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In silico Analysis of Molecular Mechanisms of *Galanthus nivalis* Agglutinin-Related Lectin-Induced Cancer Cell Death from Carbohydrate-Binding Motif Evolution Hypothesis

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Abstract *Galanthus nivalis* agglutinin-related lectins, a superfamily of strictly mannose-binding-specific lectins widespread amongst monotyledonous plants, have drawn a rising attention for their remarkable anti-proliferative and apoptosis-inducing activities toward various types of cancer cells; however, the precise molecular mechanisms by which they induce tumor cell apoptosis are still only rudimentarily understood. Herein, we found that the three conserved motifs "QXDXNXVXY," the mannose-specific binding sites, could mutate at one or more amino acid sites, which might be a driving force for the sequential evolution and thus ultimately leading to the complete disappearance of the three conserved motifs. In addition, we found that the motif evolution could result in the diversification of sugar-binding types that *G. nivalis* agglutinin-related lectins could bind from specific mannose receptors to more types of sugar-containing receptors in cancer cells. Subsequently, we indicated that some sugar-containing receptors such as TNFR1, EGFR, Hsp90, and Hsp70 could block downstream anti-apoptotic or survival signaling pathways, which, in turn, resulted in tumor cell apoptosis. Taken together, our hypothesis that carbohydrate-binding motif evolution may impact the *G. nivalis* agglutinin-related lectin-induced survival or anti-apoptotic pathways would provide a new perspective for further elucidating the intricate relationships between the carbohydrate-binding specificities and complex molecular mechanisms by which *G. nivalis* agglutinin-related lectins induce cancer cell death.

Keywords *Galanthus nivalis* agglutinin-related lectin · Evolution · Cancer · Apoptosis · Carbohydrate-binding motif

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Introduction

Plant lectins are reversible carbohydrate-binding proteins or glycoproteins of non-immuno origin that can agglutinate cells or precipitate polysaccharides and glycoconjugates [1]. The term "*G. nivalis* agglutinin-related lectins" refers to a superfamily of strictly mannose-binding specific lectins widespread among monotyledonous plants, possesses a broad range of biological functions, especially the antitumor activities [2, 3]. Previous studies held the idea that *G. nivalis* agglutinin-related lectins without any mutation of the active sites normally showed the mannose-binding specificity in order to maintain some important biological functions such as the anticancer property [4, 5]. And other previous reports demonstrated that there might be a close correlation between their carbohydrate-binding and antitumor activities [6, 7].

However, neither of these opinions was convincing enough to allow for any conclusion of the relationship between the carbohydrate-binding motif and antitumor activity. Interestingly, our previous reports demonstrated that the divergent evolved *G. nivalis* agglutinin-related lectins such as *Polygonatum cyrtonema* lectin [8] with two mutated motifs out of three, *Ophiopogon japonicus* lectin [9] with only one mutated exhibited more remarkable antitumor activity than *Polygonatum odoratum* lectin with three conserved active motifs [10]. More importantly, our studies further demonstrated that these *G. nivalis* agglutinin-related lectins could induce cancer cell death mainly through several important apoptotic-signaling pathways [3, 6, 7]. Of note, the development of cancer is close associated with apoptosis (type I programmed cell death), because it is a cell-intrinsic mechanism for suicide that can be regulated by several important cellular pathways [11]. Thus, based upon these aforementioned findings, whether the mutated carbohydrate-binding types bear on the generation of the multi-specificity and thus influencing on the antitumor activities should be further clarified.

In this study, we put forward a hypothesis that carbohydrate-binding motif evolution from *G. nivalis* agglutinin-related lectins could lead to the diversification of sugar-containing receptors that *G. nivalis* agglutinin-related lectins could bind, which blocked several anti-apoptotic or survival signaling pathways; thereby, ultimately culminating in cancer cell death.

Materials and Methods

Phylogenetic Analysis

The sequences of 81 *G. nivalis* agglutinin-related lectins were aligned using the ClustalW program [12]. The MEGA4 package [13] was used to build the phylogenetic trees. Alignment of proteins was performed with the Clustal W method, and the phylogenetic tree was calculated by using the neighbor joining method. The reliability of each branch was evaluated with bootstrap (1,000 times repeat).The phylogenetic tree is visualized using Treeillustrator [14].

Molecular Modeling

Domains of these lectin sequences were acquired by Prosite (http://www.expasy.ch/prosite/) [15], and these domains were further utilized to construct the three-dimensional structures of *G. nivalis* agglutinin-related lectins by MODELLER9v7 software [16] with the structure

of *G. nivalis* agglutinin (*G. nivalis* lectin, PDB ID: 1jpc) as the template. All the structures were optimized using Chimera program, including hydrogen atoms addition and energy minimizations, bound crystal water molecule, or other organic compounds elimination [17].

Molecular Docking

All molecular docking calculations were performed using the UCSF DOCK6.3 program [18] with the flexible ligand docking to a rigid receptor with grid-based scoring method. The flexible ligand docking algorithm in DOCK6.3 allows the ligand (sugars) to structurally rearrange in response to the receptors (*G. nivalis* agglutinin-related lectins) using the anchor-and-grow feature, which firstly identified the largest rigid substructure (the anchor) and the flexible layers of the ligand, then the flexible layers of the ligand were built onto the best anchor orientations within the context of the receptor (the growth stage). In our docking progress, we increased the maximum number of orientations to 1,000. In addition, the interactions were further analyzed between some typical *G. nivalis* agglutinin-related lectins and sugar-binding receptors by using RosettaDock Protein–protein docking server [19].

Results and Discussion

The Evolution from Carbohydrate-Binding Motif Mutations

In the previous studies, Els et al. have reported that *G. nivalis* agglutinin-related lectins do not represent a monophylogenetic group but most probably result from multiple independent domain duplications or in tandem insertion events [20–22]. Els et al. further indicated that plants used domain duplication followed by divergent evolution as a defense mechanism to generate multi-specific lectins from a single mannose-binding domain [2, 20]. In contrast to these previous studies, we found that *G. nivalis* agglutinin-related lectin evolved per se due to their sugar-binding motif mutation, which finally resulted in the disappearance of three conserved motifs. In regard to the so-called domain duplication, we indicated that the carbohydrate-binding motif evolution might occur firstly and then could keep this motif mutation-oriented evolutionary mode going on, accompanied with the subsequent occurrence of domain duplication. This hypothesis for carbohydrate-binding motif evolution of the present *G. nivalis* agglutinin-related lectins including 81 members could be supported by our phylogenetic analysis (Fig. 1). Therefore, these aforementioned results were in good agreement with our viewpoint that the evolutionary driven force might be from the motif mutation(s) of *G. nivalis* agglutinin-related lectins, which could occur before their domain duplication(s). In addition, we found that the orientation of evolution was from the three conserved motifs to three mutation motifs.

Motif Evolution Leads to Binding from Sole Mannose to More Types of Sugars

This motif evolution mode could result in the mutation of the conserved motif "QXDXNXVXY" and thus decreasing or losing mannose-binding capability. However, one or several amino acid mutation could link other neighboring amino acids to form a novel sugar-binding motif which might bind other types of sugars such as sialic acids. More importantly, this diversification of sugar-binding types would make *G. nivalis* agglutinin-related lectins have more possible opportunities to recognize more types of sugar-containing receptors on the surface of tumor cells or inside. As a result, we found that

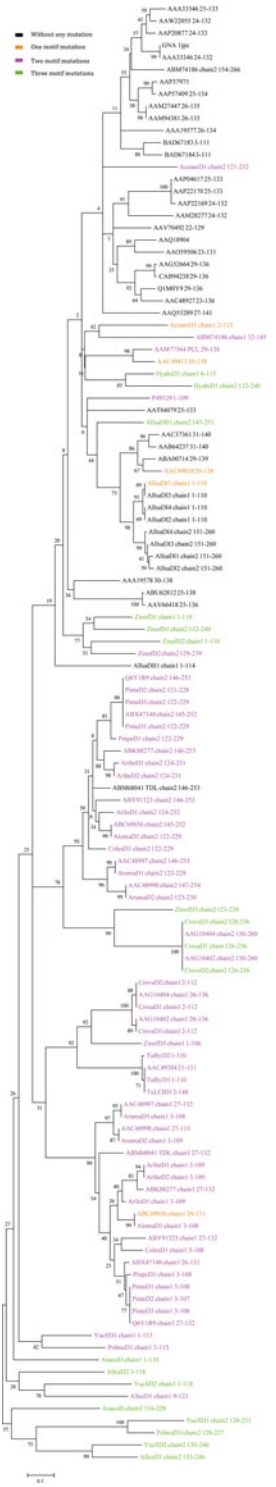

Fig. 1 Phylogenetic analysis of the present *G. nivalis* agglutinin-related lectins. The phylogenetic evolutionary tree indicated that the present *G. nivalis* agglutinin-related lectins could mutate at one or more amino acid sites, which was an original driving force for the sequential evolution and thus leading to the complete disappearance of the three conserved motifs "QXDXNXVXY"

several important *G. nivalis* agglutinin-related lectins such as *Acorus americanus* lectin (a typical *G. nivalis* agglutinin-related lectin with one motif mutation), *P. cyrtonema* lectin (a typical *G. nivalis* agglutinin-related lectin with two motif mutations), and *Yucca filamentosa* lectin (a typical *G. nivalis* agglutinin-related lectin with three motif mutations) could bind not only sole mannose but other types of sugars such as Beta-D Glucose, *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine, and sialic acids compared to *G. nivalis* agglutinin (Fig. 2). Herein, *A. americanus* lectin could bind alpha-D-mamnose, Beta-D glucose, *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine and sialic acids with binding energy −22.78, −22.55, −26.81, −25.50, and −26.79 kJ/mol, respectively; *P. cyrtonema* lectin could bind these sugars with binding energy −19.37, −20.60, −23.48, −24.59, and −30.15 kJ/mol, respectively, and *Y. filamentosa* lectin −22.45, −24.38, −24.97, −24.07, and −38.36 kJ/mol, respectively. Additionally, we found that *P. cyrtonema* lectin could bind mannose in the conserved motif but sialic acid in the mutated motif with more strongly binding capability. Additionally, we found that *Y. filamentosa* lectin, containing three mutated motifs, could constitute a novel sugar-binding domain located in the center of its molecular structure, which may possess a better affinity to bind sugars than others with fewer mutated motifs. For instance, sialic acids, the most prevalent terminal monosaccharides of glycoconjugates in the

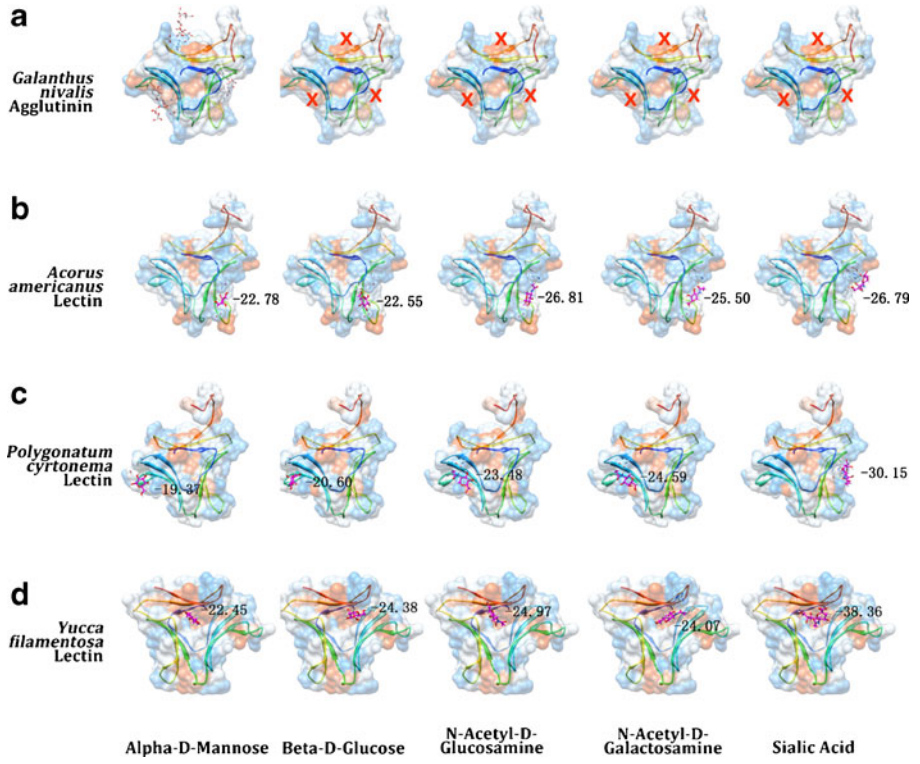

Fig. 2 Molecular modeling and docking of *G. nivalis* agglutinin-related lectins with binding different types of sugars. The primary screening sugar-binding types of *G. nivalis* agglutinin-related lectins indicated that the motif evolution could result in the diversification of sugar-binding types that *G. nivalis* agglutinin-related lectins could bind from specific mannose- to more sugar-binding types. **a** *G. nivalis* agglutinin (*G. nivalis* agglutinin) with three conserved motifs; **b** *A. americanus* lectin with one motif mutation; **c** *P. cyrtonema* lectin with two motif mutations; **d** *Y. filamentosa* lectin with three motif mutations

membrane of cells, are attached to Galβ1, 3(4) GlcNAc/Glc with different linkages such as α-2,3 [23]. Previous studies have shown that carcinogenesis, invasion, and metastasis of tumor cells are related to aberrant expression of terminal sialic acids, and in particular a 2,3-linked sialic acids [24]. In this study, we analyzed that several typical *G. nivalis* agglutinin-related lectins such as *P. cyrtonema* lectin could bind α-2,3-linked sialic acids that belonged to a number of receptors such as TNFR1 and EGFR on the surface of cancer cells. To our knowledge, several key receptors that contain α-2,3-linked sialic acids have been found to enrich on the surface of cancer cells or inside. Thus, it is suggested that *G. nivalis* agglutinin-related lectins can bind not only single mannose-containing receptors but also more types of sugar-containing receptors that may play the key roles in the carcinogenesis, invasion, and metastasis of cancer cells.

Sugar-Containing Receptors may Initiate Several Anti-apoptotic Pathways

Recent studies have reported that several sialic-containing receptors such as Hsp70, Hsp90, Hsp96, and TNFR1 that are involved in several anti-apoptotic or survival pathways [25, 26]. Due to their nature of anti-apoptosis, *G. nivalis* agglutinin-related lectins such as *Polygonatum multiflorum* lectin with one motif mutation, *P. cyrtonema* lectin with two motif mutations, and *Y. filamentosa* lectin with three motif mutations bound them, respectively, and switched off the anti-apoptotic pathways; thereby, playing the pro-apoptotic roles in downregulating-related signaling pathways (Fig. 3). For instance, TNFR1 is well known to be found on most cells in the body and activated by soluble ligand [27]. TNFR1 activation can have two different end results that are dependent on the cellular context [28]. The default pathway is induction of genes implicated in inflammation and cell survival. Ligand binding to TNFR1 induces a range of inflammatory mediators and growth factors through activation of the AP1 transcription factors or IκB kinases that, in turn, activate nuclear factor–κB (NF–κB). NF–κB activation also importantly induces negative regulators of apoptosis. If NF–κB activation is inadequate, apoptosis is mediated through caspase-8 and, through accumulation of intracellular reactive oxygen, sustained Jun amino-terminal kinase activation and mitochondrial pathways. Also, apoptosis is a late response to TNF, unlike the rapid apoptosis that is induced by other members of the TNF superfamily [29]. In our study, we indicated that *G. nivalis* agglutinin-related lectins could bind TNFR1 and thus switching off some anti-apoptotic signaling pathways in cancer cells (Fig. 4).

Furthermore, *G. nivalis* agglutinin-related could bind epidermal growth factor receptor (EGFR) and thus blocking the EGFR-activated anti-apoptotic pathways such as phosphatidylinositol 3-kinase (PI3K)-Akt, Ras-Raf, and JAK-STAT pathways. EGFR phosphorylation can directly or indirectly activate STAT1, STAT3, and STAT5. The activated STAT proteins translocate into the nucleus and directly regulate gene expression crucial for cell survival, proliferation, transformation, and oncogenesis [30]. In addition, EGFR activates PI3K that phosphorylates phosphatidylinositol 4,5-biphosphate to form phosphatidylinositol 3,4,5-triphosphate, which then activates Akt by binding at its pleckstrin homology (PH) domain. Phosphorylated Akt has several effects, both in the cytoplasm and in the nucleus, which include the inhibition of pro-apoptotic factors such as BAD, caspase9, and FOXO. Akt-mediated activation of mammalian target of rapamycin (mTOR) is also important in stimulating cell proliferation [31]. Moreover, activated Ras binds to Raf, which, in turn, triggers the phosphorylation of MEK1/2 (mitogen-activated protein kinase kinase 1/2) and ERK1/2 (extracellular signal-regulated kinase 1/2) [32]. Therefore, we indicated that *G. nivalis* agglutinin-related lectins bound EGFR and thus blocking some anti-apoptotic/survival signaling pathways (Fig. 4).

Interestingly, heat shock proteins (Hsps) are overexpressed in a wide range of human cancers and are implicated in tumor cell proliferation, differentiation, invasion, and metastasis [33]. In combination with previous studies [34], we found that *G. nivalis* agglutinin-related lectins could bind both Hsp90 and Hsp70 and thus blocking their relative anti-apoptotic pathways in cancer cells. Accordingly, in combination with previous

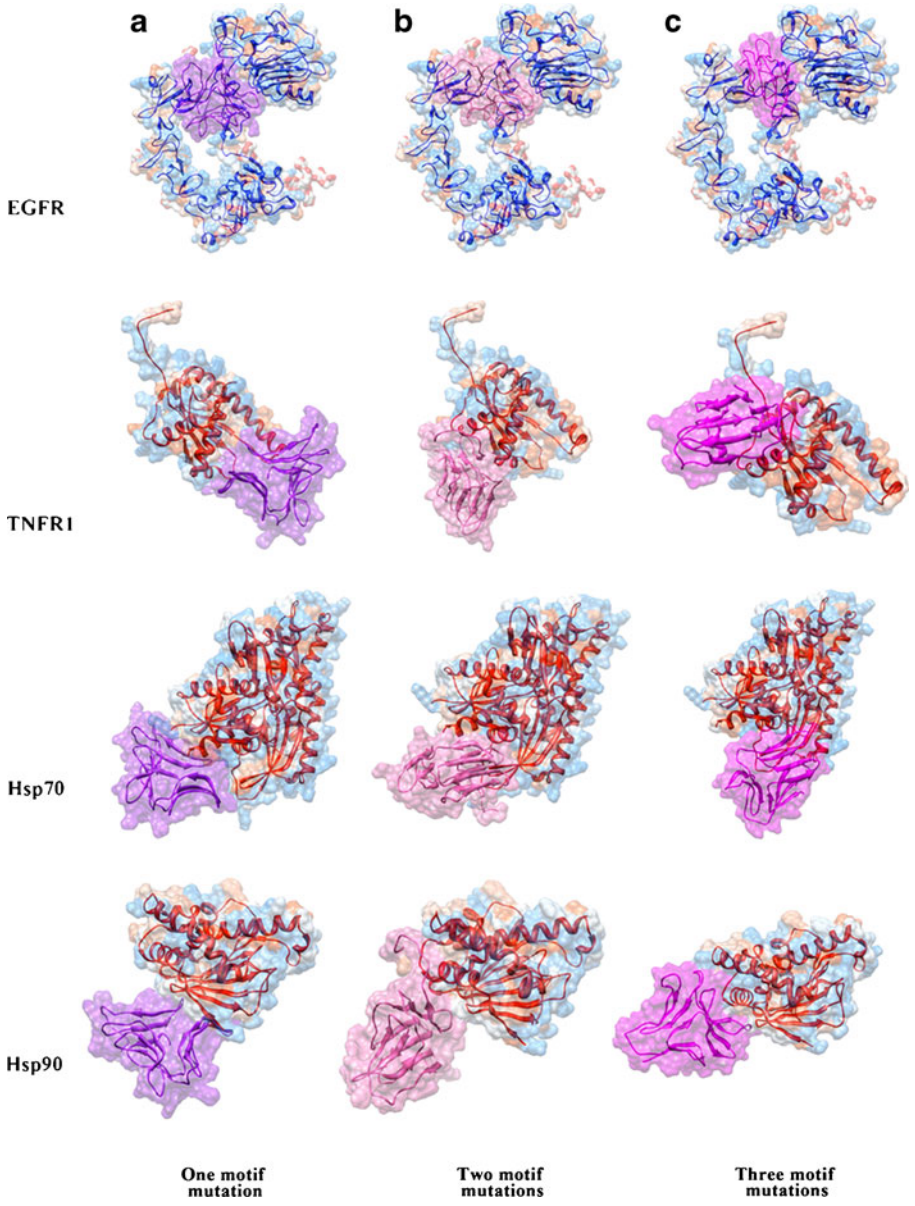

Fig. 3 The predictive interactions between some *G. nivalis* agglutinin-related lectins and sugar-containing receptors. *G. nivalis* agglutinin-related lectins could bind from specific mannose- to more typical sugar-containing receptors in a variety of cancer cells. **a** *P. multiflorum* lectin with one motif mutation; **b** *P. cyrtonema* lectin with two motif mutations; **c** *Y. filamentosa* lectin with three motif mutations

experimental data [8–10], we could explain that why several *G. nivalis* agglutinin-related lectins could induce tumor cell apoptosis and further explored their elegant apoptotic pathways (Fig. 4).

G. nivalis Agglutinin-Related Lectins may Block Some Receptor-Mediated Anti-apoptotic Pathways

Based upon these abovementioned findings, we used bibliometrics and related data mining methods to analyze a few of important sugar-containing receptors such as TNFR1, EGFR, Hsp70, and Hsp90 on or inside cancer cells. Subsequently, we further analyzed these receptor-mediated signaling pathways by using curated literature [35] and pathway databases [36]. As a result, we indicate that what kind of sugar-containing receptors are able to be associated with significant cancer-related signaling pathways and thus may seek the "best" lectin-binding targets in cancer cells. For instance, it is well known that high expression of α 2,3-linked sialic acid residues is associated with the metastatic potential of human cancer [37]. According to the sialic-acid-containing receptors, we could screen all the present *G. nivalis* agglutinin-related lectins and find the perfect match sugar-binding types that can bind the receptors and block the key anti-apoptotic signaling pathways [38–40]. In addition, we would further design the very sugar-binding types that can bind the right carbohydrate-containing receptors virtually and make the candidate lectins as what we would need. These results would provide a novel insight into how a "synthetic" *G. nivalis*

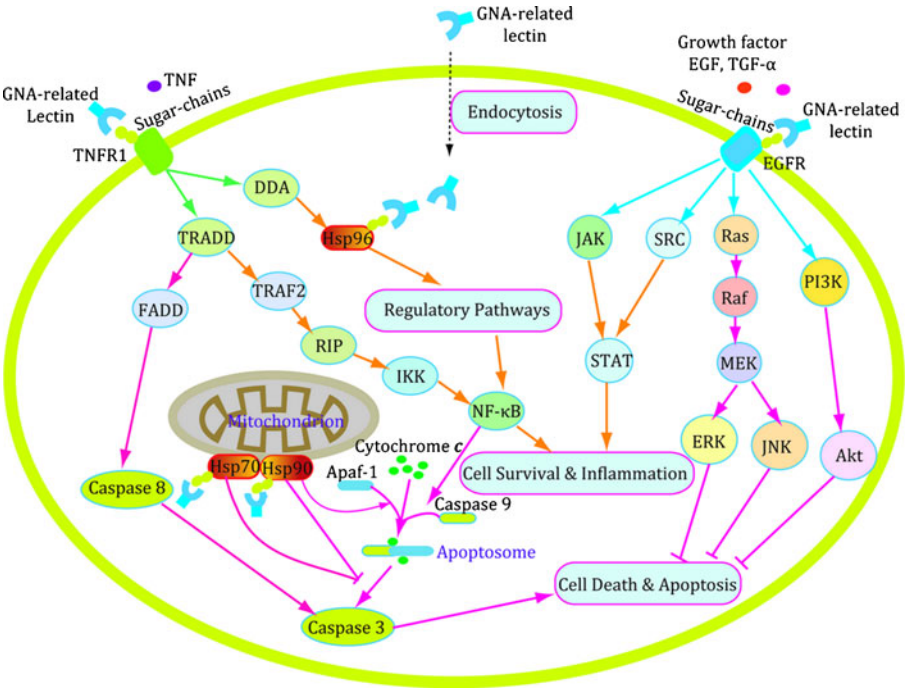

Fig. 4 *G. nivalis* agglutinin-related lectins may block receptor-mediated survival/anti-apoptotic signaling pathways. *G. nivalis* agglutinin-related lectins could block several important receptor-mediated anti-apoptotic signaling pathways involving TNFR1-mediated signaling pathways, EGFR-mediated signaling pathways, Hsp70 family-involved signaling pathways, and Hsp90 family-involved signaling pathways

agglutinin-related lectin could play its "optimal" antineoplastic role via regulating several anti-apoptotic pathways.

Conclusions

Due to the aforementioned hypothesis-driven discovery, we explored the intricate relationship between carbohydrate-binding motifs and antitumor mechanisms of the present *G. nivalis* agglutinin-related lectins. In the three sugar-binding active centers of *G. nivalis* agglutinin-related lectins, the mutations of one or more amino acids could lead to the subsequent phylogenetic evolution, which changed from the mannose-binding specificity to more diversified sugar types at molecular level. As a result, this molecular evolution of *G. nivalis* agglutinin-related lectins could give raise to that these lectins with more mutated sugar-binding sites would be speculated to possess more opportunities to bind different types of carbohydrate-containing receptors on the surface of cancer cells than those with the three conserved ones. Based upon all the present *G. nivalis* agglutinin-related lectins, we found that a few of *G. nivalis* agglutinin-related lectins with two or three mutated sites were able to bind more types of sugar chains and carbohydrate-containing receptors. More importantly, due to virtual screening of all possible carbohydrate-binding types of *G. nivalis* agglutinin-related lectins, we indicated that several "ideal" lectins with the very sugar-binding types could bind the "right" carbohydrate-containing receptors that might subsequently switch on/off downstream cancer-related signaling pathways. Lastly, these significant signaling pathways would play the crucial roles in regulating the whole cancer-related signaling pathways in *G. nivalis* agglutinin-related lectin-induced cancer cell death, respectively, or cooperatively.

In summary, these inspiring findings may provide a novel comprehensive perspective for elucidating why *G. nivalis* agglutinin-related lectins can induce tumor cell death by means of different signaling pathways and how they play the pivotal roles under different circumstances? Furthermore, it may therefore provide us more key clues to design some synthetic "ideal" *G. nivalis* agglutinin-related lectins that could initiate the very cancer-related signaling pathways to function as potential anti-neoplastic drugs for future cancer therapeutics.

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