O-glycans truncation modulates gastric cancer cell signaling and transcription leading to a more aggressive phenotype

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Abstract

Background: Changes in glycosylation are known to play critical roles during gastric carcinogenesis. Expression of truncated O-glycans, such as the Sialyl-Tn (STn) antigen, is a common feature shared by many cancers and is associated with cancer aggressiveness and poor-prognosis.

Methods: Glycoengineered cell lines were used to evaluate the impact of truncated O-glycans in cancer cell biology using in vitro functional assays, transcriptomic analysis and in vivo models. Tumor patients’ samples and datasets were used for clinical translational significance evaluation.

Findings: In the present study, we demonstrated that gastric cancer cells expressing truncated O-glycans display major phenotypic alterations associated with higher cell motility and cell invasion. Noteworthy, the glycoengineered cancer cells overexpressing STn resulted in tumor xenografts with less cohesive features and major phenotypic alterations associated with higher cell motility and cell invasion. Furthermore, truncation of O-glycans induced activation of EGFR and ErbB2 receptors and a transcriptomic signature switch of gastric cancer cells. The disclosed top activated genes were further validated in gastric tumors, revealing that SRPX2 and RUNX1 are concomitantly overexpressed in gastric carcinomas and its expression is associated with patients’ poor-survival, highlighting its prognostic potential in clinical practice.

Interpretation: This study discloses novel molecular links between O-glycans truncation frequently observed in cancer and key cellular regulators with major impact in tumor progression and patients’ clinical outcome.

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1. Introduction

Gastric cancer is a major threat to public health worldwide, with >700,000 deaths every year [1]. A common characteristic shared by many cancers is glycosylation alterations at cell surface, providing a set of diagnostic biomarkers and therapeutic targets currently being used in clinic, and known to play crucial roles during carcinogenesis [2–4]. Metastasis is often the major cause of cancer-related death. Specific altered glycosylation patterns have been reported in metastatic tissue due to changes in the expression of the enzymes controlling the synthesis of such glycans. These modifications showed to be crucial for cancer cells to adapt to a new host environment such as the metastatic site [5,6]. Alterations on specific proteins glycosylation disturb cell-cell and cell-extracellular matrix (ECM) adhesion features supporting cancer progression [7–9]. In addition, changes occurring in receptor tyrosine kinases (RTKs) glycosylation have also been reported to play key roles in cancers [10–12]. Particularly, we have previously shown that altered RTKs sialylation resulted in its hyperactivation leading to a pro-invasive phenotype of gastric cancer cells [13,14]. In other models, high levels of sialylation displayed by cancer cells have also contributed to increased invasiveness of tumor cells and concomitant higher metastatic potential [3,15–17]. Truncation of the O-glycosylation pathway, leading to expression of simple O-glycans, is one of the most common features of many cancers [18–22], including gastric cancer [23–25]. These truncated glycan structures include the T (Galβ1-3GalNAcα1-0-
Ser/Thr), Tn (GalNAc1-O-Ser/Thr) and Sialyl-Tn (STn) (NeuAcα2–6GalNAc1-O-Ser/Thr) antigens (Fig. 1a) [26–28].

The truncated STn glycan, a well-known tumor-associated antigen, is highly detected in most gastric carcinomas [29–31] as well as in other tumor tissues [32–34], and its detection is rare or absent in normal tissue [35–37]. The mechanisms underlying STn synthesis include the overexpression of the sialyltransferase ST6GalNAc1 enzyme, responsible for STn biosynthesis (Fig. 1a) [28,30], and lack of expression of the core 1 synthase C1GALT1 private chaperon COSMC, essential for O-glycans elongation. Therefore, mutations or hypermethylation of COSMC gene can also lead to STn overexpression [38,39] (Fig. 1a). In fact, genetically engineered COSMC knock-out gastric cancer cell models, named SimpleCells (SC), display overexpression of truncated O-glycan antigens Tn and STn [40,41]. Cell genetic manipulation forcing STn synthesis, either by overexpression of ST6GalNAc1 enzyme [42] or by dysregulation of C1GALT1 gene [22] led to increased metastasis and decreased survival in mice. On the other hand, silencing of ST6GalNAc1 suppressed invasion in hepatocarcinoma cells [43] and prevented metastatic potential in gastric cancer cells [44]. Although the association between STn and poor survival of cancer patients has been known for decades [45–47], the mechanisms underlying such phenotype remain unknown.

In the present study, we have evaluated the molecular and functional effects of the presence of truncated O-glycans, particularly the STn antigen, in gastric cancer using glycoengineered COSMC knock-out models, disclosing the link between STn cancer-associated phenotype and its consequences for tumor progression.

2. Materials and methods

2.1. Cell culture

The gastric cancer cell lines MKN45 and AGS were obtained from the Japanese Collection of Research Bioreresources and ATCC, respectively. MKN45 and AGS SimpleCells (MKN45 SC and AGS SC) were generated.
by targeting the COSMC gene using zinc finger nuclease precise gene editing as previously described [41]. Briefly, both MKN45 and AGS cells were transfected with 4 μg of compoZr® C1GalT1C1 DNA using an AmaxaTM Nucleofector® TM according to cell lines specific manufacture’s protocols (Lonza). The cells were grown RPMI in 1640 Glutamax, HEPES medium supplemented with 10% FBS plus 1% penicillin-streptomycin (all from Invitrogen) and maintained at 37 °C in an atmosphere of 5% CO₂.

2.2. Antibodies

All the antibodies used in this manuscript, as well as its protocol details are listed in Table 1.

2.3. Immunofluorescence confocal microscopy

Cells were seeded on ibidi µ-Slide 8-well chambers with different coatings, ibidi-treated surface (polymer-coated), collagen IV, fibronectin and poly-D-Lysine (ibidi) at 70% confluence. The cells were fixed with 4% p-formaldehyde for 20 min and labeled with tubulin and actin antibodies or with sialyl-Tn [48] and actin antibodies overnight at 4 °C (Table 1). Goat anti-rabbit Alexa 549 and goat anti-mouse Alexa 488-conjugated antibodies (Thermo Fisher Scientific) were used for 45 min at room temperature. DAPI (Sigma-Aldrich) was used for nuclei visualization. Cells were visualized using a Zeiss Imager Z.1 microscope (Zeiss).

2.4. EGF treatment

MKN45 WT and SC (5 × 10⁵ cells) were seeded on a 6-well plate in RPMI supplemented with 10% FBS. After 48 h the medium was replaced with non-supplemented RPMI medium and cells were treated with 25 ng/mL of EGF ligand. After EGF incubation for 10 min, cells were collected and protein was extracted to evaluate EGFR phosphorylation status as described in the western blot section. To evaluate gene expression regulation, cells were collected 4 h after EGF incubation. RNA was extracted and real-time quantitative PCR (RT-qPCR) for SRPX2 and RUNX1 genes was performed as described in the transcriptomic analysis section.

2.5. Transcriptomic analysis

Total RNA was extracted from MKN45 WT and SC, AGS WT and SC cell lines using TRI Reagent (Sigma-Aldrich). Ion AmpliSeq Transcriptome Human Gene Expression Kit was used to sequence the mRNAs of over 20,000 primed targets. Ion Chef system was used for templating and the loaded chips were sequenced using the Ion Proton System (Life Technologies). After sequencing, the data was automatically transferred to the dedicated Ion Torrent server and the sequencing reads were generated. Reads quality and trimming was performed using Torrent Server v4.2 before read alignment using TMAP 4.2. The TS plugin Coverage Analysis v4.2 was used to generate read counts. The sequencing was performed in two independent biological replicates and sequence reads were normalized to the total read count. Genes from MKN45 SC and AGS SC were analyzed in comparison with MKN45 WT and AGS WT, respectively. Only the genes presenting >10 reads and at least 2-fold change differences between the two sets after considering its standard deviation, were selected for analysis.

For RT-qPCR gene expression analysis total RNA was extracted from MKN45 WT and SC using TRyzol Reagent (Invitrogen). One μg of RNA was reverse transcribed with random primers using the SuperScript® IV Reverse Transcriptase Kit (Invitrogen). RT-qPCR was performed with 1 μL of cDNA, 10 μM of each primer, 10 μL SYBR® Green Master Mix (1×) (Thermo Fischer Scientific) and ultrapure water to a final volume of 20 μL using the ABI 7500 (Applied Biosystems). The following primers were used, SRPX2 (Fw: ACTGAGATGCGGCAATGTG; Rv: CCAT GATGAGTGGAGCCAGTGA; 146 bp), RUNX1 (Fw: CTGCTGGTCG TGCTTAC; Rv: AGCCATACAGTACACGTAGT; 109 bp). Relative gene expression was normalized to β-actin (Fw: AGGAAAACTGCGCACCACAC; Rv: TACGACAGCCTGAGATGCAA; 173 bp) and DeltaDelta CT was performed to compare different conditions. All conditions were normalized and compared to MKN45 WT non-treated cells. Three independent experiments with three technical replicates per condition were performed. Results are shown as average ± SEM. Two-way ANOVA with Bonferroni correction was used for statistical analysis.

2.6. Western blot

Cells were lysed in RIPA buffer and protein quantified with DC protein assay (BioRad). Total protein extracts were separated by gel electrophoresis and gels transferred onto a nitrocellulose membrane (Amersham). Membranes were probed overnight with phosphorylated FAK and FAK, phosphorylated EGFR and EGF, and phosphorylated ERBB2 and ERBB2 primary antibodies (Table 1). Membranes were developed with ECL (GE Healthcare Life Sciences). The phospho-protein band quantification was normalized for the respective total protein amount. MKN45 and AGS SC were analyzed in relation with MKN45 WT and AGS WT, respectively. Actin or tubulin (Table 1) were used as loading control. Three biological independent batches of protein extracts were analyzed for each cell model. Results are shown as average ± SEM. Student t-test was used for statistical analysis.

2.7. Proliferation assay

BrdU Labelling and Detection Kit (Roche) was used according to the manufacturer’s instructions. Briefly, cells (1 × 10⁵) were seeded in cover slips. When cells reached a 50% confluence, cells were incubated with BrdU labelling medium for 30 min. The nuclei were stained with DAPI. The percentage of dividing cells was measured by counting the number of positive BrdU cells in five different fields. Pictures were acquired using a fluorescence microscope (Carl Zeiss), and analyzed with Image J software. Three independent biological experiments were performed and cells were seeded in duplicate. Results are shown as average ± SEM. Student T-test was used for statistical analysis.

2.8. Adhesion assay

Adhesion assays were performed using the ibidi culture-inserts family. Cells were plated on ibidi µ-Slide 8-well chambers with different coatings, ibidi-treated surface (polymer-coated), collagen IV, fibronectin and poly-D-Lysine (e). Both glycoengineered MKN45 SC and AGS SC exhibited a more elongated cell shape, displaying more cytoskeletal actin and tubulin projections, which also stained positive for STn antigens.
Table 1

List of antibodies.

| Antibody                  | Clone     | Source         | Application | Dilution       |
|---------------------------|-----------|----------------|-------------|----------------|
| Tubulin                   | DM1A      | Sigma          | IF/WB       | 1:750/1:10000  |
| Actin                     | 1-9       | Santa Cruz     | IF/WB       | 1:150/1:2000   |
| Sialy-Tn                  | 3F1       | [48]           | IF/IHC      | 1:200/1:5      |
| Phosphorylated FAK (Tyr937) | #3283     | Cell Signaling | WB          | 1:1000         |
| Phosphorylated EGFR (Y1068)| D7A5      | Cell Signaling | WB          | 1:1000         |
| EGFR                      | D38B1     | Cell Signaling | WB          | 1:1000         |
| Phosphorylated ErbB2 (Y1212/1222) | 6B12     | Cell Signaling | WB          | 1:1000         |
| ErbB2                     | 29D8      | Cell Signaling | WB          | 1:1000         |
| E-cadherin                | 4A2C7     | Thermo Fisher  | IHC         | 1:50           |
| SRPX2                     | ABN488    | Millipore      | IHC         | 1:500          |
| RUNX1                     | sc-365644 | Santa Cruz     | IHC         | 1:50           |
| CD31                      | AB28346   | Abcam          | IHC         | 1:50           |

Abbreviations: IF – Immunofluorescence; WB – Western Blot; IHC - Immunohistochemistry.

2.9. In vitro wound-healing assay

Wound-healing assays were performed using the 2-well silicone ibidi inserts from ibidi culture-inserts family. The 2-well ibidi inserts were applied to ibidi μ-Slide 8-well chambers coated either with ibidi-treated surface (polymer), collagen IV, fibronectin or poly-lysine (ibidi). MKN45 WT and SC cells (8 × 10⁴) and AGS WT and SC ibidi-treated surface (polymer), collagen IV, were seeded and RPMI medium supplemented with 1% P/S (7 × 10⁴) were seeded and RPMI medium. Inserts were rehydrated for at least 1 h in RPMI medium. Cells chamber with 8 μm diameter hole of diameter pore size, in 24-well plate (BD Biosciences). Inserts were rehydrated for at least 1 h in RPMI medium. Cells were seeded in duplicate and two independent biological experiments were performed. The percentage of area with no cells was calculated by measuring the free space at each time-point, normalized to the initial area with no cells (right after insert removal) using Image J software. Results are shown as average ± SEM. Two-way ANOVA was used as statistical test.

2.10. In vitro invasion assay

Invasion assays were performed in BD Biocat Matrigel invasion chamber with 8 μm of diameter pore size, in 24-well plate (BD Biosciences). Inserts were rehydrated for at least 1 h in RPMI medium. Cells (7 × 10⁴) were seeded and RPMI medium supplemented with 1% P/S and 10% FBS was used as chemo-attractive. After 24 h, non-invading cells were carefully removed from the upper part of the insert. The inserts were washed and cells were fixed with cold methanol for 10 min on ice. Total number of invasive nuclei (DAPI labeling) was counted using a Leica DM2000 microscope (Leica). Cells were seeded in duplicate for each cell line and three independent biological replicates were performed. Results are shown as average ± SEM. Student T-test was used for statistical analysis.

2.11. In vivo chorioallantoic membrane (CAM) angiogenic assays

In vivo angiogenic activity of MKN45 WT and SC, AGS WT and SC cells was assessed by the CAM assay. Fertilized chick (Gallus gallus) eggs (8 per group) obtained from commercial sources were incubated horizontally at 37.8 °C in a humidified atmosphere and referred to embryonic day (E). On E3 a square window was opened in the shell after removal of 1.5 mm diameter) growing radially towards the ring area was counted in a blind fashion manner. Student T-test was used for statistical analysis.

2.12. In vivo survival, tumorigenesis and angiogenesis evaluation in nude mice

N:NIH(s)Ilf:nu/nu nude mice were housed at IPATIMUP Animal’s House (Medical Faculty of the University of Porto) under a pathogen-free environment, with controlled light and humidity. Two groups of female N:NIH(s)Ilf:nu/nu nude mice, aged 6–8 weeks, were subcutaneously injected between the scalpula with 1 × 10⁶ viable cells of MKN45 WT (5 mice) and MKN45 SC (4 mice). Mice were examined and tumors were measured every two days. The product of 3 major diameters was record as the tumor volume. After 21 days of cell injection, the primary tumors were surgically removed from all animals, except for one animal that died 18 days after cell injection. Surgical procedures were performed under anesthesia. The animals were left after surgery for survival analysis. Their weight was followed 25 days after surgery. The living mice were sacrificed and examined by detailed necropsy and liver, lymph nodes and lung were collected. The organs and the primary tumors were fixed in 10% buffered formalin and then embedded in paraffin for hematoxylin eosin (HE) staining and immunohistochemistry labeling. For morphological evaluation, the tumors were visualized and analyzed under a brightfield microscope and parameters such as cell morphology and tumor cell cohesion were evaluated by two independent pathologists. Necrosis was evaluated as a percentage of the whole tissue. Angiogenesis was measured by vessel number counting after overnight CD31 immunolabeling (Table 1). Kaplan-Meier method by means of Gehan-Breslow-Wilcoxon Test was used for survival curves statistical analysis.

2.13. Immunohistochemistry

Expression of STn, E-cadherin, SRPX2, RUNX1 and CD31 (Table 1) were assessed in tumor mice xenograft tissues. Detection of STn, SRPX2 and RUNX1 was measured in 25 cases of human gastric carcinoma. Heat mediated antigen retrieval using sodium citrate buffer (10 mM, pH 6.0) was required for the E-cadherin, RUNX1 and CD31 markers. Endogenous peroxidase blocking was performed in all the samples using 3% hydrogen peroxide. Tissues were incubated overnight with the respective primary antibodies (Table 1), followed by biotin-labeled secondary antibodies (Dako) and with ABC kit (Vector Labs). Sections were stained with DAB (Sigma-Aldrich) containing 0.01% H₂O₂ and counterstained with Mayer’s hematoxylin. Pictures were taken under 400× magnification with a Zeiss Optical Microscope.
The differently expressed genes between MKN45 SC and WT and between AGS SC and WT cells were analyzed with PANTHER (http://pantherdb.org) [49] for Gene Ontology (GO) functional enrichment in terms of Biological Processes (GO database release 2016.07.29). The number of mapped gene IDs was 752 out of 825 for MKN45 and 375 out of 413 for AGS cell lines. The statistical over-representation was calculated using a binomial test (release 2016.07.15) with Homo sapiens gene set as background reference and the results were considered significant at p < 0.05, after Bonferroni correction. All significantly enriched GO Biological Processes were presented as converted –log p-value and gene frequency and redundant terms clustered manually according to term similarity. Additionally, we performed a similar GO functional enrichment on a list of gene IDs corresponding to proteins previously identified to carry truncated glycans in MKN45 SC and AGS SC [41]. The protein-encoding genes associated to the GO term Adhesion are presented (Supplementary Table 4).

The Oncomine platform (https://www.oncomine.org) [50] was used to evaluate the expression and prognostic value of SRPX2 and RUNX1 in independent datasets of gastric cancer patients. SRPX2 and RUNX1 mRNA levels in normal and tumor samples were assessed in 5 independent cohorts (Wang [51], Cui [52], DeRico [53], Chen [54] and Cho [55]), comprising a total of 453 tissues, including 175 adjacent gastric mucosa and 278 gastric cancer tissues. The prognostic value of SRPX2 and RUNX1 was further evaluated in the dataset with higher number of cases and information for overall survival (Chen [54]). Categorization of patients with high and low expression was based on the log2 median-centered intensity values. The top 25% patient samples with highest expression values (>75% of all patients) were categorized as SRPX2- or RUNX1-high, and all the other patients were grouped as SRPX2- or RUNX1-low. Student t-test was used for the expression statistical analysis. Kaplan-Meier method was used for survival curves statistical analysis.

2.15. Ethics statement

Human tissue samples were obtained from IPATIMUP Diagnostics in accordance with the national regulative law for the handling of biological specimens from tumor banks, and the international Helsinki declaration.

Mice experiments followed the European Directive 2010/63/UE and the corresponding Portuguese law on animal experimentation (Portaria 113/2013), according to the FELASA (Federation of European Laboratory Animal Science Associations) guidelines and recommendations concerning laboratory animal welfare. The protocol was previously approved by the Local Animal Ethics Committee and authorized by the Portuguese Official Veterinary Department (Direccào Geral de Veterinária – DGV).

3. Results

3.1. Expression of truncated O-glycans in gastric cancer cells impacts cell shape

Two gastric carcinoma SimpleCell lines, MKN45 and AGS, were generated by targeting the COSMC gene using zinc-finger nuclease technology [41]. This manipulation resulted in the disruption of O-glycans normal elongation and increased expression of truncated O-glycans, Tn and STn (Fig. 1a).

COSMC knock-out in both MKN45 (MKN45 SC) and AGS (AGS SC) cells led to the overexpression of STn, both in the cytoplasm and plasma membrane of the targeted cells (Fig. 1b–e). Concomitantly, critical changes in MKN45 SC and AGS SC cell morphology could be observed through actin and tubulin labeling when cells were grown in different extracellular matrix (ECM) components, including collagen IV, fibronectin and poly-L-lysine (Fig. 1b–e). The MKN45 SC and AGS SC presented a mesenchymal-like morphology with more cytoskeletal actin and tubulin protrusions compared to wild-type (WT) cells. Overall, truncation of O-glycans drastically gastric cancer cell morphology.

3.2. Truncation of O-glycans in gastric cancer cells promotes invasive features

To evaluate the impact of truncated O-glycans on cellular behavior, a set of proliferation, adhesion, migration and invasion assays were performed. MKN45 SC had similar cellular proliferation rates to the WT cell line (Fig. 2a). MKN45 SC displayed higher adhesion capacity, to a polymer coated surface (Fig. 2b) and to collagen type IV and poly-L-lysine ECM components, whereas no differences were observed when cells were grown on fibronectin (Supplementary Fig. 2a–c). Furthermore, MKN45 SC revealed an increased migration capacity independently of all the tested ECM components (Fig. 2c, Supplementary Fig. 2d–f). The enhanced motility of MKN45 SC, in comparison with MKN45 WT, was accompanied by increased activation of the focal adhesion kinase (Fak) protein (Fig. 2d).

AGS SC maintained similar proliferation rates (Fig. 2a), compared to WT AGS. However, AGS SC showed decreased adhesion capacity when compared with WT cells (Fig. 2b and Supplementary Fig. 2a–c). AGS SC showed no major impact on the migration capability (Fig. 2e) except for a decreased motility noted on fibronectin component (Supplementary Fig. 3d–e). In contrast with MKN45 SC, AGS SC cells did not show Fak activation when compared with its parental cell line (Fig. 2f).

A PANTHER gene ontology (GO) analysis was performed using previously identified proteins carrying truncated glycans in MKN45 SC and AGS SC [41]. We have analyzed the truncated O-glycosylated adhesion-related proteins present in our samples (Supplementary Table 4), combining the information with the RNAseq values of the respective genes. Although some proteins were found to be commonly O-glycosylated in both models, several proteins were found to be specific of each cell line model. Interestingly, proteins belonging to the cadherin family were specifically O-glycosylated in MKN45 SC. Particular laminin and integrin subunits were found to be the O-glycosylation carriers in AGS SC. The correspondent RNAseq values corroborated the difference in expression levels of the genes belonging to the adhesion GO term in MKN45 SC and AGS SC. Regardless of the differences found in the adhesion and migration capacity between MKN45 SC and AGS SC, both models revealed an increased ability to degrade and invade the ECM in comparison to the respective parental cell line (Fig. 2g–h). Overall, these results demonstrated that premature truncation of O-glycans per se was capable of promoting a pro-invasive phenotypic behavior on gastric cancer cells.

3.3. Glycoengineering of gastric cancer cells triggers a switch in the cell transcriptomic signature

In order to identify the genetic signature underlying the increased invasive phenotype observed in the glycoengineered cell models, a transcriptomic analysis on >20,000 genes was performed in both MKN45 and AGS cell models. It was observed that COSMC knock-out resulted in a significant alteration in the transcription profile of both gastric cancer cell models when compared to their respective parental cells. From the genes that showed significant transcription alterations, 185 (63.4%) genes were upregulated in MKN45 SC and 99 (33.9%) genes in AGS SC, with 8 (2.7%) of those genes being commonly upregulated in both models. The analysis of the downregulated genes revealed 606 (66.4%) in MKN45 SC and 280 (30.7%) in AGS SC, with 26 (2.9%) genes downregulated in both SimpleCell models (Fig. 3a and Supplementary Table 1). The differentially expressed genes were analyzed with PANTHER, for functional enrichment analysis, in order to determine the GO biological processes significantly affected in the SimpleCells. Our analysis showed that the genes significantly altered were over-
represented in GO categories clustered as development and motility processes (Fig. 3b). Significantly upregulated and downregulated genes in both MKN45 SC and AGS SC were represented in a heatmap, with SRPX2 (Gene ID: 27286) and RUNX1 (Gene ID: 861) being the two most upregulated genes in the two COSMC knock-out gastric carcinoma cell models. The expression patterns of the downregulated genes were dissimilar between the two cell models (Fig. 3c). This transcriptomic analysis showed that targeting the O-glycosylation...
pathway led to significant changes in the transcriptomic profile between the isogenic cell lines in both MKN45 and AGS cell models.

3.4. Induced O-glycosylation truncation activates receptor tyrosine kinases (RTK) in gastric cancer cells

We have previously showed that RTKs could be activated due to changes in cell glycosylation [13,14]. To evaluate if in our model of gastric cancer cells expressing truncated O-glycans some particular RTKs were activated, we have analyzed the phosphorylation status of both EGFR and ErbB2 RTKs. We found that glycoengineering of gastric cancer cells led to activation of both receptors (Fig. 4a). Furthermore, we evaluated if the activation of these receptors could be responsible for inducing up-regulation of SRPX2 and RUNX1 genes. By treating MKN45 WT cells with the EGFR ligand (EGF) to force receptor activation we demonstrated that both RUNX1 and SRPX2 were up-regulated to the same levels of expression found in the SC model (Fig. 4b, c). The same treatment did not induce further transcription activation of SRPX2 and RUNX1 genes in the MKN45 SC model, where both EGFR and ErbB2 were found to be constitutively activated. These results shed light on the mechanism by which O-glycosylation truncation in our cells could lead to a switch in the cell transcriptomic profile of SRPX2 and RUNX1 genes.

3.5. Truncated O-glycans impact tumor features and survival in a mouse model

MKN45 SC and WT cells were injected in nude mice to evaluate the impact of truncated O-glycans in an in vivo environment. After 16 days of cell heterotopic injection, MKN45 SC-injected mice started to lose weight, being significantly lower at day 21 when compared with MKN45 WT-injected mice (Fig. 5a and c). Both MKN45 SC and WT cells induced tumor formation in vivo and no significant differences in tumor size were observed over time (Fig. 5b, d and e). The animals were kept after tumor removal for prognostic value evaluation and all the tumors were assessed for several histopathologic features. MKN45 SC tumors presented larger and more elongated cells characterized by a higher pleomorphism, and a large number of atypical mitosis compared to MKN45 WT tumors (Fig. 5f). Additionally, a less cohesive cellular pattern was observed in MKN45 SC tumors when comparing to the WT xenografts. This decreased cell-cell adhesion pattern was accompanied by a mislocalization of E-cadherin from the membrane to the...

Fig. 3. Transcriptomic expression profile of MKN45 SC and AGS SC discloses an enrichment in genes associated with development and motility. a) Venn diagram of upregulated (upper panel) and downregulated (lower panel) genes between MKN45 SC and MKN45 WT (in blue) and between AGS SC and AGS WT (in grey). The results are represented as gene count (and percentage) with the intersecting values indicating the common genes between MKN45 and AGS cell lines. Premature truncation of O-glycans lead to transcriptional changes in about 60% and 30% of both up- and down-regulated genes in MKN45 SC and in AGS SC model, respectively. Only about 3% of the genes were either commonly up- or down-regulated in both cell models. b) Functional enrichment analysis of GO Biological Processes for the set of differentially expressed genes between MKN45 SC and MKN45 WT (in blue) and between AGS SC and AGS WT (in grey). The results are represented as –log p-value (left axis) and gene frequency (right axis) per functional category with categories clustered based on term similarity. The genes differentially expressed were annotated to significantly enriched GO terms related to development, motility and organism processes. The statistical over-representation was calculated using a binomial test (release 2016.07.15) with Homo sapiens gene set as background reference and the results were considered significant at p < 0.05, after Bonferroni correction. c) Heatmap of the common differentially expressed genes between MKN45 and AGS cell lines. The results are represented as fold change ranked as percentile, indicated by the graded colour scale. SRPX2 and RUNX1 genes were the most upregulated genes in the two cell line models. Two biological replicates per cell line were used for transcriptomic analysis.
cytoplasm on MKN45 SC tumors (Fig. 5f), whereas MKN45 WT tumors showed a typical membrane localization of E-cadherin. IHC analysis showed that MKN45 SC tumors presented increased STn expression both at cytoplasmic and membrane cell compartments when compared to MKN45 WT tumor cells (Fig. 5f and Supplementary Table 2). Furthermore, MKN45 SC xenografts displayed less necrotic areas (Fig. 5g) together with less angiogenesis (Fig. 5h), in comparison to MKN45 tumors. A standard assay to measure angiogenesis in vivo was used to assess the capacity of both SimpleCell models to induce de novo vessel formation. CAM assay confirmed that both MKN45 SC and AGS SC had decreased capability to promote angiogenesis in vivo than their respective WT parental cells (Fig. 5f). Remarkably, concomitant with all the differences found between MKN45 SC and MKN45 WT tumors, mice injected with MKN45 SC revealed a significant poor-survival rate when compared to the mice injected with MKN45 WT cells (Fig. 5i). Nevertheless, it is important to consider that the aggressiveness of MKN45 SC tumor model resulted in a very short survival period preceding the time required for metastasis development. Altogether, in vivo data support that premature truncation of O-glycans with concomitant overexpression of STn O-glycoform, resulted in tumors with more oncogenic features as well as in mice poor-survival.

3.6. Glycoengineered cells expressing truncated O-glycan revealed new potential biomarkers for gastric cancer patient survival

In order to translate our previous findings into a clinical setting, we tested whether the top upregulated genes in our cell models would also be upregulated in human gastric tumors and whether the identified genes hold potential to be used as novel prognostic biomarkers in gastric cancer. For this analysis, we selected the SRPX2 and RUNX1 genes, since these markers showed the highest fold increase in both MKN45 SC and AGS SC (Fig. 3c).

Firstly, we evaluated the expression of SRPX2 and RUNX1 in different datasets of human gastric tissues, from Oncomine database, including both adjacent mucosa and tumor samples. Through a microarray expression data analysis, it was possible to determine that both SRPX2 and RUNX1 mRNA levels were significantly upregulated in gastric cancer tissues when compared with adjacent gastric mucosa (Fig. 6a and Supplementary Fig. 3a and b). These findings were further validated at the protein level in a cohort of gastric cancer tissue samples. SRPX2 showed higher expression in the cytoplasm of both intestinal and diffuse subtypes of gastric tumor cells in comparison with adjacent mucosa. Similarly, high RUNX1 expression was observed in the nucleus of
intestinal and diffuse gastric carcinoma cells, whereas adjacent mucosa showed low RUNX1 expression. Finally, STn expression on the evaluated tumors varied from negative or low to high expression, being absent in adjacent mucosa (Fig. 6e and Supplementary Table 3). Co-expression of SRPX2, RUNX1 and STn was observed in 84% of the analyzed human gastric carcinoma tissue samples (Fig. 6e and Supplementary Table 3). The analysis of STGALNAC1, CIGALT1 and CIGALT1C1 from Oncomine database did not show major differences in the expression levels between adjacent mucosa and gastric carcinoma samples (Supplementary Fig. 3d). However, an inverse correlation was observed between SRPX2 and CIGALT1C1, and between RUNX1 and CIGALT1C1 expression levels in gastric carcinoma samples (Fig. 6d). Furthermore, a correlation analysis based on microarray expression data on tumor and adjacent mucosa of the same patient samples from the Oncomine database corroborated the tissue co-expression results, showing a significant correlation between SRPX2 and RUNX1 expression levels within gastric cancer (Fig. 6b and Supplementary Fig. 3c) but not in adjacent gastric mucosa (Fig. 6c). Similarly, the in vivo tumor MKN45 SC xenografts displaying high STn expression levels also showed high SRPX2 and RUNX1 expression (Fig. 4f and Supplementary Table 2).

Finally, the prognostic value of SRPX2 and RUNX1 was evaluated in a dataset with survival data available for both genes. This analysis revealed that patients with high expression of SRPX2 or RUNX1 presented a worse prognosis when compared to patients with low SRPX2 or RUNX1 levels (Fig. 6f). These results indicate that SRPX2 and RUNX1 genes, associated with O-glycan truncation, could serve as novel prognostic biomarkers in gastric cancer patients.

4. Discussion

Glycosylation is involved in several regulatory mechanisms controlling cellular physiological and pathological processes. Tumor cells commonly express truncated O-glycans, as a consequence of the impairment of the normal extension of O-glycans, usually present in healthy tissues [19,27,28,30,56]. Sialylation is a major glycosylation modification which has critical impact in cell recognition, adhesion, signaling and is remarkably elevated in metastatic tumor cells [13,14,16,57]. The overexpression of the truncated STn has been described to occur in many epithelial cancers, including gastric tumors [58–60], and to be associated with more aggressive tumors and patients' poor-prognosis [45–47]. However, the molecular mechanisms underlying these high malignancy features remain unclear.

In order to evaluate the biological impact of truncation of O-glycans in gastric cancer cells behavior we have performed a systematic analysis of genetically engineered gastric cancer cell models expressing homogeneous truncated O-glycans. Our findings revealed that aberrant expression of the simple O-glycan antigen STn induced major phenotypic alterations in gastric cancer cells, characterized by the acquisition of a mesenchymal-like morphology, together with a less cohesive cell monolayer characteristic of an epithelial to mesenchymal transition [61]. The enhanced expression of STn observed in the cellular membrane extensions of the SimpleCells highlights its relevance in cell-cell and cell-ECM interactions, suggesting that STns might be responsible for mediating interactions between tumor cells and the surrounding environment during cancer progression. In agreement, STn overexpressing cells displayed reduced cell-cell adhesion contacts in the in vivo tumor xenograft model. This phenotype was accompanied with a mislocalization of E-cadherin from the membrane to the cytoplasm. Overall, these observations are in accordance with previous studies showing that expression of STn reduces cell-cell adhesion and impairs the formation of cohesive solid tumors [62].

Importantly, truncation of O-glycans showed to impact gastric cancer cell-matrix adhesion and motility. It is important to highlight that COSMC KO affects both STn and Tn levels, as we previously reported [41], resulting in a different ratio of STn/Tn expression in both MKN45 SC and AGS SC. This ratio was found to be higher in MKN45 SC cells together with increased Fak activation. These results help to explain why MKN45 SC acquired an enhanced capability to adhere and migrate on different components of the ECM, in contrast with the AGS SC cell line. Although STn expression usually accompanies with increase cell adhesion capacities, opposite results showing decrease cell-ECM adhesion in breast cancer cells have been reported [63]. The origin of these discrepancies might be related to the specific cell surface O-glycosylated proteins that are affected after glycoengineering of the cells. Supporting this hypothesis, the list of adhesion-related proteins previously identified as carrying truncated O-glycans in MKN45 SC and AGS SC models was found to be distinct [41]. Glycoproteins belonging to the cadherin family of proteins were O-glycosylated in MKN45 SC whereas in AGS SC, O-glycosylation was present in some particular laminin and integrin subunits [41]. Further biochemical and functional studies would help to clarify this matter. Independently of the genetic background of the gastric cell model, both MKN45 SC and AGS SimpleCells acquired a significant higher capacity to degrade and invade the ECM. This observation is in agreement with other COSMC modulation cell models overexpressing simple O-glycans that also acquired in vitro and in vivo oncogenic features [39,64]. On the same direction, silencing STGALNAc1, the enzyme responsible for STn production, reduced tumor growth, cell migration and invasion on hepatocellular cells [43], as well as in gastric carcinoma cells [44].

Our in vivo assays revealed that tumor xenografts from MKN45 SC displayed less necrotic areas accompanied with reduced tumor neoangiogenic features. These findings suggest that STn on tumor cells, besides increasing aggressiveness, supports enhanced biological capacity to survive in a less angiogenic environment. This was corroborated by the in vivo CAM model, where both MKN45 SC and AGS SC cells showed decreased capacity to induce de novo vessel formation. Although the molecular mechanism behind such findings is still unknown, SimpleCells appeared to be able to survive on the pre-existing blood vessels, avoiding the need to form new vessels. Expression of truncated O-glycans revealed to promote survival by decreasing apoptosis in
pancreatic cancer cells after COSMC knock-down [64]. It is possible that the massive O-glycosylation alterations occurring in a variety of proteins after COSMC KO, changes the transcription of several genes and affecting new vessel formation.

Supporting the clinical implications discussed above, truncation of O-glycans in gastric cancer cells had a remarkable impact in the transcriptomic signature of the glycoengineered cell lines. The enrichment analysis of the differentially expressed genes for GO biological processes showed a significant association with development and motility processes. These genetic signatures support the oncogenic characteristics found in both MKN45 SC and AGS SC cancer cells and demonstrate that truncation of O-glycans affects multiple systems simultaneously that sustain tumor progression. Similarly, genes involved in cellular movement and proliferation, together with genes linked to differentiation and apoptosis, were also found to be significantly changed in pancreatic and in human immortalized keratinocyte HaCaT SimpleCell models [39]. The effect of truncated O-glycans on these different cancer cell models showed that aberrant glycosylation was able to influence several important pathways in tissue homeostasis and oncogenesis, including adhesive and signaling molecules [39].
transcriptomic analysis showed that SRPX2 and RUNX1 were the genes with highest fold change in both MKN45 and AGS SimpleCells. Both SRPX2 and RUNX1 have been previously implicated in cancer progression, but their association with O-glycan expression has, to our knowledge, never been addressed. We observed that the O-glycosylation by COSMC KO resulted in activation of EGFR and ErbB2 RTKs. Interestingly, we demonstrated in our model that EGFR activation regulated both SRPX2 and RUNX1 genes transcription. A recent report showed that RUNX1 was able to positively regulate ErbB2 signaling [65]. This raises the hypothesis of a potential loop of signaling between this cell receptor and the RUNX1 gene. Furthermore, both SRPX2 and RUNX1 proteins were highly present in the gastric cancer tissues when compared with adjacent non-neoplastic gastric mucosa. Interestingly, co-expression of SRPX2, RUNX1 and STn was observed within gastric carcinoma samples. Also, an inverse correlation between CIGALTIC1 and both SRPX2 and RUNX1 was found in the analyzed gastric cancer tissues. Our findings, describe one possible mechanism by which changing the glycosylation affects several cellular biological processes. Alterations in O-glycosylation occurring in tumors can influence cell signaling leading to the transcription activation of specific genes that confer increased oncogenic features favoring the process of cancer progression.

In accordance, SRPX2 has been previously described as upregulated in gastric cancer [54,66,67]. SRPX2 was found to promote cell motility and adhesion through Fak phosphorylation [66], and capable of inducing cell invasion and aggressiveness in several types of cancer [66,68–70]. RUNX1, a hematopoietic regulator, is known to be expressed in the isthmus of human stomach, where stem cells reside [71]. Interestingly, RUNX1 gene was found to be a master regulator of carbohydrate metabolism and knocking down this gene led to decreased glucose metabolic activity [72]. Other authors have reported RUNX1 expression to be decreased in gastric cancer [73]. In order to scale up the correlation between the expression of both SRPX2 and RUNX1 in gastric cancer, we analyzed the transcriptomic data based on independent cohorts from the Oncomine database. Both SRPX2 and RUNX1 expression levels were significantly upregulated in gastric cancer in multiple gastric carcinoma datasets. Interestingly, we show for the first time a significant correlation between SRPX2 and RUNX1 expression levels in gastric cancer patients through a microarray expression data analysis from Oncomine database. Furthermore, our analysis showed that SRPX2 and RUNX1 expression was significantly associated with poor-survival of gastric cancer patients, highlighting their importance in tumor biology and in cancer progression. These results were further supported by the decreased mouse survival observed in the MKN45 SC-injected mice that also displayed high SRPX2 and RUNX1 expression levels. Taking into consideration our findings, we envision that inhibitors targeting tumor specific glycosylation pathways and/or glycan epitopes could harbor potential for therapeutic strategies. Further studies addressing this hypothesis are warranted.

Altogether, our results demonstrate a novel molecular link between premature truncation of O-glycans and concomitant STn overexpression occurring in gastric tumors and its relation with patients’ poor survival (Fig. 7). Our work sheds light on the mechanisms underlying the biological advantage of STn expressing cancer cells through regulation of key biological processes, highlighting the impact of changing cell glycosylation per se in a cancer context. These findings also revealed new genes with potential clinical application as prognostic biomarkers in gastric cancer patients.

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Declaration of Interest

All the authors have nothing to disclose.

Author contributions

DF designed and performed the experimental work, analyzed the data and wrote the manuscript. DC developed the cell line models. DC, DF and AM conceived the hypothesis, interpreted the data and wrote the manuscript. SM contributed for the transcriptomic analysis and illustrated the data. AP and FG performed histopathology and analyzed IHC results. AM and AC conceived the hypothesis, interpreted the data and wrote the manuscript. DC, DF and Reis CA. Aberrant glycosylation in cancer: a novel molecular basis for the progression of cancer. Biochim Biophys Acta 2004;1682(1):57–67.

References

[1] Stewart BW, Wild CP. World Cancer Report 2014; 2014.

[2] Pinho SS, Reis CA. Glycosylation in cancer: mechanisms and clinical implications. Nat Rev Cancer 2015;15(9):540–55.

[3] Hakomori S. Glycosylation defining cancer malignancy: new wine in an old bottle. Proc Natl Acad Sci U S A 2002;99(16):10231–3.

[4] Taniguchi N, Hancock W, Luhman DM, Ridd PM. The second golden age of glycobiology: from functional glycobiology to clinical applications. J Proteome Res 2009;8(2):423–6.

[5] Agrawal P, Fontanals-Cirera B, Sokolova E, Jacob S, Vaiana CA, Argibay D, et al. A systems biology approach identifies FTUT as a driver of melanoma metastasis. Cancer Cell 2017;31(6):894–917.

[6] Magalhaes A, Duarte HO, Reis CA. Aberrant glycosylation in cancer: a novel molecular mechanism controlling cancer. Cancer Cell 2017;31(6):733–5.

[7] Bassaganya R, Carvalho S, Dias AM, Perez-Garay M, Ortiz MR, Figueras J, et al. Pancreatic cancer cell glycosylation regulates cell adhesion and invasion through the modulation of alpha2beta1 integrin and E-cadherin function. PLoS One 2014;9(5):e88595.

[8] Guo H, Nagy T, Pierce M. Post-translational glycoprotein modifications regulate colon cancer stem cells and colon adenoma progression in ApoE(-/-) mice through altered Wnt receptor signaling. J Biol Chem 2014;289(45):31343–49.

[9] Hart CW, Copeland RJ. Glycomics hits the big time. Cell 2010;143(5):672–6.

[10] Lau KS, Partridge EA, Grigorian A, Silivcsan CL, Reithold VN, Demetriou M, et al. Complex N-glycan number and degree of branching cooperate to regulate cell proliferation and differentiation. Cell 2007;129(1):123–34.

[11] Contessa JN, Rhonjai MS, Freeze HH, Rehentulla A, Lawrence TS. Inhibition of N-linked glycosylation disrupts receptor tyrosine kinase signaling in tumor cells. Cancer Res 2008;68(10):3803–9.

[12] Duarte HO, Balmana M, Mereiter S, Osorio H, Gomes J, Reis CA. Gastric cancer cell glycosylation as a modulator of the ErbB2 oncoprotein. Int J Mol Sci 2017;18(11).

[13] Mereiter S, Magalhaes A, Adamczyk B, Jinc C, Almeida A, Drici L, et al. Glycomic analysis of gastric carcinoma cell lines discloses glycans as modulators of RON receptor tyrosine kinase activation in cancer. Biochem Biophys Acta 2016;1860(8):1795–808.
[44] Tamura F, Sato Y, Hirakawa M, Yoshida M, Ono M, Osuga T, et al. RNAi-mediated gene silencing of ST6GalNAc I suppresses the metastatic potential in gastric cancer cells. Gastric Cancer 2016;19(1):85–97.

[45] David L, Carneiro F, Sobrinho-Simoes M. Sialosyl Tn antigen expression is associated with the prognosis of patients with advanced gastric cancer. Cancer 1996;78(1):177–8.

[46] Nakagoe T, Sawai T, Tsuji T, Jibiki M, Nanashima A, Yamaguchi H, et al. Pre-operative serum levels of sialyl Tn antigen predict liver metastasis and poor prognosis in patients with gastric cancer. Eur J Surg Oncol 2001;27(8):731–9.

[47] Victorzon M, Nordling S, Nilsson O, Roberts PJ, Haglund C. Sialyl Tn antigen is an independent predictor of outcome in patients with gastric cancer. Int J Cancer 1996;65(3):295–300.

[48] Sorensen AL, Reis CA, Tarp MA, Mandel U, Ramachandran K, Sankaranarayanan V, et al. The Progesterone Receptor Modulates Cell Survival and Invasion by Inducing Glucose Importation and Clinical Relevance of Metabolic Expression Subtypes in Human Cancers. Cell 2017;152(1):218–31 e14.

[49] Chen X, Leung SY, Yuen ST, Chu KM, Ji J, Li R, et al. Variation in gene expression patterns in human gastric cancers. Mol Biol Cell 2003;14(8):3208–16.

[50] D’Errico M, de Rinaldis E, Blasi MF, Viti V, Falchetti M, Calcagnile A, et al. Genome-wide expression profile of sporadic gastric cancers with microsatellite instability. Eur J Cancer 2009;45(3):461–9.

[51] Chen X, Leung SY, Yuen ST, Chu KM, Ji J, Li R, et al. Variation in gene expression patterns in human gastric cancers. Mol Biol Cell 2003;14(8):3208–15.

[52] Cho JY, Lim JY, Cheong JH, Park YY, Yoon SL, Kim SM, et al. Gene expression signature-based prognostic score in gastric cancer. Clin Cancer Res 2011;17(7):1850–7.

[53] Brockhausen I. Mucin-type O-glycans in human colon and breast cancer: glycodynamics and functions. EMBO Rep 2006;7(6):599–604.

[54] Schultz MJ, Holdbrooks AT, Chakraborty A, Grizzle WE, Landen CN, Buchsbaum DJ, et al. The tumor-associated glycosyltransferase ST6Gal-I regulates stem cell transcription factors and confers a cancer stem cell phenotype. Cancer Res 2016;76(13):3978–88.

[55] Ohuchi N, Thor A, Nose M, Fujita J, Kyogoku M, Schlom J. Tumor-associated glycoprotein (TAG-72) detected in adenocarcinomas and benign lesions of the stomach. Int J Cancer 1986;38(5):643–50.

[56] David L, Carneiro F, Sobrinho-Simoes M. Simple mucin-type carbohydrate antigens (Tn, sialosyl-Tn and T) in gastric mucosa, carcinomas and metastases. APMIS Suppl 1992;27:162–72.

[57] Heerboth S, Housman G, Leary M, Longacre M, Byler S, Lapińska K, et al. EMT and tumor metastasis. Clin Transl Med 2013;4(6).

[58] Munkley JE, Sugars DJ. Cell adhesion: the role of ST6GalNAc1 in prostate cancer progression. Cancer Cell Microenvironment 2016;3:1174–83.

[59] Julien S, Lagadec C, Krzewinski-Rocchi MA, Courtand G, Le Bourhis X, Delannoy P. Stable expression of sialyl-Tn antigen in T47-D cells induces a decrease of cell adhesion and an increase of cell migration. Breast Cancer Res Treat 2005;90(1):77–84.

[60] Hofmann BT, Schluter L, Lange P, Mercanoglu B, Ewald F, Folster A, et al. COSMC knockdown mediated aberrant O-glycosylation promotes oncogenic properties in pancreatic cancer. Mol Cancer 2015;14:109.

[61] Mitsuda Y, Morita K, Kashiwazaki G, Taniguchi J, Bando T, Obara M, et al. RUNXI positively regulates the Erbb2/HER2 signaling pathway through modulating SOS1 expression in gastric cancer cells. Sci Rep 2018;8(1):6423.

[62] Tanaka K, Arao T, Maegawa M, Matsumoto K, Kanedo H, Kudo K, et al. SRPX2 is overexpressed in gastric cancer and promotes cellular migration and adhesion. Int J Cancer 2009;124(5):1072–80.

[63] Tanaka K, Arao T, Tamura D, Aomatsu K, Furuta K, Matsumoto K, et al. SRPX2 is a novel chondroitin sulfate proteoglycan that is overexpressed in gastrointestinal cancer. Plast Reconstr Surg 2012;129(1):279–85.

[64] Tang H, Zhao J, Zhang L, Zhao J, Zhuang Y, Liang P. SRPX2 enhances the epithelial-mesenchymal transition and temozolomide resistance in glioblastoma cells. Mol Neurobiol 2016;53(7):1087–97.

[65] Yamada T, Oshima T, Yoshikawa K, Sato T, Nozaki A, Shiozawa M, et al. Impact of overexpression of Sushi repeat-containing protein X-linked 2 gene on outcomes of gastric cancers. J Surg Oncol 2014;109(8):836–40.

[66] Liu KL, Wu J, Zhou Y, Fan JH. Increased Sushi repeat-containing protein X-linked 2 is associated with progression of colorectal cancer. Med Oncol 2015;32(4):95.

[67] Matsuo J, Kimura S, Yamamura A, Koh CP, Hossain MZ, Heng DL, et al. Identification of stem cells in the epithelium of the stomach corpus and antrum of mice. Gastroenterology 2017;152(1):218–31 e14.

[68] Peng X, Chen Z, Farshidfar F, Xu X, Lorenzi PL, Wang Y, et al. Molecular characterization and clinical relevance of metabolic expression subtypes in human cancers. Cell Rep 2018;23(1):65–76 e4.

[69] Li N, Zhang QY, Zou JY, Li ZW, Tian TT, Dong B, et al. mSir-215 promotes malignant progression of gastric cancer by targeting RUNX1. Oncotarget 2016;7(4):4817–28.