A study on TRAIL receptor DR4 modification for the regulation of cell death in cancer
DR4 Ser424 mutation avoids O-GlcNAcylation and cell death in TRAIL-resistant cancer

TRAIL (TNF-related apoptosis-inducing ligand) death receptors are critical starting points for triggering cancer cell-selective death. However, cancer cells have evolved a defensive pathway that escapes from and suppresses tumor cell death, increasing expression of antiapoptotic Bcl2 family and impairing function of proapoptotic cell death machinery. Among two cognitive receptors, DR4 and DR5, I discover a novel DR4-specific modification from cell-based screen using cancer patient-derived cDNA library. From the functional screen, a mutant form of DR4 was isolated as a suppressor of TRAIL-induced cell death. Here I show that O-GlcNAcylation of DR4 Ser424 within death domain (DD), but not of DR5, plays an important role for both apoptosis and necrosis induced by TRAIL ligation. Mutants of DR4 in which Ser424 was replaced by either proline (S424P) or alanine (S424A) lost
their cytotoxic activity due to $O$-GlcNAcylation impairment. TRAIL stimulation augmented DR4 $O$-GlcNAcylation via increased assembly with OGT. Compared to wild-type DR4, $O$-GlcNAcylation-defective DR4 mutant was less translocated to the aggregated compartment, showed reduced receptor clustering and was incapable of DISC formation. Interestingly, I found that TRAIL-resistant and DR4-preferring cancer cells were defective to DR4 $O$-GlcNAcylation, showing significant association between $O$-GlcNAcylation and cell’s sensitivity to TRAIL. Moreover, promoting DR4 $O$-GlcNAcylation by combinational treatment with TRAIL and either 2-DG or high glucose sensitized TRAIL-resistant cancer cells to cell death. In conclusion, our findings demonstrate that mutation of DR4 Ser424 and subsequent absence of $O$-GlcNAcylation can cause TRAIL resistance in cancer. The strategies for elevating DR4 $O$-GlcNAcylation should be considered for therapeutic approach of TRAIL.

Key word: TRAIL, DR4, $O$-GlcNAcylation, cell death, TRAIL-resistant cancer

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1. INTRODUCTION

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand, or TRAIL, has initially received attention as a promising antitumor therapeutics that can kill selectively cancer cells without causing lethal toxicity in normal cells or in vivo (von Karstedt et al., 2017). Given this attractive ability, TRAIL research has expanded to the study of TRAIL receptor and subsequently to development of various TRAIL-receptor agonists as therapeutic candidates for cancer prevention (Lemke et al., 2014). Specific agonists of each receptor have been tested for clinical usages, such as Mapatumumab for DR4 and Lexatumumab for DR5 (Naoum et al., 2017). It revealed, however, that clinical trials of TRAIL application failed to achieve beneficial anticancer activity and agonist therapies exhibited limited efficacy (Holland, 2014). Normal cells or tissues are damaged from TRAIL application with unexpected hepatotoxicity in preclinical studies (Zuch de Zafra et al., 2016), while many cancer cells or primary cancers are resistant to TRAIL owing to multiple causes (LeBlanc et
al., 2002). TRAIL itself also displays several weak points including instability and inefficient delivery (Stuckey and Shah, 2013), and even TRAIL receptors have pleiotropic effects on tumorigenesis and metastasis (von Karstedt et al., 2015). Clinical trials of agonists have not been advanced into phase III studies (Lemke et al., 2014). Given these disadvantages, overcoming strategies are needed to optimize the activity and efficacy of TRAIL as well as agonists, and to translate this promising ligand successfully into the cancer clinic. Combining of TRAIL with radio- or chemotherapies has been evaluated in preclinical validations (Alexiou et al., 2015). Exploring genetic or proteomic profiles to explain the resistance mechanisms in cancer cells is also one of approaches in an effort to develop innovative TRAIL-based therapeutic concepts (Lemke et al., 2014).

Two human TRAIL receptors, DR4/TRAIL-R1 and DR5/TRAIL-R2, are required for TRAIL-induced apoptosis, necrosis and gene-activatory pathways, including NF-κB signaling (Wajant, 2017). In general, after ligation with TRAIL, receptors induce the recruitment of proteins, such as adaptor protein Fas-associated
death domain protein (FADD) and caspase-8/10, to organize a death-inducing signaling complex (DISC) at the membrane-associated regions (Ashkenazi, 2008). This enables subsequent activation of downstream cytosolic events via enhancing the cleavage of effector caspases resulting in cell elimination (Wajant, 2017; Bodmer et al., 2000). To elicit adequate outcomes from TRAIL signaling, it is very important to understand more precisely how receptors are regulated and what kinds of characteristics they have. Particularly, death domain (DD) in receptors, shared with members of the TNF-receptor superfamily, is vitally required for utilizing the same set of signaling proteins to induce cytotoxic and pro-inflammatory activities (Lafont et al., 2017).

Since two receptors are similar but also different as well, they are regulated distinctively. First, several posttranslational modifications of receptors have been reported to date. For example, N-glycosylation of DR4 Asn156 and mouse TRAIL receptor plays important regulatory roles in TRAIL-mediated apoptosis and immune surveillance (Dufour et al., 2017), and ubiquitination of DR4 at Lys273 decreases
TRAIL sensitivity in human gastric cancer cells (Wang et al., 2017; van de Kooij et al., 2013). Defective S-nitrosylation of DR4 Cys336 in the cytoplasmic domain reduces TRAIL sensitivity (Tang et al., 2006), and S-palmitoylation, lipid modification, of DR4 on the cysteine triplet (Cys261-263) enables efficient DR4-mediated death signaling (Rossin et al., 2009). O-glycosylation of DR5 by GALNT14 is essential for the formation of TRAIL DISC and subsequent apoptosis (Wagner et al., 2007).

Second, spatial dynamics of receptors strongly affect TRAIL sensitivity of cancer cells. The deficiency of surface expression is somewhat responsible for causing resistance (Austin et al., 2006), and translocation into lipid rafts, specialized membrane microdomains comprised of glycolipoproteins (Rossin et al., 2009), facilitates receptor-mediated signaling (Song et al., 2007). Since lipid rafts serve as platforms for organizing proteins to initiate various signaling efficiently and synergistically, these compartmentalized regions are used for recruiting and activating of protein complexes (Rietveld and Simons, 1998). In addition, cancer cells show different preferences of receptor to trigger TRAIL signaling, indicating that receptor primarily mediating
TRAIL cytotoxicity varies in cell type-dependent manner (van Roosmalen et al., 2014). Specific targeting and manipulation of each receptor, thus, should be considered for clinical practice.

*O*-linked $\beta$-N-acetylgalactosamine (O-GlcNAc) modification, termed *O*-GlcNAcylation, is a conserved posttranslational modification at serine or threonine residues regulated by enzymes *O*-GlcNAc transferase (OGT) and *O*-linked $\beta$-N-acetylgalactosaminase (O-GlcNAcase) (Hart et al., 2007). This sugar modification serves as a nutrient sensor for metabolic status and is responsible for basic cellular regulations and disease processes in response to nutritional and hormonal contexts (Wells et al., 2001; Guo et al., 2014). *O*-GlcNAcylation is widely involved in almost all pathways, including cell cycle regulation (Sakabe and Hart, 2010), transcription (Iyer et al., 2003) and translation (Zeidan et al., 2010), protein trafficking (Chun et al., 2017), stress signaling and autophagy (Guo et al., 2014). UDP-GlcNAc derived from glucose via the hexosamine biosynthetic pathway (HBP) is a main high-energy donor which is added to target proteins by OGT and is removed hydrolytically by *O*-
GlcNAcase (Ruan et al., 2013). Cross talks between \( O\)-GlcNAcylation and other posttranslational modifications are well established. In particular, phosphorylation has extensive cross talks reciprocally, sequentially, or competitively, because the serine/threonine sites of \( O\)-GlcNAcylation and phosphorylation are often the same or proximal (Hart et al., 2011). Although \( O\)-GlcNAc modification occurs primarily on nuclear and cytoplasmic proteins, this modification is capable of mediating the critical signaling at the plasma membrane via interaction of OGT with phosphatidylinositol trisphosphate (Yang et al., 2008). However, the modification of DR4 and DR5 by \( O\)-GlcNAcylation and their roles in tumorigenesis are unknown.

In the present study, I conducted cell-based functional screen to identify a novel causative factor for TRAIL resistance in cancer cells and isolated a DR4 Ser424 mutant as a suppressor of TRAIL-mediated cell death. Using biochemical and molecular analysis, I demonstrate that \( O\)-GlcNAcylation of DR4 Ser424 functions as a crucial interface between DR4 and tumor-selective killing mechanism of TRAIL. Deficiency of DR4 \( O\)-GlcNAcylation renders cancer cells resistant to both apoptosis
and necrosis triggered by TRAIL-DR4 axis, suggesting that DR4 O-GlcNAcylation status may be a determining factor of TRAIL sensitivity in DR4-preferred cancer cells.

Together, I propose a prospective overcoming strategy to clinical application of TRAIL and provide a reliable biomarker to select cancer patients.
2. MATERIALS AND METHODS

2.1. Cell culture and DNA transfection

Human stomach cancer cells (SNU lines) were obtained from Cancer Research Institute of Seoul National Hospital, Seoul National University, and all other cells were from the American type culture collection (ATCC, LGC standards, Molsheim, France). Human colon cancer cells DLD-1 and HT-29, human stomach carcinoma cells SNU lines and lung cancer cells H460 were cultured in Roswell Park Memorial Institute medium (RPMI-1640; Hyclone, Logan, UT, USA). Other cells we used were maintained in Dulbecco’s Modified Eagles Medium (DMEM; Hyclone), and all cells were cultured with 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA) and penicillin/streptomycin (Gibco) in 5% CO2. For experiments with various glucose concentrations, cells were incubated in either no Glucose RPMI-1640 or DMEM medium (Gibco), supplemented with or without indicated concentration of D-(+)-Glucose (Sigma-Aldrich, St. Louis, MO, USA). Transfection was performed with
iN-fect (iNtRON Biotechnology, Korea) or polyethylenimine (PEI; Sigma-Aldrich) following the manufacturer’s instructions.

2.2. Generation of stable cell lines

OGT-deficient cell lines were generated using the CRISPR/Cas9 gene editing technique as described by Shalem et al., 2014. Briefly, lentiviral delivery of both Cas9 and sgRNA targeting OGT (sgOGT) into cells were carried out and then cells were selected with puromycin (Sigma-Aldrich). For generation of HeLa/RIPK3-HA stable cells, HeLa cells were transfected with RIPK3-HA using iN-fect reagent for 24 h and then grown in selection medium containing 1 mg/ml G418 (Invitrogen, Carlsbad, CA, USA) for 2 weeks. After single cell cloning, the clones were screened by western blot analysis.

2.3. Reagents and antibodies
The following chemicals were used: TRAIL (Invitrogen); SM-164 (APExBIO, ApexBio Technology Corp., Houston, TX, USA); 6-diazo-5-oxo-L-norleucine (DON), 2-deoxy-D-glucose (2-DG), cycloheximide (CHX), A23187 (Sigma-Aldrich); TNFα (Merck Millipore, Burlington, MA, USA); Thiamet G (TMG; Cayman Chemical, MI, USA). For western blotting, following antibodies were used:

anti-\textit{O}-GlcNAc (RL2) antibody (Thermo Fisher Scientific, Waltham, MA, USA); anti-\textit{O}-GlcNAc (CTD110.6), anti-DR4, anti-DR5, anti-caspase-8, anti-RIP3 (E1Z1D) antibodies (Cell Signaling, Beverly, MA, USA); anti-caspase-3, anti-OGT, anti-MLKL, anti-vimentin antibodies (Genetex, Irvine, CA, USA); anti- PARP-1, anti-GAPDH, anti-tubulin, anti-TOMM20, anti-calnexin antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-FLAG, anti-phospho-serine antibodies (Sigma-Aldrich); anti-phospho-MLKL (p-S358), anti-caveolin1 antibodies (Abcam, Cambridge, UK); and anti-RIP antibody (BD Biosciences, San Jose, CA, USA). For blocking DR5, recombinant human TRAIL R2/Fc chimera (αDR5; Diaclone, Fracne) were used.
2.4. Plasmid construction

DR4 wild-type constructs have been reported previously (Jeon et al., 2008; Oh et al., 2012). DR4 Ser424 mutants (S424P, S424A, S424P and S424L) were generated by site-directed mutagenesis through PCR using primers containing the corresponding mutations: S424P-5', 5'-CGG AAC GCC CCG ATC CAC ACC-3'; S424P-3', 5'-GGT GTG GAT CGG GCC GTT CCG-3'; S424A-5', 5'-CGG AAC GCC GCG ATC CAC ACC-3'; S424A-3', 5'-GGT GTG GAT CGC GGC GTT CCG-3'; S424D-5', 5'-GGA CGG AAC GCC GCG ATC CAC ACC CTG-3'; S424D-3', 5'-CAG GGT GTG GAT ATC GCC GTC GTT CCG-3'; S424L-5', 5'-GGA CGG AAC GCC GCG ATC CAC ACC CTG-3'; S424L-3', 5'-CAG GGT GTG GAT ATC GCC GTC GTT CCG-3'. DR4 SNPs were also generated by PCR using following primers: G422A-5', 5'-GAT CAG AAC ATC CTG GAG CC-3'; G422A-3', 5'-GGC TCC AGG ATG TTC TGA TC-3'; C626G-5', 5'-AGT GCA GCA GAG GGT GCC CC-3'; C626G-3', 5'-GGG GCA CCC TCT GCT GCA CT-3'; A683C-5', 5'-GTG ACA TCG
CGT GTG TCC AC-3'; A683C-3', 5'-GTG GAC ACA CGC GAT GTC AC-3';
A1322G-5', 5'-AGA CAT GCA AGA GAG AAG AT-3'; A1322G-3', 5'-ATC TTC TCT CTT GCA TGT CT-3'. All mutants were verified through DNA sequencing analysis. DR4 deletion mutant (1-423) was subcloned into pcDNA3-HA using following primers: DR4[1-423]-5'-HindIII, 5'-CCC AAG CTT GGG ATG GCG CCA CCA CCA GCT-3'; DR4[1-423]-3'-KpnI, 5'-CGG GGT ACC CCG GGC GTT CCG TCC AGT TTT-3'.

2.5. CRISPR/Cas9 gene editing

For genomic engineering of OGT through CRISPR/Cas9 system, following single guide RNA sequences (OGT sgRNAs) were used: sgOGT#1-5', 5'-CAC CGT GCA GTG TTA TAC GCG TG C C-3'; sgOGT#1-3', 5'-AAA CGG CAC GCG TA T AAC ACT GCA C-3'; sgOGT#2-5', 5'- CAC CGC TCT GTA A TG GGC ACA CCA C-3'; sgOGT#2-3', 5'- AAA CGT GGT GTG CCC ATT ACA GAG C-3'. sgRNA oligos were annealed and cloned into the BsmBI (Fermentas, Waltham, MA, USA)
sites of lentiCRISPR transfer plamid (pXPR_001; Addgene, Cambridge, MA, USA) for virus production. Viral vectors were produced in HEK293T cells, and cells of interest were infected.

2.6. Patient-derived cDNA library screen

HeLa cells were co-transfected with pEGFP-N1 plasmid together with human cDNA library from various cancer cell lines of patients. This cDNA library was obtained from Korea Research Institute of Bioscience & Biotechnology (KRIIBB, Daejeon, Korea), prepared by cloning of full-length cDNAs in a mammalian expression vector and purchase of membrane cDNA expression library. After 24 h transfection, cells were treated with TRAIL for additional 6 h. Bcl-2 and c-FLIP_L were used as positive controls. Viability of GFP-positive cells was assessed as a readout under a fluorescence microscope (Olympus, Tokyo, Japan).

2.7. Cell death assays
Cell viability was examined as described by Jouan-Lanhouet et al., 2012. Briefly, microscopic detection of apoptotic or necrotic death after TRAIL-apoptosis or TRAIL-necrosis stimulation was performed in both adherent and floating cells. Nuclear chromatin staining with 1 μg/ml Hoechst 33342 (Molecular Probes, Oregon, USA) and 1 μg/ml propidium iodide (PI; Sigma-Aldrich) for 5-15 min. Apoptotic ratio was measured from cells with fragmented blue nuclei among PI-positive signals, while necrotic cells were counted with non-fragmented red nuclei among total population (n = 300-600 cells). Cell death was also determined by trypan blue exclusion assays.

2.8. O-GlcNAcylation assays and fractionation analysis

For O-GlcNAc detection, cells were pre-incubated in glucose-free medium for 30 min and then exposed to TRAIL in glucose-supplemented medium. Cell extracts were prepared as described by Wang et al., 2012, with some modifications. Briefly, cells were washed twice with PBS, harvested with lysis buffer (50 mM Tris-
HCl (pH 8.0), 150 mM NaCl, 10% Glycerol, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 10 mM TMG and protease inhibitor cocktail from Quartett, Berlin, Germany), and incubated for 20 min at 4°C with agitation. After centrifugation at 20,000 × g for 10 min at 4°C, the supernatant was collected as Sup fraction. The pellet was further lized with Urea-SDS lysis buffer (8 M Urea, 20 mM Tris-HCl (pH 7.0), 150 mM NaCl, 10% Glycerol, 1% SDS, 5 mM DTT, 10 mM TMG and protease inhibitor cocktail) for 30 min at room temperature. Nine volumes of lysis buffer were added for dilution the final SDS concentration to 0.1% and samples were resuspended through pipetting several times for relieving viscous state. After centrifugation at 20,000 × g for 10 min at 4°C, the supernatant was harvested as P-SDS fraction. O-GlcNAcylated proteins were immunoprecipitated primarily from this P-SDS fraction using anti-DR4 antibody.

2.9. Immunoprecipitation (IP) and western blotting

Cell extracts were mixed with corresponding antibodies for overnight
rocking at 4°C and pulled down by protein G Sepharose beads (GE Healthcare, Fairfield, CT, USA). For FLAG pull-down and sWGA pull-down analysis, cell extract was incubated with anti-FLAG M2-agarose beads (Sigma-Aldrich) or GlcNAc-specific agarose-conjugated succinylated wheat germ (sWGA; Vector Laboratories, Burlingame, CA, USA) for 3 h rocking at 4°C. The beads were pelleted and washed with PBS or lysis buffer 3-5 times repetitively. For immunoblotting, the same amounts of protein were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto the polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). Immunoblot analysis was then carried out and visualized by the enhanced chemiluminescence method.

2.10. DISC isolation

Cells were stimulated with TRAIL in either glucose-free or complete medium for 20-60 min at 37°C. For crude exclusion of cytosolic fraction, following steps were performed. Cells were washed twice with ice-cold PBS and then lysed
immediately in 0.1% NP-40 lysis buffer for 5-10 min on ice. After centrifugation at 1,000 \times g for 10 min at 4°C, pellets were resuspended in 1% Triton X-100 lysis buffer for 20-30 min at 4°C with agitation, and centrifuged at 20,000 \times g for 20 min at 4°C. The supernatant was subjected to DISC-IP with anti-DR4 antibody for selective isolation of specific TRAIL DISC comprised of DR4, not DR5, namely DR4-bound membrane-associated complex.

2.11. Generation of DLD-1/Low and DLD-1/R cell lines

DLD-1/Low cells, adapted to low-glucose condition, were generated by selecting healthy population by incubating cells in culture medium containing low glucose concentration (5.5 mM) for 1 month to mimic the chronic glucose-starved system. DLD-1/R cells, TRAIL-resistant DLD-1 cells, were obtained by selecting cells that survived from 50-100 ng/ml TRAIL exposure over a period of 2 months.
2.12. Statistical analysis

Data are expressed as mean ± S.E.M. \( (n = 3) \). Statistical comparisons between groups were performed using one-way or two-way analysis of variance (ANOVA) followed by either Bonferroni’s post-hoc test or Tukey’s post-hoc test as appropriate. All analyses were performed using Prism version 5.03 software (GraphPad Software, San Diego, CA, USA). *\( P<0.05 \) were considered statistically significant.
3. RESULTS

3.1. DR4 Ser424Pro mutant identified from cancer-derived cDNA library blocks cytotoxicity

To isolate a regulator of TRAIL-mediated cell death, I performed gain-of-function screen using cell death-rescue assay. Total 1,099 full-length cDNAs in mammalian expression vector were prepared from cancer patient-originated cancer cells. For a cell death-rescue assay, HeLa cells were transfected with the cDNA together with EGFP-N1 and then treated with TRAIL. Viability of GFP-positive cells was measured as a readout (Figure 1a). Among 1,099 clones, seven cDNA clones were isolated to affect the cell death from the primary and secondary screens (Figure 1b). Unexpectedly, DR4 was identified as a suppressor of TRAIL-induced cell death. Since DR4 is a proapoptotic receptor, I investigated DNA sequence of this DR4 clone and found that this DR4 clone has four mutations (DR4 MUT) (Figure 2a), three mutations (L81R, H141R and R209T) in the extracellular region and one mutation
In the cytosolic domain.

In order to identify which mutation among those four of DR4 was responsible for loss of cytotoxicity, I reverted these mutations back to wild-type residue one by one, generating four DR4 revertants harboring three original mutations and one wild-type residue (Figure 2a; MUT→WT). I then tested their ability to induce cytotoxicity in DLD-1 human colon cancer cell line in which TRAIL signaling cascade is triggered dominantly through DR4 (data not shown). Like DR4 MUT, three DR4 MUT→WT revertants, including R86L, R141H and T209R, were not cytotoxic (Figure 2b). In contrast, only DR4 P424S revertant fully restored its cytotoxic activity as much as wild-type DR4. Apparently, unlike DR4 MUT and three revertants (R86L, R141H, T209R), DR4 P424S revertant induced proteolytic activation of caspase-3 and 8 even in the presence of TRAIL (Figure 2c). The results show that mutation in Ser424 residue is important for the resistance to TRAIL-induced apoptosis.

Inversely, I directly introduce a mutation in DR4 Ser424 residue with Pro,
generating DR4 S424P mutant (DR4 T1270C) and tested its effect on cell death. As expected, DR4 S424P mutant failed to mediated TRAIL-induced cell death (Figure 2d). Since DR4 polymorphisms associated with cancer risk have been described in various human cancers, such as stomach (Kuraoka et al., 2005), bladder (Wolf et al., 2006), lung (Ulybina et al., 2009) and ovarian (Kim et al., 2012) cancer, I further investigated apoptotic activity of other polymorphisms. Particularly, DR4 G422A (rs6557634), C626G (rs4871857), A683C (rs17088993) and A1322G (rs2230229) showed a higher frequency of polymorphism in various tumors of different origin (Chen et al., 2009, Geng et al., 2015). Compared to DR4 Ser424Pro mutant (DR4 T1270C; S424P), these mutants were effective to induce apoptotic cell death with caspase activation and PARP-1 cleavage in DLD-1 cells (Figure 2d and e). Together, these results indicate that Ser424 within the cytosolic region of DR4 is critical for TRAIL-induced cell death.

3.2. Mutation of DR4 Ser424 residue blocks necrosis as well as apoptosis
triggered by TRAIL

To test whether the mutation of DR4 Ser424 to Pro was critical for interfering TRAIL-induced cell death, I also generated DR4 S424A mutant replacing Ser424 with Ala and compared its activity with that of DR4 S424P mutant in various cancer cell lines. In HeLa cervical carcinoma cells, expression of wild-type DR4 induced caspase-dependent apoptosis (Figure 3a). On the other hand, overexpression of DR4 S424P or S424A mutant resulted in significant resistance to apoptosis induced by TRAIL (Figure 3b and c). By reconstituting DR4 wild-type and S424 mutants, I also examined the inhibitory effect of Ser424 mutants in DR4-negative stomach cancer cells (SNU-216) or glioblastoma cells (T98G) (Figure 14a and b). Expression of wild-type DR4 in SNU-216 and T98G cells induced apoptosis with substantial activation of caspases and subsequent cleavage of PARP-1 upon TRAIL stimulation (Figure 3d and e). In contrast, DR4 S424P or S424A mutant was unable to induce the apoptosis and failed to activate caspases in both DR4-null cancer cells (Figure 3d and e). Together, these data emphasize the notion that Ser424 of DR4 is the essential
residue for triggering TRAIL-induced apoptosis.

According to recent studies, TRAIL can induce not only apoptosis but also programmed necrosis in certain conditions, such as low pH or co-treatment with the pan-caspase inhibitor and SMAC mimetics in the presence of RIPK3 (Meurette et al., 2007; Voigt et al., 2014). Thus, I tested whether Ser424 mutation could also affect necroptosis triggered by TRAIL. HeLa cells stably expressing RIPK3 (HeLa/RIPK3-HA) were generated and treated with TRAIL in the presence of IDN-6556 (IDN), a pan-caspase inhibitor, and SM-164 (SM; SMAC mimetic). As reported, the incidence of cell death triggered by these treatment was blocked by pretreatment of necrostatin-1 (Nec-1), a pharmacological inhibitor of RIPK1 (Figure 4a). Unlike the stimulation effect of wild-type DR4, expression of S424P mutant did not potentiated the necroptosis but rather significantly suppressed it (Figure 4a). In addition, examination of necroptosis mediators revealed that the phosphorylated forms of active MLKL and RIPK3 were detected in HeLa/RIPK3-HA cells expressing wild-type DR4, but not in the cells expressing DR4 S424P or S424A (Figure 4b). Consistently, the assembly of
necrosome comprising RIPK1, RIPK3, and MLKL was not also observed in HT-29 colon cancer cells expressing DR4 S424P mutant (Figure 4c). Moreover, under non-reducing conditions, the formation of phosphorylated MLKL trimers (Cai et al., 2014), an active complex, and DR4 clusters was detected in HeLa/RIPK3-HA cells expressing DR4 wild-type, but not much in the cells expressing DR4 S424P mutant (Figure 4d). These results show that Ser424 of DR4 is also the key residue triggering necroptosis as well as apoptosis in response to TRAIL application.

3.3. Ser424 of DR4 is O-GlcNAcylated depending on TRAIL stimulation

Serine is one of the representative amino acid residues that contribute to catalytic function of many enzymes commonly through its posttranslational modification, including phosphorylation by kinases and \(O\)-GlcNAcylation by OGT (Hart et al., 2011). I thus hypothesized that DR4 might play its cytotoxic role during TRAIL signaling via posttranslational modification of Ser424. To check this possibility, I monitored the phosphorylation and \(O\)-GlcNAcylation of DR4 during
TRAIL application. The immunoprecipitation (IP) analysis of FLAG-tagged DR4 wild-type or DR4 Ser424 mutants revealed that exposure to TRAIL induced the phosphorylation of DR4 wild-type, but not in DR4 S424P mutant, in HEK293T cells (Figure 5a). However, an observation that DR4 S424A and DR4 S424D mutants were also phosphorylated by TRAIL revealed that the phosphorylation of DR4 did not occur at Ser424 residue.

Interestingly, with immunoprecipitation assay followed by western blotting, I found that DR4 wild-type was O-GlcNAcylated (Figure 5b). In contrast, DR4 S424P and DR4 S424A mutants were not. When I examined this modification of endogenous DR4 in DLD-1 cells, I could similarly observe that DR4 was a little modified by O-GlcNAc at basal level in untreated control cells and the amount of O-GlcNAcylated DR4 was markedly increased in response to TRAIL (Figure 6a). Confirmatively, this O-GlcNAcylation of DR4 was abolished by CRISPR/Cas9-mediated OGT knockdown (Figure 6b). Similarly, O-GlcNAcylation of DR4 was observed in Hela/RIPK3 cells exposed to TRAIL/SM/IDN and undergoing necroptosis but was
abolished by OGT knockdown in the same cells (Figure 6c). Further, immunoprecipitation assay revealed that upon TRAIL stimulation, the binding of DR4 to OGT was enhanced in apoptotic DLD-1 cells (Figure 6d) and necrotic SNU-16 cells (Figure 6f) or OGT bound to DR4 *vice versa* in DLD-1 cells (Figure 6e), indicating that DR4 interacts with OGT and this interaction is promoted when TRAIL is ligated to DR4. Consistently, the interaction between OGT and DR4 was abolished by OGT knockdown (Figure 6e). Taken together, I concluded that DR4 is O-GlcNAc modified at Ser424 in an OGT-dependent manner.

3.4. DR4 Ser424 O-GlcNAcylation is required for TRAIL-induced cell death by facilitating receptor translocation, clustering and DISC assembly

In order to examine whether O-GlcNAcylation of DR4 Ser424 was required for cytotoxic function of DR4, I first investigated the effect of OGT-knockdown on death rates of cells. Compared to that of control cells, TRAIL-induced cell death was noticeably delayed in OGT knockdown DLD-1 cells (Figure 7a). Accordingly, caspase
activation and PARP cleavage were decreased in OGT-knockdown DLD-1 cells upon TRAIL treatment (Figure 7b). Also, the phosphorylation-dependent band shift of RIPK1 and RIPK3, and level of phosphorylated MLKL were greatly diminished in OGT-knockdown HeLa/RIPK3-HA cells (Figure 7c). In HeLa/RIPK3-HA cells, I blocked DR5-mediated signaling by incubating cells with DR5 neutralizing antibody (αDR5), allowing only DR4-mediated TRAIL signaling. These observations revealed that O-GlcNAcylation is of potent benefit to TRAIL-induced DR4-mediated apoptosis and necroptosis.

To answer the question about how O-GlcNAcylation of DR4 could contribute cell death in response to TRAIL, I also checked subcellular distribution of DR4 in O-GlcNAc modification-defective conditions. Given ligated trimeric TRAIL receptors generally form clusters in compartmentalized and Triton X-100 insoluble lipid rafts during onset of TRAIL signaling (Song et al., 2007; Ouyang et al., 2013; Linderoth et al., 2013), I examined the presence of DR4 in Triton X-100 soluble fraction (Sup) and insoluble pellet (SDS-soluble, P-SDS) harboring lipid raft-
cytoskeleton compartment (Figure 8a-e). Since vimentin, a cytoskeletal protein, locates at aggregation-rich cages (Johnston et al., 1998), it is a marker protein representing SDS-extractable P-SDS fraction (Figure 8a and c). Cell fractionation assay revealed that wild-type DR4 was markedly translocated into the P-SDS fraction upon TRAIL stimulation (Figure 8a and b). Unlike wild-type DR4, DR4 S424P mutant was detected mainly in the Sup fraction (Figure 8a and b), indicating that the translocation of this DR4 S424P mutant into lipid rafts was delayed or quite incapable. In addition, translocation of DR4 into the P-SDS fraction was abrogated by OGT knockdown (Figure 8c). OGT was also present in the P-SDS fraction together with DR4 (Figure 8c) and DR4 O-GlcNAcylation occurred in the P-SDS fraction (Figure 8d). Thus, O-GlcNAcylated DR4 is likely present in the lipid rafts following TRAIL stimulation.

As reported, analysis the clustering of DR receptor under non-reducing conditions revealed that wild-type DR4 could form trimeric receptor complexes with molecular weight 150-160 kDa in HEK293T cells (Figure 8f). This type of receptor
clustering, however, were hardly detected in all DR4 Ser424 mutants (Figure 8f and 4d). More directly, I investigated the ability of the DR4 mutant to form DISC using DISC immunoprecipitation analysis. The results showed that unlike wild-type DR4, O-GlcNAcylation-deficient DR4 S424P mutant failed to form DISC consisting of caspase-8 and DR4, and to induce proteolytic activation of capase-8 (Figure 8g). Therefore, O-GlcNAcylation of DR4 is an essential prerequisite for DR4 translocation into active DISC platforms to initiate TRAIL signaling via receptor clustering.

3.5. O-GlcNAcylation occurs in DR4, not DR5, and DR4 Ser424 mutant inhibits DR5 activity

Although two functional receptors of TRAIL, DR4 and DR5, generally share the same co-worker proteins on the identical pathway, they are dissimilarly regulated and often drive different consequences owing to their distinct characteristics, expression, distribution and different preference in various cancer cells. Amino acid sequence comparison showed a corresponding residue of DR4 Ser424 in DR5,
When I checked whether DR5 was also O-GlcNAcylated by OGT, I found that DR5 was not O-GlcNAcylated (Figure 9a), differing from DR4. In addition, I examined TRAIL-induced cytotoxicity in O-GlcNAcylation-deficient HeLa cells and SK-Hep1 hepatic adenocarcinoma cells which transmit TRAIL signaling preferentially via DR5 rather than DR4. As expected, TRAIL-induced cell death (Figure 9c) and caspase activation (Figure 9b) were not affected by OGT knockdown in HeLa cells and SK-Hep1 cells. On the other hand, blocking of DR5 signaling by incubating with DR5-neutralizing antibody αDR5 resulted in inhibition of TRAIL-induced cell death in SK-Hep1 cells (Figure 9c). Consistent with these results, overexpressed DR5 triggered cell death (Figure 9d) and caspase activation (Figure 9e) in OGT-deficient SK-Hep1 cells as much as in control cells, while DR4 showed reduced apoptotic activity in OGT-deficient SK-Hep1 cells. DR4-mediated incidence of apoptosis (approximately 20-25%) by OGT knockdown is almost equivalent to that of apoptosis by O-GlcNAcylation-defective DR4 S424P mutant (Figure 9d and e). Thus, O-GlcNAcylation is required only for DR4-signaling,
not for DR5 cytotoxicity.

I then figured out how $O$-GlcNAcylation-defective DR4 S424P mutant took advantage of cell death-escaping ability during tumorigenesis. Previous studies have revealed that, although homotrimers are much favored over heterotrimers, DR4 and DR5 may form not only homotrimers but also heterotrimers each other upon TRAIL stimulation (Duiker et al., 2009). Accordingly, I observed assembly between DR4 and DR5 in cells (data not shown). In addition, immunoprecipitation assay revealed that DR4 S424P mutant could form protein complex with both DR4 and DR5 in the transfected cells (Figure 10a). More, expression of $O$-GlcNAcylation-defective DR4 S424P mutant disturbed DR5- and DR4-mediated cell death in a dose-dependent manner in HeLa cells (Figure 10b and c). Our data suggest that the lack of DR4 $O$-GlcNAcylation in DR4 S424P mutant mitigates TRAIL-signaling in net through binding to both DR5 and DR4, lowering the efficacy of TRAIL application and ultimately giving rise to TRAIL resistance.
3.6. Glucose level determines TRAIL sensitivity of cancer cells via DR4 O-GlcNAcylation

Given that O-GlcNAcylation is regulated according to glucose level (Guo et al., 2014), I assessed the effect of glucose on DR4 O-GlcNAcylation and on DR4-dependent TRAIL signaling. When HEK293T cells transfected with FLAG-DR4 were exposed to TRAIL in the presence of increasing concentrations of glucose, I found that O-GlcNAcylation of DR4 was increased in a glucose dose-dependent manner and vice versa (Figure 11a and b). DR4 O-GlcNAcylation was totally abrogated in a glucose-deprived condition (Glucose 0 mM) or was remarkably reduced by the treatment of 6-diazo-5-oxo-L-norleucine (DON), a glutamine analogue decreasing levels of UDP-GlcNAc by inhibition of glutamine fructose-6-phosphate amidotransferase (GFAT) (Figure 11a). In contrast, DR4 O-GlcNAcylation was increased by the treatment with thiamet G (TMG), a potent inhibitor of OGA (Figure 11a). Again, O-GlcNAcylation signal of S424P mutant was not detectable regardless of glucose existence (Figure 11b). Consistently, I observed O-GlcNAcylation of
endogenous DR4 in DLD-1 cells that were exposed to glucose and TRAIL, but not in DLD-1 cells incubated with glucose-free medium (Figure 11c). Thus, glucose is the critical metabolic factor which has a great influence on TRAIL sensitivity of cancer cells by exerting a direct impact upon DR4 modification O-GlcNAcylation.

Definitely, nutrient deprivation, such as glucose starvation, is a stress and eventually triggers cell death in general. Thus, long-lasting low glucose or inhibition of glucose metabolism augments TRAIL cytotoxicity (Muñoz-Pinedo et al., 2003; Iurlaro et al., 2017). Our experimental results under these harsh conditions for a long time coincided with the previous studies. When several TRAIL-sensitive cancer cells were exposed to TRAIL under glucose deprivation condition for long time more than 12 h, DLD-1 cells became more vulnerable to TRAIL (Figure 12a). Unlike long-lasting effect of glucose deprivation, when DLD-1 cells were pre-incubated with glucose-free condition for short time, such as 30 min or 1 h, prior to cell death analysis, cell death triggered by TRAIL was markedly mitigated within glucose-starvation at early time point (Figure 12b). Under this condition of glucose starvation, however,
there was no significant difference in the incidence of cell death triggered by other cytotoxic stimuli, such as TNFα plus cycloheximide (CHX), an extrinsic death signal, or A23187, a calcium ionophore that causes intrinsic death (Figure 12b). Accordingly, compared glucose-supplemented condition, caspase-8 activation was reduced by glucose deprivation in DLD-1 cells upon TRAIL stimulation (Figure 12c). Furthermore, the formation of caspase-8- and DR4-containing DISC upon TRAIL stimulation was also impaired in the absence of glucose (Figure 12d). Confirmatively, DR4 recruited into the DISC was O-GlcNAcylated in the presence of glucose, but not in the absence of glucose (Figure 12d).

Interestingly, TRAIL-induced cell death was even accelerated after nourishing with higher glucose concentration more than 25 mM, a given concentration in the high-glucose medium I used (Figure 16a and b). To evaluate the effect of glucose on TRAIL efficacy, I generated cancer cells adapted to either high- and low-glucose medium. DLD-1 cancer cells were incubated with high (25 mM, DLD-1/High) or low (5 mM, DLD-1/Low) glucose medium for a month and were tested for their
TRAIL sensitivity. Compared to control DLD-1/High cells cultured in high glucose medium, DLD-1/Low cells were less sensitive to the same dosage of TRAIL than DLD-1/High cells (Figure 13a), implying that TRAIL efficacy would be significantly decreased in cancer cells exposed to continuous nutrient-lacking environment like core regions of tumor. I also confirmed significant differences in the level of DR4 O-GlcNAcylation between DLD-1/High and DLD-1/Low cells (Figure 13b). All these evidences suggest that cancer cells under glucose starvation are less susceptible to DR4-mediated cell death triggered by TRAIL owing to O-GlcNAcylation impairment.

3.7. O-GlcNAcylation of DR4 Ser424 is dysregulated in TRAIL-resistant cancer cells, which is overcome by handling with either high glucose or 2-DG application

Given our data, it seems that, at least in cancer cells preferred TRAIL signaling through DR4, TRAIL efficacy is associated with glucose level and ultimately with O-GlcNAcylation of DR4 Ser424. To inquire whether impeded O-GlcNAcylation system could be a novel causative factor of TRAIL resistance in
cancer cells, I examined receptor preference and the extent of DR4 O-GlcNAcylation upon TRAIL application in various cancer cells. For accurate comparative analysis relevant to pathophysiological context, I checked the expression of each receptor in different cancer cells (Figure 14a-c). Total eight stomach cancer cell lines (Figure 14a and d), four glioblastoma cell lines (Figure 14b and e), two lung cancer cell lines (Figure 14c and f) and two colon cancer cell lines were assessed for their expression of DRs and sensitivity to TRAIL. I then classified cancer cell lines into TRAIL-sensitive and TRAIL-resistant groups with their preferential receptor engagement, DR4- or DR5-dominant signaling, in TRAIL signaling (Figure 14g). DR4- or DR5-dominant group was classified by analyzing cell death after the treatment of TRAIL with αDR5 (Figure 14d-f). The expression of OGT was ubiquitously detected by western blotting in almost of cancer cells (Figure 14a-c).

Then, the status of DR4 O-GlcNAcylation in DR4-positive and negative cells was examined in the immunoprecipitates enriched by either DR4 antibody or GlcNAc-specific agarose-conjugated succinylated wheat germ (sWGA) upon TRAIL
stimulation (Figure 15). Surprisingly, the results revealed that DR4 \( O\)-GlcNAcylation was markedly promoted by TRAIL stimulation only in the DR4-positive and TRAIL-sensitive group – two of stomach cancer cells (SNU-16, SNU-608) (Figure 15a and d), one of colon cancer cells (DLD-1) (Figure 15b) and one of lung cancer cells (H460) (Figure 15c). I did not observe the \( O\)-GlcNAcylated signal or the increase of DR4 \( O\)-GlcNAcylation upon TRAIL treatment in all of the DR4-positive TRAIL-resistant cells – SNU-638, SNU-719, HT-29, A549 (Figure 15a-c) and U-87 MG (Figure 15e). Two of glioblastoma cells in the DR4-negative group (U-373 MG, and T98G) were used as negative controls (Figure 15e). To make sure of more direct correlation and causality between this modification and cytotoxicity of DR4, I attempted to explore this discovery in a single cell line in depth by sorting cell population according to their susceptibility to TRAIL. TRAIL-resistant DLD-1 cells (DLD-1/R) were obtained by selecting cells which have survived from TRAIL-containing medium for 2 months (Figure 15f). I observed interestingly that unlike in TRAIL-sensitive DLD-1 (DLD-1/S) cells, both DR4 \( O\)-GlcNAcylation and interaction with OGT were abrogated in
TRAIL-resistant DLD-1 (DLD-1/R) cells upon TRAIL stimulation, whereas the expression levels of OGT and DR4 were not much changed (Figure 15f). From these results, I suggest that the impairment of DR4 O-GlcNAcylation after TRAIL ligation would lead to TRAIL resistance of cancer cells.

As shown earlier in this study, cellular glucose level has significant influence on O-GlcNAcylation-mediated DR4 cytotoxicity. Thus, I hypothesized that elevating glucose concentration made TRAIL-resistant cancer cells vulnerable to TRAIL application by enhancing DR4 O-GlcNAc modification. To test this hypothesis, TRAIL-resistant SNU-638 and SNU-719 stomach cancer cells were incubated in culture medium containing 25 mM glucose I usually used in the laboratory or higher concentrations of glucose, such as 100 mM (Figure 16a and b) or 250 mM (data not shown). Higher concentrations of glucose alone did not affect cell viability and TRAIL did not affect cell viability in these two TRAIL-resistant cell lines growing on 25 mM-glucose medium (Figure 16a, b and 14d). Notably, the treatment with TRAIL in the presence of 100 mM glucose in culture medium resulted in striking increase of
cell death in both cell lines from 3% to 59% in SNU-638 cells and from 5% to 45% in SNU-719 cells (Figure 16a and b). At this time, O-GlcNAcylation of DR4 in these cells was also consistently increased according to increase of glucose dose (Figure 16c), showing that enhancing DR4 O-GlcNAcylation improves TRAIL-sensitivity of TRAIL-resistant cancer cells and again confirming our idea that DR4 O-GlcNAcylation is critical for TRAIL-mediated cancer cell death.

Apart from applying 100 mM glucose that may not be available under physiological condition, I thus tested the combination effect of TRAIL and 2-deoxy-D-glucose (2-DG), a glucose analog that has been safely used as a pharmacological agent to inhibit glycolysis (Pusapati et al., 2016) and to increase TRAIL sensitivity in clinical studies (Liu et al., 2009; Huang et al., 2013; Carr et al., 2016), on TRAIL-resistant SNU-638 and SNU-719 cancer cells (Figure 16a and b). Given that 2-DG is effective as an anti-tumor reagent, cell death was pretty triggered by the treatment of 2-DG alone (Figure 16a and b). The combination of TRAIL and 2-DG, as well-established, caused markedly increase of cell death consistently in our experiments in
a dose-dependent manner (Figure 16a and b). Under this condition, compared to
TRAIL-treated control, immunoprecipitation assay revealed significant induction of
DR4 O-GlcNAcylation after stimulated by TRAIL and 2-DG (Figure 16d), indicating
that 2-DG sensitized TRAIL-resistant cancers to TRAIL by influencing DR4 O-
GlcNAcylation.

3.8. Genetic alterations of DR4 Ser424 in cancer cells avoid O-GlcNAc
modification and cell death

Lastly, I tried to search genomic changes on DR4 Ser424 residue from
database of various cancer cell lines and tissues from cancer patients. Although the
frequency was low (1 of 530 case, 0.19%), I found one more mutation in the studies of
of 1271\textsuperscript{th} cysteine of DR4 to thymidine results in amino acid change at Ser424 to Leu
(S424L) in uterine corpus endometrial carcinoma. In addition, I found SNP database
(dbSNP; rs145301145) showing that DR4 is abnormally terminated at 423\textsuperscript{th} amino
acid due to substitution of 1271$^{\text{th}}$ Cys to Ala. Based on these data, I generated DR4 mutants, DR4 S424L and DR4 deletion (1-423), harboring these mutations and compared their cytotoxic capacity in comparison with DR4 S424P mutant. Consistent with the phenotype of DR4 S424P mutant, both DR4 S424L and DR4 deletion (1-423) mutants exhibited loss-of-cytotoxicity in cell death analysis with TRAIL stimulation (Figure 17a). Moreover, O-GlcNAcylated DR4 was not detected from the cells expressing these mutants (Figure 17b). Taken together, I propose again that DR4 Ser424 is the critical residue for DR4 functioning via O-GlcNAc modification in TRAIL signaling and that the screen of genetic alteration of DR4 Ser424 in cancer patients provides clinical clues for the sensitivity to TRAIL therapy or even cancer prevention.
Figure 1. Functional screen identifies genetic regulators that cause TRAIL resistance.

(a) Representation of gain-of-function screen strategy using cell death-rescue assay. Total 1,099 full-length cDNAs from various cancer patients were collected for this screen. HeLa cells were transfected with each cDNA of the library together with pEGFP-N1 for 24 h. After 100 ng/ml TRAIL treatment for 6 h, death rates of GFP-positive cells were examined under a fluorescence microscope. (b) Seven clones were selected from the final screen.
Co-transfection w/ cDNA expression library and EGFP

Treatment w/ TRAIL

Live cells

Dying cells

Observation of cell death

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Figure 2. DR4 Ser424 mutant isolated from cancer cells blocks TRAIL-induced apoptosis.

(a) Schematic diagram depicting DR4 mutant and its revertants. DR4 wild-type (WT), DR4 mutant harboring 4 mutations (MUT), DR4 revertants harboring a resubstituted WT residue in DR4 mutant (MUT→WT). Numbers indicate amino acid residues on DR4 WT. Cytotoxicity of these mutants is summarized (+, -). Red letters correspond to WT residue in each revertant. (b) DLD-1 cells were transiently transfected with pcDNA-HA (Empty; control), HA-DR4 WT, HA-DR4 MUT or HA-DR4 revertants (MUT→WT) for 18 h and then left untreated (NT) or treated with 100 ng/ml TRAIL for 6 h. The ratio of cell death was determined as described in MATERIALS AND METHODS. The ability to trigger cell death was represented as percentage of apoptosis and Cytotoxicity in (a). Bars indicate mean ± S.E.M. (n = 3, two-way ANOVA followed by Bonferroni’s post-hoc test, ***P < 0.001). (c) Cell extracts from (b) were analyzed by immunoblotting using the indicated antibodies. The arrowheads correspond to the cleaved products of caspase-8, caspase-3 or PARP-1. GAPDH,
loading control. (d and e) DLD-1 cells were transfected with DR4 WT, DR4 T1270C (S424P) or other SNP mutants for 24 h and then subjected to apoptosis analysis (mean ± S.E.M., n = 3, one-way ANOVA followed by Tukey’s post-hoc test, ***P < 0.001) (d) and western blot analysis (e).
### Table

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### Figures

**Figure a**

Graph showing % apoptosis in various cell lines.

**Figure b**

Graph showing % apoptosis in DLD-1 cells with different treatments.

**Figure c**

Western blot analysis in DLD-1 cells showing various proteins.

**Figure d**

Graph showing % apoptosis in DLD-1 cells with different treatments.

**Figure e**

Western blot analysis in DLD-1 cells showing various proteins.
Figure 3. Mutation at Ser424 of DR4 blocks apoptosis upon TRAIL stimulation.

(a) HeLa cells were transfected with DR4 WT or DR4 S424P for 18 h, washed twice, and then left untreated (NT) or treated with 10 μM IDN-6556 (IDN) for 1 h. Cells were treated with 100 ng/ml TRAIL for 6 h prior to quantification of apoptosis using PI and Hoechst 33342 double staining. Bars indicate mean ± S.E.M. (n = 3, two-way ANOVA followed by Bonferroni’s post-hoc test, **P < 0.001). (b) HeLa cells were transiently transfected with pcDNA-FLAG (Empty; control), DR4 WT-FLAG, DR4 S424P-FLAG or DR4 S424A-FLAG for 18 h. After 100 ng/ml TRAIL treatment for 6 h, cell death rates were examined by PI and Hoechst 33342 double staining (mean ± S.E.M., n = 3, one-way ANOVA followed by Tukey’s post-hoc test, **P < 0.01, ***P < 0.001). (c) HeLa cells were transiently transfected with DR4 WT-FLAG, DR4 S424A-FLAG or DR4 S424P-FLAG and then treated with 100 ng/ml TRAIL for additional 6 h. Cell extracts were analyzed by the immunoblotting. (d and e) DR4-negative cancer cell lines, SNU-216 (stomach cancer) (d) and T98G (glioblastoma) (e), were reconstituted with DR4 WT-FLAG, DR4 S424P-FLAG or DR4 S424A-FLAG,
after which cell extracts were subjected to western blot analysis.
Figure 4. Mutation at Ser424 of DR4 suppresses TRAIL-induced necroptosis.

(a) HeLa/RIPK3-HA cells were transfected with pcDNA-FLAG (control), DR4 WT-FLAG or DR4 S424P-FLAG for 18 h and then treated with 100 ng/ml TRAIL, 10 μM IDN and 50 nM SM-164 (SM) for 6 h in the presence or absence of 50 μM Necrostatin-1 (Nec-1) within fresh medium. Quantification of necrosis was described in MATERIALS AND METHODS. In this case, cells were pretreated with 10 μM IDN and 50 nM SM for 30 min and with 50 μM Nec-1 for 1 h. (b) HeLa/RIPK3-HA cells were transfected with pcDNA-FLAG (control), DR4 WT-FLAG, DR4 S424P-FLAG or DR4 S424A-FLAG for 18 h and then exposed to 100 ng/ml TRAIL, 10 μM IDN and 50 nM SM. Cell extracts were analyzed by immunoblotting. Black arrowhead indicates the phosphorylated RIPK3. (c) HT-29 cells were transfected with pcDNA-FLAG (control) or DR4 S424P-FLAG for 18 h and then treated with 100 ng/ml TRAIL, 10 μM IDN and 50 nM SM. Cell extracts were subjected to immunoprecipitation (IP) assay using anti-RIPK1 antibody for analyzing necrosome. Whole cell lysates (Input) and the immunocomplex were analyzed by western blotting.
(d) HeLa/RIPK3-HA cells were transfected with DR4 WT-FLAG or DR4 S424P-FLAG and then treated with 100 ng/ml TRAIL, 10 μM IDN and 50 nM SM for 6 h. Whole-cell extracts were subjected to SDS-PAGE under non-reducing condition followed by western blotting. White and black arrowheads correspond to monomer and oligomers, respectively, of MLKL or DR4 (FLAG).
Figure 5. Ser424 of DR4 is not phosphorylated but O-GlcNAcylated.

(a and b) HEK293T cells were transfected with FLAG-tagged DR4 WT, S424P, S424A or S424D and cell extracts were subjected to immunoprecipitation (IP) assay using anti-phospho-serine antibody (p-Serine) (a) or anti-FLAG M2 affinity gel (b). Whole cell lysates (Input) and the immunocomplex were analyzed by western blotting.
Figure 6. O-GlcNAcylation of DR4 Ser424 is promoted in response to TRAIL stimulation.

(a and b) DLD-1 cells (a) or DLD-1 control (-; OGT sg, sgRNA targeting OGT) and DLD-1/OGT knockdown (OGT sg #1 and sg #2) cells (b) were treated with 100 ng/ml TRAIL for 30 min, followed by O-GlcNAcylation assay. (c) HeLa/RIPK3-HA control (OGT sg-) and HeLa/RIPK3-HA/OGT knockdown (OGT sg+) cells were exposed to TRAIL/SM/IDN for 1 h and then analyzed by O-GlcNAcylation assay. (d and e) DLD-1 cells (d) or DLD-1/OGT knockdown (OGT sg+) cells (e) were treated with 100 ng/ml TRAIL for 30 min and subjected to immunoprecipitation (IP) assay using anti-DR4 antibody (d) or anti-OGT antibody (e). (f) SNU-16 cells were treated with 100 ng/ml TRAIL, 10 μM IDN and 50 nM SM. Cell extracts were subjected to immunoprecipitation (IP) assay with anti-DR4 antibody.
Figure 7. DR4 O-GlcNAcylation by OGT is required for TRAIL-induced cell death.

(a and b) DLD-1 control (OGT sg-) cells and DLD-1/OGT knockdown (OGT sg #1 and sg #2) cells were left untreated or treated with 100 ng/ml TRAIL for 6 h and then cell death rates were determined by PI and Hoechst 33342 double staining (mean ± S.E.M., n = 3, two-way ANOVA followed by Bonferroni’s post-hoc test, ***P < 0.001) (a) and caspase-8 activation was examined by western blotting (b). (c) HeLa/RIPK3-HA (OGT sg-) and HeLa/RIPK3-HA/OGT knockdown (OGT sg+) cells were treated or not with TRAIL/SM/IDN in the presence of DR5-neutralizing antibody (αDR5). Cell extracts were analyzed by western blotting.
Figure 8. DR4 O-GlcNAcylation facilitates DR4 translocation, clustering and DISC assembly.

(a and b) HeLa (a) and HeLa/RIPK3-HA (b) cells were transfected with DR4 WT-FLAG or DR4 S424P-FLAG (SP) and then incubated for 3 h with 100 ng/ml TRAIL (a) or TRAIL/SM/IDN (b). After fractionation into supernatant (Sup) and pellet (SDS-soluble fraction, P-SDS) by centrifugation, each fraction was analyzed by immunoblotting. (c) HeLa control (OGT sg-) and HeLa/OGT knockdown (OGT sg #1 and sg #2) cells were transfected with DR4 WT-FLAG and were analyzed by western blotting after fractionation. (d) HEK293T cells were transfected with pcDNA-FLAG (-; control), DR4 WT-FLAG, DR4 S424P-FLAG or DR4 S424A-FLAG for 24 h and then subjected to fractionation. Each fraction was analyzed by O-GlcNAcylation assay. (e) HEK293T cells were transfected with FLAG-tagged DR4 WT, S424P, S424A or S424D for 24 h and then separated by SDS-PAGE under non-reducing condition for western blotting. The white and black arrowheads correspond to DR4 monomer and oligomers respectively. (f) HeLa cells expressing DR4 WT-FLAG or DR4 S424P-
FLAG were exposed to 100 ng/ml TRAIL for 1 h and DR4-containing DISC was pulled-down by DISC isolation assay using anti-FLAG antibody.
Figure 9. O-GlcNAcylation is essential for cell death by DR4, not by DR5.

(a) HEK293T cells expressing FLAG-tagged DR4 or DR5 were treated with 100 ng/ml TRAIL and analyzed by immunoprecipitation (IP) assay and western blotting.

(b) HeLa control (OGT sg-) and HeLa/OGT knockdown (OGT sg #1 and sg #2) cells were treated 100 ng/ml TRAIL for 6 h. Cell extracts were analyzed by western blotting. (c) SK-Hep1 control (OGT sg-) and SK-Hep1/OGT knockdown (OGT sg #1 and sg #2) cells were treated with 100 ng/ml TRAIL in the presence or absence of αDR5 for 12 h. Bars represent mean values ± S.E.M. (n = 3, two-way ANOVA followed by Bonferroni’s post-hoc test, ***P < 0.001). (d and e) SK-Hep1 control (OGT sg-) and SK-Hep1/OGT knockdown (OGT sg #1 and sg #2) cells were transfected for 24 h with pcDNA-FLAG (Empty), FLAG-tagged DR4 WT (DR4WT), DR4 S424P (DR4S424P) or DR5 WT (DR5WT). Cell death rates (mean ± S.E.M., n = 3, two-way ANOVA followed by Bonferroni’s post-hoc test, **P < 0.01, ***P < 0.001) (d) and caspase activation (e) were examined.
Figure 10. O-GlcNAcylation-defective DR4 Ser424 mutant disturbs DR5 cytotoxicity in a dominant-negative manner.

(a) HEK293T cells were co-transfected with HA-DR4 S424P and either DR4 WT-FLAG or DR5 WT-FLAG and then subjected to immunoprecipitation (IP) assay using anti-FLAG M2 affinity gel. (b and c) HeLa cells were co-transfected for 18 h with different doses of HA-DR4 S424P and either DR5 WT-FLAG (b) or DR4 WT-FLAG (c). Cells were analyzed for percentage of cell death (mean ± S.E.M., n = 3, one-way ANOVA followed by Tukey’s post-hoc test, *P < 0.05, ***P < 0.001) (upper) and by western blotting (lower).
Figure 11. DR4 O-GlcNAcylation is affected by glucose metabolism.

(a) After transfection with DR4 WT-FLAG for 16 h, HeLa cells were pretreated for 6 h with either 50 μM thiamet G (TMG) or 100 μM 6-diazo-5-oxo-L-nor-leucine (DON) and then incubated with different concentrations of glucose (0-25 mM of glucose). Immunoprecipitation (IP) assay using immunoglobulin G (IgG) or anti-FLAG antibody was performed. (b) After transfection with pcDNA-HA (control), HA-DR4 WT or HA-DR4 S424P, HeLa cells were cultured in either glucose-deprived medium (-) or high-glucose (25 mM glucose) medium (+) with 100 ng/ml TRAIL, followed by immunoprecipitation assay (IP) with anti-HA antibody. Asterisk (*) indicates nonspecific signals. (c) DLD-1 cells were incubated in either glucose-free medium (-) or high-glucose (25 mM glucose) medium (+) for 1 h and then exposed to 100 ng/ml TRAIL for additional 30 min. O-GlcNAcylation assay was performed.
Figure 12. TRAIL efficacy is lowered in cancer cells exposed to glucose-lacking environment.

(a) DLD-1 cells were incubated in either glucose-free (-) or high-glucose (25 mM glucose) (+) medium for 12 h in the presence or absence of 100 ng/ml TRAIL. Cell viability was assessed by trypan blue exclusion assay (mean ± S.E.M., n = 3, two-way ANOVA followed by Bonferroni’s post-hoc test, **P < 0.01, ***P < 0.001). (b) DLD-1 cells pre-incubated in glucose-free or 25 mM-glucose medium for 30 min were left untreated (NT) or treated for additional 3 h with 100 ng/ml TRAIL, 10 ng/ml TNFα plus 20 μM cycloheximide (CHX) or 5 μM A23187. Cell death rates were estimated and are represented as bars with mean values ± S.E.M. (n = 3, two-way ANOVA followed by Bonferroni’s post-hoc test, ***P < 0.001). (c) DLD-1 cells cultured in glucose-free (-) or 25 mM-glucose (+) medium for 1 h were treated with 100 ng/ml TRAIL for additional 2 h. Cell extracts were analyzed by western blotting. (d) DLD-1 cells were pre-incubated in either glucose-free (-) or 25 mM-glucose (+) medium for 1 h and then exposed to 100 ng/ml TRAIL for additional 1 h. DR4-
containing DISC was isolated by immunoprecipitation (IP) assay using anti-DR4 antibody.
Figure 13. Glucose level determines TRAIL susceptibility of cancer cells via regulating DR4 O-GlcNAcylation.

(a) DLD-1/High (grown in 25 mM-glucose medium) and DLD-1/Low (adapted in 5.5 mM-glucose medium) cells were treated with 50 ng/mL TRAIL for the indicated times. Cell death ratio was quantified by trypan blue exclusion assay. Bars with mean values ± S.E.M. (n = 3, two-way ANOVA followed by Bonferroni’s post-hoc test, **P < 0.01, ***P < 0.001). (b) DLD-1/High (H) and DLD-1/Low (L) cells were exposed to 100 ng/ml TRAIL for 30 min and then the O-GlcNAcylation signals of each cell type was detected by immunoprecipitation (IP) assay.
Figure 14. DR4-preferred cancer cells are classified into TRAIL-sensitive and TRAIL-resistant groups.

(a-c) Eight stomach cancer cell lines (a), four glioblastoma cell lines (b) and two lung cancer cell lines (c) were assessed by western blotting for the expression levels of DR4, DR5 and OGT. (d-f) All cells mentioned above were exposed to 200 ng/ml TRAIL for 24 h in the presence or absence of 1 μg/ml αDR5 and then subjected to cell death assay using PI and Hoechst 33342 double staining. (g) Classification of cancer cells according to their receptor distribution and the sensitivity to TRAIL. DR4⁺, DR4-positive cells; DR4⁻, DR4-null cells. DR4⁺ cells were subdivided by their receptor preference, the receptor primarily ligated upon TRAIL stimulation.
Figure 15. O-GlcNAcylation of DR4 Ser424 is dysregulated in TRAIL-resistant cancer cells.

(a and b) DR4-positive stomach cancer cells (a) and colon cancer cells (b) were incubated in the presence or absence of 100 ng/ml TRAIL for 30 min and then analyzed by O-GlcNAcylation assay. TRAIL\textsuperscript{S}, TRAIL-sensitive cells; TRAIL\textsuperscript{R}, TRAIL-resistant cells. (c-e) Lung cancer cell lines (H460 and A549) (c), SNU-608 stomach cancer cells (d) and glioblastoma cell lines (U-87, T98G, U-373) (e) were exposed to 100 ng/ml TRAIL for 30 min and then immunoprecipitation (IP) assay was performed by sWGA (GlcNAc-specific agarose-conjugated succinylated wheat germ).

(f) Acquired TRAIL-resistant DLD-1 cells (DLD-1/R) were generated and then their O-GlcNAcylation status was compared to control TRAIL-sensitive DLD-1 cells (DLD-1/S) through O-GlcNAcylation assay.
Figure 16. Defective O-GlcNAcylation of DR4 in TRAIL-resistant cancer cells is overcome aided by either high glucose or 2-DG application.

(a and b) TRAIL-resistant SNU-638 (a) and SNU-719 (b) stomach cancer cells were treated with 100 ng/ml TRAIL for 24 h in the medium containing the indicated glucose concentration with or without dose-dependent 2-DG. Cells were subjected to cell death assay (mean ± S.E.M., n = 3, two-way ANOVA followed by Bonferroni’s post-hoc test, **P < 0.01, ***P < 0.001). 2-DG, 2-deoxy-D-glucose. (c and d) SNU-638 cells of (c) were cultured in either 25 mM- or 100 mM-glucose medium for 12 h and then treated with 200 ng/ml TRAIL for additional 1 h. SNU-638 cells of (d) were pre-incubated to 10 mM 2-DG for 2 h and then exposed to 200 ng/ml TRAIL for additional 2 h. Cell extracts were analyzed by O-GlcNAcylation assay.
Figure 17. DR4 Ser424 mutations found in cancer tissues can escape from O-GlcNAcylation and subsequent cell death triggered by TRAIL.

(a) HA-DR4 Ser424Leu (S424L) and HA-DR4 deletion (1-423), searched mutations from database of cancer tissues, were generated by site-directed mutagenesis. HeLa cells were transfected with pcDNA-HA (Empty), HA-DR4 WT, HA-DR4 S424P, HA-DR4 S424A, HA-DR4 S424L or HA-DR4 deletion (1-423) mutant and then treated with 100 ng/mL TRAIL for 6 h. Cell death was quantified (mean ± S.E.M., n = 3, one-way ANOVA followed by Tukey’s post-hoc test, **P < 0.01, ***P < 0.001) (upper) and the expression of each DR4 protein was detected by western blotting (lower). (b) HEK293T cells were transfected with pcDNA-HA (-), HA-DR4 WT, HA-DR4 S424P, HA-DR4 S424A, HA-DR4 S424L or HA-DR4 deletion (1-423) mutant for O-GlcNAcylation assay. Cell extracts were subjected to immunoprecipitation (IP) assay using anti-HA antibody.
4. DISCUSSION

Here, I show new modification of DR4 and its potential prospect for the advanced clinical practice of TRAIL. From functional screen, I discovered several clones which suppressed TRAIL-mediated cytotoxicity. Among them, unexpectedly, DR4 was included as a potent suppressor of the cell death. From a subsequent DNA sequencing analysis of the full-length clone, I found that DR4 was mutant form harboring mutations at several places. In our analysis, I figured out the functional consequence of the mutation, showing that only DR4, not DR5, is O-GlcNAcylated by OGT on Ser424 in death domain, which is facilitated after TRAIL ligation which enables to initiate active death signaling. This modification is required for receptor aggregation and subsequent amplification of DR4-mediated cytotoxic signaling via DISC or necrosome formation. Initially, I focused on the phosphorylation of DR4 Ser424 residue but DR4 turned out to be O-GlcNAcylated upon TRAIL stimulation. In various cancer cell lines, I observed a strong correlation between DR4 O-
GlcNAcylation status and TRAIL sensitivity of the cell.

To characterize the possible modification in DR4 Ser424, I first took advantage of modified cell lysis methods for stepwise separation of crude fractions. Ligated trimeric TRAIL receptors generally form clusters during onset of TRAIL signaling (Song et al., 2007; Ouyang et al., 2013; Linderoth et al., 2013) and a series of these processes can be facilitated through the translocation of receptors into the compartmentalized lipid rafts. Lipid raft-cytoskeleton region is tightly packed and aggregated so that this compartment is Triton X-100 insoluble (Wang et al., 2012). O-GlcNAcylated DR4 was detected primarily in the Triton X-100-insoluble fraction, suggesting that this modification is crucial for DR4 translocation into the lipid raft-cytoskeleton compartments and receptor clustering. Moreover, O-GlcNAcylated DR4 was detected in the DISC containing FADD and caspase-8 locating in the lipid raft.

It appears that DR4 O-GlcNAcylation might be a nutrient-dependent checkpoint that licenses DR4 to transmit death signals from TRAIL stimulation.
Tumor cells very often display altered glucose metabolism, increased glucose uptake and glycolysis switch, which is considered to be one of the hallmark of cancer (Annibaldi and Widmann, 2010; Yu et al., 2017). It cannot be emphasized enough that understanding of the reprogrammed glucose metabolism in cancer has been closely related with cancer therapy. Furthermore, O-GlcNAcylation is highly dependent on glucose and is generally increased as a communicator linking the nutritional status to various metabolic pathways in cancer cells (Yang and Qian, 2017). Thus, I speculate that elevated glucose uptake and HBP flux of tumors may cause the enhancement of DR4 O-GlcNAcylation and may subsequently induce cancer cell-specific sensitization to TRAIL. Basal level of DR4 O-GlcNAcylation is also observed in our experiments, but this extent may not reach the threshold to trigger cell death in non-transformed or non-stimulated cells. In tumor cells, however, glucose uptake becomes greatly increased so that strongly up-regulated DR4 O-GlcNAcylation may help TRAIL to kill cancer cells selectively. That is, O-GlcNAcylation status may determine the sensitivity of cancer cells to TRAIL.
Furthermore, I also found that the increased DR4 O-GlcNAcylation by providing extensive high-glucose condition even enables TRAIL-resistant cancer cells to overcome their tolerance. On the other hand, acute glucose deprivation can activate or upregulate oncogenic pathways and make favorable environments for tumor cells (Yun et al., 2009; Mayers and Vander Heiden, 2015). Consistently, absence of DR4 O-GlcNAcylation by glucose shortage rendered tumor cells more resistant to apoptosis in our study. Previous reports, however, have shown that TRAIL application within glucose-deprived system makes cancer cells more vulnerable to TRAIL (Iurlaro et al., 2017; Muñoz-Pinedo et al., 2003). According to our research, duration of glucose-free condition is the key factor that elicits different responses to TRAIL in in vitro systems. Therefore, I propose that manipulation of glucose level can provide a new way for chemotherapy against the TRAIL-resistant cancer cells but it needs to be handled carefully with proper time point and to depend on the context of cells, such as DR4-dependence, for relevant TRAIL therapy.

The precise mechanism remains unexplored how DR4 O-GlcNAcylation is
further promoted by TRAIL stimulation. In our observations, TRAIL ligation does not alter the expression level of OGT but significantly enhances DR4 O-GlcNAcylation and the interaction of DR4 with OGT. It is well known that O-GlcNAcylation is deeply involved in other modifications, including phosphorylation (Hart et al., 2011) or ubiquitination (Han et al., 2017), and proteolysis (Capotosti et al., 2011). Because I detected a possibility for DR4 phosphorylation at other residues (Figure 5a), it will be interesting to investigate whether DR4 O-GlcNAcylation has a close cross talk with the phosphorylation or something other. In addition, I observed that the inhibitory effects among DR4 Ser424 mutants, DR4 S424P and DR4 S424A, on their cytotoxicity are not equally same. In the immunoprecipitation assay using phosphorylated serine antibody, DR4 S424P also displays a little different phenotype. I infer the reason of this result from the unique structural characteristic of proline, because proline has an exceptional conformational rigidity due to its distinctive cyclic structure of its side chain compared to other amino acids (Reid Alderson et al., 2017). The secondary structure of proteins near a proline residue is affected by this trait of
proline, Proline is commonly located in turns of secondary structures such as at the first site of an $\alpha$-helix or in the edge strands of $\beta$-sheets (Buevich et al., 2000). In other words, in the secondary structures, proline can cause conformational changes of proteins.

Since TRAIL monotherapy has revealed indisputable limitations in clinical trials, combined chemotherapies with TRAIL have received attention. It is required to provide the effective TRAIL-comprising therapeutic concepts. Given this need, our study addresses the mechanism of combined TRAIL therapy with 2-DG or extensive high glucose. 2-DG has the advantage since its safety is well-established and its specific effect on cancer cells is also proven (Pusapati et al., 2016). As recently reported, 2-DG metabolism has effect on augmenting sensitivity to TRAIL in TRAIL-resistant cancers as well (Huang et al., 2013; Liu et al., 2009). However, the importance of DR4 participation in synergistic cell death by 2-DG plus TRAIL remains unclear, while DR5 contributes partly, but not all, to 2-DG effect via increase of surface expression (Carr et al., 2016). Surprisingly, 2-DG also leads to hyper-$O$-
GlcNAcylation of a certain protein despite of its inhibitory role for glycolysis (Kang et al., 2003). I thus report for the first time that encouraging DR4 O-GlcNAcylation by 2-DG or extensive high glucose is the key mechanism to restore the effectiveness of TRAIL in TRAIL-resistant cancer cells. This point can provide a missing link between elevated O-GlcNAcylation by 2-DG and 2-DG-mediated enhancement of TRAIL death. The detailed strategies, however, should be sufficiently discussed to design safe approaches for specific clinical application. In addition, since I found shorter DR4, which seems to be a cleaved product or unmodified form by N-glycosylation (Dufour et al., 2017), after 2-DG treatment (Figure 16d), there is still another mechanism unrevealed yet.

Although several modifications of DR4 and DR5 have been investigated in depth, recent studies highlight particularly the importance of sugar modifications. All of modifications widely known, however, spotlighted on triggering only apoptotic elimination of cancer cells, despite TRAIL can induce programmed necrosis. In this study, I apparently demonstrate that DR4 O-GlcNAcylation plays an important role in
TRAIL-necrosis sensitization as well. Tumors capable of escaping from both apoptotic and necrotic defenses can have quite aggressive and metastatic phenotypes. Because $O$-GlcNAcylation is responsible for DR4-associated necrosis as well as apoptosis, cancer cells harboring Ser424-mutated DR4 gene are at risk for becoming more tolerant of TRAIL therapy. Moreover, until now, the reason why human requires particularly two receptors unlike a mouse is not understood, and, furthermore, how their differential tuning mechanisms are relevantly in close connection within the physiological context is one of the main concern in the studies on TRAIL receptors. I supposed that $O$-GlcNAcylation is a novel modification which clearly enables to distinguish DR4 signaling from that of DR5. On the other hand, $O$-GlcNAcylation-defective DR4 Ser424 mutant can disturb not only the function of wild-type DR4 (Figure 10c) but also that of DR5 (Figure 10d) via direct interaction. Given that DRs can form heterotrimer upon TRAIL ligation (Evelien et al., 2009), the recruitment of DR4 Ser424 mutant to wild-type DR5 can undermine DR5-mediated TRAIL signaling in a dominant-negative manner, which might cause tumorigenesis in cells.
Thus, I provide evidences that DR4 Ser424 residue deserves careful considerations to a reliable diagnostic marker for genetic test of cancer patients. To identify changes on DR4 Ser424 is likely to be a potential therapeutic approach for TRAIL administration to cancer patients. Even though it needs to be more explored whether Ser424 mutation can gain the tumorigenic potential, promoting DR4 O-GlcNAcylation obviously may have synergistic effect on TRAIL remedy and may improve TRAIL-based clinical fields by enabling to overcome TRAIL resistance in certain circumstances, when the clinical application of TRAIL could not be translated successfully into patients.
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국문 초록

암환자 샘플에서 발견된 DR4의 돌연변이에 의한 TRAIL 내성
조절 기작 규명

TRAIL은 암세포만을 선택적으로 제거할 수 있는 치료제로서 주목받아 온 리간드이다. DR4는 TRAIL을 인식하여 결합하는 수용체 중의 하나로서, 암세포 특이적인 세포사멸을 유도하는 데 있어서 중요한 역할을 한다. 하지만 상당히 많은 암세포들이 TRAIL에 내성을 가짐으로써 세포사멸을 회피한다는 것이 밝혀지면서, 다양한 치료제와의 융합을 통해 TRAIL의 효율성을 높이는 방안에 관한 많은 연구가 필요한 실정이다. 뿐만 아니라, TRAIL을 인식하여 신호를 전달하는 수용체들의 조절 메커니즘에 관한 규명 역시 매우 중요하다.

본 논문에서는 암환자에서 유래한 DNA들의 기능을 검사하여 TRAIL 내성을 유발하는 요인을 찾고자 하였다. 검사 결과 홍미롭게도
TRAIL의 두 가지 수용체들 중 하나인 DR4의 돌연변이체가 발견되었으며, DR4 돌연변이체의 424번째 serine에 유전적 변이가 있으면 TRAIL에 의한 세포사멸을 억제하는 것을 확인하였다. 이 야미노산은 DR4에서만 특이적으로 발견된 새로운 조절 기작인 O-GlcNAcylation의 타겟 잔기였으며, 이 돌연변이는 TRAIL에 의해 유도되는 세포사멸뿐만 아니라 괴사 역시 저해하는 것을 관찰하였다. DR4가 O-GlcNAcylation에 의해 조절되는 정도는 TRAIL과 수용체가 결합하였을 때 더 가속화되며, 이는 세포 내의 glucose의 농도에 영향을 받는다는 사실을 확인하였다.

DR4 424번째 serine의 돌연변이체는 O-GlcNAcylation의 결함으로 인해 DR4의 정상적인 암세포 독성 기능을 수행할 수 없으며, 이는 암세포의 TRAIL 내성과 밀접한 관계가 있다. DR4가 발현되는 암세포들을 수집하여 TRAIL 내성을 갖는 집단과 그렇지 않은 집단으로 분류한 뒤, 각 집단의 세포들에서 DR4 O-GlcNAcylation 양상을 살펴보았을 때, TRAIL 내성을 갖는 암세포들에서만 O-GlcNAcylation이 관찰되지 않는 것을 확인하였다. 또한 이 집단의 세포들에서 DR4의 O-GlcNAcylation을
인위적으로 촉진시켰을 때, TRAIL에 의한 세포사멸이 유의미하게 증가되는 것을 관찰할 수 있었다. 이러한 결과들을 통해, DR4 424번째 serine의 O-GlcNAcylation을 조절함으로써 TRAIL 내성을 갖는 암환자들에게 대한 효율적인 치료 방안을 마련할 수 있다는 가능성을 제시한다.