I surface expression. These results were further confirmed in human HER-2/neu model systems (Figure S4).

BGN-mediated upregulation of DCN expression

Since the SLRP member DCN has been shown to exhibit a tumor-suppressive activity,[24, 25] it was analysed whether BGN

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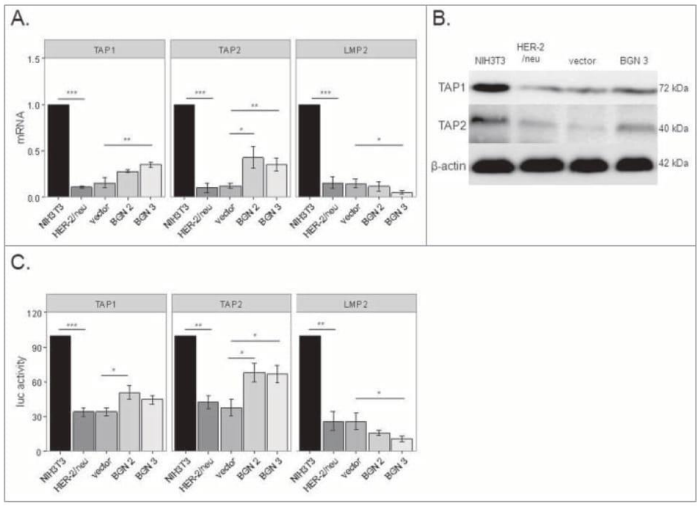

Figure 2. Transcriptional upregulation of MHC class I APM components by BGN overexpression in BGN$^{low/neg}$ HER-2/neu^{+} cells. A. Relative mRNA expression levels of selected APM components in BGN$^{low/neg}$ and BGN^{high} HER-2/neu^{+} cells. The mRNA expression levels of APM components in BGN$^{low/neg}$ vs. BGN^{high} HER-2/neu^{+} cells were analysed by qPCR as described in Materials and Methods. The results represent the mean of 3 independent experiments. B. Western blot analysis. Protein expression was determined as described in Materials and Methods. A representative Western blot staining is presented. C. Transcriptional upregulation of APM components by BGN overexpression. The activity of selected APM promoters was determined in BGN$^{low/neg}$ HER-2/neu^{+} cells, mock transfectants and two independent BGN transfectants (clones 2 and 3) as described in Materials and Methods. APM promoters were transiently co-transfected with the β-gal vector using lipofectamine in the respective cells 48 h prior to determination of the luc activity. The data were normalized to β-gal activity. The experiments were performed at least 3 times. Error bars in all panels indicate standard error. rel luc activity, relative luc activity.

overexpression influences DCN expression. As shown in Fig. 6A, BGN^{high} HER-2/neu^{+} cells expressed higher levels of DCN at mRNA and protein levels than BGN^{low} HER-2/neu^{+} cells (Fig. 6A) suggesting a link between BGN and DCN expression in these cells. The BGN-induced DCN expression was also found in *in vivo* in BGN^{high} HER-2/neu^{+} tumor lesions (Fig. 6C) and in the human melanoma model systems (Fig. 6B). Analogous to recombinant BGN, treatment of BGN$^{low/neg}$ HER-2/neu^{+} cells with recombinant DCN enhanced MHC class I surface antigens (Fig. 6D, S1A).

Correlation of BGN and DCN expression with clinical parameters

In order to determine the clinical relevance of ERBB2, BGN, DCN, HLA-B, HLA-C, CD3, CD8, CD25, TGF-β1 and Foxp3

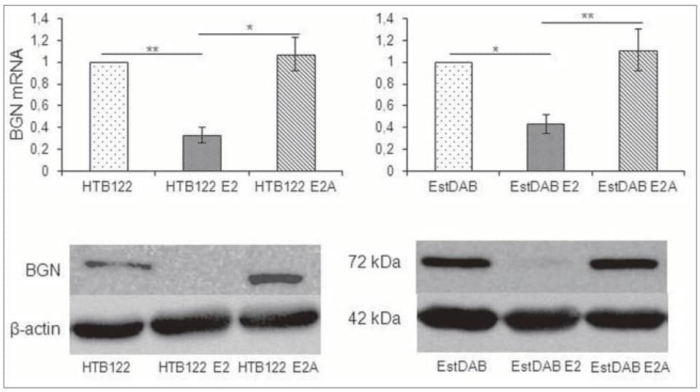

Figure 3. Downregulation of BGN expression in HER-2/neu overexpressing human tumor cells. A. Effect of HER-2/neu overexpression on BGN transcription in melanoma cells. Wt and mut HER-2neu-overexpressiong melanoma cells were subjected to qPCR as described in Materials and Methods. The qPCR data are expressed in bar charts relative to parental cells (set 1) and represent the mean $\pm$ SE format from 3 independent experiments. B. Effect of HER-2/neu overexpression on BGN protein expression in melanoma cells. For Western blot analysis 50 μg protein/cell line was separated by 10% SDS-PAGE and transferred onto a nitrocellulose membrane, before immunostaining with a BGN specific mAb was performed as described in Materials and Methods. Staining with a mAb directed against β-actin served as control.

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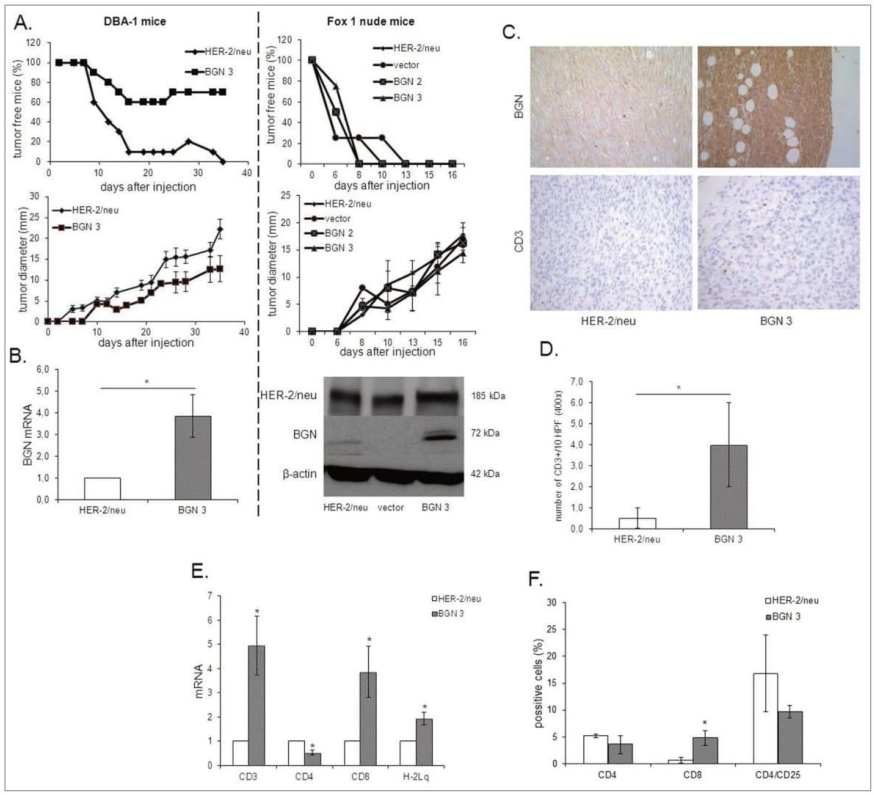

Figure 4. Reduced tumor growth of BGN transfectants *in vivo* in immune competent mice. A. Frequency and diameter of tumors 1 × 10^6 $BGN^{low/neg}$ and BGN^{high} HER-2/neu$^+$ cells/mouse were subcutaneously injected as described in Material and Methods. Tumor growth was monitored overtime regarding frequency and diameter of the tumors. B. mRNA and/or protein expression of HER-2/neu and BGN in $BGN^{low/neg}$ vs. BGN^{high} HER-2/neu$^+$ tumors. The mRNA and/or protein expression of HER-2/neu and BGN was determined in the$BGN^{low/neg}$ and BGN^{high} tumors as described in Materials and Methods using qPCRand Western blot analysis. C. Immunohistochemical staining of BGN and CD3 in $BGN^{low/neg}$ and BGN^{high} HER-2/neu$^+$ cells. IHC of tumors was performed as described in Materials and Methods using anti- BGN, anti-HER-2/neu and anti-CD3 specific mAbs, respectively. D. Analysis of the number of CD3$^+$ immune cells. The number of CD3$^+$ immune cells was determined upon staining with an anti-CD3 mAb at 400 x magnification (HPF) considering preferentially areas with higher intra-tumoral immune cell density. E. Determination of the mRNA expression of immune markers and MHC class I expression in $BGN^{low/neg}$ and BGN^{high} HER-2/neu$^+$ cells. MRNA expression of the different immune cell markers CD3, CD4, CD8, IL-2, FoxP3 and the MHC class I HC was determined by qPCR as described in Materials and Methods. The expression levels of these markers were compared in $BGN^{low/neg}$ vs. BGN^{high} HER-2/neu$^+$ tumor lesions. F. Immune marker expression in peripheral blood of tumor bearing mice. The expression of levels of percentages of CD4, CD8 and CD4/CD25 were determined after injection of BGN^{low} vs. BGN^{high} HER-2/neu$^+$ tumor cells in the peripheral blood of mice using flow cytometry as described in Materials and Methods.

expression, *in silico* analysis of TCGA data from BC patients was performed by correlating the mRNA expression levels with the clinical outcome of BC patients. As shown in Figure S5, high BGN (nearly significant; p 0.061) and DCN (significant p 0.008) expression levels along with nearly significant expression levels of HLA-B, HLA-C, CD3 and CD8 had prognostic value and were considerably associated with an increased progression free survival (PFS) of patients. This was not the case for ERBB2, TGF-β1 and Foxp3, where their lower expression levels might support a higher patient's survival.

Discussion

HER-2/neu amplification and/or overexpression has been shown to be associated with altered growth properties and a reduced immunogenicity of tumors, which might be at least partially mediated by a HER-2/neu-induced downregulation of MHC class I surface expression due to transcriptional suppression of major APM components leading to escape from immune surveillance.[39, 40] Furthermore, the tumor-induced modification of the tumor microenvironment (TME) is accompanied by a reduced activation, migration and cytotoxic activity of T cells, while the frequency of immune suppressive cells, e.g. Treg, M2 macrophages and myeloid suppressor cells, is increased.[41] Thus, there is an urgent need to recover the immune escape phenotype of tumors to enhance the efficacy of T cell-based immunotherapies.[42]

The SLRP BGN has a broad range of functions. It links soluble matrix with innate immune responses via TLR 2 and 4 thereby inducing "danger" signals[14, 43]. Furthermore, BGN has

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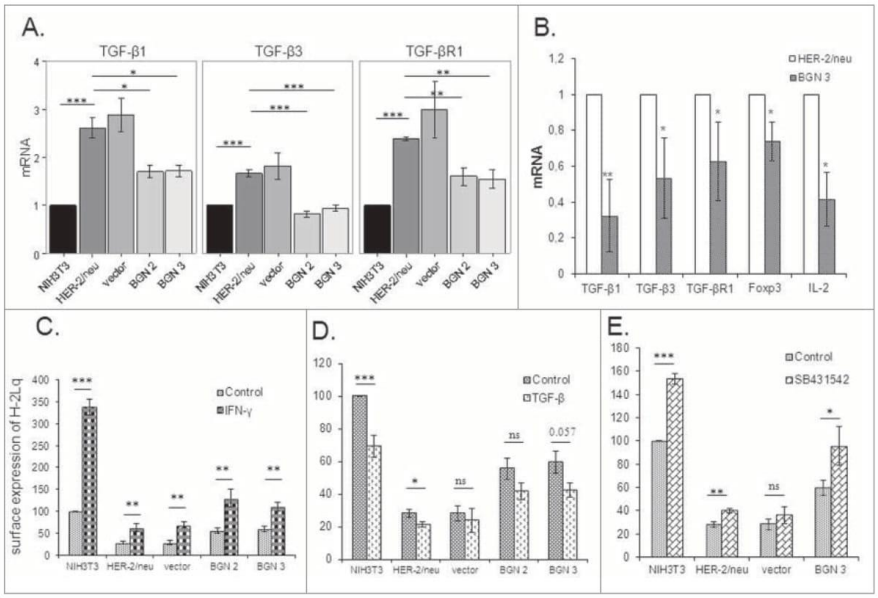

Figure 5. Changes of the TGF-β pathway and MHC class I surface antigens by treatment with TGF-β and IFN-γ. A. mRNA expression of TGF-β isoforms 1 (TGF-β1 and 3) and the TGF-βR1 in BGN$^{low/neg}$ and BGN^{high} HER-2/neu^{+} cells. The expression of components of the TGF-β pathway was analysed by qPCR as determined in Materials and Methods. The results represent the mean of three independent experiments and expressed relative to NIH3T3 cells (set 1). B. Analysis of TGF-β and Treg in BGN^{low} vs. BGN^{high} HER-2/neu^{+} cells. The expression levels of TGF-β isoforms (TGF-β1 and 3) and receptor (TGF-βR1), were analysed in BGN^{high} HER-2/neu^{+} tumor lesions *in vivo* using qPCR. C. Enhanced MHC class I surface expression upon IFN-γ treatment. Untreated and 20ng/ml IFN-γ-treated cells were subjected to flow cytometry as described in Materials and Methods. MFI was determined and expressed relative to NIH3T3 cells (100%). D. Effects of TGF-β on MHC class I surface expression. Untreated and 40ng/ml TGF-β-treated cells were subjected to flow cytometry as described in Materials and Methods. E. Influence of the TGF-β inhibitor on MHC class I surface expression. NIH3T3, BGN$^{low/neg}$ and BGN^{high} HER-2/neu^{+} cells were either left untreated or treated with 20 ng/ml TGF-β inhibitor (SB431542), before MHC class I surface expression was determined by flow cytometry. MFI of MHC class I surface expression of untreated and SB431542-treated BGN$^{low/neg}$ and BGN^{high} HER-2/neu^{+} cells was correlated to MFI of untreated NIH3T3 cells, which was set 100%. The experiments were at least performed three times and results represent the mean of these experiments.

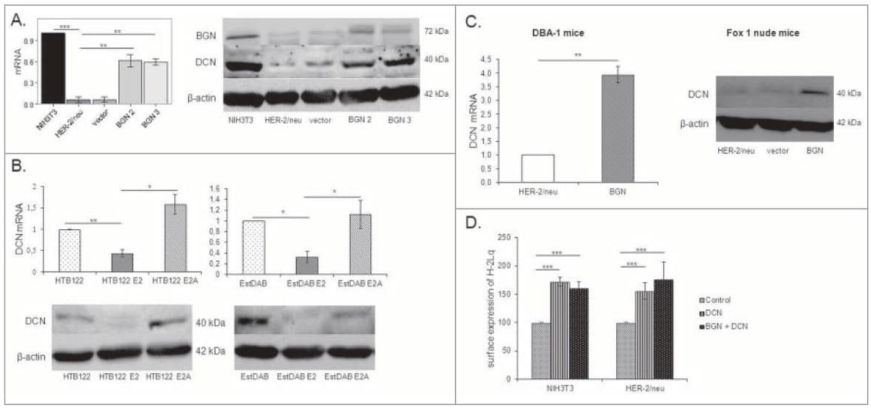

Figure 6. Induction of DCN expression in BGN transfectants. A. Determination of DCN expression in BGN^{high} HER-2/neu^{+} cells. Relative mRNA and protein expression of DCN were determined by qPCR and Western blot, respectively. DCN mRNA expression was analyzed in the NIH3T3 BGN$^{low/neg}$ and BGN^{high} HER-2/neu^{+} cells and transcription was correlated to NIH3T3 cells (set 1). For protein expression, 50 μg protein/cell line was separated by 10% SDS-Page, transferred onto a nitrocellulose membrane, before immunostaining was performed with an anti-DCN-specific mAb as described in Materials and Methods. Staining of the Western blot with an anti-β-actin-specific mAb served as loading control. B. Downregulation of DCN expression by HER-2/neu overexpression in human tumor cells. The mRNA and protein expression of DCN was determined in human HER-2/neu model systems as described in Materials and Methods. C. Enhanced expression of DCN in BGN^{high} HER-2/neu^{+} cells *in vivo*. DCN mRNA and protein expression was determined in BGN^{low} and BGN^{high} HER-2/neu^{+} tumors as described in Materials and Methods. D. Increased MHC class I surface expression in the presence of DCN and/or BGN. Cells were treated with recombinant DCN (1.5 μg/mL) alone or in combination with recombinant (1 μg/mL) BGN for 23h, before MHC class I expression of untreated DCN and DCN/BGN-treated cells was determined by flow cytometry using an anti-H-2L^{q} mAb. MFI of untreated NIH3T3 cells and BGN^{low} HER-2/neu^{+} cells was set 100%.

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been shown to promote angiogenesis via VEGF signaling[44] and tumorigenesis via the wnt/β-actin pathway.[11] In contrast, HER-2/neu-transformed cells with high angiogenic activity expressed decreased levels of BGN in a PKI/CREB-dependent manner.[28] In the present study, the lack of BGN expression in HER-2/neu transformants was associated with reduced MHC class I surface expression, which could be reverted by BGN overexpression or by the addition of exogenous BGN. *In vivo*, BGN overexpression in HER-2/neu$^+$ cells resulted in reduced tumorigenicity of these cells in immune competent mice when compared to BGN$^{low/neg}$ HER-2/neu$^+$ cells suggesting that BGN acts as a tumor suppressor and enhances immunogenicity. This might be explained by a stronger immune cell infiltration as shown by an increased frequency of CD3$^+$ cells and higher mRNA expression levels of CD3 and CD8 in BGN^{high} vs BGN$^{low/neg}$ HER-2/neu$^+$ tumors. Particularly the presence of T cells (CD3$^+$) and T cell subpopulations (CD8$^+$) are indicators for a better prognosis,[45] strongly suggesting that the antitumoral immune responses could be exploited as a therapeutic option. It is noteworthy that CD4 transcription was low in BGN^{high} HER-2/neu$^+$ cells. These data suggest that the reduced frequency and size of BGN^{high} HER-2/neu$^+$ tumors might be due to an increased immunogenicity of these cells accompanied by a strong infiltration of effector T cells when compared to BGN$^{low/neg}$ HER-2/neu$^+$ tumors. Since CD4 transcription was reduced in BGN^{high} HER-2/neu$^+$ tumors, one might speculate that BGN restoration downregulates the frequency of immune suppressive CD4$^+$ FoxP3$^+$ Treg. In addition, overexpression of BGN in HER-2/neu$^+$ cells was accompanied by an upregulation of DCN expression suggesting a link between BGN and DCN expression. Treatment of BGN$^{low/neg}$ HER-2/neu$^+$ cells with recombinant BGN or DCN resulted in an upregulation of MHC class I surface antigens due to increased expression of major APM components including TAP1 and TAP2 demonstrating that both SLRPs have an immune modulatory potential.

Neoplastic malignancies often overexpress TGF-β and its receptor.[46] In a murine neu-driven BC model, TGF-β can accelerate metastasis formation possibly through the synergistic activation of PI3K/AKTand Ras/MAPK pathways with neu-dependent signaling.[47] Furthermore, TGF-β signaling is activated in HER-2/neu-overexpressing BC cells,[48] which is accompanied by increased tumor cell motility and metastatic progression. The crosstalk between HER2 and TGF-β not only alters intracellular signaling in cancer cells, but also influences components of the TME through the induction of several pro-invasive growth factors. In BGN$^{low/neg}$ HER-2/neu$^+$ cells high transcript levels of TGF-β and of the TGF-β receptor were detected, while BGN expression in HER-2/neu$^+$ cells reduced their expression. This is in line with the regulation of BGN by the ALK5-Smad2/3 TGF-β1 signaling pathway[49] and its function as a TGF-β repressor.[50] Thus, BGN expression could be linked to changes in the TGF-β pathway known to negatively interfere with MHC class I surface expression[51] and antitumoral immune responses.[52] These data confirm the TGF-β-mediated escape from immune surveillance due to downregulation of MHC class I expression[53] and an induction of the epithelial mesenchymal transition[53] as demonstrated by increased SNAIL expression and activation of MMP9[54]. So far, there exist no data on the effect of BGN in this context. Interestingly, TGF-β inhibition induced MHC class I expression in BGN$^{low/neg}$ HER-2/neu$^+$ cells. Such data highlight the important the role of microenvironmental TGF-β signaling on escape of tumor cells from immune surveillance leading to progression.

On the other hand, DCN has also been shown to block TGF-β transcription and protein expression in glioblastoma cells, which was accompanied by a strong inhibition of tumor formation *in vivo*.[55] DCN-expressing glioblastoma showed an altered TME characterized by an increased frequency of infiltrating T and B cells. Furthermore, the DCN-induced inhibition of TGF-β was accompanied by significantly enhanced anti-glioblastoma immune responses *in* tumor necrosis factor-α-converting enzyme signaling.[55] These data are in line with our BGN^{high} HER-2/neu model demonstrating an increased DCN expression in these cells. Thus, a better understanding of the contextual networks of BGN and DCN in tumors is required to modulate immunogenicity by targeting the MHC class I surface expression.

TGF-β1 binds to its receptors (TGF-βRI and TGF-βR2) and induces the phosphorylation of the tumor necrosis factor-α-converting enzyme (TACE) (Fig. 7), which resulted in its translocation to the cell surface, where TACE induces integrins and cleaves the epidermal growth factor receptor (EGF-R) pro-ligands[56]. EGF-R ligands will initiate an autocrine and paracrine EGF-R signaling, which is amplified in HER-2/neu-overexpressing cells (BGN$^{low/neg}$ HER-2/neu$^+$ cells). In BGN^{high} HER-2/neu$^+$ cells, BGN and DCN bind to TGF-β1 and restrict HER-2/neu signaling, which might induce tumor suppression (Fig. 7). It can be suggested that TGF-β by signaling via the TGF-β receptor enhances the HER-2/neu-initiated signal transduction by increasing HER-2/neu ligand shedding, HER-2/neu-containing heterodimers, and their cross talk with integrins.[57] In our study, an enhanced expression of TGF-β1 and its receptor TGF-βR1 was found in BGN^{low} HER-2/neu, which could be reverted to normal levels by BGN overexpression. Both BGN and DCN regulate the TGF-β availability in BGN^{high} HER-2/neu cells. In BGN^{high} HER-2/neu$^+$ cells, the amount of BGN and DCN in the ECM increases and both proteoglycans bind to TGF-β and sequester it to the ECM. In this way both DCN and BGN translocate TGF-β from the membrane thereby reducing the binding to its receptor resulting in a decreased TGF-β

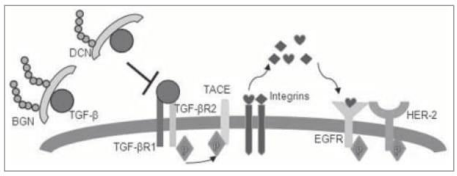

Figure 7. Schematic representation of BGN- and DCN-mediated inhibition of the TGF-β pathway and restriction of HER-2/neu signaling. TGF-β1 binds to its receptors (TGF-βRI and TGF-βR2) and induces phosphorylation of TACE, resulting in its translocation to the cell surface, where TACE induces integrins and cleaves EGFR pro-ligands. EGFR ligands will initiate autocrine and paracrine EGFR signaling, which is amplified in HER2-overexpressing cells (BGN$^{low/neg}$ HER-2/neu$^+$ cells). In BGN^{high} HER-2/neu$^+$ cells, BGN and DCN bind to TGF-β1 and restricts HER-2/neu signaling, thus allowing tumor suppression to occur.

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signaling and restricting HER-2/neu-mediated carcinogenic effects.

In cancer, contradicting data exist regarding the clinical significance of BGN expression. In some tumors an increased BGN expression was linked to poor prognosis,[58] whereas in others its overexpression was associated with inhibition of cancer cell growth and a good prognosis.[10,59] The expression of BGN was increased in liver, ovarian, endometrial, pancreatic and gastric cancer[60] suggesting an important role of BGN in the pathogenesis of these malignancies. In contrast, several other studies demonstrated an anti-tumoral activity of BGN associated with anti-proliferative capacity. Similar results were obtained in our HER-2/neu model system suggesting that BGN has tumor suppressive activity, which might be associated with an increased immunogenicity. *In silico* analysis of TCGA data from BC patients demonstrated a prognostic value of BGN and DCN, which is in line with our *in vitro* results. Based on these reports BGN displays very contradicting roles, which might depend on the cellular context. Thus further *in vitro* and *in vivo* studies are required to elucidate the precise underlying molecular mechanisms of BGN in the tumor development and progression.

Material and methods

Cell culture and treatment

Murine NIH3T3 fibroblasts were purchased from ATCC, while the HER-2/neu-overexpressing NIH3T3 cells (termed $BGN^{low/neg}$ HER-2/neu^{+}) were kindly provided by H. Bernhard (University Hospital of the Technical University, Munich, Germany) and have been described elsewhere.[32] BGN-overexpressing HER-2/neu^{+} cells (HER-2/neu^{+} BGN^{+} Clone 2 and HER-2/neu^{+} BGN^{+} Clone 3, termed BGN^{high} HER-2/neu^{+}) were cultured in Eagle's modified essential medium (EMEM; Lonza) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS), 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin (PAA). Human breast cancer cells, HTB122 transfected with wild type (wt) HER-2/neu (HTB122 E2) and mutant (mut) HER-2/neu (signal transduction deficient; HTB122 E2A) were employed and have been previously described.[35]

All experiments were carried out during the logarithmic growth phase of the cells. For IFN-γ stimulation, cells were treated for 24 to 48 h at 37°C with 20 – 40 ng/mL murine or human recombinant IFN-γ (Roche Diagnostics), respectively. In addition, cells were treated with 20 ng/mL human and 50 ng/mL murine TGF-β1 for 24 h, respectively, 1.0 μg/mL recombinant BGN (R&D Systems), 1.5 μg/mL recombinant DCN (R&D Systems) and 20 ng/mL TGF-β1 inhibitor (SB431542).

RNA extraction and real-time quantitative PCR

Total cellular RNA was isolated using the NucleoSpin RNA II kit (Macherey-Nagel). An equal amount of total RNA (2 μg) was reverse transcribed into cDNA using the Revert H Minus First Strand cDNA synthesis kit (Fermentas) and oligo(dT)18 primer according to manufacturer's instructions. Real-time PCR was performed as previously described[37] using gene-specific and control primers (Table S1). Comparative quantification of gene expression was performed as previously described.[61] The experiments were independently performed three times with two technical replicates.

Western blot analyses

For Western blot analysis 5×10^6 cells were harvested as previously described[26], proteins were solubilized according to Laemmli[62]. 50 μg protein/lane were separated in 10% SDS-PAGE gels[29], transferred onto nitrocellulose membranes (Schleicher & Schuell) and stained with Ponceau S as previously described.[61] Membranes were incubated over night at 4°C with primary monoclonal antibodies (mAb) directed against BGN (Proteintech), DCN (Sigma-Aldrich), TAP1 (Santa Cruz Biotechnology Inc.), TAP2 (kindly provided by K. Früh (Howard Johnsson, La Jolla, CA)), β-actin (Sigma-Aldrich) and GAPDH (Cell Signaling Technology) followed by incubation for 1h with horseradish peroxidase-linked secondary antibody and developed using the ECL method. Chemiluminescence signals were visualized with the Lumi-Light Western Blotting Substrate (Roche Diagnostics) and recorded with a LAS3000 system (Fuji). For quantification of the protein expression, the respective area of the signal was integrated using an AIDA image analyser (Raytest) and subsequently normalized to β-actin or GAPDH.

Flow cytometry

The mAbs used for flow cytometry were the phycoerythrin (PE)-labeled anti-H-$2L^{d/q}$ (Cedarlane Laboratories LTD) and the respective PE-labeled isotype mouse immunoglobulin (Beckman Coulter). Whereas, human cells were stained with a FITC-labeled HLA class I-specific mAb (Beckman Coulter) using a FITC-labeled lgG2a mAb (Beckman Coulter) as control. Flow cytometric analysis was performed as recently described.[26] Briefly, 5×10^5 cells were incubated with the appropriate amount of antibodies at 4°C for 30 minutes, before the stained cells were measured on a FACScan unit (Becton Dickinson) and subsequently analyzed with the CellQuest software (Becton Dickinson). The data are represented as mean specific fluorescence intensity (MFI) from three independent experiments.

Promoter assay

TAP1/LMP2, TAP2, and TPN promoter sequences were amplified from genomic DNA and then cloned into the pGl3 luciferase (luc) vector (Promega) as recently described.[32] For transient transfections, 1×10^5 cells were incubated overnight in 100 μL OptiMEM (Invitrogen), followed by transfection with 0.3 μg promoter construct and 0.016 μg β-galactosidase (β-gal) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Forty-eight hours after transfection, the luc activity was determined with the luc substrate (Promega) using a luminometer and normalized to the transfection efficiency determined by β-gal enzyme activity.[26]

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In vivo *tumor formation*

All animal experiments described were approved by the Regional Council of Halle (Germany). The animals were maintained in accordance with the Guides for the Care and Use of Laboratory Animals. Adult (2-3-month old, 20 ± 4g body weight, male and female), specific pathogen-free in-bred and immune competent DBA/10IaHsd mice (Harlan Laboratories) and immune deficient mice (Fox 1 nude) were used for analysis of tumor formation. These mice were randomly split into three groups, with 10 mice in groups I and II [group I: HER-2/neu$^+$ cells; group II: BGN-transfected HER-2/neu$^+$ cells (Bgn clone 3) and 5 mice in group III (mock-transfected HER-2/neu$^+$ cells), and 1 × 10^6 cells in 200 μL PBS/mouse were subcutaneously injected into the left lateral abdominal wall. The right lateral abdominal wall was used for sham injections with PBS. Tumor diameter was monitored three times a week by caliper measurements of the greatest longitudinal diameter.

Immunohistochemistry staining and analysis

For staining with anti-HER-2/neu, anti-BGN and anti-CD3 antibodies 5 μm tissue sections of the tumors were deparaffinized with xylol and transferred via alcohol into aqua dest (Elix 5 Filter System, Merck-Millipore). Antigen decloaking for mAb CD3 was performed by steaming the slides with a preheated T-EDTA buffer (ZUC029-500, 1:10 dissolved, Zytomed Systems) at pH 6.0 at 98°C for 30 minutes in an oven (Braun, type 3216). No antigen decloaking was required for staining with BGN and HER-2/neu mAbs. The slides were blocked for 7-10 minutes with 3% H_2O_2. Following a rinsing step and application of washing buffer (ZUC202-2500, 1:20 solution, Zytochem Plus HRP Kit / Plus Polymer System, Zytomed), the primary mAbs were added dropwise on the tissue area. For BGN the primary mAb 16409-1-AP (Proteintech) was incubated at 1:50 for 60 minutes at room temperature (RT). The HER-2/neu staining was performed as previously described.[63] For CD3 the primary mAb SP7 (RM-9107-S, Thermo-Fisher) was incubated at 1:200 for 60 minutes at RT. After vacuuming and washing off the primary Ab, the slides were incubated with a HRP-polymer secondary antibody (POLHRP-100, Zytochem Plus HRP Polymer System Mouse/Rabbit, Zytomed) for 15 (BGN, HER-2/neu) and for 30 minutes (CD3), respectively, at room temperature. After a washing step, the epitopes were visualized with DAB (10 minutes of DAB Substrate Kit, Zytomed), followed by a counterstain with hemalaun (Dr. K. Hollborn & Sons) for 30 seconds, then transferred into xylol and slip covered (Eukitt, ORSAtec). Negative controls were obtained by omitting the primary antibody. Microscopic analysis of the staining was independently performed by two pathologists (CW, DB). The staining intensity of BGN and HER-2/neu expression was scored as absent, weak, moderate or strong. The distribution of intra-tumoral CD3$^+$ T cells was scored as homogenous or non-homogenous, while their density was scored as number of CD3$^+$ cells per 10 high power fields (HPF, 400x).

Blood preparation and analysis

Between day 36 - 41 tumor bearing mice were anesthetised with 2.5% (v/v) isofluran and blood was collected by cardiac puncture into heparin containing tubes. Following lysis of erythrocytes in erythrocyte lysis buffer (c-c-pro GmbH, Germany), the cells were incubated with the rat anti-mouse CD16/32 (Beckman Coulter, Brea, CA, USA) to block non-specific antibody binding. Anti-CD4 PeCy7 (eBioscience/ThermoScientific), anti-CD8α FITC (Beckman Coulter) and anti-CD25 eFluor450 (eBioscience/ThermoScientific) were used. Before acquisition on a Navios flow cytometer (Beckman Coulter), the cells were stained with propidium iodide to exclude dead cells. Analysis was performed using the Kaluza software package (Beckman Coulter).

In silico *analysis*

The R2 web tool (http://r2.amc.nl) was used to predict the association of ERBB2, BGN, DCN, HLA-B, HLA-C, CD3, CD8, CD25, TGF-β1 and Foxp3 expression with survival of patients with breast cancer. R2 calculates the optimal cut-off in the expression level for each gene and are divided into two groups. The statistical differences in the gene expression values between the patient groups with 'High' and 'Low' mRNA expressions were evaluated by ANOVA tests implemented in the R2 web tool. The p-values were corrected for multiple testing according to the false discovery rate. All of the cut-off expression levels and their resulting groups are analyzed according to the patients' survival. The cut-off level is reported and was used to generate the Kaplan-Meier curves, which allowed to discriminate patients into 'good' and 'bad' prognosis cohorts[59]. Kaplan scan analysis was performed to estimate the overall survival according to the breast cancer microarray dataset called 'Mixed Tumor Breast' that included 104 breast cancer and 17 normal breast biopsies with different clinical characteristics.

Statistical analysis

Microsoft Excel version 2010, R (programming language), GenStat 15th Edition were used for student's t-test and one-way ANOVA. A p-value of < 0.05 was considered as significant result (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

Disclose of interest

The authors have no conflict of interest.

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5.2. Publication - Two

2. **Subbarayan K,** Massa C, Lazaridou MF, Ulagappan K, Seliger B. Identification of a novel miR-21-3p/TGF-β signaling-driven immune escape via the MHC class I/biglycan axis in tumor cells. Clinical and translational medicine. 2021 Mar;11(3). Available from: https://pubmed.ncbi.nlm.nih.gov/33783999/

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CLINICAL AND TRANSLATIONAL MEDICINE

WILEY

LETTER TO EDITOR

Identification of a novel miR-21-3p/TGF-β signaling-driven immune escape via the MHC class I/biglycan axis in tumor cells

Dear Editor,

For the first time, a miR-21-3p-mediated downregulation of MHC class I surface antigens was shown in different model systems, which is linked to the expression of the extracellular matrix (ECM) constituent biglycan (BGN) and transforming growth factor (TGF)-β signaling in HER-2/neu-positive (HER-2/neu$^+$) cells. HER-2/neu transformation induces the expression of miR-21-3p, which interferes with the expression of immune-modulatory molecules, thereby accelerating immune suppression and reducing tumor immunogenicity.

The oncogenic miR-21 is overexpressed in many cancers associated with altered growth characteristics leading to tumor progression and reduced patients' survival.[1,2] Recently, we have shown that HER-2/neu transformation resulted in a downregulation of BGN, causing a reduced MHC class I surface expression.[3] However, a relationship between miR-21-3p overexpression, enhanced TGF-β signaling, impaired BGN, and MHC class I expression in HER-2/neu$^+$ cells has not yet been investigated.

Using murine in vitro models of HER-2/neu transformation and human tumor cells with a distinct HER-2/neu status, the role of miR-21 in the HER-2/neu-mediated downregulation of the MHC class I surface antigens, which is accompanied by a reduced BGN expression and increased TGF-β signaling, was analyzed. The expression of MHC class I antigen processing machinery (APM) and TGF-β pathway components, BGN and miR-21-3p, were determined by qPCR using primers listed in Table S1, Western blot and surface expression by flow cytometry. MiR-21-3p targets were identified and validated by binding miR-21-3p to the respective 3′ untranslated region (UTR). The function of miR-21-3p was assessed upon its overexpression and inhibition. CD107a degranulation assay was used to determine the NK cell activity upon co-culture of miR-21-3p overexpressing cells with NK cells.

High miR-21-3p expression levels in both murine and human cells were accompanied by an increased proliferation (Figure S1). BGN^{low} HER-2/neu$^+$ cells constitutively expressed high levels of miR-21-3p, while HER-2/neu$^-$ NIH3T3 cells exhibited low miR-21-3p expression levels (Figure 1A). BGN overexpression in HER-2/neu$^+$ (BGN^{high}/ HER-2/neu$^+$) cells decreased miR-21-3p expression (Figure 1A), while the siRNA-mediated BGN downregulation in NIH3T3 cells increased miR-21-3p levels (Figure 1B). Similar results were obtained in vivo demonstrating that miR-21-3p expression was higher in BGN$^{low/neg}$ compared to BGN^{high} HER-2/neu$^+$ tumors[3] (Figure 1C). The inverse correlation of the expression of miRNA-21-3p and HER-2/neu to BGN was confirmed in HTB122 cells expressing functional (E2) or signaling defective (E2A) HER-2/neu (Figure 1D), but also upon miR-21-3p overexpression in NIH3T3 cells (Figure 1E) as well as in the breast cancer (BC) cell line MCF-7 (Figure 1F), which could be reverted by a miR-21-3p inhibitor leading to a statistically significant twofold upregulation of BGN expression.

BGN is modulated by and can modulate the TGF-β pathway by regulating the expression/activity of SMAD family members.[3] TGF-β treatment of the murine in vitro models and human BC cell lines caused a significantly enhanced miR-21-3p expression in BGN^{low} HER-2/neu$^+$ cells, a heterogeneous increase in BC cell lines, but only a marginal upregulation in BGN^{high} HER-2/neu$^-$ cells (Figure S2). SMAD2 expression is downregulated in BGN^{low} HER-2/neu$^+$ cells, but significantly enhanced in BGN-transfected HER-2/neu$^+$ cells (Figure 2A). Vice versa, SMAD2 transfectants of HER-2/neu$^+$ cells caused an increased BGN mRNA expression (Figure 2B), while miR-21-3p overexpression leads to a downregulation of SMAD2 (Figure 2C and D). This mechanism was due to the binding of miR-21-3p to the 3′ UTR of SMAD2 as demonstrated by in silico analysis,[4] a high binding energy of

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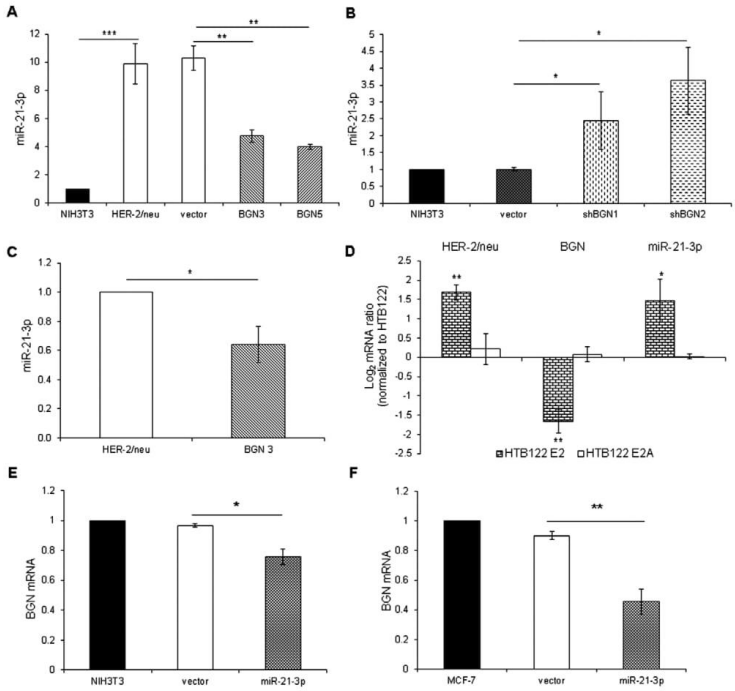

FIGURE 1 BGN-mediated reduction of miR-21-3p expression in HER-2/neu$^{\pm}$ cells. (A) Reduced expression of miR-21-3p by BGN overexpression. The constitutive expression of miR-21-3p was determined in HER-2/neu^{-} BGN^{high} NIH3T3 cells, BGN^{low} HER-2/neu^{+} cells, HER-2/neu^{+} vector controls as well as two BGN^{high} HER-2/neu^{+} transfectants by qPCR. The results are presented as x-fold induction of miR-21-3p expression in transfectants compared to parental NIH3T3 cells (set 1). (B) Effect of sh-mediated BGN silencing on miR-21-3p expression in NIH3T3 cells. The BGN expression in NIH3T3 cells was silenced by shRNA and miR-21-3p expression was determined by qPCR in shBGN transfectants, vector control, and parental cells. The results are expressed as x-fold induction of miR-21-3p expression in transfectants compared to parental NIH3T3 cells (set 1). (C) Basal miR-21-3p expression in BGN^{low} HER-2/neu^{+} and BGN^{high} HER-2/neu^{+} tumor cells. The miR-21-3p expression in BGN^{low} HER-2/neu^{+} and BGN^{high} HER-2/neu^{+} tumor lesions was determined by qPCR analysis and data were presented as x-fold downregulation of miR-21-3p expression. (D) Required HER-2/neu signaling for miR-21-3p upregulation. The expression of HER-2/neu, BGN, and miR-21-3p was determined in HTB122 cells, HTB122 E2, and HTB122 E2A transfectants by qPCR. Data are expressed as log mRNA ratio normalized to parental HTB122 cells. (E) miR-21-3p-mediated effects on BGN expression in NIH3T3 cells. NIH3T3 cells were transfected with the empty vector and miR-21-3p, before miR-21-3p and BGN expression was determined by qPCR miR-21-3p transfection resulted in an x-fold upregulation of miR-21-3p in NIH3T3 cells (set to 1). (F) miR-21-3p-mediated effects on BGN expression in BC cells. MCF7 cells were transfected with miR-21-3p and vector control, respectively, before miR-21-3p and BGN expression was determined by qPCR. Transfection resulted in a 3.07-fold upregulation of miR-21-3p. Data were normalized to parental MCF7 cells (set to 1)

miR-21-3p to the SMAD2 3′ UTR (Figure 2E) and luciferase (luc) reporter assays (Figure 2F). Next to the effect of miR-21-3p on SMAD2 expression in the murine model system, miR-21-3p overexpression in the human MCF-7 cell line downregulated TAP1 expression (Figure 2G), which supported our recent in vitro experiments in melanoma,[5] the in silico data of miR-21-3p binding to the 3′ UTR of human TAP1 (Figure 2H), and the luc reporter assays demonstrating a binding of miR-21-3p to the wild-type TAP1 3′ UTR (Figure 2I).

Flow cytometric analysis revealed lower MHC class I surface levels in miR-21-3p transfected BGN^{high} HER-2/neu^{-} NIH3T3 cells compared to control transfectants (Figure 3A), while MHC class I surface expression was enhanced in NIH3T3 cells upon transfection with a miR-21-3p inhibitor (Figure 3B). Similar results were obtained with human miR-21-3p-transfected MCF-7 (Figure 3C) and MDA-MB-231 (Figure S3A) cells demonstrating downregulated HLA class I surface expression, which was reverted a miR-21 inhibitor (Figure 3D; Figure S3B). In this

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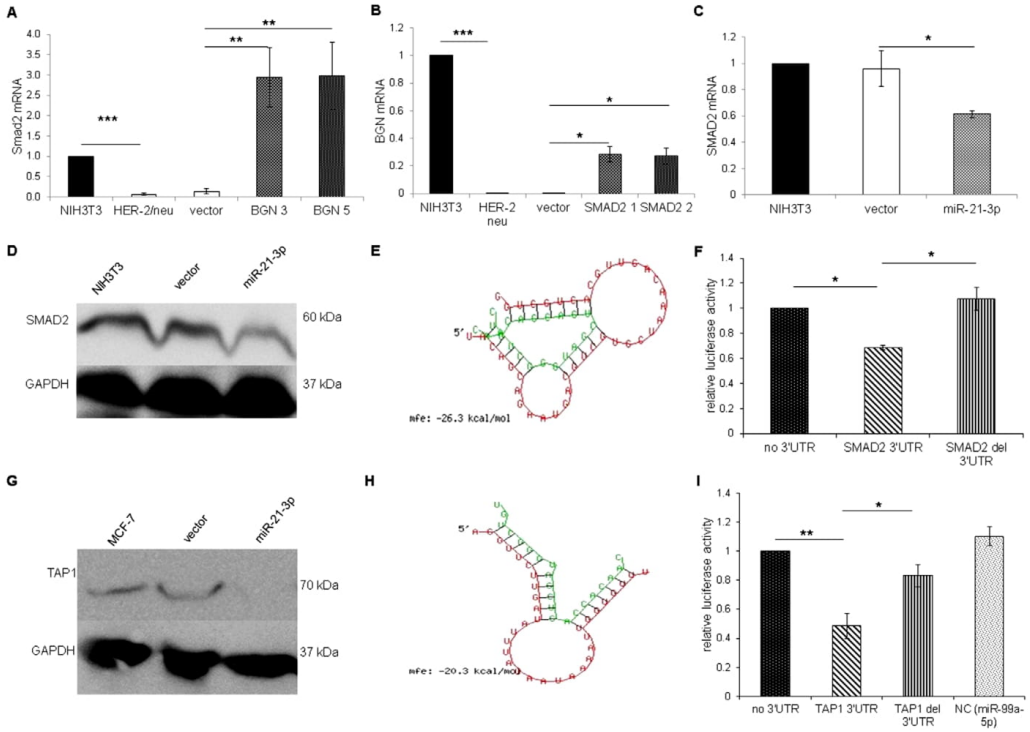

FIGURE 2 Mouse SMAD2 and human TAP1 as targets of miR-21-3p. (A) Correlation of SMAD2 and BGN expression in the murine model system. SMAD2 mRNA expression was determined in HER-2/neu$^-$ cells, BGN^{low} HER-2/neu$^+$ cells and vector controls BGN^{high} HER-2/neu$^+$ using qPCR. Data were normalized to parental NIH3T3 cells (set 1). (B) Restoration of BGN expression in SMAD2 overexpressing HER-2/neu$^+$ cells. BGN expression was analyzed in SMAD2 overexpressing HER-2/neu$^+$ cells by qPCR. Data were normalized to parental NIH3T3 cells set to "1." (C) Link of SMAD2 with miR-21-3p expression. MiR-21-3p was transfected into NIH3T3 cells and SMAD2 mRNA expression was determined in miR-21-3p transfectants by qPCR. Data were normalized to parental NIH3T3 cells (set 1). (D) Effect of miR-21-3p on SMAD2 protein expression SMAD2 protein expression was determined by Western blot analysis of NIH3T3 cells and miR-21-3p transfectants using a SMAD2-specific antibody. An anti-GAPDH antibody served as loading control. (E) In silico analysis of SMAD2 as a target of miR-21-3p. RNA hybrid was used as a tool to determine the binding energy. The high binding energy using RNA hybrid suggested that the 3′ UTR of SMAD2 targets miR-21-3p. (F) Direct binding of miR-21-3p to the SMAD2 3′ UTR. Luciferase reporter assays were independently performed three times using the wt and del 3′ UTR of SMAD2 and data are expressed as luc activities normalized to constructs lacking the 3′ UTR. (G) Reduced TAP1 protein expression upon miR-21-3p overexpression. MCF-7 cells were transfected with vector or miR-21-3p, before TAP1 protein expression was determined by Western blot. Staining with an anti-GAPDH antibody served as control. (H) *In silico* analysis of the 3′ UTR of TAP1. *In silico* analysis using various algorithms postulated that miR-21-3p targets the 3′ UTR of human TAP1. RNA hybrid was used as a tool to determine the binding energy of miR-21-3p to the 3′ UTR of TAP1. (I) Direct binding of miR-21-3p to the 3′UTR of TAP1. Luc activities were determined in a luc reporter assay using wt TAP1 3′ UTR, del TAP1 3′ UTR and a nonsense control. The data are expressed as the mean of luc activity normalized to MCF-7

context, it is noteworthy that SMAD2 overexpression caused an upregulation of H2-Lq (Figure S4).

Since the impaired TAP1 expression reduced MHC class I surface expression,[6] the influence of the miR-21-mediated MHC class I downregulation on NK cell responses was determined using the CD107a degranulation assay. As expected, the impaired MHC class I expression of miR-21-3p transfectants enhanced NK cell recognition of MCF-7 (Figure 3E) and MDA-MB-231 cells (Figure 3F).

Using The Cancer Genome Atlas (TCGA) data of invasive BC, a link between miR-21-3p, HLA class I, and HER-2/neu expression to clinical parameters was detected. High

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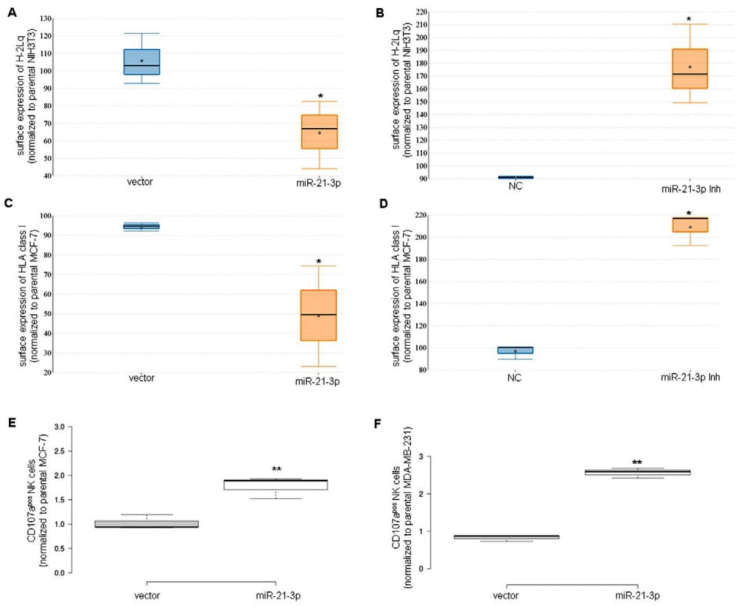

FIGURE 3 miR-21-mediated downregulation of MHC class I antigens and functional relevance. Both murine of NIH3T3 cells and BC cells transfected with miR-21-3p or a miR-21-3p inhibitor, respectively, were monitored for the expression of MHC class I surface antigens using flow cytometry with MHC class I-specific antibodies. The results are presented as MFI and normalized to parental cells set to 100. (A) Downregulation of MHC class I surface expression by a miR-21-3p transfection of NIH3T3 cells. (B) Upregulation of MHC class I surface expression by a miR-21-3p inhibitor transfection of NIH3T3 cells. (C) Reduced HLA class I surface expression in miR-21-3p transfected MCF-7 cells. (D) Reversion of HLA class I surface expression by miR-21-3p inhibitor transfection in MCF-7 cells. (E and F) Functional relevance of miR-21-3p-mediated downregulation of MHC class I. Degranulation assays were performed using NK cells isolated from PBMC obtained from four different donors. Shown are the mean ± standard error of the fold increase in CD107a+ NK cells upon normalization to parental cells, MCF7 (E) and MDA-MB-231 (F)

miR-21 expression was found in primary BC compared to the normal mammary epithelium (Figure 4A), which correlated to high HER-2/neu, but low HLA class I expression levels as representatively shown for HLA-B (Figure 4B) and was accompanied by a worse BC patients' outcome (Figure 4C).

In summary, the murine miR-21-3p enhances the TGF-β signaling by binding to the 3′UTR of SMAD2, resulting in a reduced MHC class I surface expression, while hsa-miR-21-3p binds to the human TAP1 3′UTR thereby restricting the peptide transport and loading of MHC class I antigens (Figure S5A). The BGN-mediated overexpression in BGN$^{low/neg}$ HER-2/neu^{+} cells induced MHC class I expression and reduced miR-21-3p (Figure S5B), underlining its critical role on the MHC class I-mediated tumor immune escape. The crosstalk between HER-2/neu and miR-21-3p alters the intracellular signaling in cancer cells by promoting cell proliferation, enhancing pro-invasive growth factors, like TGF-β and inhibiting immune stimulatory molecules or ECM components, like BGN.

These data postulate the pharmacological targeting of the miRNA-proteoglycan-MHC class I axis as a novel, innovative therapeutic concept for HER-2/neu^{+} cancers.

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CONFLICTS OF INTEREST

All authors declare that there is disclose competing interest.

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DATA AVAILABILITY STATEMENT

The authors will make the data and material used available upon request.

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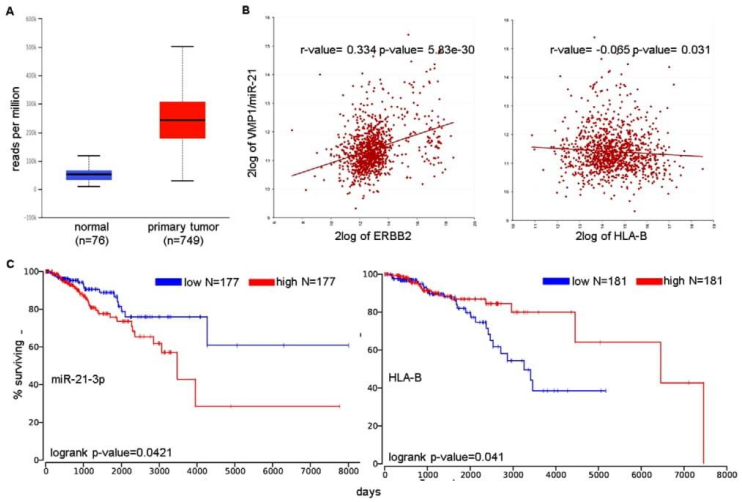

FIGURE 4 TCGA data analysis of breast cancer samples. (A) miR-21 expression in normal ($n = 76$) and BC samples ($m = 749$). Box-whisker plot showing the miR-21 expression in BC dataset was detected by the UALCAN database. (B) Correlation analysis of the TCGA invasive breast carcinoma dataset (1097 patients) using R2 Genomics was performed between miR-21 and HER-2/neu (ERBB2) and HLA-I genes (representatively shown for HLA-B). MiR-21 correlated positively with ERBB2 and negatively with HLA class I antigens. (C) Kaplan Meyer curve demonstrated a higher survival of HLA^{high} and miR-21-3p groups as described. The assessment of clinical relevance was performed in a patient survival analysis using OncoLnc database (http://www.oncolnc.org/). While HLA^{high} (HLA-B) indicated the higher survival, miR-21-$3p^{high}$ displayed lower survival for TCGA Breast Invasive carcinoma dataset followed for 20 years

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Subbarayan K, Massa C, Lazaridou MF, Ulagappan K, Seliger B. Identification of a novel miR-21-3p/TGF-β signaling-driven immune escape via the MHC class I/biglycan axis in tumor cells. Clinical and translational medicine. 2021 Mar;11(3). Available from: https://pubmed.ncbi.nlm.nih.gov/33783999/

5.3. Publication - Three

3. **Subbarayan K,** Seliger B. Tumor-dependent effects of proteoglycans and various glycosaminoglycan synthesizing enzymes and sulfotransferases on patients' outcome. Current cancer drug targets. 2019 Mar 1;19(3):210-21. Available from: https://pubmed.ncbi.nlm.nih.gov/29984655/

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Current Cancer Drug Targets, 2019, *19*, 210-221

RESEARCH ARTICLE

Tumor-dependent Effects of Proteoglycans and Various Glycosaminoglycan Synthesizing Enzymes and Sulfotransferases on Patients' Outcome

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Abstract: ***Background*:** The small leucine-rich proteoglycans (SLRPs) biglycan (BGN) and decorin (DCN) linked with sulfated glycosaminoglycan (GAG) chains exhibit oncogenic or tumor suppressive potentials depending on the cellular context and association with GAGs.

***Objective*:** We hypothesized that structural alterations and expression levels of BGN, DCN and their associated chondroitin sulfate (CS) polymerizing enzymes, dermatan sulfate (DS) epimerases and various sulfatases might be correlated with the tumor (sub)type and patients' survival.

***Methods*:** We acquired breast cancer (BC) and glioma patients' datasets from cBioPortal and R2 Genomics. Structural alterations and the expression pattern of CS polymerizing enzymes, DS epimerases and carbohydrate sulfotransferases (CHST) were compared to that of BGN and DCN and correlated to their clinical relevance.

***Results*:** In BC, no mutations, but amplifications (0.2 – 2.1 %) and deletions (0.05 – 0.4 %) were found in BGN, DCN and CS/DS enzymes. In contrast, missense and/or truncated mutations (0.1 – 0.5 %), but a reduced amplification rate (0 – 1.5 %) were found in glioma. When compared to BC, the structural abnormalities caused altered mRNA expression levels of BGN, DCN, GAG synthesizing enzymes and CHST. Mutations in SLPRs, CHSY1, CHST4 and CHSY3 were correlated with a poor prognosis in glioma, while lack of mutations and copy number variations in the SLRPs, CHSY3, CHST15 and DSE displayed an increased survival in BC.

***Conclusion*:** A distinct association of BGN and DCN with CHST, CS polymerizing enzymes and DS epimerases was found in BC and glioma. Thus, a unique pattern of structural alterations and expression, which has clinical relevance, was found for PGs and GAG synthesizing enzymes and CHST in BC and glioma, which might help to identify high-risk patients and to develop personalized therapeutics.

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1. INTRODUCTION

Cancer development and progression is associated with genetic alterations. Although many genomic events with direct phenotypic impact have been identified [1], the role of well-known glycosylation-associated genes, like proteoglycans (PGs) and glycosaminoglycan (GAG) chain modification enzymes has not yet been studied in detail in cancer. Due to differences in the type of monosaccharides in the repeating unit as well as in their sulfation, GAGs were categorized into hyaluronan (HA), chondroitin sulfate (CS) and dermatan sulfate (DS), heparan sulfate (HS) and keratan sulfate (KS) [2]. The small leucine-rich PGs biglycan (BGN) and decorin (DCN) are present in the extracellular matrix (ECM), on the cell surface and in the cytoplasm and represent the best-studied class of matrix molecules. BGN consists of a core protein and two CS chains; DCN has a core protein with a > 65 % homology to BGN and a single CS/DS chain. BGN and DCN expression and localization are substantially divergent and under some conditions mutually exclusive [3]. Due to the presence of sulfate groups at various positions along GAG chains and the resulting high density of negative charges, PGs are able to interact electrostatically with numerous soluble ligands and matrix proteins involved in cell adhesion [4]. Concerning their function, alterations in the synthesis of PGs can stimulate tumorigenic growth by decreasing the adhesion of transformed cells to the ECM [5], although tumor formation is also dependent on the biosynthesis of PGs [6].

Recently, controversial functional roles of BGN and DCN were described in cancers, which might depend on the microenvironmental context [7, 8]. BGN and DCN bind to the transforming growth factor (TGF)-β1 [9], restrict signaling via the HER-2/neu pathway and induce tumor suppression [10]. Based on the tumor suppressive potential, DCN was suggested as a "guardian of the matrix" [11]. For example, in breast cancer, DCN inhibitory roles were observed [10] and its lower expression was associated with a worse patients' prognosis [12]. In contrast, in pancreatic cancer, an

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increased DCN expression was found [13]. This is referred to as the PG paradox since an incredible versatility of BGN/DCN structures and functions in cancers were reported.

Although few reports are available on PG-GAG associations in the tumor microenvironment (TME) [14, 15], information regarding CS/DS chain modulating enzymes, structural alterations, expression pattern or post-translational modifications of BGN and DCN are not available. It is hypothesized that genetic alterations and/or impaired expression levels of BGN and DCN, as well as an altered association of these SLRPs with different GAG chain modulating enzymes, might affect neoplastic transformation and thus has clinical relevance. Therefore, the aims of this study were to analyze (i) the structural abnormalities and expression status of BGN and DCN and (ii) CS/DS-modifying enzymes in different cancer types using 'The Cancer Genome Atlas' (TCGA) data with focus on BC and glioma, (iii) to determine the neighboring genes of BGN and DCN using network analysis and (iv) to correlate these data with clinicopathological parameters of the selected tumor entities, such as tumor grading, staging, disease progression and poor patients' outcome. To address the above issues, we assembled different TCGA datasets of BC and glioma from cBioPortal and R2 Genomics. These analyses demonstrated a distinct frequency of structural alterations including amplifications, mutations and deletions in BC and glioma. Network analysis showed that the BGN/DCN and CS/DS modification enzymes were neighboring in BC and glioma, while co-occurrence of BGN/DCN was encountered with different genes. Interestingly, the co-occurrence of mutations was only found in glioma, in which higher expression levels of BGN/DCN and their associated enzymes correlated with a poor patients' outcome. These data further suggest that both SLRPs alone or in combination with their various GAG synthesizing enzymes and sulfotransferases represent biomarkers for diagnosis and prognosis of tumors and might be used also as therapeutic targets.

2. MATERIALS AND METHODS

2.1. Cancer Datasets and OncoPrint Analysis

The breast cancer (Dataset name: 'Breast Cancer (METABRIC, Nature 2012 & Nat Commun 2016)' [16, 17] and glioma (Dataset name: 'Merged Cohort of LGG and GBM (TCGA, Cell 2016)' [18]) datasets were investigated. These data sets, from August 31, 2017, contain the genomic sequencing data encompassing gene mutations, deletions, copy number alterations (CNA) and loss of heterozygosity (LOH) [19, 20]. The BC and glioma cohorts consist of 2051 and 794 cases, respectively. All TCGA data were assessed and analyzed using cBioPortal (http://www. cbioportal.org/).

The OncoPrint algorithm in cBioPortal database was used for analysis of genetic alterations such as amplification, deep deletions and mutations. A concise and compact graphical summary was generated for genomic alterations in multiple genes across a set of tumor samples in BC and glioma. All DNA mutations are standardized to the canonical RefSeq isoform (using Oncotator, http://www.broadinstitute.org/oncotator/).

The DNA copy number and mutation data were downloaded from cBioPortal in which the corresponding TCGA data were analyzed. "–2" in copy number indicates "deep deletion", "–1" indicates "shallow deletion", and "0" indicates "diploid" [21].

2.2. Network Analysis

The Network section in cBioPortal was used for interactive analysis and visualization of networks that are altered to specific cancer types. The network consists of pathways and interactions from the Human Reference Protein Database (HPRD) [22], Reactome [23], National Cancer Institute (NCI)–Nature [24], and the Memorial Sloan-Kettering Cancer Center (MSKCC) Cancer Cell Map (http://cancer. cellmap.org) as derived from the open source Pathway Commons Project [25].

2.3. Correlation Analysis

In mutually exclusive studies, gene-related events associated with particular cancer; one genetic event is often mutually exclusive in one tumor sample, while in co-occurrence multiple genes are altered in the same cancer sample [20]. This strategy was used to gather information about the different gene signaling in BC and glioma. To explore patterns of co-alterations and mutual exclusivity, pairwise associations between somatic events were analyzed using Fisher's exact test. The changes were quantified concerning the presence or absence of alterations in gene A in correlation to the presence or absence of alterations in gene B in the selected tumors using Fisher's exact test and p-values were calculated between genes.

2.4. Survival Analysis

The R2 web tool (http://r2.amc.nl) was used to predict the association of BGN, DCN, CHST and CS/DS modification enzymes with the survival of patients with BC and glioma. R2 calculates the optimal cut-off of the expression level for each gene and is divided into two groups. The statistical differences in the gene expression values between the patients' groups with 'high' and 'low' mRNA expression levels were evaluated by ANOVA tests implemented in the R2 web tool. The p-values were corrected for multiple testing according to the false discovery rate. All cut-off expression levels and their resulting groups are correlated to the patients' survival. The cut-off level is reported and was used to generate Kaplan-Meier curves, which allowed to discriminate patients into 'good' and 'bad' prognosis cohorts. Kaplan scan analysis was performed to estimate the overall survival (OS) according to mRNA expression status using the two microarray datasets: the 'Mixed Tumor Breast' dataset that included 104 BC and 17 normal breast biopsies [26]; and the 'Glioma' dataset that included 276 glioma samples and 8 control samples [27].

2.5. Statistical Analysis

Web-based bioinformatics statistics was automatically calculated by each website and the results were displayed on the webpage. The results for continuous variables are

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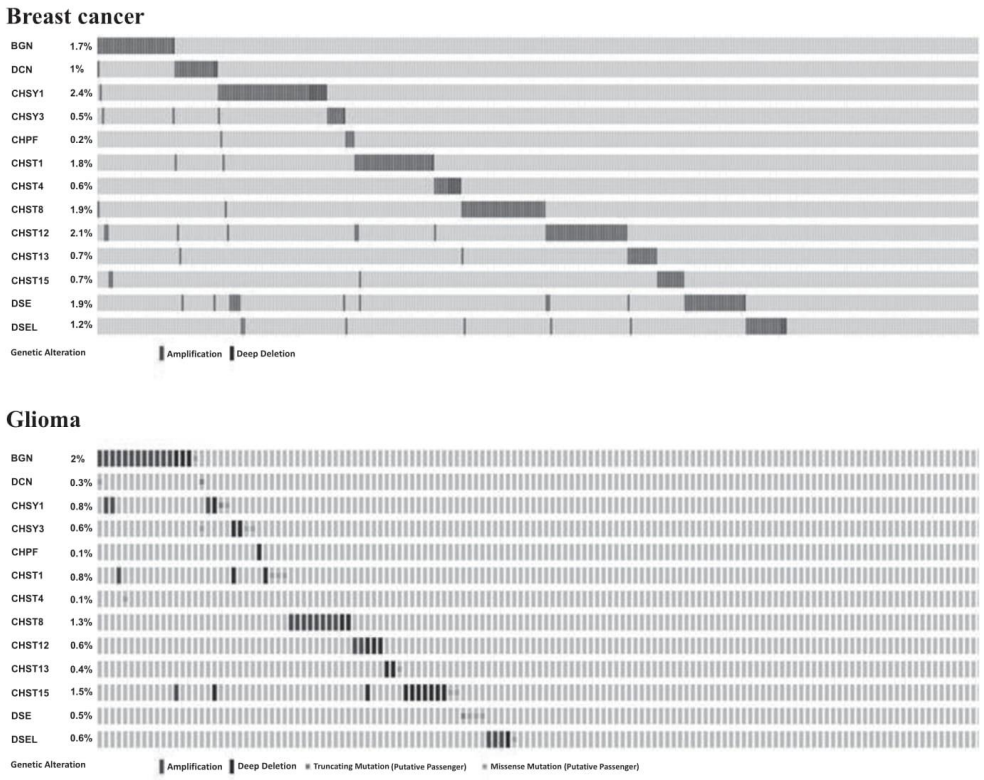

Fig. (1). Distinct mode and frequency of structural abnormalities in BGN, DCN and sulfate-modification enzymes. A schematic diagram of the frequency and mode of structural alterations in SLRPs and sulfate modification enzymes is shown. **A**). Genetic alterations of BGN and DCN with sulfate modification enzymes; the BC data were obtained from the cBioPortal and plotted using Oncoprint. **B**). Genetic alterations of BGN and DCN with sulfate modification enzymes; the glioma data were obtained from the cBioPortal and plotted using Oncoprint.

presented as mean ± SD, and the results for the categorical variables are presented as a number of cases and percentage. The significance of the differences between the groups was assessed using Student's t-test for continuous variables and the Fisher's exact test for categorical variables. All tests were 2-tailed, and a p-value of 0.05 was considered to be statistically significant. The survival differences were analyzed by a Log-rank test using the GraphPad Prism 6 software (GraphPad Software, Inc, La Jolla, CA). The data were analyzed with XLSTAT for Windows, version 2016.02 (Addinsoft, New York, NY).

3. RESULTS

3.1. Differences in the Mode and Frequency of Structural Alterations of BGN, DCN, CHST and CS/DS Modification Enzymes

In order to analyze the frequency and mode of structural alterations in PGs and the sulfate-modification enzymes as well as their tumor-dependent occurrence, BC and glioma were selected as tumor types. 15 % of BC cases were found to have structural alterations in BGN, DCN, CHST and CS/DS modification enzymes, while in glioma 8 % abnormalities were detected, which include mutations, deletions and amplifications (Fig. **1A**, **1B**). As shown in Fig. (**1B**) mutations in BGN and DCN, CHSY1, CHSY3, CHST1, CHST4, CHST13, CHST15, DSE and DSEL, which represent either truncated or missense mutations, were detected in glioma. Furthermore, in this disease amplifications were found in BGN, CHSY1, CHST1, CHST8, CHST12, CHST15, DSE and DSEL, but also deletions in BGN, CHSY3 and DSE. The mutations in DCN with a frequency 0.3 % were localized in the leucine-rich repeat (LRR) domain (Fig. **2B**). No mutations were found in both LLR and leucine-rich repeat N-terminal domain (LRRNT) of BGN, but mutations occurred in the p57L amino acid adjuvant to the LRRNT domain (Fig. **2A**; Table **1**).

For the CS enzymes CHSY1 and CHSY3, two mutations in the chondroitin N-acetylgalactosaminyltransferase (CHGN) domain were detected, while CHST4 had a

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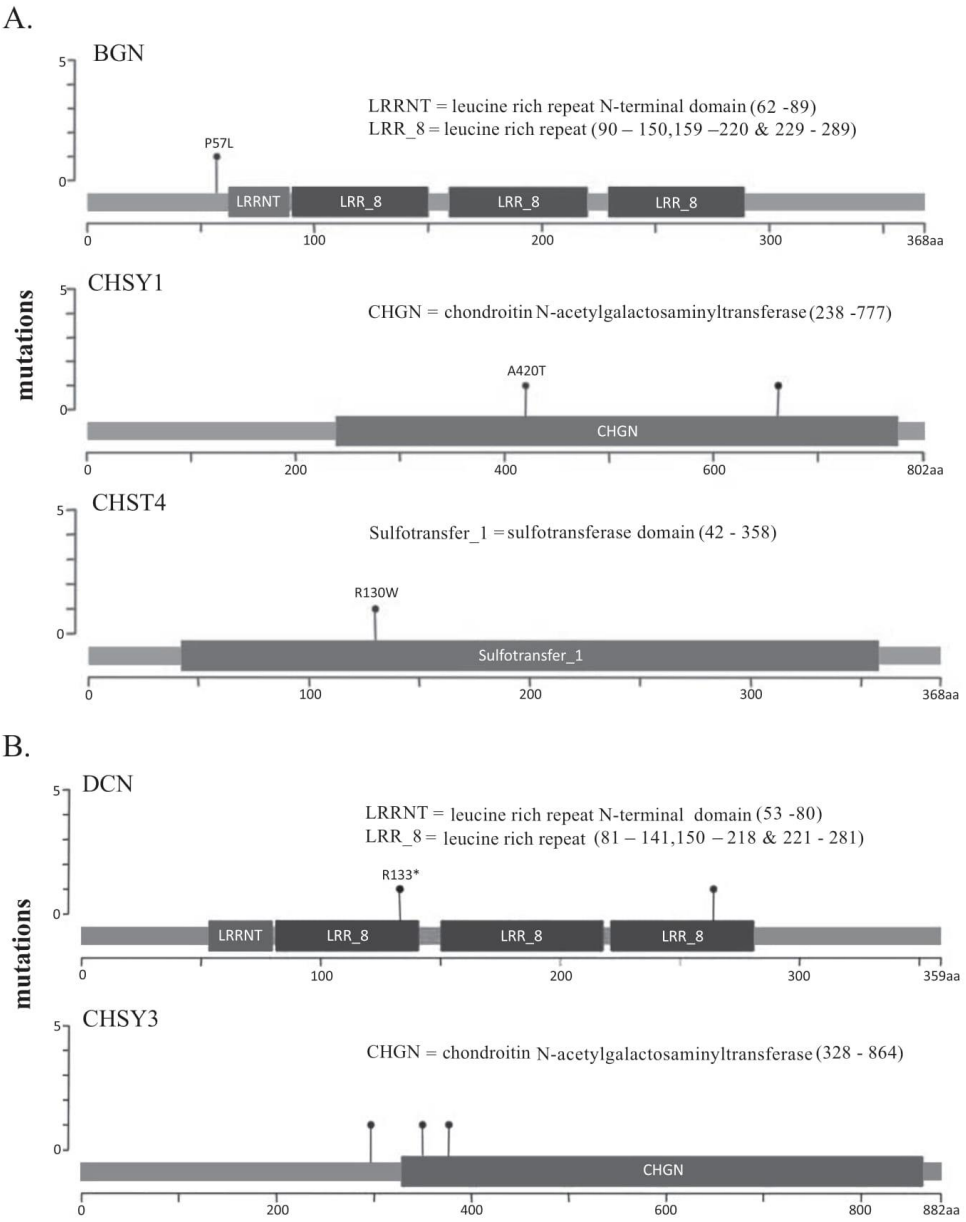

Fig. (2). Distribution of gene mutations obtained from the glioma dataset. **A**). Distribution of gene mutations of BGN and its associated CHSY1 and CHST4. **B**). Distribution of gene mutations of DCN and its associated CHSY3.

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Table 1: Description of gene mutation with protein change, mutation type and allele frequency using TCGA dataset (http://www.cbioportal.org).

Gene	Protein Change	Type	Copy #	# in COSMIC	Allele Freq (T)
BGN	P57L	Missense	Diploid	1	0.42
DCN	R133	Nonsense	Diploid	2	0.3
	S264C	Missense	Diploid	-	0.34
CHSY1	Q662	Nonsense	Diploid	1	0.12
	A420T	Missense	ShallowDel	-	0.21
CHST4	R130W	Missense	Gain	1	0.42
CHSY3	L297V	Missense	Diploid	2	0.26
	R350C	Missense	Diploid	4	0.4
	G377S	Missense	Diploid	3	0.45

aa: amino acid; COSMIC: the Catalogue Of Somatic Mutations In Cancer.

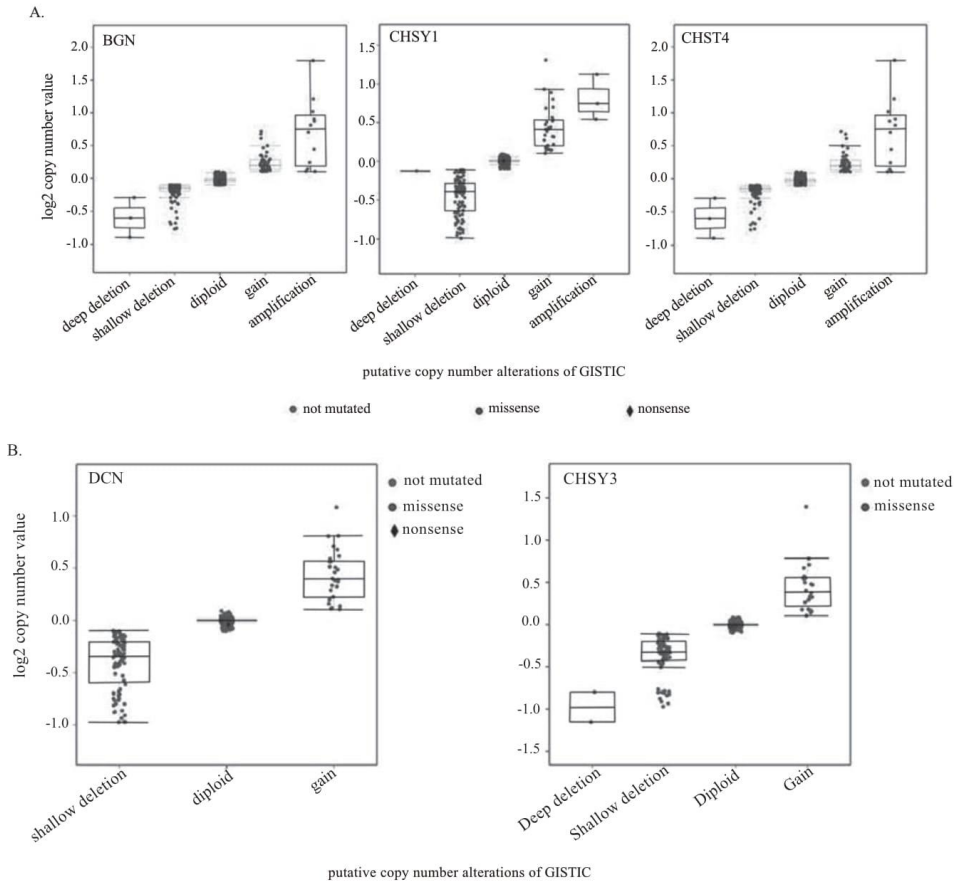

Fig. (3). Correlation plot of genes is shown putative copy number alterations against their respective copy number value from glioma dataset. **A**). Correlation plot for BGN and its associated CHSY1 and CHST4. **B**). Correlation plot for DCN and its associated CHSY3.

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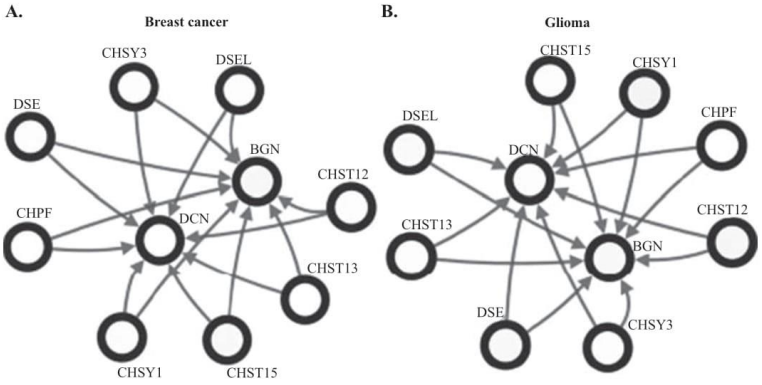

Fig. (4). Network analysis of SLRPs with neighboring genes in BC and glioma. **A**). Network analysis showing neighbor genes and gene interactions in breast cancers. **B**). Network analysis showing neighbor genes and gene interactions in glioma.

Table 2. Correlation studies of the SLRPs BGN/DCN and in BC and glioma using TCGA dataset (http://www.cbioportal.org).

		CHPF	CHST1	CHST4	CHST8	CHST12	CHST13	CHST15	CHSY1	CHSY3	DSE	DSEL
Breast cancer	BGN	0.92	0.536	0.818	0.482	0.158	0.778	0.024	0.563	0.013	0.518	0.668
	DCN	0.952	0.306	0.889	0.32	0.347	0.137	0.863	0.615	0.898	0.05	0.789
Glioma	BGN	0.98	0.115	0.02	0.815	0.903	0.941	0.218	0.005	0.903	0.922	0.903
	DCN	0.997	0.985	0.997	0.975	0.987	0.992	0.97	0.985	0.013	0.99	0.987

A tendency towards co-occurrence between BGN/DCN and CS/DS modification enzymes, $p<0.05$ was found.

missense mutation in the sulfotransferase domain with an R130W amino acid change (Fig. **2A**, **2B**; Table **1**). In the next step, somatic changes were validated by comparing mutant allele frequencies (Table **1**). Missense mutations in BGN, CHST4 and CHSY3 had the highest mutant allele frequency of ~0.4 % and non-sense mutations in CHSY1 with the lowest mutant allele frequency of 0.12 % in glioma. The missense and nonsense mutations were plotted for different mutations for their copy number value against different genetic alterations. As shown in Fig. (**3A-3B**), five types of structural alterations (deep deletion, shallow deletion, diploid, gain, amplification) were detected in the different genes analyzed. Diploid mutated copy number alterations were recorded in BGN, DCN, CHSY1 and CHSY3; shallow deletion was observed in CHSY1 and in particular amplifications and gain of copy numbers documented for CHST4 (Fig. **3A**, **3B**). Interestingly, no mutations were identified in BGN, DCN, CHST and CS/DS modification enzymes in BC, but the frequency of amplification was higher when compared to glioma. Furthermore, deletions were found with a much lower frequency (0.05 – 0.4 %) in BGN, DCN, CHSY1, CHSY3, CHST1, CHST4, DSE and DSEL. These data suggest tumor type dependent genetic changes of these genes, which might be associated with lack or enhanced expression, and thus might serve as a diagnostic biomarker or as a therapeutic target.

3.2. Network Analysis of BGN, DCN, CHST and CS/DS Modification Enzymes

Network analysis of BGN, DCN and respective CHST and CS/DS modification enzymes in BC (Fig. **4A**) and glioma (Fig. **4B**) gives insights into the complex association and highlights the genes most relevant to the cancer type analyzed. Using this approach, associations between BGN, DCN, CHST and CS/DS modification enzymes were detected in both BC and glioma. Notably, BGN and DCN were not directly interacting with either cancer types. BGN and DCN were in the center and neighbor genes of CHST and CS/DS modification enzymes, such as CS enzymes (CHSY1, CHST3 and CHPF), DS enzymes (DSE and DSEL) and carbohydrate sulfotransferases (CHST12, CHST13 and CHST15) associate with BGN and DCN in BC and glioma. Furthermore, the CHST and CS/DS modification enzymes (CHPF, CHST12, CHST13, CHST15, CHSY1, CHST3, DSE and DSEL) control changes of BGN and DCN expression in both malignancies (Fig. **4A**, **4B**).

3.3. Co-occurrence of BGN and DCN Expression with CHST and CS/DS Modification Enzymes

Using the mutual exclusivity analysis, co-occurrence among BGN, DCN and their neighboring genes was investigated (Table **2**) and pairwise associations between somatic

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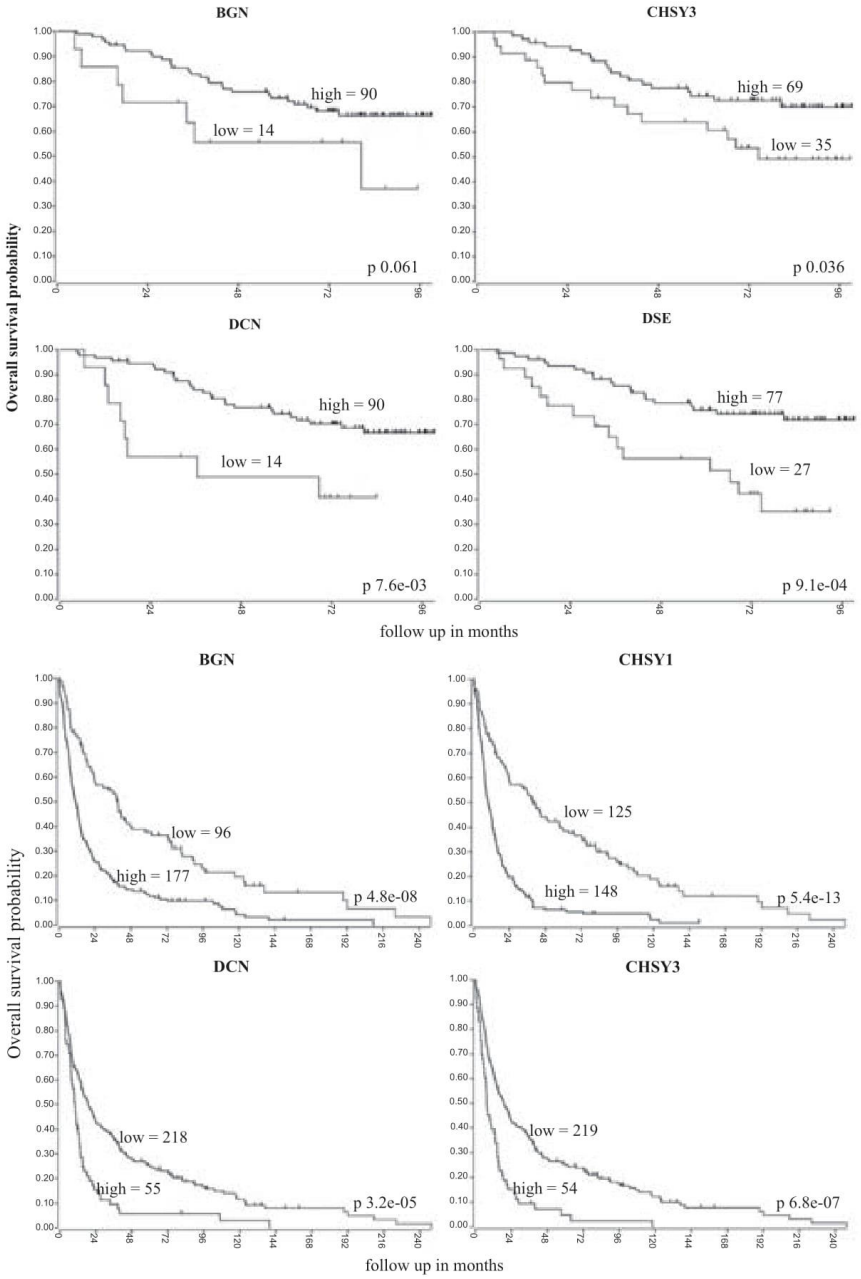

Fig. (5). Correlation of BGN/DCN expression levels with sulfate-modification enzymes. **A**). Higher expression levels of BGN and its associated CHSY3 as well as DCN and its associated DSE showed higher survival rates using TCGA data from the BC dataset. **B**). Lower expression levels of BGN and its associated CHSY1 as well as DCN and its associated CHSY3 showed higher survival rates using TCGA data from the glioma dataset.

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events were analyzed using Fisher's exact test. As shown in Table **S1**, BGN showed a tendency towards co-occurrence with CHST15 (log odds ratio=2.265, p=0.024) and CHSY3 (log odds ratio=2.635, p=0.013) in BC and with CHST4 (log odds ratio >3, p=0.02) and CHSY1 (log odds ratio >3, p=0.005) in glioma. BGN is co-mutated with two categories of GAG enzymes, CS and carbohydrate sulfotransferases in both BC and glioma. DCN is associated with the DS modification enzyme DSE (log odds ratio=1.79, p=0.05) in BC and the CS modification enzyme CHSY3 (log odds ratio >3, p=0.013) in glioma.

The mutual exclusivity analysis showed that events in DCN were likely to co-occur with either CS or DS dependent on the cancer type. The quantified log odds ratios demonstrate how strongly the presence or absence of BGN/DCN alterations is associated with abnormalities in CS/DS modification enzymes and in BC and glioma (Table **S1**). Only genes with at least one significant association (Fisher's exact test; p=0.05) are shown, and only associations with absolute log odds $\geqslant$ log (2) were considered.

In contrast to the sulfate modification genes, CHST4 did not directly interact with BGN/DCN (Fig. **4A**, **3B**). BGN and DCN were networking with TGF-β1 in glioma and TGF-β2 in BC, which follows the same networking pattern as the SLRP member fibromodulin (FMOD) (Fig. **S1**). CHST4 controls the networking with PGs, such as FMOD, prolargin (PRELP) and aggrecan (ACAN), respectively.

3.4. Prognostic Associations of Mutations in co-occurred Genes

To determine whether there exists a prognostic relevance of mutations in co-occurred genes, the expression profiles of BGN, DCN and their CHST and CS/DS modification enzymes have been analyzed using the R2 Genomics database in BC and glioma tissues in comparison to their matched normal tissues. As shown in Fig. (**5A**), higher BGN (90 out of 104, p=0.061) and DCN (90 out of 104, p=7.6e-03) mRNA transcript levels were correlated with higher survival rate. Interestingly, higher expression of their co-mutated genes CHSY3 (69 out of 104, p=0.036) (Fig. **5A**), CHST15 (96 out of 104, p=8.7e-04) (Fig. **S2A**) and DSE (77 out of 104, p=9.1e-04) (Fig. **5A**) were also significantly associated with an increased survival rate of BC patients. In contrast, high BGN (177 out of 273, p=4.8e-08) and DCN (218 out of 273, p=5.4e-13) mRNA levels were correlated with a worse survival of glioma patients (Fig. **5B**), which is in line with higher expression levels of co-occurred CHSY1 (148 out of 273, p=5.4e-13), CHSY3 (219 out of 273, p=6.8e-07) (Fig. **5B**) and CHST4 (232 out of 273, p=9.4e-04) (Fig. **S2A**). Furthermore, mutations in BGN and DCN were associated with higher expression levels, which were accompanied by a worse survival of glioma patients. Furthermore, concordant mutations found in CHST15, CHSY1 and CHSY3 resulted in lower expression and poor patients' prognosis.

4. DISCUSSION

The first cancer-related gene mutation was discovered nearly thirty years ago which was a point mutation in the HRAS gene that causes a glycine-to-valine mutation at codon 12 [28]. In the era of individualized therapies, grouping patients based on their co-association pattern of genetic alterations is the first step towards the implementation of genomics-based personalized medicine. Our report describes how the genetic alterations co-occur between proteoglycans and CS/DS modification enzymes in every individual BC and glioma patients.

Tumor cells display a wide range of glycosylation alterations compared to their non-transformed counterparts and have already been described a long time ago [29, 30]. Changes in the connective tissue within and around tumors are increasingly recognized as important contributors to tumor development and progression [31]. A central role for PGs, containing at least one GAG chain, such as heparan, dermatan, chondroitin, or keratan sulfate covalently attached to the protein core, has been described in tumors [32]. Among PG family members, the expression profiles of DCN and/or BGN are well-studied in different cancers and have been associated with the clinical outcome of tumor patients [33-35]. However, their function *e.g.* their tumor-suppressive or tumor-promoting potential is controversially discussed.

In this study, the structural alterations of the ECM components BGN and DCN in BC and glioma with special emphasis on their GAG chains focusing on CHST and CS/DS modification enzymes were investigated. It is well-known that mutations and aberrant glycosylation occur in all major human cancers [30-32]. The solid tumor types BC and glioma were selected to unravel the distinct roles of BGN, DCN, CHST and CS/DS modification enzymes as the cancer entities have already been shown for the RNA-binding protein Musashi-1 [36] and STAT3 inhibitors [37]. We have addressed the frequency of glycosylation modifications and structural abnormalities of CS/DS chains, BGN and DCN and their associations between genomic, transcriptomic and clinical features. Computational approaches allow to correlate gene expression with mutation rates in order to identify frequently mutated genes while eliminating many false-positive cells made in several single-tumor-type projects [38]. A better understanding of the relevant alterations will help to identify glycan-based biomarkers with prognostic and diagnostic value.

Using network analyses, the mechanisms of interaction among the different genes could be determined. For example, mutual exclusivity analyses have been published for *K-ras* and *p53* and co-occurred in pancreatic adenocarcinoma (log odds ratio = 1.599, p=0.006), where K-ras and p53 were not neighboring genes [39]. In our investigation, BGN and DCN were not neighboring genes; but many CS, DS and carbohydrate sulfotransferases enzymes were reported as neighboring genes of BGN and DCN. BGN was associated with CS modifying enzymes, CHSY3 in BC and CHSY1 in glioma and carbohydrate sulfotransferases, CHST15 in BC and CHST4 in glioma. Except for CHST4, all these CHST and CS/DS modification enzymes were directly interacting with BGN and DCN suggesting that the CHST and CS/DS modification enzymes influence and control the expression of BGN and DCN in both cancer types. CHST4 is interacting with FMOD, while BGN and DCN were interacting with TGF-β2 in BC and TGF-β1 in glioma, respectively. In this context, it is noteworthy that it has already been reported that BGN, DCN and FMOD are potent modulators of TGF-β activity [40]. BGN and DCN showed differential co-occurrence in BC and glioma, while DCN co-

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occurs with DSE in BC and CHSY3 in glioma. The specificities of glycosylation depend on various intrinsic factors of the glycosylation process within a given cell or tissue type [41].

Since we hypothesized that the prognostic effects may be influenced by mutations, the mutation rates of BGN, DCN and modification enzyme genes and their survival analyses were correlated with the patients' survival by R2 Genomics. There is an association between the occurrence of mutations and poor patients' outcome. No mutations were found in BGN/DCN and their co-occurred CHST and CS/DS modification enzymes in BC, which were linked with good clinical outcome of patients. Germ-line mutations in DCN and p53 have been reported to cooperate in the transformation of lymphocytes and ultimately lead to a more aggressive phenotype by shortening the latency of lymphoma [42]. DCN was mutated in the LRRNT and LRR domains of nonsense and missense mutations, respectively. Along with DCN, the co-occurred CS modification enzyme CHSY3 was mutated in the chondroitin CHGN domain with two missense mutations occurring with allelic frequencies of 0.4 and 0.45 %, respectively. BGN and their associated CHSY1 and CHST4 were also mutated in glioma, which was also in line with a statistically significant increase in variant allele frequency in melanoma and bladder cancer [43, 44].

BGN and their associated CHST and CS/DS modification enzymes were over-expressed in both BC and glioma. However, overexpression of BGN in BC was correlated with higher patients' survival, whereas in glioma, it was associated with a worse prognosis. Many studies have already shown an association of BGN and DCN with cancer in the context of oncogenic [45, 46] and anti-tumor properties [47-49]. Different sulfate modification genes were co-associated with BGN and DCN in BC and glioma; notably, DCN co-occurred with DSE in BC and CHSY3 in glioma. Expression levels of CS or genes required for CS biosynthesis reportedly correlate positively with poor prognosis of some malignant tumors [50, 51]. CHSY1 expression is closely associated with the malignant potential of soft tissue sarcomas [52], while high expression levels of CHSY1 and CHSY3 were found in colon cancer [53]. The changes in the glycosylation pattern will increase the molecular heterogeneity as well as the functional diversity of both diseases [54].

We encountered mutations in glioma based on their copy-number alterations. Cancer drivers identified only by integrating less frequent events across tumor types with mutations and copy number changes and aggregating genes using gene network and pathway analyses [55].

CONCLUSION

Targeting altered glycosylation as an immunotherapeutic strategy provides an appealing option for cancer treatment [56, 57]. While the HA-CD44 was the most studied GAG associations in cancer leading to an altered signal transduction [2, 58], we here report the first evidence of BGN and DCN interacting with different CHST and CS/DS modification enzymes. Understanding of the structure and specific biological roles of GAGs might lead to the development of novel therapeutic approaches, including the development of mimics as well as delivery systems for anti-cancer drugs targeting over-expressed BGN/DCN and their associated enzymes in the TME. Therefore, an increased knowledge of BGN/DCN and GAG chains in tumors of distinct origin is important for diagnosis, prognosis, drug delivery and design of novel treatment of tumor patients.

LIST OF ABBREVIATIONS

ACAN	=	Aggrecan
BC	=	Breast Cancer
BGN	=	Biglycan
CHGN	=	Chondroitin N-acetylgalactosaminyltransferase
CHST	=	Carbohydrate Sulfotransferase
CHSY	=	Chondroitin Sulfate Synthase
CNA	=	Copy Number Alterations
CS	=	Chondroitin Sulfate
DCN	=	Decorin
DS	=	Dermatan Sulfate
ECM	=	Extracellular Matrix
EGFR	=	Epidermal Growth Factor Receptor
FMOD	=	Fibromodulin
GAG	=	Glycosaminoglycan
HA	=	Hyaluronan
HS	=	Heparan Sulfate
KS	=	Keratan Sulfate
LOH	=	Loss of Heterozygosity
OS	=	Overall Survival
PG	=	Proteoglycan
PRELP	=	Prolargin
SLRP	=	Small Leucine-rich Proteoglycan
TCGA	=	The Cancer Genome Atlas
TGF-β1	=	Transforming Growth Factor-beta 1
TLR	=	Toll-like Receptor
TME	=	Tumor Microenvironment

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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SUPPLEMENTARY MATERIAL

Supplementary material is available on the publishers web site along with the published article.

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Other publications (not included in this dissertation)

4. **Subbarayan, K.**, Ulagappan, K., Wickenhauser, C., Bachmann, M. and Seliger, B., 2021. Immune interaction map of human SARS-CoV-2 target genes: implications for therapeutic avenues. *Front. Immunol.*, 12:597399. doi: 10.3389/fimmu.2021.597399.
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Conference Presentations

1. **Subbarayan, K.**, Sturm, G., Trajanoski, Z. and Seliger, B. (2021) Identification of genes and pathways associated with immune modulatory properties of biglycan in colorectal cancer cells. World Immunotherapy Council's 4th Young Investigator Symposium. Washington, D.C., USA.
2. **Subbarayan, K.**, Massa, C., Schäfer, H., Karapetian, E., Vaxevanis, C., Ulagappan, U., Yang, B. and Seliger, B. (2021) Modulation of immunogenicity by SARS-CoV-2 entry molecules in tumor cells. World Immunotherapy Council's 4th Young Investigator Symposium. Washington, D.C., USA.
3. **Subbarayan, K.**, Seliger, B. (2020). Transcriptional and post-transcriptional regulation of MHC class I antigen in HER-2/neu expressing tumor cells. Spring School on Immunology - German Society for Immunology (DGfI). Ettal, Germany.
4. **Subbarayan, K.**, Leisz, S., Massa, C., Balina, S., Müller, A., Wickenhauser, C. and Seliger, B. (2020). Improved growth properties and immune surveillance in K-RAS G12V-transformed cells through overexpression of biglycan. http://dx.doi.org/10.1136/jitc-2020-SITC2020.0828, SITC 2020, USA.
5. Schäfer, H., **Subbarayan, K.**, Seliger, B. (2020). PRELP-facilitated enhancement of MHC class I surface expression in B16F10 melanoma cells. http://dx.doi.org/10.1136/jitc-2020-SITC2020.0868, SITC 2020, USA.
6. **Subbarayan, K.**, Seliger, B. (2019). Novel mechanism of miR-21-3p on shaping the MHC class I mediated immune escape in tumor. EFIS-IL Tumor immune escape and resistance (TIMER), 8-9 October 2019, Halle(Saale), Germany.
7. **Subbarayan, K.** and Lazaridou, M. (2019). Transcriptional and post-transcriptional regulation of MHC class I expression in breast cancer and melanoma. Tumor immunology meets oncology (TIMO XIV), 25-27 April 2019, Halle(Saale), Germany.
8. **Subbarayan, K.**, Leisz, S., Balina, S., Wickenhauser, C., Bethmann, D., Bukur, J., Steven, A., and Seliger, B. (2018). Different modes of modulation of MHC class I antigens in HER-2/neu expressing tumor cells. Tumor immunology meets oncology (TIMO XIV), 24-26 May 2018, Halle(Saale), Germany.
9. **Subbarayan, K.**, and Seliger, B. (2017). Mutational landscape and co-expression of biglycan and chondroitin sulfate modifying enzymes in breast and brain cancers. 7 Lakes Proteoglycans Conference, 10-14 September 2017, Varese, Italy.

10. Lazaridou, M., **Subbarayan, K.,** Rahn, J., Handke, D., Mueller, A., Tretbar, S., Friedrich, M., Huettelmaier, S., Meyer, S., and Seliger, B. (2017). Post-transcriptional regulation of HLA class I APM components and its clinical relevance in tumors. Proceedings of 3rd Symposium on Advances in Cancer Immunology and Immunotherapy, Athens, Greece.
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12. **Subbarayan, K.**, Leisz, S., Steven, A., and Seliger, B. (2016). Expression, interaction and antitumor activities of biglycan in HER-2/neu mediated carcinogenesis. Proceedings of 12th EFIS-EJI Tatra Immunology conference, Slovakia.

Declarations

1. I declare that I have not completed or initiated a doctorate procedure at any other university.
2. I declare that all information given is accurate and complete. The thesis has not been used previously at this or any other university in order to achieve an academic degree.
3. I declare under oath that this thesis is my own work entirely and has been written without any help from other people. I met all regulations of good scientific practice and I used only the sources mentioned and included all the citations correctly both in word and in content.

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