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Review

Genomic-Glycosylation Aberrations in Tumor Initiation, Progression and Management

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Abstract: Post-translation modifications of proteins alter their functional activity and thus are key contributors of tumor initiation and progression. Glycosylation, one of the most common post-translational modifications of proteins, has been associated with tumorigenesis for decades. However, due to complexity in analysis of the functional effects of glycosylation, definitive information on the role of altered glycosylation in cancer is lacking. Importantly, imputing changes in glycosylation in proteins from analysis of DNA mutations has not been attempted globally. It is thus critical to elucidate the role of glycosylation in tumor pathophysiology as well as potential roles of altered glycosylation as cancer biomarkers and therapeutic targets. In this review, we summarize the evidence that glycosylation regulates functions of a set of frequently mutated oncogenes and tumor suppressors. Moreover, we explore the potential that protein sequence changes engendered by genomic mutations broadly alter glycosylation and thus promote tumor initiation and progression.

Keywords: glycosylation; tumor suppressors; oncogene; mutation; tumorigenesis

1. Introduction

Glycosylation is a common and diverse post-translational modification mediated by enzymatic addition of carbohydrates to proteins or lipids. Large varieties of proteins, including transmembrane

receptors, secreted proteins, surface ligands, and organelle-resident proteins as well as many others are modified by glycosylation to regulate their structure, stability, subcellular localization, and function. Glycosylation is categorized by the glycosidic linkage involved, including N-linked, O-linked, C-linked glycosylation, and glypiation, in which N- and O-linked glycosylation of proteins are the most common. For N-glycosylation, the carbohydrate is usually attached to asparagine in a N-glycosylation motif with a consensus sequence Asn-Xaa-Ser/Thr/Cys where Xaa is not proline. In contrast, the carbohydrate is attached to serine, threonine, tyrosine, hydroxylysine, or hydroxyproline for O-glycosylation. Although there is no known consensus sequence for O-glycosylation, many experimental and statistical studies propose preference for the presence of proline near the O-glycosylation site [1–7]. There is also a suggestion that accessibility of the enzymes to potential O-glycosylation sites rather than a specific sequence is the dominant regulator of O-glycosylation [8].

The association between altered glycosylation and cancer was first described more than six decades ago [9,10]. Oncogenic transformation leads to the aberrant expression of enzymes, including glycosyltransferase and glycosidases [11,12], and altered glucose metabolism [13] resulting in aberrant or tumor specific glycosylation, that not only have functional consequences, but raise the potential for the application as cancer biomarkers and therapeutic targets. More recent studies support the contention that altered glycosylation plays an essential role in regulating different pathophysiological steps in cancer progression, including proliferation, metastasis and invasion, angiogenesis, and immune modulation [14].

Genomic aberrations are the key drivers of tumorigenesis. Indeed, mutation-induced inactivation of tumor suppressors or activation of oncogenes are frequent events in tumorigenesis and are likely obligatory steps in acquisition of the malignant phenotype. The altered glycan structure and enzyme expression observed in tumor cells may be a consequence of specific genomic aberrations or may be due to integrated effects of the malignant transformation process. Indeed how specific genomic aberrations contribute to altered glycosylation and whether this enables tumor initiation and progression is a gap in knowledge that requires extensive additional study.

In this review, we focus on the known and potential roles of glycosylation in regulating function of protein products of key frequently mutated tumor suppressor genes and oncogenes that contribute to tumorigenesis. In addition, patient-derived mutations that alter the presence of glycosylation sites, a fundamental element for glycosylation, and their possible impact on protein function and tumor progression are explored. Each section is organized with an introduction to the function of the cancer gene, followed by what has been experimentally demonstrated in terms of glycosylation and then a concluding section on the potential impact of patient-derived mutations on glycosylation and how changes in glycosylation sites or glycosylation could potentially alter other forms of post-translational modifications and protein function.

2. Tumor Suppressors

2.1. p53

p53, which is encoded by *TP53*, is the most frequently mutated gene across human cancers [15]. Under normal conditions, the level of p53 is low due to continuous ubiquitin-proteasome mediated degradation [16]. In response to cellular stresses, including DNA damage and oncogenic stimuli [17], wild type p53 is activated and stabilized. The accumulation of active p53 induces transcription of its target genes that promote cell cycle arrest, senescence, apoptosis, DNA repair, and altered metabolism [17–19]. One of the key roles of p53 is to provide cells an opportunity to repair DNA damage or alternatively if the damage cannot be repaired, die. The stability of wild type p53 is regulated by post-transcriptional modifications including phosphorylation, O-GlcNAcylation [20,21], acetylation [22,23], and methylation [24]. Phosphorylation at the N-terminal of p53 by Ataxia telangiectasia mutated kinase (ATM) and RAD3-related kinase interrupts the interaction of p53 with murine double minute-2 (MDM2), which acts as an E3 ubiquitin ligase, targeting p53 for proteasome degradation [25–27]. In contrast, phosphorylation of the DNA-binding domain by COP9 signalosome (CSN)-associated kinase at Thr155 promotes ubiquitin-proteasome degradation of p53 [28]. The balance between phosphorylation mediated by these two kinases ensures that p53 is activated and inactivated in a tightly regulated manner in response to external stimuli.

The role of O-glycosylation of p53 was first described by Shaw et al. with the demonstration that O-glycosylation enhances DNA binding and sequence specific transcriptional activity of p53 [21]. More recently, Yang et al. showed that stress increases the level of O-GlcNAcylation of p53 thus enhancing p53 stability in breast cancer cells. O-GlcNAcylation at Ser149 in the DNA-binding domain of p53 antagonizes phosphorylation at Thr155 and also decreases the interaction of p53 with MDM2. Hence, Ser149 O-GlcNAcylation decreases p53 ubiquitination and protects p53 from proteasome degradation resulting in increased p53 levels and activity [20]. Inhibition of O-GlcNAcase (OGA), an enzyme that catalyzes the removal of O-GlcNAc, by the OGA inhibitor, streptozotocin (STZ), decreases breast cancer cell viability in the presence of DNA-damage compared to DNA-damaging agents alone consistent with O-GlcNAc being critical to optimal p53 function [20]. Although another study showed that STZ decreases activity of the proteasome resulting in p53 accumulation [29], the decrease in Thr155 phosphorylation and p53 ubiquitination could be reversed by an Ser149 to Ala mutation, showing that O-GlcNAcylation at this site could both directly and indirectly regulate p53 stability [20]. Ser149 mutation alone did not significantly reduce the level of O-GlcNAcylation of p53 suggesting that there are multiple O-GlcNAcylation sites on p53 in addition to Ser149 [20].

TP53 missense mutations in cancers predominantly cluster within the DNA-binding domain. Mutations in this region change the folding of p53 protein that either directly suppress DNA binding and the transcription of tumor suppressor genes, or indirectly alters the interactions with transcriptional factors or co-factors [30]. In some cases, this results in inactivation of p53 signaling but there are suggestions that many of these aberrations are gain of function. This may occur through altering the genomic targets of wild type p53 or alternatively of the related p63 and p73 molecules [31,32]. Mutation of Ser149 has been reported in several types of tumors (Table 1). It may contribute to tumor initiation or progression as, indicated above. The loss of the O-GlcNAcylation site could decrease the stability of p53 and thus its DNA binding activity and tumor suppressor activity. Whether Ser149 mutations alter the spectrum of DNA binding sites or affinity for p53 binding

partners or specific DNA binding sites requires further elucidation. As O-GlcNAcylation can occur on serine or threonine residues, further studies on whether Ser149Thr [33] as compared to other Ser149 alterations alters O-GlcNAcylation of p53 and its stability and activity are needed.

Table 1. Loss of glycosylation site caused by nonsynonymous substitution in patients.

Protein	Wild type AA	Mutant AA	Tumor type	Reference
p53	Ser149	Pro	Ethmoidal intestinal-type adenocarcinoma	[181]
			Head and neck squamous cell carcinoma	[182,183]
		Phe	Bowen's disease (squamous cell carcinoma in situ)	[184]
		Thr	Esophageal adenocarcinoma	[33]
PTEN	Thr398	Ser	Glioblastoma	[59]
	Thr401	Ile	Glioblastoma	[60]
			Leiomyosarcoma	[185]
	Ser294	Asn	Lung squamous cell carcinoma	[61]
NF1	Ser821	Arg	Pheochromocytoma	[186]
BRAC1	Asn913	Tyr	Epithelial ovarian cancer	[187]
	Ser915	Cys	Invasive lobular carcinoma	[188]
	Asn916	Ile	Epithelial ovarian cancer	[187]
EGFR	Asn175	Asp	Myelodysplastic syndromes (hematological malignancies, a risk to acute myeloid leukemia)	[189]
	Ser198	Arg	Endometrioid carcinoma	[190]
	Thr363	Ile	Glioblastoma	[191]
	Thr446	Lys	Small cell lung cancer	[192]
ErbB2	Ser573	Leu	Urothelial bladder carcinoma	[193]
ErbB3	Asn126	Ile	Esophageal adenocarcinoma	[194]
	Asn126	Lys	Colon adenocarcinoma	[195]
	Thr355	Ala	Colorectal adenocarcinoma	[195]
		Ile	Breast invasive carcinoma	[196]
			Stomach adenocarcinoma	COSU541
	Asn522	Tyr	Aggressive cutaneous squamous cell carcinoma	[197]
	Ser568	Leu	Colon adenocarcinoma	COSU376
	Asn616	Lys	Biliary tract carcinoma	COSU658
ErbB4	Thr140	Ile	Colon adenocarcinoma	COSU28
	Asn181	Ser	Lung adenocarcinoma	[65]
	Asn 253	Tyr	Oesophageal squamous cell carcinoma	COSU582
	Asn 358	Lys	Stomach adenocarcinoma	[198]
	Asn 473	Lys	Colorectal adenocarcinoma	[199]
	Thr475	Pro	Chronic lymphocytic leukemia	[200]
	Asn 548	Thr	Gastric adenocarcinoma	[201]
	Ser550	Phe	Cutaneous squamous cell carcinoma	[202]
β-catenin	Ser23	Arg	Wilms' tumor (nephroblastoma)	[203]
			Hepatocellular carcinoma	[204,205]
		Asn	Cutaneous adnexal tumor	[206]
			Hepatocellular carcinoma	[207]

	Gly	Lymphoma	[208,209]
Thr40	Ile	Thyroid carcinoma	[210]
		Liposarcoma	[211]
		Odontogenic tumor	[212]
		Endometrial carcinoma	[213]
		Desmoid-type fibromatosis	[214]
		Ovarian endometrioid carcinoma	[215]
		Melanoma	[216]
	Aln	Hepatocellular carcinoma	[216]
		Appendiceal mucinous tumor	[217]
	Ser	Primitive neuroectodermal tumor	[218]
		Gastric tumor	[219]
		Desmoid-type fibromatosis	[220]
	Pro	Colorectal tumor	[219]
Thr41	Pro	Wilms' tumor (nephroblastoma)	[221–223]
		Colon adenocarcinoma	[224]
		Hepatocellular carcinoma	[225]
		Salivary gland basal cell adenoma	[226]
	Aln	Multiple tumor types	[15]
	Ser	Endometrial carcinoma	[227,228]
	Asn	Hepatocellular carcinomas, cervical carcinoma, melanoma, non–hodgkin lymphomas	[229–232]
	Ser	NK/T-cell lymphoma, thyroid carcinoma	[208,210]
	Ile	Multiple tumor types	[15]

2.2. PTEN

PTEN is the 3rd most frequently mutated gene (about 10%) across cancer lineages in The Cancer Genome Atlas (TCGA) [15]. PTEN is primarily a plasma membrane phosphoinositide 3-phosphatase dephosphorylating the signaling lipids PIP(3,4,5)P3 to PI(4,5)P2 and PI(3,4)P2 to PI(4)P. Through this activity, PTEN antagonizes the action of phosphatidylinositide-3-kinase (PI3K) and subsequent activation of PH domain and other lipid domain containing proteins. Activation of Akt, one of the critical PH domain containing proteins, promotes cell proliferation, survival, cell cycle progression, differentiation, motility, and metabolism [34–36]. Moreover, PTEN also appears to have a tumor suppressor role in the nucleus, in which the catalytic activity of PTEN may not be critical, wherein PTEN increases p53-dependent apoptosis and maintains genomic stability through preserving heterochromatin structure and potentially other less clear mechanisms [37,38].

PTEN is subjected to multiple post-translation modifications, including phosphorylation, acetylation, ubiquitination, sumoylation, oxidation, and S-nitrosylation, across four functional domains in the protein. Post-translation modification of PTEN regulates its enzymatic activity, subcellular localization, stability, and protein-protein interactions [39,40]. Recently, Hopkins et al. identified a secreted 576-amino acid translational variant of PTEN (~75 kDa), named PTEN-Long, which adds an additional 173 N-terminal amino acids to the 403-amino acid classical PTEN (55 kDa)

resulting in higher phosphatase activity [41]. Moreover, PTEN-Long, but not classical PTEN is secreted from cells and subsequently taken up by other cells in which it antagonizes PI3K signaling and induces breast and brain cancer cell death both *in vitro* and *in vivo*. Using an antibody that recognizes an epitope at the C-terminus that is common to both PTEN-Long and classical PTEN, both variants of PTEN were detected in concanavalin A pull down eluents suggesting that both classical and the long variant are glycosylated. Furthermore, glycosylated PTEN binds heparin-sulphate-modified cell surface proteins in the glypican and syndecan families suggesting that PTEN glycosylation may have a role in facilitating its interaction with and entry to cells. PTEN has 5 potential O-GlcNAcylation sites (Ser83, Thr536, Thr539, Thr571, and Thr574, numbers based on PTEN-Long) and 1 potential N-glycosylation site (Asn465), of which Ser83 is PTEN-Long specific. The N-glycosylation site Asn292 in classical PTEN is located in the C2 phosphatase domain, which is located close to an ubiquitination site Lys289 (Figure 1) that regulates PTEN nuclear localization [37] and stability [42]. Thus N-glycosylation at Asn292 has the potential to alter PTEN stability and function. The O-glycosylation sites, Thr363, Thr366, and Thr398, are located in the C-terminal tail; while Thr401 is located in the C-terminal PDZ domain (Figure 1). The potential O-glycosylation sites in the C-terminal tail are close to a cluster of regulatory serine and threonine phosphorylation sites (Ser361, Ser362, Thr363, Thr366, Ser370, Ser380, Thr382, Thr383 and Ser385). In general, phosphorylation of the C-terminal tail increases the half-life of PTEN, but significantly decreases it catalytic activity, prevents its plasma membrane translocation to antagonize PI3K signaling and masks the PDZ protein-protein interaction domain [43–48]. However, phosphorylation of Thr366 is contextual in that Glycogen Synthase Kinase 3β (GSK3β)–induced phosphorylation promotes PTEN degradation [49] while Polo-like kinase 3-induced phosphorylation enhances PTEN stability [50]. In addition to plasma membrane localization, the cluster of phosphorylation sites (Ser370–Ser385) has been shown to contribute to cytosol/nuclear localization balance [51]. The Thr401 potential O-glycosylation site is next to an acetylation site Lys402 that regulates interaction of PTEN with other PDZ-domain containing proteins [52]. At this moment, the type of O-glycosylation and also the glycan structure on both N- and O-glycosylation sites in PTEN have yet to be determined. However by using computational prediction tools of O-GlcNAc site, YinOYang 1.2 (http://www.cbs.dtu.dk/services/YinOYang/), Thr363, Thr366, Thr398, and Thr401 in classical PTEN are predicted to be potential O-GlcNAcylation sites. As increasing evidence suggests that O-GlcNAcylation and phosphorylation appear to have a reciprocal relationship in many proteins [53–55], glycosylation may have a role in altering the stability, subcellular localization, and the protein-protein interactions of PTEN.

Although the N-terminal phosphatase domain is primarily responsible for the catalytic activity of PTEN, approximately 40% of PTEN mutations occur in the C2 and C-terminal tail domain, suggesting the importance of the C-terminus in functional regulation of PTEN [56]. An extensive analysis of tumor-derived missense mutations revealed that the majority of mutations abolish (81%) or largely decrease (10%) the phosphatase activity of PTEN [57]. As discussed above, although the C-terminus is not directly responsible for the catalytic activity of PTEN, mutations in this region have marked effects on protein stability, enzymatic activity, and possibly interaction with critical binding partners [58]. Tumor-derived mutations at the potential Thr398 and Thr401 O-glycosylation sites in

PTEN (classical PTEN numbering) have been reported in glioblastoma and leiomyosarcoma [59,60] (Table 1), suggesting that loss of O-glycosylation on these sites could alter PTEN activity and thus contribute to oncogenesis. In addition, a tumor-derived mutation that disrupts the Asn292 N-glycosylation consensus sequence has been detected in lung cancer [61] (Table 1).

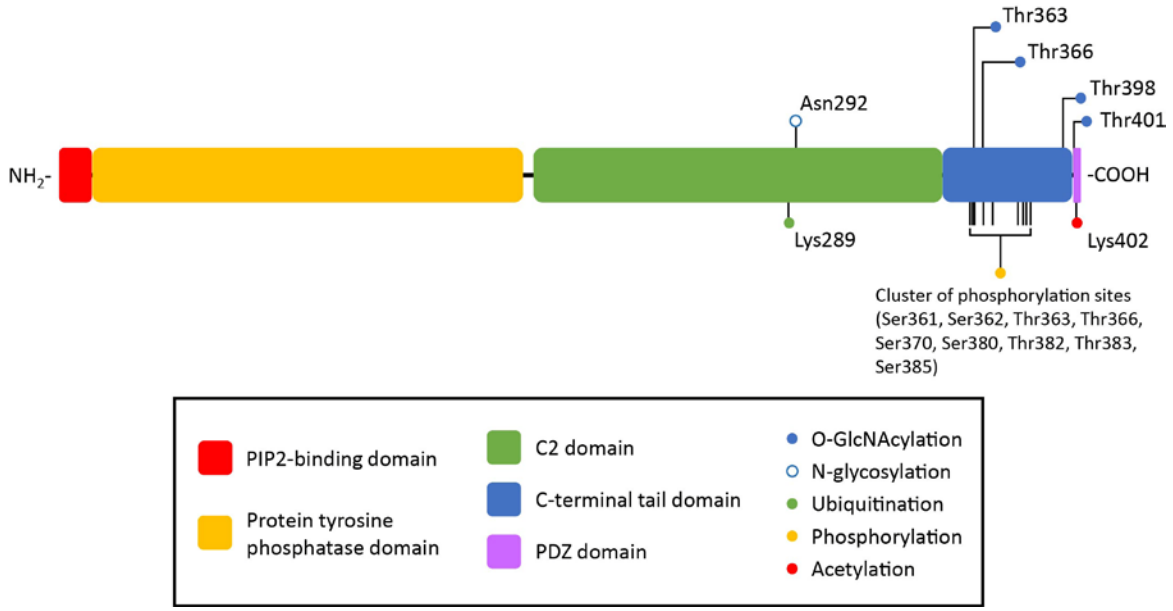

Figure 1. The potential glycosylation sites and other post-translational modification sites around the glycosylation sites in classical PTEN.

2.3. Neurofibromin 1 (NF1)

NF1, transcribed from the *NF1* gene, is responsible for Neurofibromatosis, an autosomal dominant congenital disease. NF1 aberrations have also been associated with different types of cancer [62–66]. *NF1* is one of the most significantly mutated genes across human cancers with glioblastoma and lung cancer having *NF1* mutation frequencies over 10% [15]. NF1, which consists of 2839 amino acids (~319 kDa), is a Ras GTPase-activating protein (GAP) that binds Ras through its GAP-related domain (GRD) and negatively regulates oncogenic Ras signaling by converting Ras from an active GTP-bound form to an inactive GDP-bound form [67,68]. NF1 is highly phosphorylated which indirectly regulates the interaction of NF1 and Ras [69,70]. Recently, an in silico study using the YinOYang 1.2 and Netphos 2.0 sites (http://www.cbs.dtu.dk/services/NetPhos/) identified a series of potential O-GlcNAcylation sites of which some could either be modified by O-GlcNAcylation or phosphorylation suggesting that O-GlcNAcylation could alter the ability of NF1 to be phosphorylated (Table 2) [71]. More than half of the potential O-GlcNAcylation sites localize in the C-terminal domain (CTD) of NF1. Phosphorylation of the serine/threonine residues (Thr 2556, Ser2576, Ser2578, Ser2580, and Ser2813) in the CTD of NF1 by cAMP dependent protein kinase A (PKA) directs NF1 binding with 14-3-3 with subsequent degradation thus relieving the inhibitory effect of NF1 on Ras [69,70]. Of the potential O-GlcNAcylation sites in the CTD, Thr2560, is close

to the PKA-mediated phosphorylation sites Ser2576 and Ser2578 [70], suggesting that O-GlcNAcylation could block the phosphorylation of NF1 by PKA and enhance the binding of NF-1 with Ras rather than 14-3-3 and thus antagonize Ras signaling. Cross talk between PKA or protein kinase C (PKC) and O-GlcNAc transferase (OGT) has been previously shown in model systems [72]. Phosphorylation of the Cys/Ser-rich domain (CSRD) of NF1 by PKCα alters subcellular localization, facilitates the association of NF1 with actin cytoskeleton and enhances NF1 GAP activity [73,74]. There is also evidence that phosphorylation of the CSRD inhibits NF1 lysosomal degradation [75]. Sites of phosphorylation mediated by PKC in the CSRD are still unknown; therefore, whether the three potential O-GlcNAcylation sites in the CSRD crosstalk with phosphorylation by PKC and regulate NF1 subcellular localization remains open for further investigation.

Table 2. NF1 glycosylation sites.

Domain	Glycosylation site
Cys/Ser-rich domain (CSRD)	Ser821, Ser871, Ser892
Ras-GAPs related domain (GRD)	Ser1399
Leu-repeat domain (LRD)	Ser1813
C-Terminal domain (CTD)	Thr2423, Ser2475, Ser2500, Ser2502, Ser2509, Thr2560, *Ser2576, *Ser2578, Ser2739

Asterisks indicate serine residues which are also phosphorylation sites for PKA.

2.4. Ataxia telangiectasia mutated kinase (ATM)

The protein kinase ataxia-telangiectasia mutated (ATM) is a key component of DNA damage response (DDR), cell cycle checkpoint activation, DNA repair, and metabolic changes in response to DNA double strand breaks (DSB) and oxidative stress [76–78]. Patients with germline loss of ATM are susceptible to the pleiotropic neurodegeneration disorder ataxia-telangiectasia, immunodeficiency, and a high rate of malignancies due to genomic instability. The majority of ATM mutations are inherited compound heterozygotes or homozygotes that would inactivate ATM function [79–81]. Individuals with ataxia-telangiectasia are estimated to possess a 100-fold increased risk of cancer in comparison to other populations [82], which matches the high mutation rate of ATM across different cancer types, such as liver (~18%), colon (~17%), and stomach (~15%) cancer.

ATM encodes a protein consisting of 3056 amino acids (~350 kDa). ATM belongs to the family of PI3K-related protein kinases (PIKK) [83] due to the homology of its C terminal kinase domain to the catalytic domain of PI3K, which confers the ability of ATM to phosphorylate inositol phosphate molecules [84,85]. However, the main function of ATM appears to be as a protein kinase and linker molecule. As a member of the PIKK family, ATM acts as a regulator in DNA repair and damage signaling through phosphorylation of p53 on Ser15, Chk2 on Thr68, and MDM2 on Ser39, which are required for DNA-damage induced check point responses to provide cells with sufficient time to repair DNA breaks and to regulate the decision to survive or undergo apoptosis. ATM also

phosphorylates BRCA1 and CtIP, a BRCA1 binding protein that inhibits BRCA1 function, contributing to effects on DNA damage repair [86].

Upon the introduction of DSB, ATM undergoes autophosphorylation [87] and is acetylated [88] to transform from an inactive dimer to an active monomer. The monomer is subsequently recruited by the MRE11-RAD50-NBS1 complex to DNA breaks where it phosphorylates downstream effector proteins. Among all autophosphorylation sites, phosphorylation on Ser1981 is involved in most of the biological functions of ATM [87,89]. Interestingly, O-GlcNAcylation has been implicated in modulating Ser1981 phosphorylation. Using immunoprecipitation, Miura et al. showed that ATM interacts with OGT and is modified by O-GlcNAcylation in HeLa cells and primary mouse neurons. Moreover, enhancing O-GlcNAcylation by PUGNAc, a GlcNAc analogue that potently inhibits OGA, enhances ATM phosphorylation at Ser1981 upon irradiation [90] through weakening the association between ATM and protein phosphatase 2A, resulting in autophosphorylation of ATM at Ser1981. Similar observations have been found in MEF cells where phosphorylation of ATM at Ser1987 (corresponding to Ser1981 in human ATM) is the most significantly upregulated event (approximated 2.5 fold change) induced by OGT [91]. Phosphorylation of ATM on Ser1981 was detected after 1h of oxidative stress induced by H_2O_2 while the level of O-GlcNAcylated ATM was not markedly changed; therefore, at least in response to oxidative stress, Ser1981 is unlikely an O-GlcNAcylation site [90]. However, the effects of O-GlcNAcylation and autophosphorylation at the same Ser1981 in ATM are likely to inversely alter ATM function. Indeed, given that phosphorylation of Ser1981 is a hallmark of ATM activation, localization of ATM to DSBs, it is likely that O-GlcNAcylation at this site when it occurs plays a crucial role in ability of ATM to alter the function of downstream proteins involved in cell cycle regulation, DDR, checkpoint arrest.

2.5. BRCA1 and BRCA2

Inherited mutations in the tumor-suppressor genes *BRCA1* and *BRCA2* are powerful predictors for the likelihood of developing breast and ovarian cancers. Approximately 5%-10% of all female breast cancers are considered to have a hereditary component. Mutations in *BRCA1* and *BRCA2* account for the majority of this population [92,93]. In addition to germline mutations, somatic mutations as well as loss of protein expression contribute to tumorigenesis [94,95]. *BRCA1* and *BRCA2* genes play pivotal roles in maintaining genomic integrity and exert their tumor suppression function primarily through involvement in cell cycle checkpoint control and DNA damage repair [96] to ensure genome stability.

Human *BRCA1* and *BRCA2* encode two large, structurally related proteins consisting of 1,863 (~220 kDa) and 3,418 (~380 kDa) amino acids, respectively. BRCA1 contains an N-terminal RING-finger domain, two nuclear localization signals (NLS), and two copies of a C-terminal BRCT domain [97]. BRCA1 is an important participant in pathways regulating DNA repair, cell cycle progression, ubiquitination, and transcriptional regulation through direct or indirect binding to various proteins through different domains. The binding of the RING-finger domain of BRCA1 and BRCA1-Associated Protein 1 (BAP1) increases the ubiquitin-ligase function of BRCA1 and enhances BRCA1-mediated inhibition of breast cancer cell growth [98]. Proteins involved in DNA

repair and cell cycle regulation, such as MRE11, RAD50, NBS1, MDC1, ATM, Chk2 and Cdk2, directly bind to the central region of BRCA1. The two BRCT domains allow BRCA1 to attenuate DNA replication and maintain heterochromatin structure through their interaction with proteins such as RNA polymerase II, p300, BACH1, histone deacetylases 1 and 2, etc. [99]. In contrast to *BRCA1*, *BRCA2* encodes for a larger transcript with 27 exons but a simpler protein domain structure. It contains two NLS at the C-terminal and eight BRC-repeat motifs, which are located in the middle region of BRCA2. The BRC motifs are essential for its direct interaction with an important recombinase, RAD51, making BRCA2 a key regulator of Homologous Recombination (HR) [100].

Post-translational modification by phosphorylation is required for normal BRCA1 and BRCA2 function and it is one of the first modifications of BRCA1 in the DNA damage response [96,101]. The kinases that phosphorylate and regulate BRCA1 and BRCA2 vary dependent on the stimulatory process [99,102]. Interestingly, the first team demonstrated BRCA1 to act as a granin, Jensen et al. also demonstrated that BRCA1 was deglycosylated upon the treatment of PNGase F [103], consistent with specific N-glycosylation of BRCA1. By mass spectrometry, Whelan et al. confirmed that BRCA1 is N-glycosylated at amino acid residues Asn909, Asn913, and Asn916, with Asn913 being in a N-glycosylation consensus sequence, while Asn909 and Asn916 of BRCA1 do not have a consensus NXS/T motif [104]. Asn913 and Asn916 are mutated in ovarian carcinomas (Table 1); however, the function of these point mutations has yet been elucidated. Residues 758-1064 of BRCA1, which covers the locations of the three identified N-glycosylation sites, forms RAD51-containing complexes *in vitro* and mediates direct binding between BRCA1 and RAD51 [105]. It is possible that N-glycosylation on these sites may play a role in regulating the function of BRCA1 in HR through interfering with binding of BRCA1 to RAD51, indicating potential roles of N-glycosylation of BRCA1 in tumorigenesis of breast and ovarian cancers. Similar to BRCA1, BRCA2 is also deglycosylated by PNGase F, suggesting that BRCA2 is also modified by N-glycosylation. Binding of CREB binding protein (CBP), a transcriptional cofactor with HAT, to the BRCA2 N-terminus is required for N-glycosylation on Asn272 [106]. However, how CBP functionally regulates BRCA2 glycosylation and function remains unclear. Siddique et al. have reasoned that CBP-mediated N-glycosylation is likely to play essential roles in BRCA2 stability due to associations with ubiquitination and proteasomal degradation. Although evidence demonstrating crosstalk between N-glycosylation and ubiquitination or phosphorylation of BRCA2 is yet lacking, this represents a reasonable model that should be explored experimentally.

2.6. Retinoblastoma protein (pRB)

pRB is encoded by *RB1* and contains 928 amino acids (~110 kDa). Inactivation of pRb by mutation or deletion is one of the most frequent and early events in initiation of cancers including retinoblastoma, osteosarcoma, and small cell lung carcinoma [107–109]. pRb plays a pivotal role in negatively regulating cell cycle progression at the G1 checkpoint through blocking S phase entry [110]. In early G1, pRb is hypophosphorylated and binds with E2F transcriptional factors. As the cell cycle progresses from G1 towards S-phase, pRb is sequentially phosphorylated by cyclinD/Cdk4/6 at the C-terminus [111] and then by cyclinE/Cdk2 at the pocket region [112] resulting in relief of E2F

binding that allows the E2F-dependent transcription of genes required for S-phase. Further pRb-E2F interactions are controlled by the interplay between phosphorylation and other post-translational modifications, including acetylation and methylation, which negatively regulate cyclin/Cdk-dependent phosphorylation [113,114] and SUMOylation, which enhances the ability of pRB to repress E2F-dependent transcription [81,115].

A recent study provided evidence that pRb is heavily O-GlcNAcylated, especially when the cell cycle is arrested at G1. Moreover, as O-GlcNAcylated pRb interacts with E2F at the G1 phase of the cell cycle [116], O-GlcNAcylation of pRb may have a role in the interplay in the post-translational regulated suppression on E2F-dependent transcription by maintaining pRb in the active and E2F bound state. One possible mechanism by which O-GlcNAcylation regulates pRb-E2F interactions is by preventing cyclin/Cdk-mediated phosphorylation by blocking phosphorylation sites at the C-terminus and the pocket region. This hypothesis is supported by the reciprocal status of O-GlcNAcylation and phosphorylation at the G1 phase where pRb is highly O-GlcNAcylated, but hypophosphorylated. Identification of the sites of O-GlcNAcylation on pRb and their effect on pRB structure and accessibility are needed to elucidate how O-GlcNAcylation regulates pRb activity.

Stabilization of pRb by phosphorylation and ubiquitination has also been implicated in activation of transcription of proapoptotic genes [117]. Whether O-GlcNAcylation of pRb plays a role in this and other cellular functions of pRb remains to be determined.

3. Oncogenic proteins

3.1. EGFR and ErbB family

The ErbB family of receptor tyrosine kinases (RTKs) consists of four members, epidermal growth factor receptor (EGFR/ErbB1), ErbB2, ErbB3, and ErbB4. The activation of ErbBs and their downstream signaling networks regulate diverse cellular events, including proliferation, differentiation, motility, apoptosis, and adhesion. In general, autoinhibitory tethering in the subdomain IV is relieved upon ligand binding to the extracellular subdomain I and III resulting in a conformational change. The receptors become active and form homo- or heterodimers through the dimerization arm in subdomain II, which subsequently leads to auto- and cross phosphorylation of tyrosines in the intracellular C-terminal domain and subsequent activation of downstream signaling pathways. Aberrant activation of ErbBs due to gene mutation or amplification that contribute to ligand-independent activation or increased response to ligand stimulation plays a central role in tumorigenesis of various human cancers, including brain, lung, and breast cancer [118]. Indeed, this family is being extensively explored as therapeutic targets.

The extracellular domain of ErbB receptors is heavily N-glycosylated (Table 3). N-glycosylation of ErbBs plays a critical role in regulating receptor trafficking, conformation of the ligand-binding domain, ligand affinity, cell surface protein-protein interactions, receptor activation, tumorigenesis, and also the sensitivity towards RTK inhibitors. Treatment with N-glycosylation inhibitors, such as tunicamycin, results in retention of the hypoglycosylated receptor in the ER and Golgi [119], remarkably reducing ligand binding to receptors and subsequent kinase activation [119–121].

The role of N-glycosylation in receptor cell surface expression was further elucidated by mutagenesis of all N-glycosylation sites in subdomain III resulting in significantly reduced cell surface expression of ErbB3 [122]. Furthermore, loss of N-glycosylation at Asn444 (Asn420 in an alternative numbering of the human EGFR sequence) in EGFR, but not other glycosylation sites in subdomain III, impaired ligand binding ability. This resulted in spontaneous dimerization and constitutive phosphorylation in the absence of ligand [123]. Similar observations were reported with ErbB3 with mutation of Asn437 (Asn418 in an alternative numbering of the human ErbB3), a site corresponding to Asn444 in EGFR [122]. Moreover, loss of glycosylation at Asn437 in ErbB3 promotes cell proliferation, anchorage-independent cell growth, and tumorigenesis *in vivo* [122]. Thus Asn444 in EGFR and Asn437 in ErbB3 prevent constitutive receptor dimerization in addition to controlling ligand sensitivity rendering mutations at these sites oncogenic.

Table 3. EGFR and ErbBs glycosylation sites.

Domain	ErbB	Type of glycosylation	Glycosylation site
Subdomain I	EGFR	N-linked	Asn56 (non-canonical), Asn128, Asn175
	ErbB2		Asn68, Asn124
	ErbB3		Asn126
	ErbB4		Asn138, Asn174, Asn181
Subdomain II	EGFR	N-linked	Asn196
	ErbB2		Asn187, Asn259
	ErbB3		Asn250
	ErbB4		Asn253
Subdomain III	EGFR	N-linked	Asn352, Asn361, Asn413, Asn444
	ErbB2		N/A
	ErbB3		Asn353, Asn408, Asn414, Asn437, Asn469
	ErbB4		Asn358, Asn410, Asn473, Asn495
Subdomain IV	EGFR	N-linked	Asn528, Asn568, Asn603, Asn623
	ErbB2		Asn530, Asn571, Asn629
	ErbB3		Asn522, Asn566, Asn616
	ErbB4		Asn548, Asn576, Asn620
Juxtamembrane	EGFR	O-GlcNAc	Thr678
Catalytic domain	EGFR	O-GlcNAc	Ser1070, Ser1071

A study on EGFRvIII, a constitutively active truncated EGFR mutation commonly found in glioblastoma, suggested that glycosylation of subdomain III positively regulates receptor self-dimerization [124]. EGFRvIII has only eight glycosylation sites due to in-frame deletion of subdomain I and II (amino acid residue 6-273). Although the deletion impairs ligand binding ability, the kinase is constitutively active due to self-dimerization [125–127]. Ligand-independent dimer formation and kinase activity of EGFRvIII were impeded by inhibition of N-glycosylation by tunicamycin [124]. Further, when compared with the type II truncated EGFR, another tumor-derived

EGFR truncation mutant, which has high affinity EGF and TGF-α binding and enhanced kinase activity [128], the lack of three glycosylation sites in the subdomain IV due to deletion of amino acid residue 520–603 did not affect ligand binding and kinase activation, suggesting that the glycosylation sites in subdomain III, but not in subdomain IV, are critical for both ligand binding and receptor dimerization [124]. Saxon et al. proposed that partial deletion of subdomain IV greatly reduced the affinity of EGF for the receptor [129]. In contrast, binding studies revealed that EGFR without subdomain IV has a higher affinity for ligand [130,131]. Mutagenesis of Asn603 (Asn579 in an alternative numbering of the human EGFR sequence) in EGFR, a glycosylation site located in the subdomain IV, showed that loss of this glycosylation site weakened tethering of the receptor and increases the affinity between receptors; however, the mutation-induced untethering was not sufficient to drive receptor dimerization or 32D cell survival in the absence of interleukin-3 [132]. Based on the above findings, receptor conformation and kinase activity are likely modulated by the cooperation between glycosylation sites rather than a single site of glycosylation.

In addition to conformational changes that affect kinase activation, N-glycosylation on the ectodomain of the EGFR also interacts with other cell surface glycoproteins or glycolipids through the glycan termini that prevents ligand-binding and subsequent receptor activation [133,134]. On the other hand, interaction of EGFR with galectins, a glycoprotein whose affinity is proportional to the N-glycan branching modified by N-acetylglucosaminyltransferase V (GnTV) on EGFR [135], sustains EGFR signaling by preventing ligand-induced receptor endocytosis, and thereby, retains the presence of EGFR at the cell surface [136] and the association of receptor with caveolin-1 that suppresses EGFR signaling [137]. Knockdown of GnTV reduces the N-glycan branching on EGFR that attenuates the invasive phenotype of cancer cells induced by EGFR signaling [138–140], and also delays the tumorigenesis induced by Erbb2 signaling [141,142]. Six out of twelve glycosylation sites (Asn56, Asn175, Asn413, Asn444, Asn528, and Asn603) on EGFR appear to have complex-type glycans where the glycan termini is sialylated and fucosylated [143]. By comparing two cell lines with differential invasive capability isolated from same parental lung cancer cell line, Liu et al. found that the more invasive cell line has a higher level of sialylation on EGFR and expression of α1,3- and α1,6-fucosyltransferases that contribute to glycan terminal and core fucosylation respectively [143]. Removal of sialic acid by sialidase and fucose by fucosidase enhances EGF-induced WT receptor dimerization, phosphorylation, and autophosphorylation of EGFR mutants [143,144]. Furthermore, overexpression of FUT4 and FUT6, α1,3-fucosyltransferases, in A549 cells suppresses EGF-induced receptor dimerization and activation. In contrast, knockdown of FUT8, an α1,6-fucosyltransferase in the more invasive cell line, reduces EGFR dimerization and activation in the presence of ligand [143], which is consistent with another study using A549 cells where FUT8 knockdown reduces EGF-induced cell proliferation [145]. Similarly, fucosylation on EGFR has been shown to protect EGFR from degradation upon ligand activation and thus enhance downstream signaling activation and cell motility in oral cancer cells [146]. Taken together, modification of N-glycans can alter the binding and consequences of binding of EGF to the EGFR and of the interaction of other cell surface glycoprotein with ErbB family receptors.

EGFR glycosylation not only affects receptor activity, but also the sensitivity of the activated EGFR to EGFR inhibitors. Tunicamycin significantly increases the inhibitory effect of erlotinib on

EGFR phosphorylation and cell proliferation in the erlotinib resistant lung cancer cell lines, A549 and H1650 [147]. Moreover, knockdown of FUT8 in A549 cells decreases the inhibitory effect of gefitinib on cell survival and colony formation, showing that EGFR fucosylation also affects sensitivity to gefitinib [145]. Further, sialylation of EGFR inhibitor resistant mutants with secondary T790M mutations is significantly higher compared to inhibitor sensitive mutants. Sialyltranserase inhibitor against α2,3-sialyltransferases and α2,6-sialyltransferase (ST6GalI) increases gefitinib sensitivity in lung cancer cells with EGFR L858R/T790M mutations consistent with this observation [144]. An increase in sensitivity to gefitinib was also observed in ST6GalI-deficient colon cancer cell lines [148]. Long-term treatment with AG1478, an EGFR inhibitor, disrupts EGFR glycosylation and facilitates the binding of mAb 806, a monoclonal antibody that binds to a short cysteine loop on the extracellular EGFR domain that is exposed in the untethered conformation, resulting in synergistically inhibition of *in vivo* tumor growth [149]. These findings raise a new potential direction for treating patients with drug-resistance EGFR mutations by combining EGFR inhibitors and with drugs that intervene at specific steps in the glycosylation pathway.

Recent data suggest that contrary to previous suggestions that the EGFR is O-glycosylated as well as N-glycosylated. O-GlcNAcylation of the EGFR was first identified in cytosolic extracts of Drosophila S2 cells by metabolic GlcNAc labeling and mass spectrometry [150]. In the human cancer cell lines A431 and A549, O-GlcNAc was detected on the EGFR and N-deglycosylation did not affect the presence of O-GlcNAc on EGFR [151]. Furthermore, the level of O-GlcNAcylation on EGFR increased when OGA activity was inhibited. OGT could be immunoprecipitated with EGFR and the level of O-GlcNAcylation of EGFR increased when the EGFR was incubated with OGT *in vitro*, showing that EGFR is a substrate of OGT [151]. There are three EGFR O-GlcNAcylation sites predicted by the YinOYang 1.2 O-GlcNAc prediction website, one in the juxtamembrane region (Thr678; Thr654 in an alternative numbering of the human EGFR sequence) and two in the catalytic domain (Ser1070 and Ser1071; Ser1046 and Ser1047 in an alternative numbering of the human EGFR sequence) [152]. Phosphorylation of EGFR Thr678 by PKC prevents desensitization of EGFR after activation by directing recycling of the endocytosed receptor back to cell surface instead of lysosomal degradation [153]. Thus glycosylation of this site would prevent access of PKC and could interfere with recycling. Further phosphorylation of Ser1070/1071 by Ca^{2+}/calmodulin-dependent kinase II (CaMKII) decreases ligand affinity [154,155] and O-GlcNAcylation of Ser1070 and Ser1071 would prevent phosphorylation of these sites. Thus, EGFR O-GlcNAcylation may regulate its recycling, stability and ligand affinity.

Other ErbBs can be targets for glycosylation (Table 1). ErbB3 Thr355, which is in N-glycosylation motif of Asn353 in subdomain III, is a mutational hot spot across human cancers [156]. Whether this mutation alters N-glycosylation and whether the altered glycosylation contributes to selection of this mutation is as yet unknown. Although the role of ErbB4 in oncogenesis is the least well characterized of the ErbBs, mutation of ErbB4 is frequently activating in melanoma [157] and lung cancers [158]. There are eight tumor-derived mutations that could result in loss of glycosylation of ErbB4. Asn181 mutation did not significantly affect neuregulin-1-induced ErB4 activation but did enhance ligand-dependent ErB2 phosphorylation [158], suggesting the loss of glycosylation at Asn181 could be oncogenic and may rewire downstream signaling.

3.2. β-catenin

β-catenin is encoded by *CTNNB1* and contains 781 amino acids (~92 kDa). β-catenin is the key regulator of canonical WNT signaling which plays a pivotal role in embryogenesis. Deregulation of WNT signaling has also been implicated in tumorigenesis of several cancers, including colon, lung, leukemia, breast, thyroid, and prostate [159]. In the absence of WNT signaling, cytosolic β-catenin is targeted to phosphorylation/ubiquitination-mediated degradation by a destruction complex consisting of adenomatous polyposis coli (APC), scaffold protein Axin, GSK3, and Casein Kinase 1α (CK1α). Sequential phosphorylation at the N-terminus destruction box of β-catenin on Ser45, Thr41, Ser37, and Ser33 by CK1α and GSK3 recruits E3 ubiquitin ligase β-transducin repeats-containing proteins to phosphorylated Ser33 and Ser37 [160] and triggers ubiquitination on Lys19 and Lys49 followed by proteasome degradation [161]. Upon WNT stimulation, the activity of GSK3 in the destruction complex is inhibited resulting in cytosolic β-catenin accumulation and nuclear translocation where β-catenin subsequently interacts with transcription factor TCF and activates gene transcription [162]. In addition to phosphorylation and ubiquitination, the stability and transcriptional activity of β-catenin can also be regulated by acetylation (reviewed in [163]) and also O-GlcNAcylation [164–167]. The role of O-GlcNAcylation in regulating β-catenin function, however, is controversial.

Stichelen et al. showed that the level of β-catenin O-GlcNAcylation in colon tumors is significantly higher when compared to normal tissues and the level of O-GlcNAcylation is positively correlated with the level of β-catenin [166]. Using mass spectrometry, they found four O-GlcNAcylation sites (Ser23, Thr40, Thr41, and Thr112) at the N-terminus destruction box (Figure 2). They further showed that increase in β-catenin O-GlcNAcylation by OGA knockdown decreases the level of phosphorylation of β-catenin on Ser33/Ser37/Thr41 and ubiquitination and enhances the cellular level of β-catenin, suggesting that O-GlcNAcylation positively regulates the stability of β-catenin. Site-directed mutagenesis found that Ser23, Thr40, and Thr112 are not involved in regulating β-catenin ubiquitination and stability. On the other hand, loss of Thr41 or all four O-GlcNAcylation sites significantly decreases ubiquitination and enhances cytosolic accumulation of β-catenin when compared with WT β-catenin, suggesting that O-GlcNAcylation and phosphorylation on Thr41 is critical in controlling the stability of β-catenin. In contrast, the Persad group proposed that normal prostate cells have a higher level of O-GlcNAcylated β-catenin when compared to prostate cancer cells and O-GlcNAcylation of β-catenin in cancer cells does not alter stability but rather alters subcellular localization and transcriptional activity of β-catenin. PUGNAc induces β-catenin plasma membrane translocation, decreases the amount of nuclear β-catenin and TCF transcriptional activity, without affecting the degradation rate and cellular level of β-catenin [165,168]. Moreover, they showed that the level of O-GlcNAcylation of cytosolic β-catenin is higher than that of nuclear β-catenin in prostate cancer cells, suggesting that O-GlcNAcylation negatively regulates β-catenin nuclear localization and transcriptional activity [165]. Later, they showed that O-GlcNAcylation regulates β-catenin subcellular localization and transcription activation through Ser23 O-GlcNAcylation [168]. Apart from the stability and transcriptional activity of β-catenin, both groups have also investigated the effect of β-catenin O-GlcNAcylation on the interaction with α-catenin and E-cadherin. Stichelen et al. showed that β-catenin O-GlcNAcylation interferes with the

interaction with α-catenin, but not E-cadherin in breast cancer cells [166], which is consistent with another study [164]. On the other hand, the Persad group showed that Ser23 mutation attenuates β-catenin-E-cadherin interaction induced by PUGNAc, suggesting that β-catenin O-GlcNAcylation regulates E-cadherin binding in prostate cancer cells [168]. Further evidence is needed to conclude whether the effect of O-GlcNAcylation on β-catenin is tissue or cancer type specific. These controversial results however do indicate that O-GlcNAcylation of β-catenin does alter its function, albeit through unclear mechanisms.

Apart from the N-terminus, the C-terminus of β-catenin has also been shown to have high basal level of O-GlcNAcylation [168]. The YinOYang 1.2 server predicts only one potential O-GlcNAcylation site (Thr779) in the C-terminus of β-catenin (Figure 2).

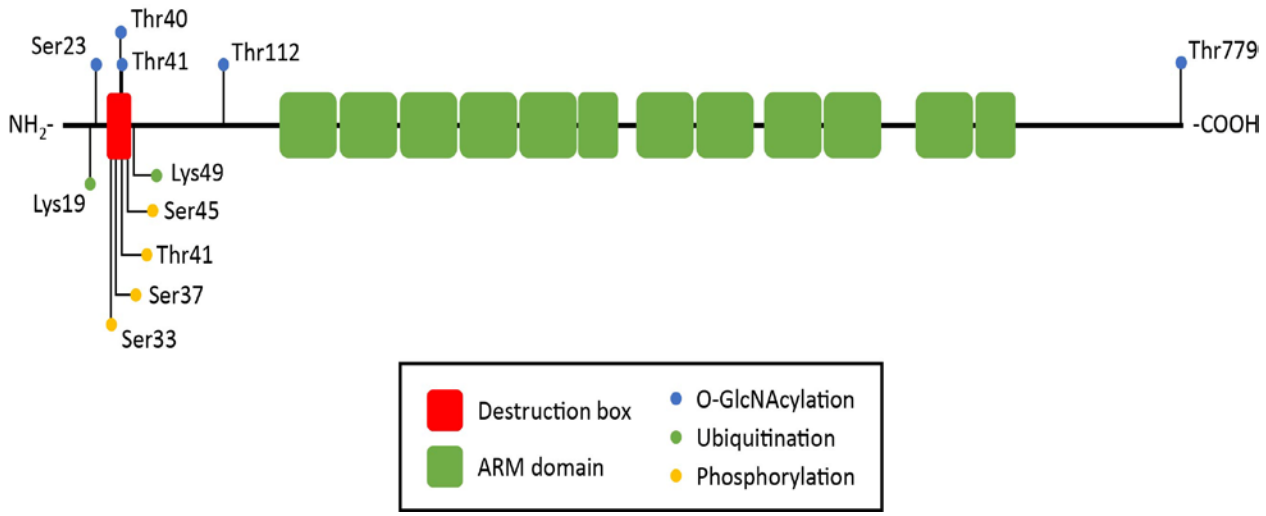

Figure 2. The glycosylation sites and the nearby post-translational modification sites in β-catenin.

CTNNB1 is one of the most frequent mutated genes in human cancers, especially in endometrial cancer where the frequency is over 25% [15]. There are mutations along the whole protein, but recurrent mutations, including missense mutation and in-frame deletion, concentrate at the N-terminus destruction box. Hot spot mutation residues include the key phosphorylation sites, Ser33, Ser37, Thr41, and Ser45. Mutation of these residues protects β-catenin from degradation. Frequent mutations at O-GlcNAcylation sites, Ser23 and Thr40, are commonly found in tumors. Overexpression of Ser23Arg, one of the patient-derived Ser23 mutants, in Jurkat cells did not induce transcriptional activation of the Tcf reporter [169], suggesting O-GlcNAcylation on Ser23 may affect transcriptional activity of β-catenin. This observation is opposing to that of the Persad group and needs resolution. A double mutation, Thr40Aln/Ile35Asn, found in hepatocarcinoma is mainly localized in the nucleus suggesting that loss of O-GlcNAcylation on Thr40 may alter transcriptional activation [170]. As the role of O-GlcNAcylation on β-catenin remains controversial, the contribution of loss of O-GlcNAcylation mutation on tumorigenesis needs further investigation.

4. Conclusion

In this review, we have summarized the role of glycosylation, one of the most frequent post-translational modification of proteins, in the potential regulation of the functions of some frequently mutated tumor suppressors and oncogenes. While in some cases a role for glycosylation has been clearly demonstrated, for most of the genes, the studies are in their infancy. Further the pleomorphic effects of glycosylation on function of the targets, and the lack of understanding of the rules regulating glycosylation of particular cancer drivers suggests that additional work will be needed prior to application as a target for therapy or as biomarkers. We also provided theoretical and supportive data suggesting that patient-derived mutations can lead to the loss of glycosylation sites potentially altering protein function including stability, localization and catalytic activity thus contributing to tumorigenesis. Single nucleotide variation (SNV)-mediated loss of glycosylation sites, especially N-glycosylation sites, is in fact a frequent event in human cancers [171–174], yet subsequent follow up biochemical studies on the impact of loss of glycosylation sites on protein function is lacking. Furthermore, systematic analysis of SNV-mediated gain of glycosylation sites is still limited [171].

As protein glycosylation is complex, altered glycosylation sites on tumor suppressor genes and oncogenes is only one of the possibilities on how altered glycosylation plays a role in carcinogenesis. Not only alterations in glycosylation sites, but aberrant expression [175] or mutation of glycosyltransferases or glycosidases [176] could also affect glycan structure that may play a role in tumorigenesis or drug sensitivity [177]. Moreover, a number of studies suggest that glycosylation has a role in regulating tumor related signaling pathways, such as cell cycle, genomic stability [91], and PI3K/Akt pathway [178–180]. Intensive studies on the correlation between genomics and glycosylation aberrations in cancer progression may shed light on novel opportunities for cancer therapy.

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Conflict of Interest

All authors declare no conflicts of interest in this paper.

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