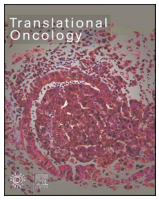
Translational Oncology 31 (2023) 101643

Contents lists available at ScienceDirect

Translational Oncology

journal homepage: www.elsevier.com/locate/tranon



N-glycolylneuraminic acid as a carbohydrate cancer biomarker

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ARTICLE INFO

Keywords:
N-glycolylneuraminic acid
Neu5Gc
Cancer biomarker
Detection
Prognosis

ABSTRACT

One of the forms of aberrant glycosylation in human tumors is the expression of *N*-glycolylneuraminic acid (Neu5Gc). The only known enzyme to biosynthesize Neu5Gc in mammals, cytidine-5′-monophosphate-*N*-acetylneuraminic acid (CMAH), appears to be genetically inactivated in humans. Regardless, low levels of Neu5Gc have been detected in healthy humans. Therefore, it is proposed that the presence of Neu5Gc in humans is from dietary acquisition, such as red meat. Notably, detection of elevated Neu5Gc levels has been repeatedly found in cancer tissues, cells and serum samples, thereby Neu5Gc-containing antigens may be exploited as a class of cancer biomarkers. Here we review the findings to date on using Neu5Gc-containing tumor glycoconjugates as a class of cancer biomarkers for cancer detection, surveillance, prognosis and therapeutic targets. We review the evidence that supports an emerging hypothesis of *de novo* Neu5Gc biosynthesis in human cancer cells as a source of Neu5Gc in human tumors, generated under certain metabolic conditions.

Introduction

Glycosylation is an enzymatic process that covalently attaches a carbohydrate to proteins or lipids and plays important roles in physiological and pathological conditions such as signaling delivery, cellular interactions and recognition, and immunological responses [1]. A prominent feature of all cancer cells is aberrant glycosylation, contributing to carcinogenesis, metastasis and cancer progression [2–6]. The expression levels and glycosylation patterns of glycoconjugates secreted or shed from tumor cells change dramatically during tumor progression and malignant transformation [2–6]. Exploiting tumor-associated glycosylation changes can facilitate the design of tests for cancer detection and monitoring, and cancer-specific glycosylations may also serve as therapeutic targets [7–10].

It has been well-documented that one of the predominant forms of aberrant glycosylation in tumors is the expression of *N*-glycolylneuraminic acid (Neu5Gc) [11–15]. In mammals, the only known *de novo* biosynthetic pathway of Neu5Gc is from the activated precursor cytidine-5′-monophosphate-*N*-acetylneuraminic acid (CMP-Neu5Ac) catalysed by a CMP-Neu5Ac hydroxylase (CMAH) [16–19] (Fig. 1). Although humans lost the ability to express an active CMAH to synthesize Neu5Gc due to a 92-bp deletion in the *CMAH* gene [20–24], very low levels of Neu5Gc have been detected in healthy human tissues [13, 25–27]. In the absence of any known alternative pathways for the biosynthesis of Neu5Gc, dietary Neu5Gc (mainly found in red meat and dairy products) is proposed to explain the low amounts existing in normal humans [25]. In contrast to the small amounts of Neu5Gc found in healthy human tissues, increased Neu5Gc levels have been consistently reported in various cancer tissues, cells and secretions [11–15]. Due to the differential expression of Neu5Gc between cancer samples and their normal counterparts, Neu5Gc-containing proteins and lipids (Neu5Gc-glycoconjugates) can be considered for use as a class of cancer biomarkers. However, the studies on developing Neu5Gc-containing tumor antigens as a class of cancer biomarkers over the past decades are limited due to the lack of sensitive and specific methods for detecting Neu5Gc in complex biological samples, such as serum, in a high-throughput manner. Recent studies have overcome this barrier and demonstrated the potential of Neu5Gc-containing antigens as cancer biomarkers [12,28–31].

In addition to the lack of effective Neu5Gc detection methods, the unclear mechanisms of Neu5Gc expression by human cancer cells has impeded the progress of biomarker discovery using Neu5Gc-containing antigens, i.e. if diet is the only source of Neu5Gc then tumors in vegetarians and vegans may be expected to express very little or no Neu5Gc-glycoconjugates. Dietary incorporation and endogenous production are two currently separate theories proposed to explain the presence of Neu5Gc in human cancer cells [32–34]. Unraveling the mechanisms of Neu5Gc expression by cancer cells and the relative contributions of exogenous and potential endogenous Neu5Gc to Neu5Gc-glycoconjugate expression in humans will pave the way for the

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https://doi.org/10.1016/j.tranon.2023.101643
Received 19 October 2022; Received in revised form 31 January 2023; Accepted 11 February 2023
Available online 18 February 2023

use of Neu5Gc-glycoconjugates as cancer biomarkers.

In this article, we review the evidence and challenges of using Neu5Gc-glycoconjugates as cancer biomarkers, and the evidence for an alternative explanation for the origin of Neu5Gc in human tumors; via *de novo* Neu5Gc biosynthesis by human cancer cells.

The potential of using Neu5Gc-containing antigens as cancer biomarkers

The expression of Neu5Gc is associated with tumor malignancy, invasiveness and metastasis [11–14], indicating that Neu5Gc-glycoconjugates may have utility as cancer biomarkers.

Neu5Gc-containing antigens for cancer detection

Early studies have demonstrated higher levels of Neu5Gc in the form of Hanganutziu-Deicher (H-D) antigen (which refers to Neu5Gc-glycoconjugates) and mammary serum antigen (MSA) in sera from patients with lymphoma, leukemia, lung cancer, breast cancer and other types of cancers compared to healthy humans [35] (Table 1). Recent studies have supported the utility of Neu5Gc-containing glycoconjugates as a serological biomarker for the detection of ovarian and breast cancer via surface plasmon resonance (SPR) employing a purpose-engineered Neu5Gc-specific lectin SubB2M [12,28–30]. The SubB2M-SPR-based assay was able to differentiate ovarian cancer patients at all stages from cancer-free controls with 100% specificity and 100% sensitivity [12,30], breast cancer patients at all stages with 98.96% specificity and 100% sensitivity [30] and melanoma cancer patients (primary and metastatic) with 85.48% sensitivity and 94.12% specificity [36] (Table 1). Teng *et al.* have recently developed another lectin-based assay using lamprey immunity protein (LIP) to detect Neu5Gc levels in urine samples from healthy individuals and patients with benign bladder diseases or bladder cancer [31,37]. Teng *et al.* reported that levels of LIP-bound Neu5Gc in urine samples from bladder cancer patients (n = 518) were significantly greater than those from healthy individuals (n = 2821) and patients with benign bladder diseases (n = 360), highlighting the value of using Neu5Gc-containing antigens for cancer detection [31] (Table 1). In addition, the presence of Neu5Gc on prostatic acid phosphatase (PAP) [38] in expressed prostatic secretions collected from urine samples has been associated with more severe prostate cancer, indicating that Neu5Gc-PAP could be used a cancer biomarker for detection of prostate cancer [39,40]. Altogether, these findings suggest that Neu5Gc-containing antigens can serve as a class of biomarkers for cancer detection.

Neu5Gc-containing antigens for cancer prognosis and monitoring

Sequential serum Neu5Gc levels measured over time from 15 breast cancer patients at high-risk for recurrence (6 patients in remission and 9 patients with recurrence) detected using a SubB2M-SPR-based assay showed a decreasing trend following the first line of treatment in those patients in remission [30] (Table 1). A similar reduction in Neu5Gc levels was found in the urine samples from bladder cancer patients after therapy (n = 518) compared to a non-treated group (n = 314) using the LIP test assay [31]. These studies demonstrate that Neu5Gc may have utility as a biomarker for monitoring response to treatment and remission status.

A Neu5Gc-terminating ganglioside, monosialodihexosylganglioside (GM3), GM3(Neu5Gc), is reported to be overexpressed in tumors, but

Table 1
The use of Neu5Gc-containing tumor antigens as potential cancer biomarkers in various cancers.

Disease	The use of Neu5Gc-containing tumor antigens as cancer biomarkers			Therapeutic targets
	Detection/ Staging	Prognosis	Surveillance	
Bladder cancer	Neu5Gc [31]		Neu5Gc [31]	
Breast cancer	Neu5Gc [30], MSA [41]	Neu5Gc [30], MSA [41]	Neu5Gc [30], MSA [41]	GM3 (Neu5Gc) [42]
Colon cancer	GM3 (Neu5Gc) [43]			
Glioma				GM3 (Neu5Gc) [44]
Lung cancer	HD antigen [45]			GM3 (Neu5Gc) [46]
Lymphoma	HD antigen [14], MSA [41]			
Leukemia	HD antigen [14]			
Melanoma	GM3 (Neu5Gc) [47], Neu5Gc [36]	GM3 (Neu5Gc) [47]		GM3 (Neu5Gc) [48,49]
Neuroblastoma				GM3 (Neu5Gc) [44]
NSCLC		GM3 (Neu5Gc) [50,51]		GM3 (Neu5Gc) [52,53]
Ovarian cancer	Neu5Gc [12]			
Retinoblastoma				GM3 (Neu5Gc) [44]
Sarcoma		GM3 (Neu5Gc) [54]		
Wilms' tumor				GM3 (Neu5Gc) [44]

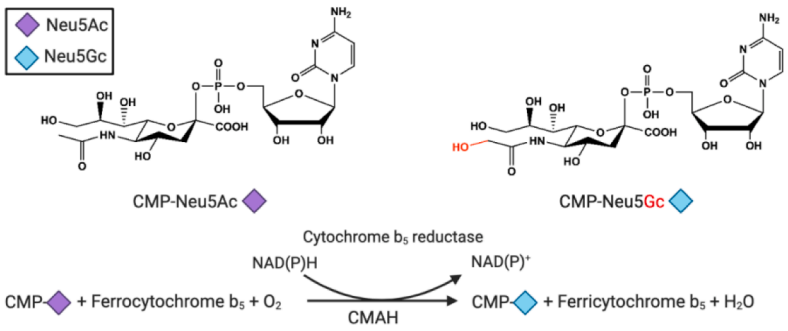

Fig. 1. Schematic representation of the CMAH enzymatic reaction. Neu5Ac and Neu5Gc differ by the presence of a single hydroxyl group on C-5 of Neu5Gc (red). Neu5Ac can be converted into Neu5Gc via CMAH by a CMP sugar nucleotide donor and CMAH requires cytochrome b_5 and cytochrome b_5 reductase, and Fe^{2+} ions for its enzymatic activity.

rare in corresponding normal tissues. Increased expression of GM3 (Neu5Gc) in the tissue specimens from sarcomas, colon, melanoma and non-small cell lung carcinomas (NSCLC) patients showed a significant correlation with a poor 5-year overall survival rate and was therefore proposed as a prognostic factor for these types of cancers [50–51] (Table 1). However, few studies have shown the opposite results that low levels of Neu5Gc correlate with a better overall 5-year survival outcome and this warrants further investigation [55,56]. Collectively, several studies in multiple diseases have indicated that Neu5Gc has potential as a cancer biomarker to monitor and predict disease progression for various cancers.

Neu5Gc-containing antigens as a class of immunotherapy targets

Several immunotherapies have been developed using Neu5Gc-glycoconjugates as immunotherapy targets. Based on the cancer-specific expression of GM3(Neu5Gc), it has been exploited as an antigen to develop active and passive cancer immunotherapy [57]. Two active immunotherapies were developed as cancer vaccines employing GM3(Neu5Gc) as an immunogen: Vaxira (Racotumomab) and Glyco-VaxGM3, to treat multiple cancers including NSCLC, and malignant breast cancer and melanoma [42,44,48,49,52,53,58] (Table 1). Racotumomab has been tested in clinical trials: an ongoing phase 2 trial for treating patients with high-risk neuroblastoma and a phase 3 trial for treating patients with advanced NSCLC. A passive immunotherapy was also developed using a monoclonal humanized anti-GM3(Neu5Gc) antibody, 14F7hT mAb, to directly attack the tumor cells expressing GM3(Neu5Gc) antigens [59,60]. Another active cancer immunotherapy using Neu5Gc-containing antigens as targets to generate a biomimetic glyconanoparticle cancer vaccine was developed by Reuven *et al.* [61]. This vaccine was modified from engineered αGal knockout swine erythrocytes expressing Neu5Gc-containing glycoconjugates, which induced anti-Neu5Gc IgG immune responses and inhibited tumor growth in Neu5Gc-deficient *Cmah*$^{-/-}$mice [61]. As the expression of Neu5Gc on urinary PAP correlates with a more advanced stage of prostate cancer and PAP is highly expressed in prostate tissues, Neu5Gc in combination with PAP has the potential to be used as a target to develop treatments for prostate cancer [39,40,62].

Current barriers to the use of Neu5Gc-glycoconjugates as cancer biomarkers

Given that the presence of Neu5Gc has also been reported in the sera from patients with other pathological diseases such as leprosy, infectious mononucleosis and rheumatoid arthritis [11], cancer-specific Neu5Gc-glycoconjugates, by delineating underlying linkages and/or scaffolds, are required to refine the specificity and sensitivity for detection and prognostic purposes. In addition, according to the hypothesis that human Neu5Gc levels originate from their diet [25], only individuals who have consumed red meat and/or other Neu5Gc-containing food would be suitable for Neu5Gc-based detection and monitoring tests. Likewise, the dietary source hypothesis suggests that Neu5Gc levels present in individuals may be affected by the level of consumption of the relevant animal food products, and further affect diagnostic test results, potentially leading to false positive results. Further investigations of short and long-term Neu5Gc-dietary impacts on the levels of Neu5Gc in both cancer patients and healthy individuals, especially their sera, are needed to provide more insight into the development of Neu5Gc-based detection assays.

Controversy surrounding the mechanisms for Neu5Gc expression by cancer cells

Over the past two decades, the only explanation for the presence of Neu5Gc in humans has been dietary acquisition from Neu5Gc containing foods [25]. However, an alternative emerging hypothesis has suggested that human cancer cells can biosynthesize Neu5Gc *de novo* under hypoxic conditions [63]. Here we review the evidence for both propositions.

Proposition: dietary acquisition is the only source of Neu5Gc in humans

Incorporation of exogenous Neu5Gc is reported to be the only pathway contributing to the presence of Neu5Gc in humans. This is supported by observations from an oral Neu5Gc intake study involving three healthy individuals [25], Neu5Gc supplementation cell culture studies [25,33,34], and a Neu5Gc feeding study on the human-like, Neu5Gc-deficient *Cmah*$^{-/-}$ mice [64]. In the human study, Neu5Gc was detected in the subjects' urine and saliva after consuming ~150 mg of Neu5Gc-containing porcine submaxillary mucin [25]. In a Neu5Gc-deficient *Cmah*$^{-/-}$ murine model study, increased Neu5Gc was found in tissues from MMTV-PyMT transgenic *Cmah*$^{-/-}$ mice (the MMTV-PyMT transgenic mouse is a model for studying breast cancer) after feeding these mice with exogenous Neu5Gc [64]. In cell culture studies, increased Neu5Gc was also detected in cell-derived samples after supplying these cells with exogenous Neu5Gc [25,33,34]. Furthermore, expressed Neu5Gc was depleted in cells over time by simply switching from the supplementation of Neu5Gc-rich serum e.g. fetal bovine serum (FBS) to Neu5Gc-deficient, human or chicken serum or serum free media [25,33,34,64–69], and any residual Neu5Gc would be degraded via the intracellular Neu5Gc-degradative pathway [65]. In this pathway, Neu5Gc is degraded to *N*-glycolylmannosamine (ManNGc), *N*-glycolylglucosamine (GlcNGc) and GlcNGc-6-phosphate, then finally enters the glycolysis and citrate acid cycles [65].

Exogenous Neu5Gc is proposed to enter cells predominantly via macropinocytosis, with subsequent release of Neu5Gc from Neu5Gc-glycoconjugates by lysosomal sialidases and delivery to the nucleus by the lysosomal sialic acid transporter sialin. Neu5Gc is then activated by CMP sialic acid synthetase (CMAS) to form CMP-Neu5Gc [34] (Fig. 2, pathway A). CMP-Neu5Gc is then transported to the Golgi apparatus, where the Neu5Gc is attached to newly synthesized glycoconjugates, to be presented as membrane-bound or secreted. The whole process is then recycled with the help of lysosomal sialidase and sialin [34] (Fig. 2, pathway A). The hypoxia-induced over-expression of sialin and rapid growth of tumor cells are suggested to explain the high levels of Neu5Gc accumulated in tumors [32,33].

Hypothesis: de novo Neu5Gc biosynthesis in human cancer cells

In 2018, Bousquet *et al.* hypothesized that the human CMAH in cancer cells can be reactivated to produce Neu5Gc in the context of a hypoxic microenvironment by using a protein similar to the catalytic domain of CMAH, the subunit B of the succinate dehydrogenase complex, although it remains to be explained in this study why such complementation did not occur in cells cultured with FBS, but only Neu5Gc-deficient human and chicken serum [63]. In line with this hypothesis, prior studies showed that the levels of Neu5Gc expressed by cancer cells after cultivation were significantly higher than the total Neu5Gc present in the culture medium and/or FBS supplied [63,70–74], indicating that exogenous Neu5Gc does not account for all of the Neu5Gc measured, and that other mechanisms must exist in cancer cells to account for this disparity. Bousquet *et al.* proposed that *de novo* Neu5Gc biosynthesis occurs via the conversion from the cytosolic CMP-Neu5Ac pool catalyzed by activated human CMAH under hypoxia or other abnormal metabolic conditions in human cancer cells [63] (Fig. 2, pathway B). Compared to the multiple steps involved in the exogenous Neu5Gc metabolic pathway (Fig. 2, pathway A), generating endogenous Neu5Gc is a one-step reaction if CMAH is active, which is easier to meet the fast sialylation demand for highly proliferative cancer cells.

In support of this hypothesis, our lab showed that among 111 serum samples analyzed from ovarian (24) and breast (63) cancer patients with

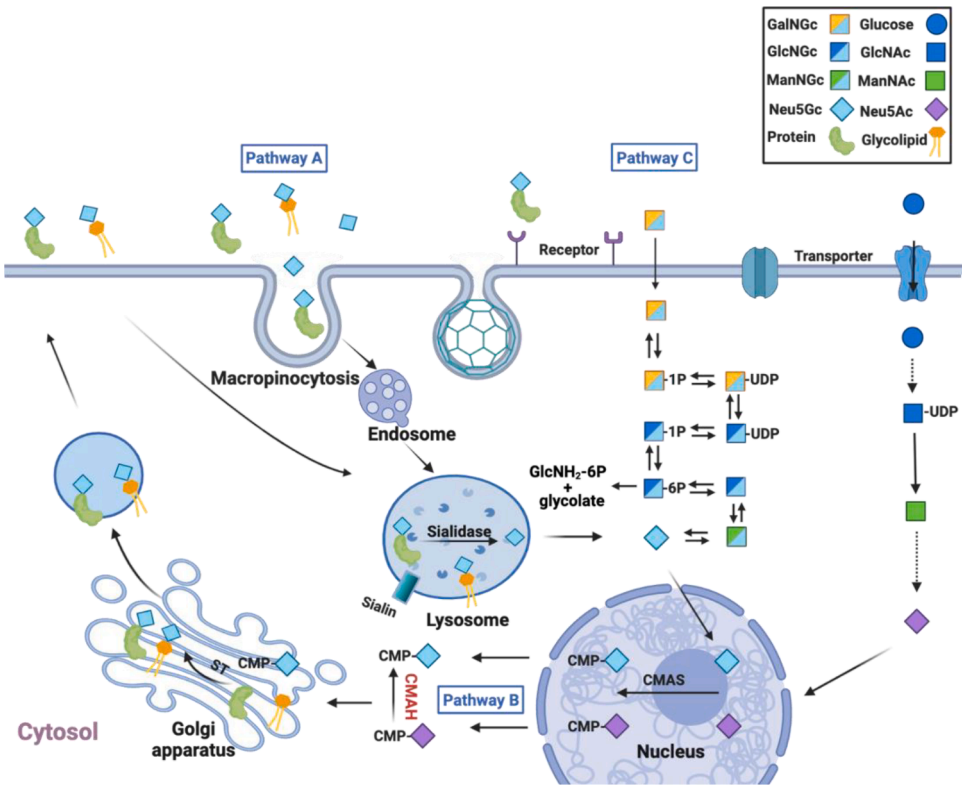

Fig. 2. Possible pathways for the incorporation of exogenous Neu5Gc (pathway A) and *de novo* biosynthesis of Neu5Gc (pathways B and C) in human cancer cells.

stage III and IV disease and primary cutaneous melanoma (24), the Neu5Gc levels are consistently higher compared to cancer-free females [12,30]. In addition, elevated Neu5Gc levels are ubiquitous in urine samples from 463 bladder cancer patients (stage II – IV) compared to healthy controls [31]. Although not recorded, it would be expected that vegans or vegetarians may exist within these large cohorts of cancer patients. Based on the hypothesis that Neu5Gc is obtained only from the diet, particularly from red meat and dairy, these vegan or vegetarian patients would be expected to have no or minimal Neu5Gc, despite having the upregulated lysosomal transporter sialin. The uniformly high Neu5Gc levels in the samples from all cancer patients within these cohorts supports the alternative hypothesis that cancer cells harbor unique mechanisms to produce endogenous Neu5Gc.

Other pathways contributing to the expression of Neu5Gc in tumors

The degradative pathway of Neu5Gc is partially reversible, as feeding human cancer cells with synthetic free or per-*O*-acetylated ManNGc, *N*-glycolylgalactosamine (GalNGc) or GlcNGc can lead to *de novo* synthesis of Neu5Gc, with enhanced uptake of per-*O*-acetylated forms [65,75]. As GalNGc-chondroitin sulfate is detectable in the serum from normal humans [76], it is possible that GalNGc may serve as a precursor for Neu5Gc biosynthesis under abnormal metabolic conditions (Fig. 2, pathway C).

In addition to the possible mechanisms already discussed, down-regulation of the downstream degradative pathways may also be contributing to the accumulation of Neu5Gc in cancers. The expression and activity of downstream enzymes for degrading Neu5Gc such as the pyruvate lyase metabolizing Neu5Gc to ManNGc may also lead to varying expression of Neu5Gc [65,77]. In terms of Neu5Gc acquired from the diet, the role of the human gut microbiota in consuming Neu5Gc for carbon, nitrogen and as an energy source, and the incorporation rate of the residual Neu5Gc after digestion also needs to be clarified [78].

Mutations in CMAH genes among humans and other species

CMAH is the only known enzyme to biosynthesize Neu5Gc in deuterostomes, thus Neu5Gc presence in deuterostomes often correlates with CMAH activity. During evolutionary history, some lineages either lost or inactivated CMAH independently, resulting in the absence of Neu5Gc, with resulting changes in their susceptibility and resistance towards diverse pathogens (see Peri et al., 2018 for a comprehensive review) [19]. A classic example of the complete loss of the *CMAH* gene is in chickens, which have no Neu5Gc expression and thus this species is used to produce anti-Neu5Gc antibodies [19,26]. In addition to the mechanism of a complete loss of the *CMAH* gene that results in no Neu5Gc production, the mechanisms of the inactivation of the *CMAH* gene are described below. Among these inactivation mechanisms of the *CMAH* gene, the mutations in human *CMAH* are unique. This has prompted others to propose a truncated form of CMAH may be expressed [23,79], offering the possibility for CMAH to be activated to synthesize Neu5Gc under certain conditions [11,63].

Complete inactivation of the CMAH gene is caused by large disruptions in the open reading frame

Typical examples of inactivation of the *CMAH* gene are the model animals exhibiting no Neu5Gc expression to study human diseases. In the New World monkey genome, a deletion spanning coding exon 3 to exon 15 has been identified in *CMAH*, leading to the inactivation of this gene. As expected, no Neu5Gc was detected in liver and spleen tissues from this species [80] (Table 2). Ferret, a human-like and naturally humanized model to study human influenza A virus, cannot express Neu5Gc due to deletion of the first nine exons and multiple stop codons in exon 11 in the genomic sequence of *CMAH* [81] (Table 2). In both instances, *CMAH* is unlikely to be transcribed and further translated into a protein due to such large mutations occurring in the gene.

Table 2
Summary of examples of the types of mutations of the *CMAH* gene. N.A.: not available; ND: not detectable.

Species (Common name)	Types of mutations	*CMAH* mRNA expression	CMAH protein expression	Evidence of Neu5Gc expression	References
Human	Deletion of 92 bp in coding exon 1 in terms of CMAH variant 1 (Accession number: NR_002174)	Detected in various tissues and cells	Detected in the cell lysates from human stem cells	Small amounts of Neu5Gc have been detected in many healthy tissues, while higher levels of Neu5Gc have been detected in tumor samples.	[11,12,15, 21,23,47,79]
Ferret	Deletion of coding exons 1–9 and multiple premature stop codons in exon 11	N.A.	N.A.	ND in serum samples and tissues including brain, lung, kidney and spleen	[81]
New World monkey	Deletion spanning coding exons 3–15	N.A.	N.A.	ND in spleen and liver tissues	[19,80]
Cat	Type A (mainly express Neu5Gc) contain two intact A alleles and type B (express Neu5Ac only) and AB (express both antigens) carried two mutated alleles with distinctive mutations which are linked to the CMAH protein expression and its activity	Detected in various tissues	Detected in the cell lysates from two feline cells	The erythrocytes in type A cats express Neu5Gc with low levels of Neu5Ac and type B cats only express Neu5Ac, while type AB cats express both antigens at similar levels.	[82–86]
European dog	The well-reserved *CMAH* gene but with SNPs	Detected in low to absent levels in canine cell lines	ND in the cell extracts of two western canine cell lines	ND in glycolipid extractions from erythrocytes	[19,81,82, 84,87,88]

Point mutations in the CMAH gene lead to polymorphisms within species with different CMAH activity

Unlike New World monkeys and ferrets where *CMAH* is completely inactivated by disruption of exons in the *CMAH* gene, the *CMAH* gene is polymorphic in cats and dogs (Table 2). The expression of Neu5Gc and Neu5Ac on the surface of erythrocytes in cats determines their blood serotypes: type A expresses mainly Neu5Gc, type B only expresses Neu5Ac, and type AB expresses both Neu5Ac and Neu5Gc antigens at comparable levels. The expression of Neu5Ac/Gc is controlled by CMAH expression due to genotypic variants of the CMAH coding region [89]. Type A cats carry an intact *CMAH* gene and resulting protein, type B cats carry two copies of the recessive *b* allele consisting of various single nucleotide polymorphisms (SNPs) upstream or in the *CMAH* gene, leading to either no protein translated or a non-functional truncated protein, while type AB cats carry variants resulting in varying CMAH expression [82,83,85,90] (Table 2).

Dogs are divided into two categories based on the presence or absence of Neu5Gc. In contrast to Asian dogs (except Japanese Hokkaido dogs), western dogs do not express Neu5Gc [87,88]. Although the canine *CMAH* gene remains well-conserved and its mRNA expression was detected in various tissues in two canine cell lines, there was no CMAH protein [19,81,82] (Table 2). The genomic and cDNA analysis of canine *CMAH* did not exhibit a loss-of-exon trait like human, ferret and New World monkey, thus it remains to be elucidated whether the absence of Neu5Gc in western dogs is a result of SNPs occurring in or upstream of the *CMAH* gene leading to CMAH inactivation, similar to the genetic mechanisms leading to the polymorphic expression in cats [84].

The CMAH mutation in humans is unique among known CMAH genes

The human *CMAH* gene is highly homologous to that of mice and six non-human primates (Fig. 3), however, all humans are homozygous for an inactivated *CMAH* gene by an *Alu*-mediated deletion of a 92-bp exon, leading to a truncation in the Rieske Fe_2-S_2-binding region, which is required for the activity of CMAH [21–23,91] (Fig. 3). This is likely due to escape from infections caused by pathogens preferring to bind Neu5Gc such as *Plasmodium reichenowi* [92] and *E. coli* K99 [93]. This trait was further fixed in the population by sexual selection whereby anti-Neu5Gc antibodies in the human female reproductive tract attack and kill sperms expressing Neu5Gc [94,95].

Unlike the mechanisms that inactivate the *CMAH* gene in other animals, the small deletion in the human *CMAH* gene could allow CMAH to be expressed [21,23,24,79]. *CMAH* mRNA expression has been widely detected in various human tissues and cells (Table 2), except the brain,

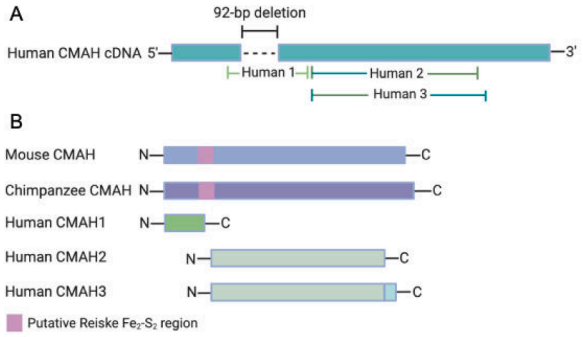

Fig. 3. Representation of (A) human CMAH cDNA and (B) the human CMAH protein sequence compared with other species. (A) Human CMAH cDNA contains a 92-bp deletion compared to other mammals exhibiting an active CMAH. Human 1, 2 and 3 represent three proposed CMAH coding sequences with two different predicted translation start codons located upstream and downstream from the 92-bp deletion [21,23,79]. (B) Comparison of the CMAH protein sequences in mouse, chimpanzee and three proposed human CMAH proteins (Accession no: AF074480, D86324, and FJ794466, respectively). Human CMAH 1, 2 and 3 proteins correspond to Human 1, 2 and 3 labelled in B respectively.

despite the presence of a 92-bp deletion in the human *CMAH* gene with a proposed resultant loss of activity of the protein [23,31]. Notably, Tringali *et al.* showed that *CMAH* mRNA expression was detected in melanoma cell lines (derived from melanoma patients with stage III and IV disease) exhibiting high Neu5Gc-containing glycolipids, but was not expressed in melanocytes [47], suggesting there is a possibility that cancer cells could regulate CMAH for functional roles. The start codon for the translation of human CMAH remains controversial as shown in Fig. 3, although it is possible that all proposed CMAH proteins may represent distinctive isoforms expressed in humans. The CMAH proteins proposed by both Irie *et al.* and Nystedt *et al.* predict the same start codon, with translation starting after the 92-bp deletion, producing a 486 amino acid (Accession no: D86324) protein and a 501 amino acid (Accession no: FJ794466) protein, respectively [23,79] (Fig. 3). Conversely, Chou *et al.* suggested a shortened 72 amino acid CMAH polypeptide (Accession no: AF074480) was made with the translation start codon located further upstream from the 92-bp deletion [21] (Fig. 3). Current evidence of the expression of the CMAH protein is limited to a western blot analysis with an anti-CMAH antibody, which demonstrated that a ~75 kDa CMAH protein (Accession no: FJ794466) was produced in human stem cells [79] (Table 2). Further evidence of

the expression of CMAH at the protein level in humans is needed and it remains to be resolved whether various isoforms of the protein are expressed which may depend on the metabolic conditions of cells.

Given the direct correlation between the expression of CMAH and the expression of Neu5Gc and the lack of alternative known pathways to produce endogenous Neu5Gc in animals, it is possible that a CMAH-dependent Neu5Gc biosynthetic pathway could account for Neu5Gc expression in human tumors when no exogenous Neu5Gc is provided.

Conclusion and future directions

The potential utility of using Neu5Gc-glycoconjugates as cancer biomarkers has been demonstrated for cancer detection, prognosis, surveillance and therapeutic targets [11,15,21,23,30,32,33,71]. The detection of Neu5Gc in bodily fluids has been shown to distinguish ovarian, breast, melanoma and bladder cancer from normal controls with high sensitivity and specificity [12,30,31,36]. It will also be interesting to see whether Neu5Gc can be utilized as a biomarker for other types of cancer, particularly rare or difficult to detect cancers. The current challenges of using Neu5Gc as a clinical biomarker are the Neu5Gc expression observed in other inflammatory diseases and unclear dietary impacts. Determining cancer-specific or even cancer type-specific Neu5Gc-containing biomarkers will improve the sensitivity and specificity of Neu5Gc-based detection and monitoring tests. Future studies on investigating the dietary impacts of Neu5Gc-containing food on cancer-free individuals and cancer patients are required to provide insight into the developments of Neu5Gc-based biomarker tests.

Uncovering the mechanisms of the expression of Neu5Gc in cancer cells and how endogenous and exogenous Neu5Gc contribute to the distribution of Neu5Gc in humans will provide the rationale for the development of Neu5Gc-based detection tests and therapies. Although this review highlights the possibility of the activation of CMAH in cancer cells induced by hypoxia to generate Neu5Gc, cancer cells may harbor other unknown pathways to explain cancer cell-Neu5Gc expression at both the cellular and tissue levels.

CRediT authorship contribution statement

Jing Wang: Conceptualization, Writing – original draft, Writing – review & editing. **Lucy K. Shewell:** Conceptualization, Writing – review & editing, Supervision, Funding acquisition. **Christopher J. Day:** Conceptualization, Writing – review & editing, Supervision, Funding acquisition. **Michael P. Jennings:** Conceptualization, Writing – review & editing, Funding acquisition, Supervision.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: The following authors (CJD and MPJ) declare that they are named inventors on a patent on the SubB2M technology (WO2018085888A1) and a second patent (CJD, LKS and MPJ) for improvements to the SubB2M test (2,021,901,444). Both of these patents were licensed to Inoviq, VIC, Australia in 2020.

Acknowledgments

The authors acknowledge the PhD scholarships (GUIPRS and GUPRS, JW) provided by Griffith University. This work was supported by the NHMRC (Principal Research Fellowship 1138466 to MPJ), Bourne Foundation (awarded to MPJ, LKS and CJD), Tour de Cure (RSP-00114–19/20 awarded to MPJ and LKS), and the US Department of Defense (Breakthrough Award Level 1 W81XWH-20–1–0527 awarded to MPJ, CJD and LKS). All figures were created with BioRender.com.

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