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Expression of the glycolipid globotriaosylceramide (Gb3) in testicular carcinoma in situ

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Abstract Changes in the cell membrane glycolipid composition and metabolism are frequently associated with carcinogenesis. The accumulation of globo-series glycolipids is the most notable change of the germ cell glycolipid composition observed in testicular tumours. In this study, the expression of the globo-series core-structure, globotriaosylceramide (Gb3) was investigated in the preinvasive stage of testicular germ cell tumours, carcinoma in situ (CIS). Seventeen tissue specimens with CIS and 12 samples of overt testicular tumours were immunostained with anti-Gb3 monoclonal antibody 38-13. The accumulation of Gb3 was detected in 12 CIS samples (70.6%) and in 8 invasive tumour samples (66.7%), including seminoma, non-seminoma and a combined germ cell tumour. Our findings indicate that the composition of glycolipids shift at the common preinvasive stage of testicular germ cell tumours and confirm that Gb3 is a tumour-associated antigen of testicular germ cell neoplasia.

Key words Testicular neoplasms · Testicular carcinoma in situ (CIS) · Globotriaosylceramide (Gb3) · Immunohistochemistry

Introduction

Glycosphingolipids (glycolipids) are important components of the cell membrane. They play a role in the differentiation and development of cells, probably by serving as membrane transducers of regulatory signals [7]. There are three major series of glycolipids: globo-, lacto- and ganglio-series. Changes in glycolipid composition and a shift from one series to another are frequently associated with carcinogenesis [7]. Specific patterns of glycolipids expressed by tumour cells are known as tumour-associated glycolipid antigens. Forssman glycolipid in human gastric cancer [11]; GD_3 ganglioside in melanoma [13], GD_2 in neuroblastoma [23] and globotriaosylceramide (Gb3) in Burkitt's lymphoma (BL) [21] are good examples.

The profile of glycolipids in human germ cell tumours has been studied previously. Globo-series glycolipids Gb5 and GL7 were described to be the carriers of stage-specific embryonic antigen (SSEA)-3 and SSEA-4, and the expression of both glycolipids was reported in human teratocarcinoma cell lines [9, 18]. Kannagi et al. [10] analysed the glycolipid composition in a human teratocarcinoma cell line, 2102Ep thoroughly, and noted high levels of globo-series glycolipids, including Gb3, Gb4, Gb5, and GL-7, but not of the ganglio- or lacto-series. SSEA-1 and SSEA-3 have also been demonstrated immunohistochemically in embryonal carcinoma, the most undifferentiated histological component of nonseminomatous testicular germ cell tumours [2]. A marked accumulation of Gb3, the globo-series core structure, was reported in seminoma, the most common histological form of all testicular germ cell tumours; and Gb3 was even proposed as a marker for seminoma [14, 15]. By high-performance thin layer chromatography (HPTLC) analysis combined with immunostaining of a large number of human germ cell tumour lines, Wenk and others [20] have recently found distinct patterns of globo-series antigens in embryonal carcinoma cell lines and their differentiated derivatives. They concluded that the switch in the glycolipid expression might be a key step in germ cell differentiation and that the differential expression of glycolipids provided a basis for better classification of germ cell tumours.

Testicular carcinoma in situ (CIS) is a precursor of germ cell tumours including classical seminoma and nonseminomatous tumours, but not spermatocytic seminoma (spermatocytoma) [19]. There are no data regarding the expression of glycolipids in this preinvasive lesion. As Gb3 has been reported in a variety of germ cell

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tumours including both seminoma and embryonal carcinoma, it was an obvious first candidate glycolipid to evaluate in CIS. CIS cells arise inside seminiferous tubules and are usually located between the tubular wall and the layer of Sertoli cells. CIS-containing tubules are very frequently found adjacent to overt tumours, sometimes as focal changes in the preserved non-malignant testicular parenchyma. In some cases CIS may also be found in the testis contralateral to a tumour. In order to localize precisely the expression of Gb3 to a specific cell type, an immunohistochemical method was chosen.

Materials and methods

Seventeen adult testicular tissue samples of CIS, 12 samples with overt tumours and 1 specimen of normal testicular tissue were examined in this study. The group of overt tumours included 8 classical seminomas, 3 nonseminomas (all pure embryonal carcinomas) and 1 combined germ cell tumour (combination of seminoma, embryonal carcinoma and mature teratoma). Specimens were obtained from patients who underwent orchidectomy for testicular neoplasia or prostatic cancer (for a normal control). The use of orchidectomy specimens for the immunohistochemical studies had been approved by the local Ethical Committee.

Table 1 Expression of globotriaosylceramide (*Gb3*) in carcinoma in situ (*CIS*) and overt testicular germ cell tumours (the histological type of tumour to which CIS was adjacent is given in brackets; *S* seminoma, *NS* nonseminoma, *EC* embryonal carcinoma)

Case number	Diagnosis	Gb3
1	CIS (NS)	+*
2	CIS (S)	+
3	CIS (NS)	+
4	CIS (NS)	+*
5	CIS (NS)	+*
6	CIS (no overt tumour)	+*
7	CIS (S and NS)	+–
8	CIS (NS)	+
9	CIS (S)	+–
10	CIS (S)	+*
11	CIS (NS)	–*
12	CIS (S)	–*
13	CIS (NS)	+–
14	CIS (S and NS)	+–
15	CIS (S)	–
16	CIS (S)	–
17	CIS (S)	–
18	S	+*
19	S	+–*
20	S	+*
21	S	+
22	S	+–
23	S	–
24	S	–
25	S	–
26	EC	+–
27	EC	+–
28	EC	–
29	S and NS	+
30	Normal testis (PC)	–*

* Frozen and paraffin sections
CIS: carcinoma in situ; S: seminoma; NS: nonseminoma; EC: embryonal carcinoma; PC: prostatic cancer

One of the CIS samples (case 6 in Table 1) contained isolated CIS (no overt tumour) which was diagnosed in a patient with one small testicle by ultrasonographic examination and a surgical biopsy followed by orchidectomy [6]. In the remaining specimens CIS was present adjacent to overt tumours (see Table 1). In these cases CIS was found during histopathological examination of macroscopically unchanged testicular tissue in the vicinity of tumours. The histological diagnosis was made according to morphological features and positive immunostaining with antibodies used as CIS markers, such as placental-like alkaline phosphatase (PLAP), M2A, TRA-1-60 and c-*kit* [4, 5, 8, 16]. Formalin-fixed and paraffin-embedded tissue blocks were used in all cases. In 11 cases (7 CIS, 3 seminomas, 1 normal testis; asterisks in Table 1) frozen tissue fragments were also available for the study.

The monoclonal antibody 38-13 is a rat IgM that recognizes Gb3. The antibody was raised by one of the authors as previously described [21].

For immunocytochemistry paraffin-embedded sections cut at 4 μm were dewaxed and rehydrated. Cryo-preserved tissues were sectioned at 6 μm and fixed in 4% buffered formalin for 15 min at 4° C. Non-specific binding of the antibody was blocked by 20 min incubation with human serum (diluted 1:4), afterwards the sections were incubated with the primary antibody (diluted 1:50 for the paraffin sections and 1:100 for the frozen sections) at 4° C overnight. Subsequent steps were performed at room temperature. After washing in four changes of 0.05 M TRIS-buffered saline (TBS), pH 7.4, the sections were incubated with either a biotinylated rabbit anti-rat antibody (DAKO, Copenhagen, Denmark) or a biotinylated goat anti-mouse antibody (Zymed Laboratories, San Francisco, USA) for 15 min. The anti-mouse antibody cross-reacted with the rat immunoglobulins and gave identical results of these obtained with the anti-rat antibody. Subsequently, the samples were incubated with a streptavidin-peroxidase conjugate (Zymed) for 10 min. Colour reaction was developed using aminoethyl carbazole or 3,3′-diaminobenzidine as substrates (diluted in TBS containing hydrogen peroxide). For negative controls, primary antibody was substituted with TBS buffer.

After cover slips were mounted, the sections were examined under a light microscope. The Gb3 staining was evaluated qualitatively, using the following symbols: (+) positive in the majority of CIS tubules or in large areas of a tumour specimen; (+–) many CIS tubules negative or very weakly stained, or large areas of a tumour negative; (–) no Gb3-positive cells found.

Results

The results are summarized in Table 1. The expression of Gb3 was detected in CIS samples as well as in overt testicular germ cell tumours. Positive Gb3 staining was mostly present in the plasma membrane. However, some diffused cytoplasmic staining was also present. In positive CIS cases, most of the CIS cells visible in a section were stained, although the intensity was variable. In most cases of overt tumours, the immunostaining was rather heterogenous, ranging from a strongly positive to a negative reaction in various areas of the same specimen.

Twelve out of seventeen CIS specimens (70.6%) showed positive immunostaining with anti-Gb3 antibody; there was no difference depending on the histological type of the adjacent tumour. No staining was seen in the non-malignant seminiferous tubules adjacent to CIS-containing tubules (Fig. 1a, 2a).

In the overt tumours, immunoreactivity was found in 8 of 12 cases (66.7%), including 5 seminomas (Fig. 3a, b), 2 embryonal carcinomas and 1 combined germ cell tumour (seminoma+embryonal carcinoma+mature tera-

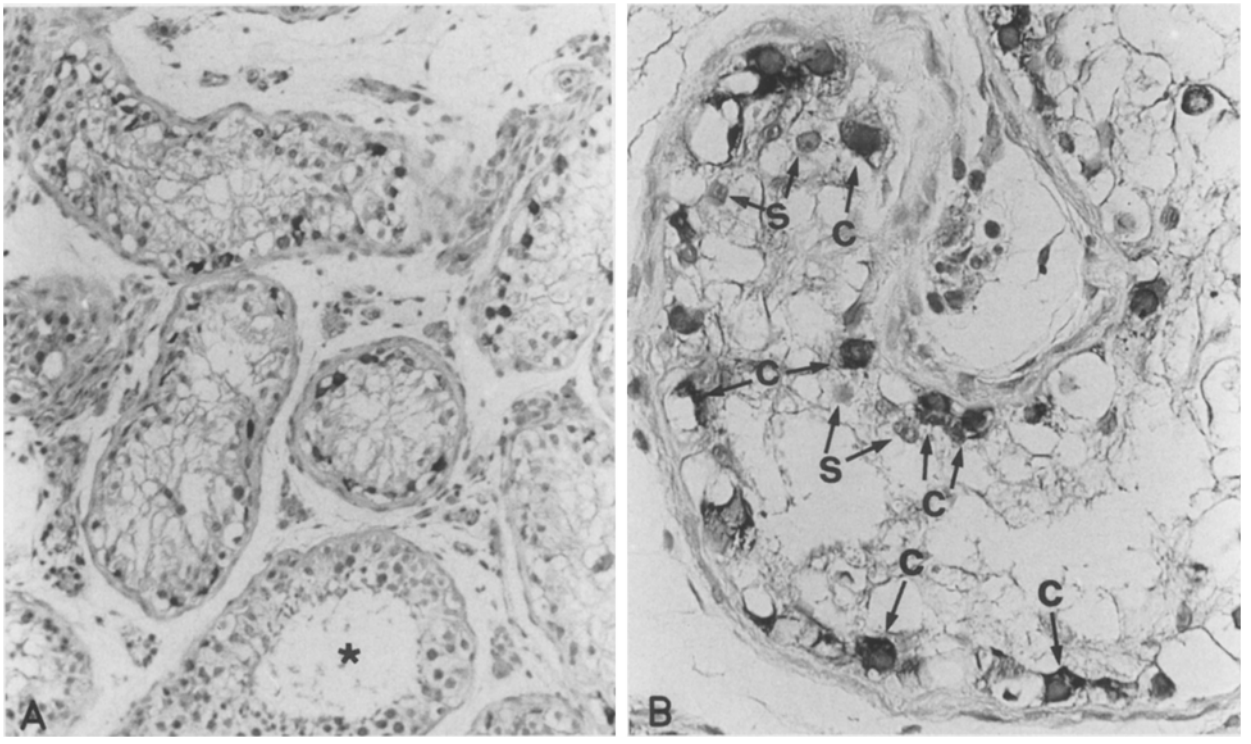

Fig. 1 **A** Immunoperoxidase staining of a carcinoma in situ (CIS) specimen with the anti-globotriaosylceramide (Gb3) antibody. Majority of CIS cells are Gb3-positive. Note absence of staining in the normal tubules (*asterisk*), ×100. **B** Higher magnification of a CIS sample; only CIS cells (*C*) are stained, Sertoli cells (*S*) show no reaction, ×250

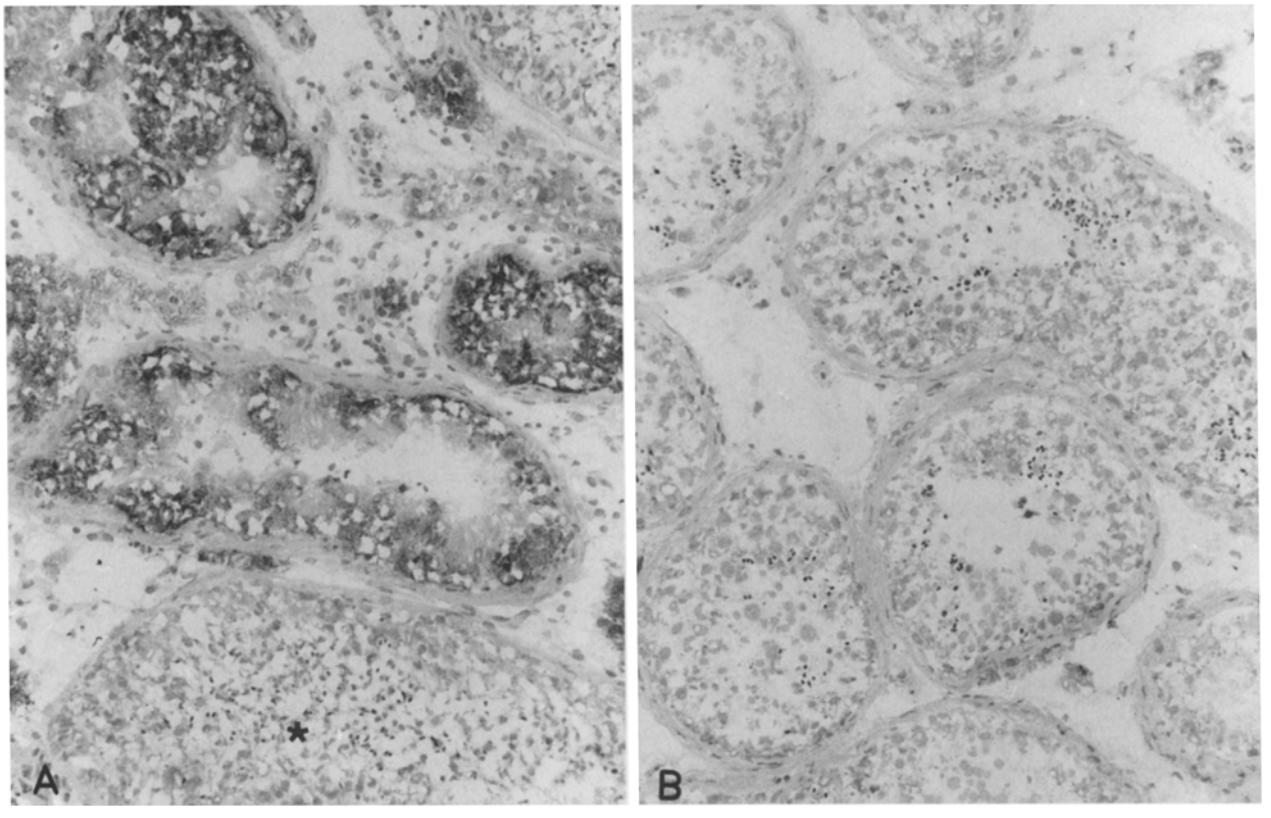

Fig. 2 **A** Frozen section of CIS, immunostaining for Gb3 is seen in seminiferous tubules with CIS. Note no reaction in adjacent non-malignant tubules (*asterisk*), ×100. **B** Frozen section of a normal testis. No immunoreactivity of Gb3 was found, ×100

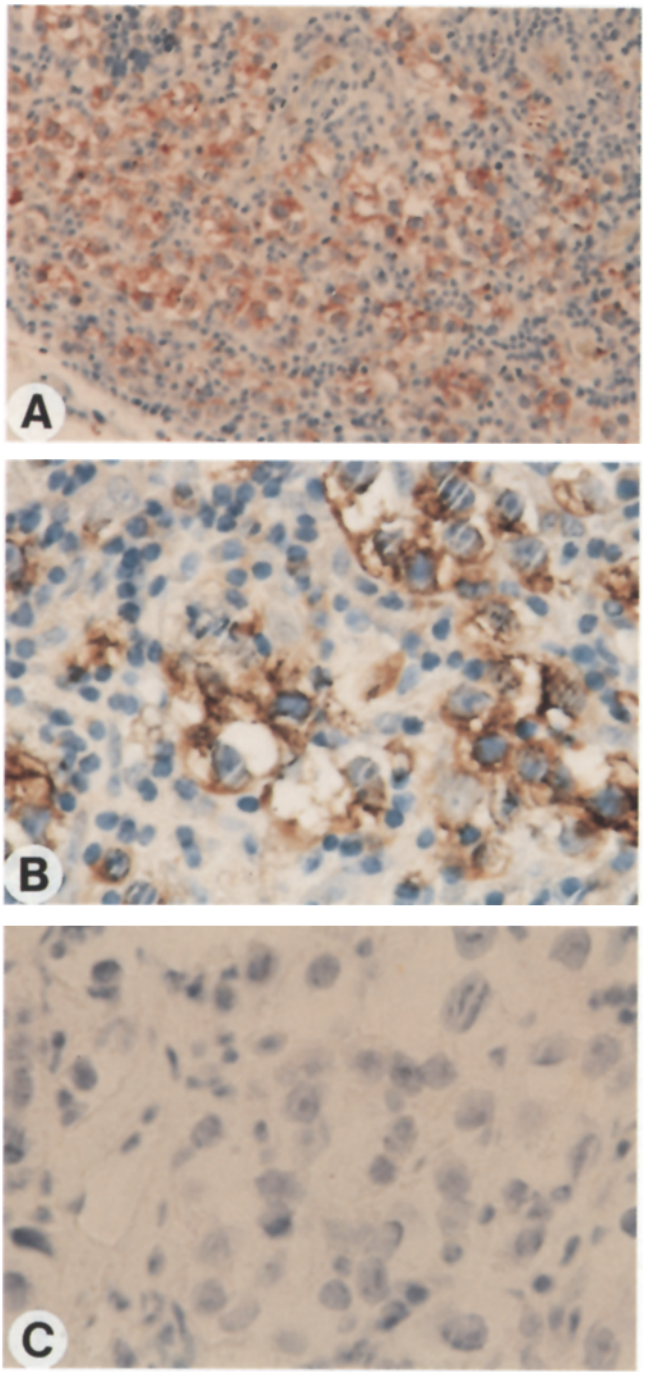

Fig. 3 **A** An example of immunohistochemical staining for Gb3 of a seminoma; lymphocytic infiltrate shows no reaction, ×100. **B** Higher magnification of a Gb3-positive seminoma, ×250. **C** Negative control, ×250

toma). In the combined tumour, teratoma showed no reactivity, except for a few gland-like structures which were weakly stained. In comparison with CIS and seminoma, embryonal carcinoma exhibited weaker immunostaining with Gb3.

In frozen samples, immunoreactivity with anti-Gb3 was seen in five of seven CIS cases (Fig. 2a). A slight, probably non-specific staining in Leydig cells was noted in some cases. All three frozen samples of seminoma showed positive immunoreactivity of tumour cells. In the frozen samples, the results were parallel to these obtained in paraffin-embedded tissue fragments, although the intensity of staining was stronger and, in some cases, the number of positive cells was greater.

In the normal testis, positive immunoreaction was found in neither paraffin-embedded nor frozen sections (Fig. 2b). Control sections immunostained with the omission of the primary antibody did not exhibit any staining.

Discussion

This study has shown the expression of Gb3 in CIS for the first time. This observation in the preinvasive stage of testicular germ cell tumours provides further insight into the histogenetic relationship between CIS and overt germ cell tumours. In our series 70% of CIS cases demonstrated immunostaining with anti-Gb3 antibody. Our study confirmed also the presence of Gb3 in overt testicular germ cell cancers.

Most of the previous reports describing the expression of glycolipids in germ cell tumours or cell lines have been based on HPTLC analysis. Few studies have provided immunohistochemical data, like that of Damjanov et al. [2] who demonstrated SSEA-1 and SSEA-3 in embryonal carcinomas. It is generally considered that paraffin-embedded samples are not suitable for the detection of cell surface glycolipid antigens. Ohyama et al. [14, 15], however, found marked expression of Gb3 in formalin-fixed, paraffin-embedded tissue in all seminomas studied. In agreement with Ohyama, comparable results were obtained in our study in both frozen and paraffin-embedded tissue fragments, although the intensity of staining was usually weaker in paraffin blocks. The absence of Gb3 in a few of our CIS cases may be due in part to the loss of antigenicity in the process of tissue preparation; for example, in case 5 the Gb3 staining was very strong in the frozen specimen, but rather weak in the paraffin sections. However, two paraffin-embedded Gb3-negative CIS specimens (cases 11 and 12) were also negative in frozen sections. Thus, there are apparently differences in Gb3 expression in CIS cells that may suggest heterogeneity of differentiation stages of CIS cell despite their morphological similarity.

The expression of glycolipid Gb3 in CIS, seminoma and nonseminoma may have some aetiopathological implications. The prevalent opinion is that the CIS lesion is the common precursor for various types of germ cell tumours. The co-expression of Gb3 in CIS and overt germ cell tumours further corroborates an histogenetic relationship between these neoplasms. In addition, the results confirm that Gb3 is a tumour-associated glycolipid antigen of testicular germ cell neoplasms and indicate that the glycolipid composition in malignant germ cells shifts by the preinvasive stage.

The biological significance of the high expression of Gb3 in germ cell tumours remains obscure. Not much is known about the role of aberrant glycosylation in human carcinogenesis in general. In the haematopoietic system, Gb3 (also called CD77), was originally described as a specific antigen for BL [22] and was then found on a subset of tonsillar germinal centre B lymphocytes which probably constitute the normal counterpart of BL cells. Although the biological role of Gb3 in these cells is not completely understood, it seems likely that this antigen play a role in cell apoptosis, since cross-linking of surface Gb3 induces apoptosis of BL cells [12]. It would thus be of interest to test whether or not Gb3 in CIS and seminoma is also able to transduce a signal leading to apoptosis of these cells.

Differential expression of glycolipids in a variety of tumours is believed to be the result of altered activities of synthesizing enzymes. Either an activation of synthesis of the core structure or a block in synthesis of derivative glycolipids can cause the accumulation of Gb3 [7]. Wiels et al. [22] reported an activation of the Gb3 synthesizing enzyme in BL cells, and similarly, GD_3 expression in human melanoma is due to high levels of GD_3 synthetase [17]. Reduced synthesis of derivative glycolipids may cause the accumulation of Gb3 in testicular neoplasms [14]; glycolipid synthesis of one series may also shift to another series upon tumourigenesis. It was observed that induction of differentiation of human embryonal carcinoma cells led to reduced levels of globo-series glycolipids and increased levels of lacto- and ganglio-series glycolipids [1, 3]. The regulation of the glycolipids-synthesizing enzymes in the process of tumour progression and differentiation requires further investigation at the molecular level.

Glycolipid Gb3 has been used as a tumour marker for BL and Ohyama has suggested Gb3 to be of value for differential diagnosis of seminoma [14, 15]. Although CIS is diagnosed on morphological features, the diagnosis has been increasingly supported by immunohistochemical staining with CIS markers. Gb3 appears to be a new CIS marker, although in comparison with PLAP, it is less suitable for routine use since PLAP is more sensitive, expressed by more than 90% of CIS cases, and is detectable in paraffin-embedded tissues fixed in various types of fixatives that preserve testicular morphology better (such as Stieve's or Bouin's fluids).

We have demonstrated the expression of glycolipid Gb3 in CIS as well as an invasive testicular germ cell tumours. The change in the glycolipid pattern occurs at the preinvasive stage of tumour development and may mark an important step in differentiation of malignant germ cells. Our results further confirm the histogenetic relationship between CIS and overt testicular germ cell tumours.

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