

## Detection of GD3 Ganglioside in Primary Melanomas Depends on Histopathologic Procedures Used for Tumor Preservation

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**SUMMARY** Gangliosides, cell surface glycosphingolipids, are implicated in diverse biologic functions potentially important for tumor growth. Because expression of the GD3 ganglioside may have an impact on the melanoma malignancy, and therefore on the patient prognosis, we evaluated the feasibility of a retrospective immunohistochemical study of GD3 in paraffin embedded biopsies of primary melanomas. Immunoperoxidase staining of frozen and deparaffinized sections of melanoma lesions with two anti-GD3 antibodies was compared using Dako biotin-streptavidin detection kit. Residual ganglioside content was evaluated in the tissues submitted to routine histopathologic procedures using HPLC. A strong and reproducible staining was obtained with both antibodies on frozen sections of all 17 melanoma samples. However, only KM641 antibody could detect GD3 on deparaffinized sections. Biochemical quantification revealed that the Bouin fixative resulted in degradation of GD3. Additionally, most of GD3 was eluted from the tissue samples during dehydration and re-hydration steps. A subgroup of tumors particularly rich in GD3 could be detected on deparaffinized sections after standard formaldehyde fixation. Clinical evolution of such melanomas can now be compared to the group with low GD3 expression. However, any Bouin-fixed, paraffin-embedded biopsies should be excluded from such a retrospective study.

**KEY WORDS:** melanoma, GD3 ganglioside, immunohistochemistry, retrospective study

### INTRODUCTION

Melanoma is a skin cancer showing an increasing incidence. At present, early surgery is the only efficient treatment of this tumor. Determination of

prognostic factors is crucial for classifying patients into homogeneous subgroups allowing development of relevant clinical trials.

Gangliosides are ubiquitous glycosphingolipids expressed on the cell surface. They have been shown to be implicated in diverse biologic functions such as attachment of cells to the extracellular matrix (1), regulation of immune functions (2), regulation of cellular growth and proliferation (3), apoptosis (4), and angiogenesis (5). Therefore, they may play a very important role in tumor growth. Normal melanocytes express gangliosides different from these of malignant melanocytes; in the former, GM3 ganglioside is mainly expressed, whereas GD3 ganglioside is more abundant in melanoma (6).

Some authors have attempted to correlate the degree of malignancy of melanoma to the ganglioside composition but have reached contradictory conclusions, especially concerning the role of GD3 (7-10). The correlation between the GD3 content in primary melanoma lesions and the clinical outcome of patients has never been investigated.

In previous studies, immunohistochemical detection of *in situ* expressed GD3 was always performed on frozen tissue sections (11-13). Such a technique is not compatible with the routine assessment of primary melanomas because of a suboptimal morphological preservation of unfixed frozen biopsies.

The aim of our study was to evaluate the feasibility of immunohistochemical detection of GD3 on paraffin-embedded biopsies. Indeed, the use of the routine histopathology block collections could allow for a retrospective study comparing initial *in situ* expression of GD3 by primary melanoma cells and clinical outcome in these patients.

## MATERIAL AND METHODS

### Tissues

The study was approved by the local Ethics Committee of the Hospices Civils de Lyon. Upon obtaining an informed consent from the patients, frozen biopsies of surgically removed large malignant melanomas were provided by Department of Dermatology, Hôtel-Dieu Hospital, Lyon, France. Sections of paraffin-embedded primary melanomas were also obtained from the same department.

A frozen large metastasis of melanoma to a lymph node was used for ganglioside extractions after various steps of histological processing.

### Monoclonal antibodies (MAb)

Two anti-GD3 monoclonal IgG3 antibodies were used. KM641 MAb (14) was provided by Dr Takao

Taki (Otsuka Pharmaceutical, Co., Tokushima, Japan). C4F6 MAb (15) was produced by Dr. J. Portoukalian in our laboratory. Anti-CD1a (Monosan® clone 66IIC7, Sanbio, Uden, The Netherlands) and anti-PS100 (DakoCytomation, Carpinteria, USA) MAb were used as negative and positive controls of melanocyte detection, respectively.

### Immunohistochemistry (IHC)

Four micrometer-thick frozen sections were stocked at -20 °C after 10-minute fixation in ice-cold acetone (-20 °C). They were defrosted immediately before IHC. For paraffin embedded tissues, IHC was preceded by microwave antigen retrieval: 2x3 minutes at 750 Watts in a citrate buffer (Buffer for Antigen Retrieval, Dako ChemMate, Glostrup, Denmark).

Sections were then rinsed in phosphate buffered saline (PBS; pH 7.2) for 15 minutes. After 5-minute incubation in 3% H<sub>2</sub>O<sub>2</sub> (K0672 Peroxidase block, DakoCytomation) and rinsing in PBS, non specific sites were blocked with an "antibody diluent with background reducing components" (DakoCytomation). A primary antibody was then added for 45 minutes at room temperature. Dilutions were as follows: for KM641, 1/100 for frozen sections and 1/50 for deparaffinized sections; for C4F6, 1/200 for frozen sections and 1/50 for deparaffinized sections; for CD1a, 1/20; and for PS100, 1/400. After washing in PBS, the secondary mouse biotinylated antibody (K0672 Biotinylated Link, DakoCytomation) was added for 15 minutes and rinsed with PBS. Sections were then incubated with streptavidin-peroxidase complex (K0672 HRP, DakoCytomation) for 15 minutes and washed in PBS. Amino-ethyl-carbazole substrate (K0672 AEC Substrate chromogen, DakoCytomation) was added for 15 minutes and after visual control of the developing coloration (red), followed by rinsing in water, the slides were counterstained with hematoxylin and mounted in Glycergel® (DakoCytomation).

### Semi-quantitative evaluation of GD3 content in tumor fragments subjected to various histological procedures

#### Sample preparation

Roughly cubic 100 mg samples were taken from a large frozen lymph node metastasis of melanoma. Some of them were fixed either in Bouin fixative (25 mL of 40% formaldehyde, 75 mL of picric acid in saturated water solution, 5 mL of glacial acetic acid) or in formol-eosin (4% water

solution of formaldehyde, pH 7, colored with eosin; Carlo Erba, Paris, France) for different time periods. Some of the fixed samples were then manually dehydrated, using the same protocol as in our routine dermatopathology laboratory: 1 hour in 70° ethanol, 1 hour in pure ethanol, 5x1 h 30' in pure ethanol, 1 hour and 1 h 30' in methylcyclohexan. Each dehydrated sample was then cut into inframillimetric slices, which were immersed in Roti®-Histol, a xylene substitute used for deparaffinization (Carl Roth, Lauterbourg, France) (2x10 minutes). At last, the samples were rehydrated in graded ethanol baths following the routine dermatopathology laboratory protocol.

Thereafter, semi-quantitative evaluation of the ganglioside content was performed in the samples that had been fixed only, and in the samples that had been fixed, dehydrated and rehydrated. Each series also included a thawed sample that had not been chemically treated. The processed samples were compared to that control.

Ganglioside analysis was performed on all tissue samples and on the solvents used for dehydration and re-hydration (ethanol and methylcyclohexane), except for the Roti®-Histol baths that could not be evaporated.

#### Lipid extraction

Total lipids were extracted from each specimen with a chloroform-methanol (C/M) mixture (1:1). The extract was then filtered and evaporated. The dry lipid extract was dissolved with 6 mL of C/M. Upon the addition of 2 mL of PBS (16) and thorough shaking, centrifugation of the mixture led to separation into two phases. The upper, aqueous phase contained gangliosides.

#### Purification of gangliosides

The gangliosides were recovered from the aqueous phase according to Popa *et al.* (17). The aqueous phase was applied to a copolymer column (Envi-Chrom® P, Supelco-Park, Bellefonte, USA) that had previously been conditioned with a methanol/PBS mixture (1:1). The column was then rinsed with distilled water and gangliosides were eluted with 4 mL of methanol and 4 mL of C/M (1:1). The ganglioside fraction was dried and dissolved in 100 µL of C/M (2:1). Ten µL (for unfixed and fixed tissue samples) or 20 µL (for fixed, dehydrated and rehydrated samples) of extracts were applied to a HPLC plate (Silica Gel 60F254 10x10 cm; Merck, Darmstadt, Germany). The plate was then placed in a glass tank and samples were

migrated in chloroform/methanol/water (55:45:10, v/v/v) solvent system containing 0.22% (w/v) of aqueous CaCl<sub>2</sub>. Visualization was performed with a resorcinol-HCl spray reagent (18) followed by heating at 120 °C.

#### Normal phase HPLC purification of neutral glycolipids

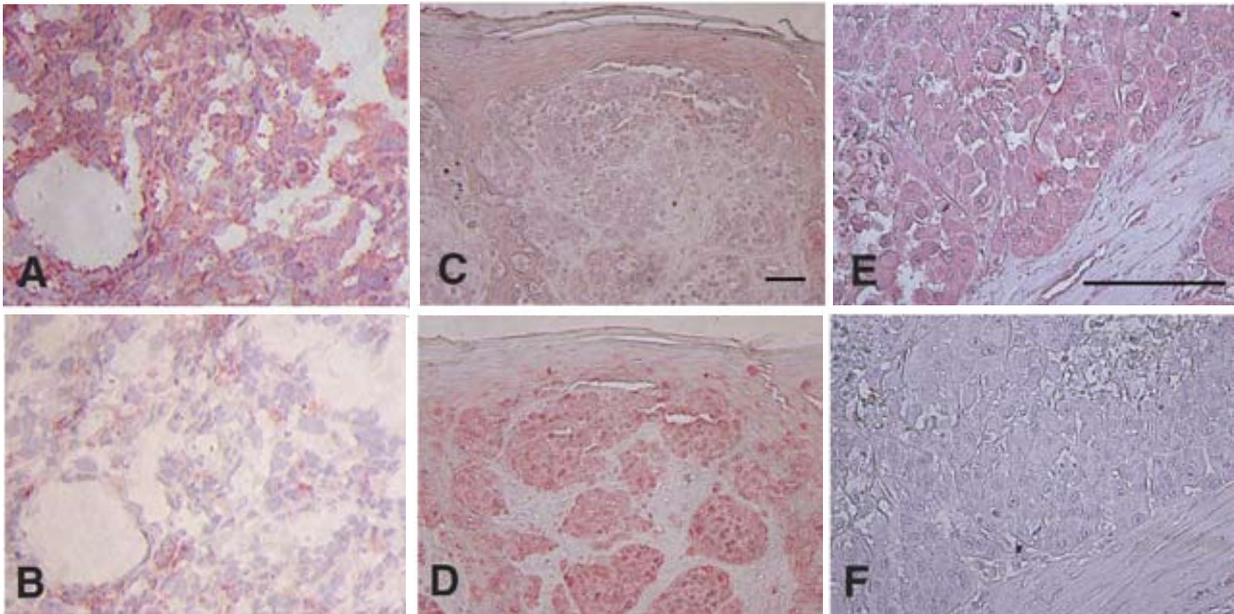
The neutral glycolipids were isolated according to Bodennec *et al.* (19). The lower organic solvent phase was dried, dissolved in 500 µL of chloroform and applied to an aminopropyl-bonded silica gel column Supelclean® LC-NH2 (Supelco, L'Isle d'Abeau, France) that had previously been conditioned with hexane. The column was first eluted with diethylether, then with a C/M mixture (23:1) and finally with a diisopropylether/acetic acid mixture (98:4) to remove the first three lipid fractions. Monohexosyl ceramides and dihexosyl ceramides were then eluted with an acetone/methanol mixture (9:1.2). This fraction was then dried and dissolved in 100 µL of C/M (2:1). Ten µL of the neutral glycolipid extract were then applied to a HPLC plate (Silica Gel 60F254; Merck) and run in chloroform/methanol/water (65:20:3, v/v/v). Visualization was performed with an orcinol-H<sub>2</sub>SO<sub>4</sub> spray reagent and heating at 150 °C.

#### Semi-quantitative evaluation and statistical analysis

The plates were scanned with a photodensitometric scanner (ChromatoScan CSD-930, Shimadzu, Kyoto, Japan). Peak areas of the gangliosides of interest were compared between the test and control samples and the ratio was calculated. The ratios obtained for Bouin fixed tissues and formol-eosin fixed tissues were compared using Student's t-test.

## RESULTS

Reactivity with KM641 antibody to GD3 could be observed on deparaffinized sections of melanomas, although it was less uniform than on the frozen tissue sections (Fig. 1). All frozen sections of melanomas included in the study were reactive with both antibodies directed to GD3. IHC performed on 17 biopsy samples from 13 patients (large primary melanomas and subcutaneous metastases) showed, in all cases, a diffuse cytoplasmic staining of malignant melanocytes with C4F6 and KM641 antibodies (Fig. 1A). Only sparse staining of individual cells was observed with the control anti-CD1a antibody (Fig. 1B). It was



**Figure 1.** Melanoma sections reacted with the KM641 antibody in a manner depending on the tissue fixation procedures. Frozen sections of malignant melanoma lesions were strongly and specifically stained with KM641 MAb, whereas deparaffinized sections showed increased heterogeneity. (A) Frozen section of a melanoma metastasis reactive with KM641 MAb (1/100), X200; (B) the same metastasis (a consecutive frozen section) showing only sparse cell staining with control CD1a MAb (1/20), X200; (C) and (E): two different formol-eosin fixed primary melanomas showing variable degrees of staining with KM641 MAb (1/50; deparaffinized sections); (D) the KM641-negative tumor was readily stained with anti-PS100 antibody (1/400; positive control on a section consecutive to C; X100); and (F) negative control with CD1a MAb (1/20; a section consecutive to E; X200).

Bars = 100  $\mu$ m; The bar in C is also valid for D; and that in E is also valid for A, B, F.

difficult to estimate relative variations in the intensity of GD3 staining within a given tumor because of the presence of GD3 reactivity not only in the melanoma cells but often also in the neighboring epithelial or connective tissues.

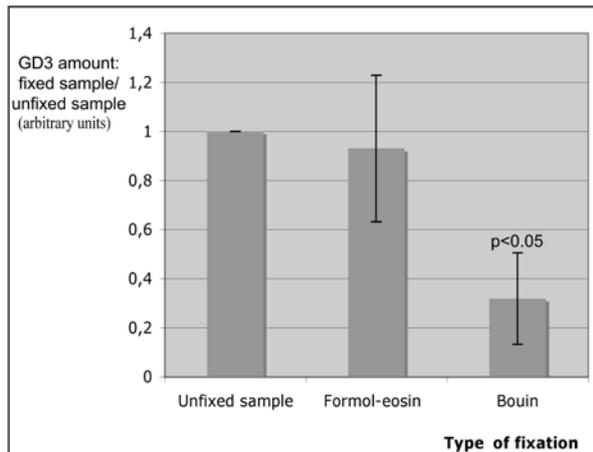
Specific staining of GD3 was less frequently observed in the deparaffinized tissues. Ten of the paraffin embedded tissues were fixed with formol-eosin. The staining with KM641 antibody was absent in 6 (Fig. 1C), weak in 2, and strong in 2 cases (Fig. 1E). Biopsies of melanoma fixed in Bouin were only occasionally reactive with KM641 antibody (not shown). No staining was observed with C4F6 MAb, whatever the fixative used.

Most of the GD3 was either hydrolyzed or eluted during routine histopathologic preparation. The quantity of samples used in this study was limited because ganglioside extractions require large amounts of tissue. The pooled results of three separate GD3 extractions from 3 distinct samples of melanoma tumors are shown in Figure 2. There were only slight variations in the measured GD3 content when extractions were repeated in the

same conditions. When the tumor samples were fixed in formol-eosin, there was only a slight decrease in the GD3 content, whereas Bouin fixation induced a significant decrease. After 88 hours of fixation, the difference in GD3 content between Bouin fixed (3 samples; mean residual quantity = 32% of the initial value) and formol-eosin fixed samples (3 samples; mean residual quantity = 93% of the initial value) was statistically significant ( $p < 0.05$ ).

When the samples were fixed for 12 hours or 88 hours in Bouin, the GM3/GD3 ratio and the lactosyl-ceramide content increased with the duration of fixation, indicating conversion of GD3 into its precursor forms. Such a phenomenon was not observed when the samples were fixed in formol-eosin (not shown).

The results of biochemical analysis of the fixed and solvent-treated samples are summarized in Figure 3. When the fixative was Bouin, practically no GD3 remained in the tissue. When the fixative was formol-eosin, the residual GD3 content was  $25.5 \pm 5.5\%$  after 12 hours of fixation and



**Figure 2.** GD3 extraction from malignant melanoma tumors yielded various quantitative results after the initial tissue fixation step. Three distinct samples were extracted before and after 88-hour fixation with formol-eosin or Bouin; the amounts of glycolipids quantified with thin layer chromatography were expressed as a ratio of GD3 in the fixed over the unfixed sample (mean residual fraction  $\pm$  SD). Fixation in Bouin induced a significant loss of GD3 ( $p < 0.05$ ).

13.5 $\pm$ 1.5% after 88 hours of fixation ( 2 samples; mean  $\pm$  standard deviation), when compared to the unfixed, untreated samples. The number of processed samples was too low to draw a conclusion concerning statistical significance of the difference observed. GD3 was found in the solvents used for dehydration and re-hydration of the tumor tissue. More GD3 was found in the pooled solvent baths when the fixative was formol-eosin compared to Bouin (not shown). We concluded that most of the GD3 was extracted from the tumor samples during the phases of dehydration and re-hydration.

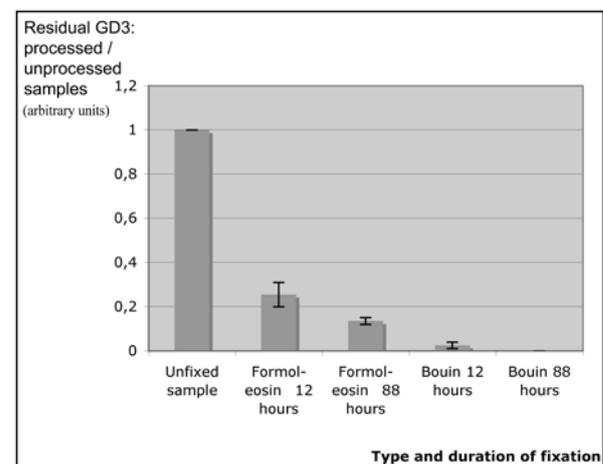
## DISCUSSION

Gangliosides are part of the lipids constituting cell membrane “rafts”, i.e. important platforms of cell signaling involved in the regulation of cell behavior. In melanoma, as in other situations, the composition of the rafts may apparently influence cell function, and thus constitutes important information from the practical point of view (20). Also, gangliosides released from the surface of melanoma cells are able to modulate the function or even induce apoptosis of other cell types, e.g., immunocompetent dendritic cells (21). The authors of previously published studies have often drawn conflicting conclusions regarding the correlation

between GD3 expression and the degree of melanoma malignancy. Results of a previous study conducted in our laboratory (9) have suggested that primary tumors with a low expression of GD3 are more prone to give metastasis than tumors with a high GD3 expression. Also, analysis of gangliosides expressed on melanoma cells issued from primary tumors and their metastases indicated that GD1a may be a better marker of metastatic phenotype than GD3 (22). On the other hand, two other groups (7,8) have found more GD3 in most aggressive tumors. In neuroblastoma, Zeng *et al.* demonstrated a decrease in GD3 content to be associated with a reduction of angiogenesis, which resulted in slower tumor growth (23). Accordingly, other authors have linked malignant properties of melanoma to GD3-dependent signaling through the integrin receptor junctions (24).

The relationship between the GD3 content in primary melanomas and the clinical outcome of the disease in humans has never been investigated and remains an important unsolved issue.

Previous studies aiming at detecting GD3 in melanocytes by IHC were always performed on frozen sections. Dippold *et al.* compared the stain-



**Figure 3.** Further loss of GD3 from the samples was observed after the treatment with organic solvents during dehydration and re-hydration steps. Residual GD3 in the samples was expressed as a ratio between the values obtained before and after histologic processing (mean residual fraction  $\pm$  SD). Bouin fixation for 88 hours followed by solvent baths resulted in complete depletion of gangliosides from the tissues. The impact of solvents on formol-eosin fixed tissues was less pronounced, although only 25.5% of the initial GD3 values were still present in the samples fixed for 12 hours.

ing with R24 anti-GD3 antibody on frozen sections of 21 primary melanomas and 37 metastases (11). They found strong staining of more than 60% of malignant melanocytes in all of the primary tumors; in most cases, the staining exceeded 80% of cells. GD3 expression in metastases was also strong but more heterogeneous. Hersey *et al.* also found strong staining of malignant cells with the same antibody (12). In both studies, there was a tendency towards a lower expression of GD3 in metastasis, although it was not statistically significant.

Our results of GD3 detection on frozen sections of melanoma samples obtained with different primary antibodies are in agreement with previous reports (11-13).

The staining intensity obtained on frozen sections was difficult to interpret because of the reactivity of adjacent tissues. The presence of GD3 labeling on cells and stromal fragments surrounding tumor masses could be due to diffusion and passive transfer of the ganglioside molecules from melanoma. However, staining of other normal and tumoral tissues, and specifically of the normal human skin, has previously been reported (25) and could result from *in situ* ganglioside production under the influence of local or distant tumor-derived stimuli.

In conclusion, evaluation of the level of GD3 expression with IHC on frozen sections appears to be difficult because nearly all tumors are intensely stained and labeling of the infiltrated and adjacent tissues represents a confusion factor. Additionally, only routine chemical fixation guarantees the reliable histopathologic diagnosis of primary melanomas, so frozen sections of small primary lesions are not available for ethical reasons.

The principal goal of our study was, therefore, to assess the feasibility of GD3 IHC on deparaffinized sections, in order to perform a retrospective study on archived blocks of melanoma samples. Indeed, we found that such staining could be observed on some deparaffinized sections but the frequency and intensity of GD3 detection was clearly much lower than on frozen samples from the same tumors. We could further verify that staining corresponded to a detectable content of GD3 in the study tissues and could check the impact of the histologic preparation process on this content.

Schwarz and Futerman demonstrated that fixation of cultured neurons in formaldehyde decreased their GD3 detection by 10% (26). Suzuki

found the ganglioside content to have dramatically decreased in brains that had been fixed for several years in formaldehyde (27). Significant variations observed from the sixth week of fixation on could have been induced by increasing acidification of formaldehyde resulting in hydrolysis of sialic acids. However, no significant variations were observed when fixation lasted for up to 10 days. Our data showed the fixation step in Bouin fixative to induce a significant decrease in GD3 content compared to the frozen tissue or formaldehyde-fixed tissue. Concomitantly, there was a relative increase in GM3 and lactosyl-ceramide, which are precursors of GD3. According to the data reported by Suzuki (27), it is likely that the acidity of Bouin fixative, that contains picric acid, causes hydrolysis of both sialic acids on GD3, thus converting these gangliosides into precursor forms. Tissues fixed that way are therefore not fit for studies on GD3 expression. On the other hand, formol-eosin with its neutral pH did not cause any significant variation of GD3 during a few days of fixation and proved compatible with such studies.

During the second step of the procedure, organic solvents partly extracted GD3 from the tissue sample, as shown by the results of biochemical analysis of the ethanol and methyl cyclohexane baths. It is logical that fewer GD3 was extracted when the fixative was Bouin since residual GD3 content was already lower in that sample after the fixation step.

Following the complete procedure, practically no residual GD3 remained in the Bouin-fixed sample; only a very small amount was detected when the fixation was very short (less than 12 hours). It may explain that some of our Bouin fixed tumors could still be stained with the KM641 antibody. We conclude that detection of GD3 ganglioside on deparaffinized sections of routinely Bouin-fixed melanoma is not reliable and retrospective studies on such tissues should not be undertaken.

When the fixative was formol-eosin, much GD3 was extracted during the dehydration, deparaffinization and re-hydration steps. Nevertheless, there still remained a detectable residual GD3 in the sample, amounting to approximately 13%-25% of the initial content, depending on the duration of fixation. We cannot exclude further variations due to the formol fixation should this step exceed 88 hours evaluated in this study; however, such variations appear to be limited compared to the *in vivo* variability of ganglioside expression. Ravindranath *et al.* (7) observed variations of GD3 comprised between 3.24 and 173 µg/g of dry tissue in their

series of lymph node metastases (28 samples; mean  $\pm$  SD = 49.2 $\pm$ 40  $\mu$ g/g). In another series of cutaneous metastatic melanomas (8), the extreme values were 7 and 907  $\mu$ g/g of dry tissue (19 samples; mean  $\pm$  SD = 261 $\pm$ 260  $\mu$ g/g). Therefore, it is likely that the intensity of anti-GD3 staining of our formol-eosin fixed samples corresponded to the real residual content in GD3 depending on the initial values expressed in the studied tumors.

Thus, the tumors that strongly express GD3 may be identified by this technique. A retrospective study on formaldehyde fixed tumors can therefore be proposed in order to evaluate the clinical outcome of patients with primary melanomas differing in GD3 expression. This should help define the prognostic value of the GD3 marker in the newly occurring melanomas.

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**DAME KOJE UPOTREBLJAVAJU TAKY  
SIGURNE SU ZA SVOJE ČARI**

Svaka dama, ako hoće da bude privlačiva, znade, da ne smije imati na rukama, nogama i zatiljku ni dlaka ni malja. Međutim britva pušta crne točkice, izazivlje bubuljice i čini da dlaka brzo raste tako, da je naskoro potrebno dnevno brijanje. Ostala sredstva bez obzira na to, što su komplicirana i što neugodno mirišu, prouzrokuju često crvenilo.

Sa Taky-em, parfimiranom kremom, koja se upotrebljava onako kako izlazi iz tube, možete se za 5 minuta, a da se ne morate prije pripremiti u Vašoj toaletnoj sobi ili u kabini za kupanje osloboditi svih suvišnih dlaka, gdje god one bile. Taky uništava dlaku do korijena i čini, da raste vrlo lagano tako da vrlo često sasvim nestane. Taky je svakoj elegantnoj dami, osobito ljeti, neophodno potreban.

Dobiva se u svim drogerijama, parfumerijama i ljekarnama.

**ZASTUPSTVO I GLAVNI DEPOT ZA JUGOSLAVIJU: POŠT. PRETINAC 310.**

Ladies using Taky are sure for their beauty; year 1934.  
(From the collection of Mr. Zlatko Puntijar)