

High expression of lactotriaosylceramide, a differentiation-associated glycosphingolipid, in the bone marrow of acute myeloid leukemia patients

Zheng Wang^{2,†}, Lijun Wen^{2,†}, Xiao Ma^{2,†}, Zijun Chen³, Yunhui Yu², Jian Zhu², Yanping Wang², Zhenming Liu⁴, Haiyan Liu², Depei Wu^{1,2}, Dapeng Zhou^{1,5}, and Yunsen Li^{1,2}

²Laboratory of Cellular and Molecular Tumor Immunology, Institutes of Biology and Medical Sciences, the First Affiliated Hospital, Soochow University, Suzhou, China; ³College of Chinese Materia Medica, Shanghai University of Traditional Chinese Medicine, Shanghai, China; ⁴State Key Laboratory of Natural and Biomimetic Drugs, School of Pharmaceutical Sciences, Peking University, Beijing, China; and ⁵Department of Melanoma Medical Oncology, University of Texas M.D. Anderson Cancer Center, Houston, TX, USA

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Glycosphingolipids (GSLs) are information-bearing biomolecules that play critical roles in embryonic development, signal transduction and carcinogenesis. Previous studies indicate that certain GSLs are associated with differentiation in acute myeloid leukemia (AML) cells. In this study, we collected bone marrow samples from healthy donors and AML patients and analyzed the GSL expression profiles comprehensively using electrospray ionization linear ion-trap mass spectrometry. The results showed that AML patients had higher expression of the GSL lactotriaosylceramide (Lc3), GM3 and neolactotetraosylceramide (nLc4) in their bone marrow than did the healthy donors ($P < 0.05$), especially the M1 subtype of AML. To further explore the molecular mechanisms of Lc3, we examined the expression of the Lc3 synthase β 1,3-*N*-acetylglucosaminyltransferase5 (β 3Gn-T5) and found that the bone marrow samples of AML patients had 16-fold higher expression of β 3Gn-T5 than those of healthy donors ($P < 0.05$). Our results suggest that AML-associated GSLs Lc3, GM3 and nLc4 are possibly involved in initiation and differentiation of AML.

Keywords: acute myeloid leukemia / glycosphingolipidomics / lactotriaosylceramide / Lc3 synthase / mass spectrometry

Introduction

Glycosphingolipids (GSLs) are essential biomolecules present in the cell membranes of all animal cells. They are composed of carbohydrates covalently linked to a ceramide (*N*-acylsphingosine) lipid tail that anchors the molecule within the cell membrane. Given the differences between carbohydrate and lipid moieties, more than 300 GSL series have been identified to date (mostly in carbohydrates) on the basis of sequencing and anomeric linkages (Ichikawa and Hirabayashi 1998). GSLs in mammalian tissues can be generally divided into three major groups: ganglio-, lacto- and globo-GSLs. The biosynthesis and degradation of these GSLs involves numerous enzymes that act at various subcellular locations (Hakomori 2008; Wennekes et al. 2009).

Biologically, GSLs play critical roles in cell signaling, apoptosis, adhesion, receptor modulation, growth and differentiation (Jarvis et al. 1996; Hakomori 1998; Kasahara and Sanai 1999). Cellular differentiation and malignant transformation are often accompanied by dramatic changes in GSL expression. Many GSLs are capable of inducing differentiation, apoptosis, bone marrow suppression and metastasis (Sietsma et al. 1999; Liang et al. 2010; Ogasawara et al. 2011). Aberrant glycosylation occurs in essentially all types of human cancers. Numerous glycosyl epitopes constitute tumor-associated antigens. However, there is a long-standing debate over whether aberrant glycosylation is a result or a cause of cancer (Hakomori 2002).

Previous studies indicate that certain GSLs are associated with differentiation in acute myeloid leukemia (AML) cells. AML is the most common acute leukemia affecting adults, and its incidence increases with age. The symptoms of AML are caused by the replacement of normal bone marrow with leukemic cells. Several risk factors and chromosomal abnormalities have been identified, but the specific cause of AML is not clear. It is suspected, however, that cell-surface carbohydrates play roles in myelopoiesis. Carbohydrate antigens have been shown to facilitate vascular adhesion, cell activation and phagocytosis of granulocytes and monocytes. Neutrophilic and monocytic differentiations are also often accompanied by changes in *N*-glycan, ganglioside and neutral

[†]To whom correspondence should be addressed: Laboratory of Cellular and Molecular Tumor Immunology, Institutes of Biology and Medical Sciences, the First Affiliated Hospital, Soochow University, 199 Ren-Ai Road, Suzhou 215123, China. Tel: +86-51265880419; Fax: +86-512-65880423; e-mail: yunsenli@suda.edu.cn (Y.L.); e-mail: dzhou@mdanderson.org (D.Z.); e-mail: wudepei@medmail.com.cn (D.W.)

[†]These authors contributed equally to this work.

GSL expression (Saito 1993; McEver et al. 1995; Hinkovska-Galcheva et al. 1998; Liu et al. 1998; Cooling et al. 2003). In leukemic cells, GSLs have been found to be biomarkers of successive maturation stages (Yu 1994; Smolenska-Sym et al. 2004). For example, in HL-60 cells, granulocytic and monocytic differentiations are accompanied by distinct changes in ganglioside synthesis and expression (Nakamura et al. 1992).

Over the past decade, mass spectrometry (MS) technologies have been applied to the characterization and quantification of GSLs (Costello and Vath 1990; Peter-Katalinic and Egge 1990; Samuelsson et al. 1990; Levery 2005). We have used the ion-trap MS (MS^n) technology for the analysis of complex mixtures of cellular GSLs, on the basis of our previous finding that signature ion fragments can be generated for each specific GSL after multiple rounds of fragmentation (Li, Teneberg et al. 2008; Li, Zhou et al. 2008). Here, we report the first cellular glycosphingolipidomics study of primary AML tumor cells using the MS^n technology. We collected bone marrow samples from healthy donors and AML patients and analyzed the GSL expression profiles comprehensively to determine which GSLs are associated with AML.

Results

Major GSL expression pattern in human bone marrow samples

We used electrospray ionization linear MS^n (ESI-LIT-MS) to analyze GSLs extracted from the human bone marrow

samples. We found a number of potential GSL molecular ions (as Na^+ adducts) in the ESI-LIT- MS^1 profile spectrum of the permethylated GSL fraction isolated from the samples (Figure 1A and B). Subsequently, we systematically acquired MS^2 spectra for every molecular precursor ion in the MS^1 spectrum from the mass-to-charge ratio (m/z) 1000–1500, covering the range of dihexosylceramides and trihexosylceramides with ceramide compositions of d18:1 sphingosine and C16–C26 fatty acids. Major GSLs have the C16 or C24 fatty acid ceramide.

The relationship among major species of GSLs that exist in mammals is shown in Scheme 1. After multiple rounds of fragmentation, signature ion fragments for each specific GSL can be generated. Using the MS^n technology, we can generally distinguish one series of GSLs from another. We analyzed the MS^2 spectrum of the major molecular ion m/z 1010 in the MS^1 spectrum and found that m/z 463 and 547 were the main fragments in the MS^2 spectrum (Figure 2A). Using the fragmentation rules shown in Figure 2A (right), we identified the m/z 463 molecular ion as the Hex-Hex fragment (Gal β 4Glc-OH; Li et al. 2009) and the m/z 547 molecular ion as the C16 fatty acid ceramide. The MS^2 spectrum of the major molecular ion m/z 1120 in the MS^1 spectrum (Supplementary data, Figure S1A) was similar to that of m/z 1010: we identified the m/z 463 molecular ion as the Hex-Hex fragment (Gal β 4Glc-OH) and the m/z 657 molecular ion as the C24 fatty acid ceramide. We concluded that m/z 1120 was lactosylceramide (LacCer) with the C24 fatty acid ceramide (Supplementary

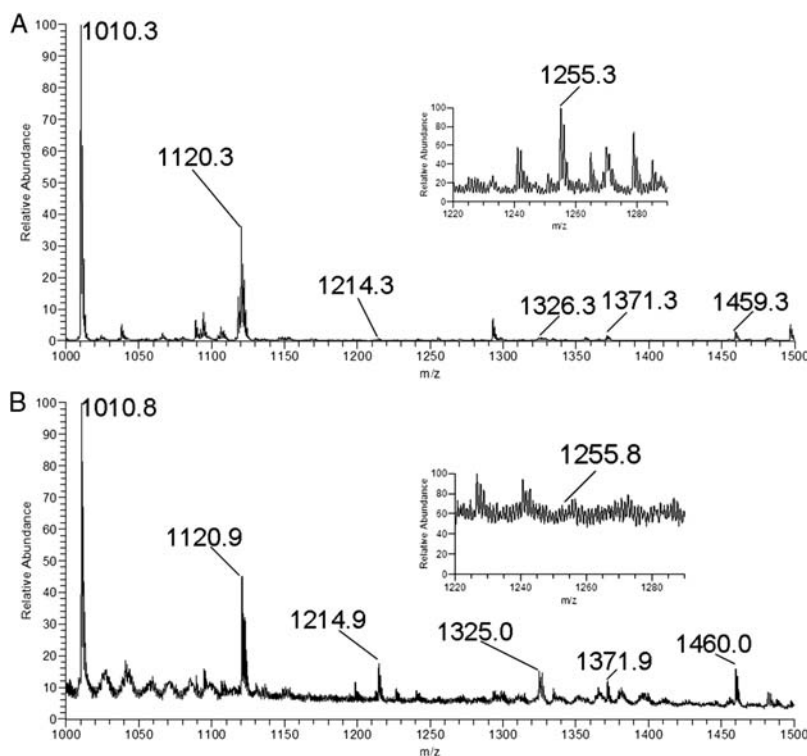
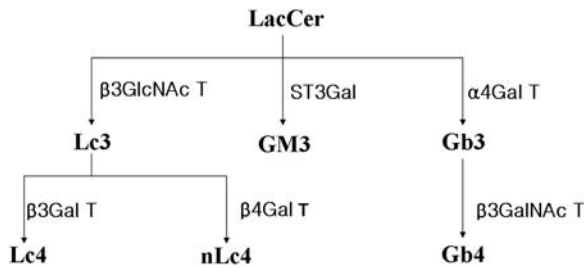


Fig. 1. Positive electrospray ionization linear MS^n analysis of bone marrow samples. (A) MS^1 profile of GSLs isolated from an AML patient bone marrow sample; the expression of Lc3 (1255) was at a high level. (B) MS^1 profile of GSLs isolated from a healthy control bone marrow sample; the expression of Lc3 (1255) was at a low level.



Scheme 1. Biosynthetic relationship of major GSL families in humans.

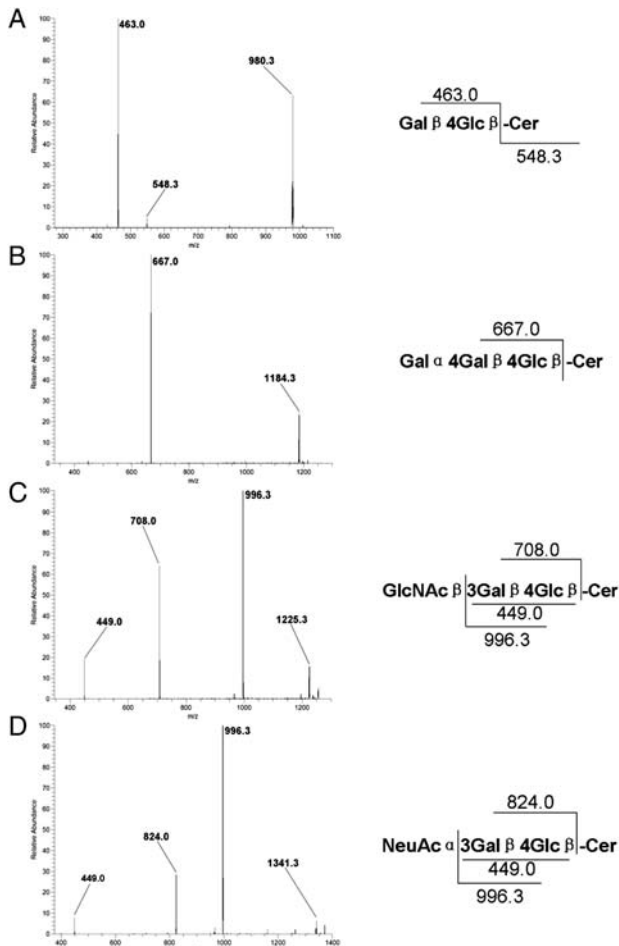


Fig. 2. Qualitative analysis of major GSLs in the human bone marrow samples. MS² spectrum, fragmentation pathway and characteristic fragmentation products for major permethylated GSLs in the human bone marrow samples in positive-mode MSⁿ are shown. (A) MS² spectrum and characteristic decomposition products of permethylated LacCer (1010) in the positive mode. (B) MS² spectrum and characteristic decomposition products of permethylated Gb3 (1214) in the positive mode. (C) MS² spectrum and characteristic decomposition products of permethylated Lc3 (1255) in the positive mode. (D) MS² spectrum and characteristic decomposition products of permethylated GM3 (1371) in the positive mode.

data, Figure S1A). These results indicated that both *m/z* 1010 and 1120 were LacCer, though with different lengths of fatty acyl chains in ceramide.

We analyzed the MS² spectra of other major molecular ions in the MS¹ spectra, including *m/z* 1214, 1255 and 1371 as well as *m/z* 1326, 1368 and 1483 (Figure 2B–D and Supplementary data, Figure S1B–D, respectively). The MS² spectra were compared with our previous study (Li et al. 2009), which revealed *m/z* 1214 and 1326 to be globotrihexosylceramide (Gb3) with C16 and C24 fatty acid ceramides, respectively; *m/z* 1255 and 1368 to be lactotriaosylceramide (Lc3) with C16 and C24 fatty acid ceramides, respectively; and *m/z* 1371 and 1483 to be GM3 with C16 and C24 fatty acid ceramides, respectively.

LacCer, Gb3, Lc3 and GM3 were present in the bone marrow samples from both AML patients and healthy donors. However, the isomers for *m/z* 1460, which represented GSL containing three hexose and one HexNAc residues, in healthy donors were significantly different from those in AML patients (Figure 3A and B). Healthy control had one major fragment, *m/z* 912, representing globotetra glycosylceramide (Gb4), whereas AML patients had *m/z* 912 plus another major fragment, *m/z* 996, representing lactotetraosylceramide (Lc4) or neolactotetraosylceramide (nLc4; Figure 3C). We hypothesized that *m/z* 1460 was a mixture comprising mainly Gb4 in healthy donors and primarily Lc4 or nLc4 in AML patients. To test this hypothesis, we generated an MS³ spectrum (*m/z* 1460 → 486 →; Figure 3D) and compared it with that published by Ashline et al. (2005); our comparison suggested that Galβ4GlcNAc-ene was the terminal linkage. Therefore, we speculated that the *m/z* 1460 mixture in AML patients was mainly nLc4 with the C16 fatty acid ceramide. Similarly, we found that the *m/z* 1572 mixture in the AML patients was mainly nLc4 with the C24 fatty acid ceramide, whereas the mixture in the healthy controls was mainly Gb4 with the C24 fatty acid ceramide.

Differential expression of major GSLs in the bone marrow samples of healthy donors and AML patients

ESI-LIT-MS revealed four major GSLs in the human bone marrow samples: LacCer, Gb3, Lc3 and GM3. We used a method of relative quantification to analyze these GSLs. Among these GSLs, LacCer was the precursor to the other GSLs and much more abundant in than the other GSLs in the bone marrows of both healthy donors and AML patients. We used differential isotope labeling to compare the amount of LacCer in bone marrow cells of healthy donors with that in the bone marrow cells of AML patients. In bone marrow cells from AML patients, GSLs were permethylated with deuterated iodomethane and mixed with GSLs from the same number of iodomethane-permethylated bone marrow cells (as determined by hemocytometer cell count) from healthy donors. We found the levels of LacCer in the bone marrow samples from AML patients to be similar to those in the bone marrow samples from healthy donors (Supplementary data, Figure S2), so we chose LacCer as the internal standard, with *m/z* 1010 and 1120 as major LacCer ion fragments, with C16 and C24 fatty acid ceramides, respectively.

We used a method of relative quantification to determine the ratios of Gb3 (Figure 4A and Supplementary data, Figure S3A), Lc3 (Figure 4B and Supplementary data, Figure S3B) and GM3 (Figure 4C and Supplementary data,

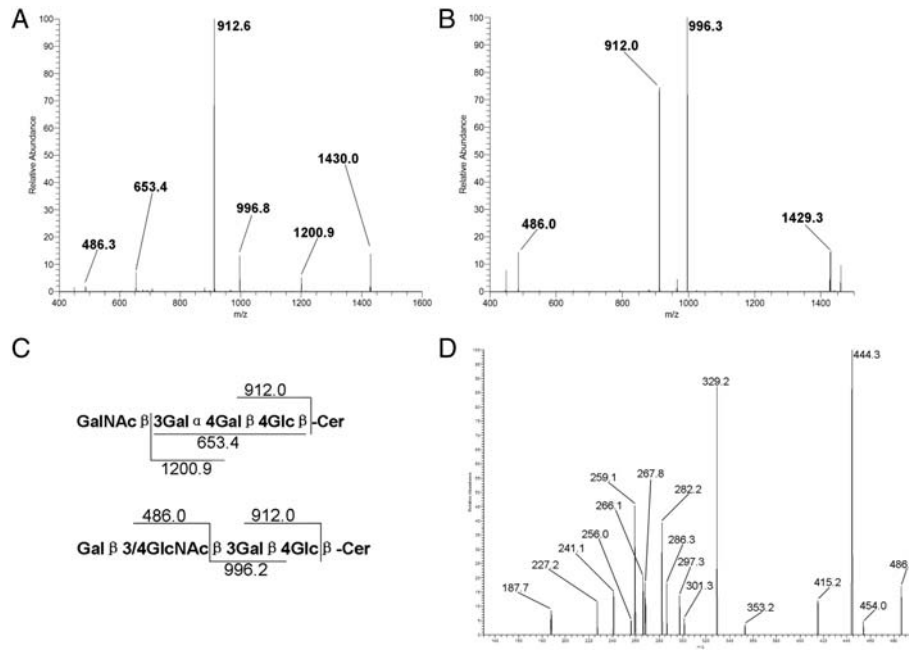


Fig. 3. Qualitative analysis of Gb4 in the bone marrow samples of healthy donors and nLc4 in the bone marrow samples of AML patients. (A) MS² spectrum of *m/z* 1460 precursor in the bone marrow samples of healthy donors. (B) MS² spectrum of *m/z* 1460 precursor in the bone marrow samples of AML patients. (C) Fragmentation pathway and characteristic decomposition products for Gb4 (top) and (n) Lc4 (bottom) in the positive-mode MSⁿ of permethylated GSLs. (D) MS³ spectrum (*m/z* 1460 → 486) in the bone marrow samples of AML patients.

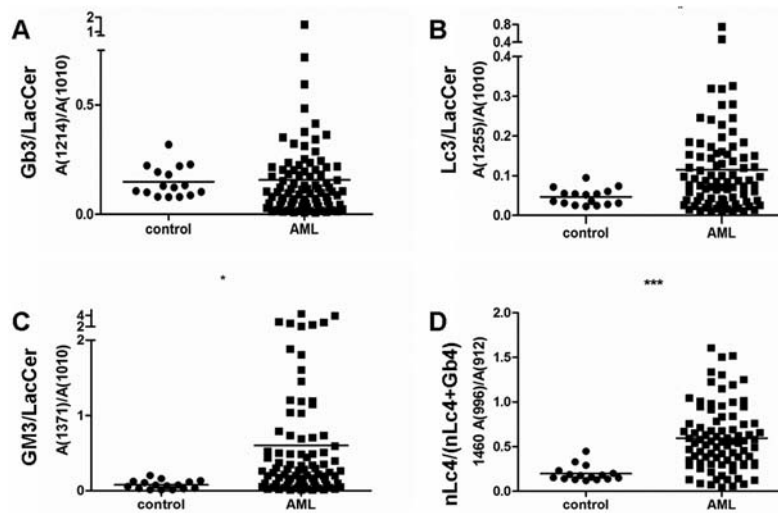


Fig. 4. Relative quantitative analysis of the bone marrow samples in healthy donors and AML patients. With LacCer (1010) as the internal standard, relative quantity was represented by the ratios of abundance of Gb3, Lc3 and GM3 to LacCer. (A) Relative quantification of Gb3 (1214) in the bone marrow samples of healthy donors and AML patients. (B) Relative quantification of Lc3 (1255) in the bone marrow samples of healthy donors and AML patients. (C) Relative quantification of GM3 (1371) in the bone marrow samples of healthy donors and AML patients. (D) Relative quantification of nLc4 (1460) in the bone marrow samples of healthy donors and AML patients. The ratios of *m/z* 996 abundance to *m/z* 912 in the MS² spectrum of *m/z* 1460 were chosen to represent the relative proportion of nLc4 (1460) in the mixture. Data represent the bone marrow samples from healthy donors (*n* = 16) and AML patients (*n* = 92). Statistical significance of differences between AML patients and controls was analyzed using the two-tailed Student's *t*-test of means. Compared with control, **P* < 0.05, ****P* < 0.001.

Figure S3C) to LacCer. The abundance of *m/z* 1460 and 1572 indicates the presence of a Gb4-and-nLc4 mixture. Although the relative quantities of Gb4 and nLc4 could not be determined from the MS¹ spectrum, they could be determined

from the MS² spectrum, which revealed *m/z* 912 to be the common fragment of Gb4 and nLc4 and *m/z* 996/1108 to be the specific fragment of nLc4 (1460/1572). Gb4 had only a small *m/z* 996/1108 fragment. Therefore, the ratio of the

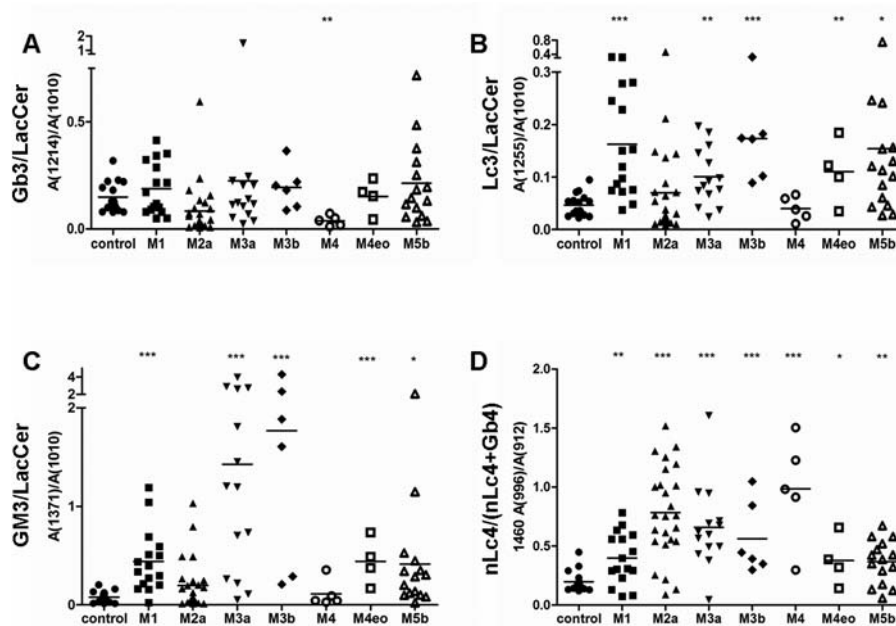


Fig. 5. Relative quantitative analysis of different AML subtypes. With LacCer (1010) as the internal standard, relative quantification was represented by the ratios of abundance of Gb3, Lc3 and GM3 to LacCer. (A) Relative quantification of Gb3 (1214) in the bone marrow samples of healthy donors and patients with different subtypes of AML. (B) Relative quantification of Lc3 (1255) in the bone marrow samples of healthy donors and patients with different subtypes of AML. (C) Relative quantification of GM3 (1371) in the bone marrow samples of healthy donors and patients with different subtypes of AML. (D) Relative quantification of nLc4 (1460) in the bone marrow samples of healthy donors and patients with different subtypes of AML. The ratios of m/z 996 abundance to m/z 912 abundance in the MS² spectrum of m/z 1460 were taken to represent the relative proportion of nLc4 (1460) in the mixture. Data represent the bone marrow samples of healthy donors ($n = 16$) and AML patients with the M1 ($n = 16$), M2a ($n = 25$), M3a ($n = 14$), M3b ($n = 6$), M4 ($n = 5$), M4eo ($n = 4$) and M5b ($n = 15$) subtypes. Statistical significance in differences between AML patient subtypes and controls was analyzed using the two-tailed Student's *t*-test of means. Compared with control, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

abundance of m/z 996/1108 to that of m/z 912 represented the relative proportion of nLc4 (1460/1572) in the mixture (Figure 4D and Supplementary data, Figure S3D). These results suggested that Lc3, GM3 and nLc4 expression are higher in the bone marrow samples from AML patients than in the bone marrow samples from healthy donors. However, Gb3 expression in healthy donors and AML patients did not differ significantly (Figure 4A).

Differential GSL expression in the bone marrow samples of different subtypes of AML patients

The French–American–British (FAB) classification system (Bennett et al. 1976) categorizes AML into eight subtypes, M0–M7, based on the type of cells from which the leukemia developed and the maturity of cells. Based on the clinical information, we identified different subtypes of AML and analyzed the expression of different GSLs in the subtypes. Differences in the relative quantities of Gb3 (Figure 5A and Supplementary data, Figure S4A), Lc3 (Figure 5B and Supplementary data, Figure S4B), GM3 (Figure 5C and Supplementary data, Figure S4C) and nLc4 (Figure 5D and Supplementary data, Figure S4D) were found in different subtypes of AML. In the M1 subtype (acute myeloblastic leukemia without maturation), Gb3, Lc3, GM3 and nLc4 were all more highly expressed than in the control group. However, in the M2a subtype (acute myeloblastic leukemia with

Table I. The amounts of major GSLs in patients with different subtypes of AML when compared with healthy controls

	Control	M1	M2a	M3a	M3b	M4	M4eo	M5b
Gb3 (1214)	1.00	1.26	0.56	1.51	1.30	0.26	1.02	1.43
Lc3 (1255)	1.00	3.47	1.49	2.15	3.70	0.86	2.36	3.29
GM3 (1371)	1.00	5.61	2.51	18.19	22.54	1.43	5.62	5.28
nLc4 (1460)	1.00	2.02	3.97	3.34	2.85	4.99	1.91	1.86

granulocytic maturation), only nLc4 expression was higher than in healthy donors, whereas Gb3, Lc3 and GM3 expression did not differ significantly from the control. In the M3a group, both Lc3 and nLc4 had higher expression than in healthy donors. In the M4 subtype (acute myelomonocytic leukemia), only nLc4 expression was higher than in the healthy donors. In the M5 subtype, Gb3, Lc3, GM3 and nLc4 were all higher than in the healthy donors (Table I).

Higher expression levels of Lc3 synthase

β 1,3-*N*-acetylglucosaminyltransferase 5 in the bone marrow samples of AML patients versus healthy donors

In our previous studies (Henion et al. 2001; Biellmann et al. 2008), β 1,3-*N*-acetylglucosaminyltransferase 5 (β 3Gn-T5) was demonstrated to be Lc3 synthase that synthesizes the precursor structure for the lacto/neolacto-series GSLs in both in

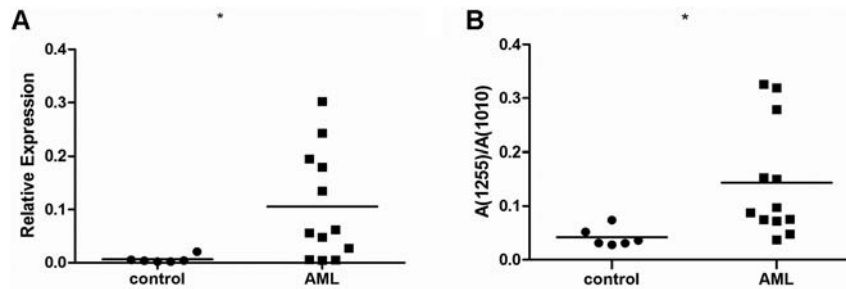


Fig. 6. Expression level of Lc3 synthase β 3Gn-T5 and corresponding relative quantification of Lc3 (1255) in the bone marrow samples of healthy donors and AML patients. (A) The expression of Lc3 synthase β 3Gn-T5 in bone marrow samples was analyzed using the real-time PCR method. (B) Corresponding relative quantification of Lc3 (1255) in the bone marrow samples of healthy donors and AML patients was analyzed as in Fig. 4. Data represent the bone marrow samples of healthy donors ($n=6$) and AML patients ($n=12$). Statistical significance in differences between AML patients and controls was analyzed using the two-tailed Student's t -test of means. Compared with control, $*P < 0.05$.

Table II. β 3Gn-T5 and Lc3 levels in each of the healthy donors (No. 1–6) and AML patients (No. 7–18, randomly selected from 104 patients)

No.	β 3Gn-T5 ^a	Lc3 ^b	No.	β 3Gn-T5	Lc3	No.	β 3Gn-T5	Lc3
1	0.0055	0.0519	7	0.0056	0.0723	13	0.0622	0.0877
2	0.0022	0.0309	8	0.1953	0.2787	14	0.1801	0.1505
3	0.0039	0.0312	9	0.0481	0.0759	15	0.0059	0.0483
4	0.0209	0.0739	10	0.0561	0.0976	16	0.2426	0.3195
5	0.0042	0.0358	11	0.0049	0.0375	17	0.0275	0.0749
6	0.0023	0.0279	12	0.3022	0.3263	18	0.1353	0.1530

^aThe expression of Lc3 synthase β 3Gn-T5 in bone marrow samples was analyzed using real-time PCR.

^bCorresponding relative quantification of Lc3 (1255) in healthy donors' bone marrow samples and AML patients' bone marrow samples were analyzed as in Fig. 4.

vitro and in vivo experiments. β 3Gn-T5 is, in fact, the key enzyme responsible for the synthesis of lacto- and neolacto-series GSLs. Therefore, we compared the mRNA levels of β 3Gn-T5 in the bone marrow samples from healthy donors and AML patients. Figure 6A shows that the expression of β 3Gn-T5 was significantly higher in AML patients than in the healthy donors, which was consistent with MS analysis results, as shown in Figure 6B. Table II shows the β 3Gn-T5 and Lc3 expression levels in each bone marrow sample of the healthy donors and the AML patients.

Discussion

In the present study, we used a specific, sensitive and generally applicable method to analyze GSLs in tumor cells (Li, Teneberg et al. 2008; Li, Zhou et al. 2008). We detected Gb3, Lc3, GM3 and nLc4 as major GSLs expressed in bone marrow cells. Furthermore, significant differences in GSL expression between healthy donors and AML patients were found. Our findings confirm previous reports that AML cells have high expression of GM3. We found that Lc3 and nLc4 were expressed at higher levels in AML patients than in healthy donors. We also found small amounts of isoglobotriosylceramide (iGb3; Zhou et al. 2004) in Gb3/iGb3 isomers in AML patients (Wang et al., unpublished data).

Our study was made possible by the use of MSⁿ, which has greatly improved the applicability of the MS technology for analyzing patient samples. The application of ESI and matrix-assisted laser desorption ionization, in conjunction with tandem collision-induced dissociation, time of flight and quadrupole ion-trap techniques, has revolutionized the sensitivity of MS (Reinhold et al. 1995; Reinhold and Sheeley 1998; Costello 1999; Harvey 1999; Dell and Morris 2001; Levery 2005). The selectivity of our method is based on the characteristic fragmentations of the sugar chain of permethylated GSLs, which differ from those of other GSL series when isolated and analyzed by MSⁿ using the MSⁿ analysis. Relative proportions of GSLs could be determined from the abundance ratios of selected characteristic fragmentation products of the GSLs.

In this study, the major GSL species in human bone marrow, including Gb3, Lc3, GM3 and nLc4, were analyzed in a semi-quantitative manner. Through statistical analysis, we minimized the deviation between the donors and the AML patients. Gb3 did not differ significantly between the control and AML groups, whereas Lc3, GM3 and nLc4 had significantly higher expression in the bone marrow samples of AML patients. GM3 serves as a precursor for most of the more complex ganglioside species, and ganglioside participates in the regulation of various cellular functions, including cell proliferation, apoptosis, migration and invasion. Hence, the finding of a large amount of GM3 in the bone marrow samples of AML patients is very valuable for further investigation.

Lc3 is composed of glucose, galactose and *N*-acetylglucosamine as carbohydrate chain constituents. This GSL is a precursor structure, which is elongated to the lacto- and neolacto-series GSLs. Lc3 is highly expressed on the cell surface of HL-60 cells (Nozaki et al. 2010; Togayachi et al. 2010). The glycosyltransferase β 3Gn-T5 (Henion et al. 2001; Togayachi et al. 2001), which synthesizes Lc3, plays critical roles in embryonic development and differentiation. Its expression in leukemia cell lines has been reported previously (Nozaki et al. 2010). Our finding that Lc3 is highly expressed in primary AML suggests that it may serve as a potential biomarker and a target for therapy.

We detected high expression of Lc3 in the AML cell line HL-60, whereas little Lc3 was detected in the acute

lymphoblastic leukemia (ALL) cell line Jurkat (Togayachi et al. 2010). We also detected only a small amount of Lc3 using MS in Jurkat cells (data not shown), and we did not find high expression of Lc3 in the bone marrow samples of T-ALL or B-ALL patients (data not shown). In K-562 cells, a chronic myeloid leukemia cell line, the expressions of Lc3 and structurally related GSL components were very low and could not even be detected by flow cytometry (Nozaki et al. 2010). In AML, both cell lines and clinical samples showed significantly higher expression of Lc3. Interestingly, the expression level of Lc3 varies in different subtypes of AML, indicating that the expression of Lc3 and/or nLc4 is closely related to cell differentiation. The expression of Lc3 in the M1 subtype (acute myeloblastic leukemia without maturation) was significantly higher than that in healthy controls. However, no significant difference was found in Lc3 expression between healthy controls and the M2a subtype (acute myeloblastic leukemia with granulocytic maturation). It indicates that the expression of Lc3 is closely related to cell differentiation in human bone marrow. Moreover, there may have differences in GSLs metabolism between the various leukemia subtypes that would be worthy of further investigation.

Inducing differentiation is currently one of the most important therapies for AML, especially for the M3 subtype, also known as acute promyelocytic leukemia (APL). Differentiation therapy with all-trans retinoic acid (ATRA) has been proven to be very effective and has become an established APL treatment (Degos and Wang 2001; Bolanos-Meade et al. 2003; Vitoux et al. 2007). In vitro studies have shown that the human leukemia cell lines HL-60 and NB4 can be induced to enter differentiation along the neutrophil lineage upon stimulation with ATRA or along the monocyte lineage after phorbol 12-myristate 13-acetate (PMA) treatment, resulting in morphological, functional and drug sensitivity changes accompanied by a loss of proliferative capacity. Thin-layer chromatography has been applied to analyze the changes in GSLs in the differentiated cells (Nakamura et al. 1992; Jasek et al. 2008). Thus, we used the MS method to analyze the GSLs in HL-60 and NB4 cells treated with ATRA and PMA and found that their Lc3 expression was lower than that of the untreated cells (Supplementary data, Figure S5).

The biosynthesis and degradation of GSLs is a complex process involving numerous enzymes that act at various subcellular locations. Among these enzymes, only one or a few key enzymes control the metabolism of GSLs. As Lc3 is a precursor structure of lacto- and neolacto-series GSLs, β 3Gn-T5 also affects lacto- and neolacto-series GSLs (Togayachi et al. 2010). In this study, Lc3 is identified as a potential biomarker of AML. However, whether the presence of Lc3 is a result or a cause of AML is still unknown. This question may be resolved by silencing the Lc3 synthase β 3Gn-T5 to reduce the expression of Lc3 and examining the effect of lacto-series GSLs on AML carcinogenesis.

Our results clearly indicate that Lc3 is abnormally expressed by AML cells, and the underlying mechanism is the overexpression of the Lc3 synthase. However, due to the semi-quantitative nature of MS assays, the exact level of Lc3 expression in AML patients must be further quantified by

other independent methods, such as staining by specific monoclonal antibodies. Similarly, the high expression of GM3 and nLc4 in the bone marrow of AML patients also need to be quantified by other independent methods. The subcellular localization of Lc3 in AML cells remain to be studied, although its expression on cell surface has been reported by Nozaki et al. (2010).

In conclusion, we show for the first time in clinical samples that the increased expression of Lc3, GM3 and nLc4 is characteristic in AML patients and Lc3 is related to cell differentiation. AML-associated GSLs Lc3, GM3 and nLc4 are possibly involved in initiation and differentiation of AML. Future studies will be focused on the transcriptional regulation of Lc3 synthase, the receptors for Lc3 and the alterations of Lc3 in patients after induction therapy, as well as developing Lc3-specific antibodies for diagnosis and therapies. Similarly, all these studies are necessary for GM3 and nLc4.

Materials and methods

Tissues and cultured cells

Bone marrow samples were obtained from AML patients and healthy donors at the First Affiliated Hospital of Soochow University (Suzhou, China) under institutional guidelines. A total of 16 healthy donors and 104 patients were enrolled (Supplementary data, Table S1 and Supplementary data, Figure S2). All the bone marrow samples were obtained at initial diagnosis without chemotherapy. Human AML cell lines HL-60 (ATTC CCL 240) and NB4 (DSM ACC 207) were cultured in RPMI 1640 (Life Technologies, Carlsbad, CA) supplemented with 10% (v/v) fetal bovine serum (Life Technologies), 2 mM L-glutamine (Life Technologies) and 50 μ g/mL of gentamicin. Cells were cultured at 37°C in a humidified 5% CO₂ atmosphere.

Total lipid extraction from leukemia cells

Cultured leukemia cells (5×10^7 cell aliquots) were stored in 16×100 mm glass tubes at -80°C . Lipids were extracted by extensive sonication four times with mixed polarity solvents (1 mL each). The first and last solvent used was chloroform-methanol 1:1 (v/v). The second and third solvent used was isopropanol-hexane-water 55:25:20 (v/v/v, upper phase removed by aspiration before use). The supernatants were pooled and dried under a nitrogen stream at 40°C .

Per-N,O-methylation of GSLs

A modification of the method of Ciucanu and Costello (2003) was employed for per-N,O-methylation of GSLs. GSLs (1–20 μ g) were introduced into a conical glass vial, and dimethyl sulfoxide (150 μ L) was added without using special drying conditions or an inert gas atmosphere. Powdered sodium hydroxide (40–60 mg) was then added to the sample solution and stirred at room temperature until completely dissolved. Iodomethane or deuterated iodomethane (80 μ L) was added with a syringe, and the mixture was shaken at room temperature for 1 h. The methylation reaction was quenched with water (2 mL). The permethylated products were extracted three times by the addition of dichloromethane (2 mL). The

combined dichloromethane extracts were then washed three times with water (2 mL each). Following the final wash, the samples were transferred to a new tube and dried under a nitrogen stream at 35–40°C.

Electrospray ionization MSⁿ of permethylated GSLs

Electrospray ionization MS (MS and MSⁿ) was carried out in a positive ion mode on a linear ion-trap mass spectrometer (LTQ, Thermo Finnigan, San Jose, CA) using an electrospray source for direct infusion of samples dissolved in methanol, with a flow rate of 0.30 µL/min at a capillary temperature of 230°C, a sheath gas flow rate of 2 arb, a spray voltage of 3.50 kV, a capillary voltage of 28.00 kV, a tube lens of 120.00 V, an injection time of 100.00 ms, an activation time of 30 ms, an activation Q-value of 0.250 and an isolation width of *m/z* 1.5. Normalized collision energies were set to leave a minimal residual abundance of precursor ion; in this case, 30% was used for all product ion scans. All ions were detected as sodium adducts.

Quantitative real-time polymerase chain reaction assays

The RNA from each sample was reverse-transcribed into first-strand cDNA with an oligo-dT primer. The quantitative reverse transcriptase–polymerase chain reaction (PCR) analysis was performed using the ABI 7500 system (Life Technologies) with a Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific, Glen Burnie, MA) according to the manufacturer's protocol. The amount of β-actin mRNA was measured as an internal standard. PCR was performed using primers specific to each gene through 40 cycles at 95°C for 15 s and 60°C for 60 s after a preincubation at 50°C for 2 min and 95°C for 10 min. The specific primers used for amplification were as follows: β3Gn-T5-F: 5'-TTTTGGATTGGTC GTGTTTCAT-3'; β3Gn-T5-R: 5'-CGGCTGTTAGTCAGGGT AAG-3'; β-actin-F: 5'-CACCATTGGCAATGAGCGGTTCC-3'; β-actin-R: 5'-GTAGTTTCGTGGATGCCACAGG-3'.

Statistical analysis

Statistical significance in differences between AML patient and control samples was analyzed using the two-tailed Student's *t*-test of means. For all tests, *P* < 0.05 was considered as significant.

Supplementary data

Supplementary data for this article is available online at <http://glycob.oxfordjournals.org/>.

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Conflict of interest

D.Z. is the President of NanoCruise Suzhou Pharmaceutical Ltd.

Abbreviations

ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; APL, acute promyelocytic leukemia; ATRA, all-trans retinoic acid; β3Gn-T5, β1,3-*N*-acetylglucosaminyltransferase5; ESI-LIT-MS, electrospray ionization linear ion-trap mass spectrometry; FAB, French–American–British; Gb4, globotetra glycosylceramide; Gb3, globotrihexosylceramide; GSL, glycosphingolipid; iGb3, isoglobotrihexosylceramide; LacCer, lactosylceramide; Lc3, lactotriaosylceramide; Lc4, lactotetraosylceramide; MS, mass spectrometry; MSⁿ, ion trap mass spectrometry; nLc4, neolactotetraosylceramide; PCR, polymerase chain reaction; PMA, phorbol 12-myristate 13-acetate.

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