厚生労働科学研究委託費

革新的がん医療実用化研究事業

未治療原発不明癌に対する次世代シークエンスを用いた原発巣推定に基づく治療効果の意義を問う第Ⅱ相試験

平成２６年度　委託業務成果報告書

業務主任者　中川　和彦

平成２７（２０１５）年　３月

様式第１８

委託業務成果報告書への標記について

委託業務に係る成果報告書の表紙裏に、次の標記を行うものとする。

本報告書は、厚生労働省の厚生労働科学研究委託事業による委託業務として、学校法人近畿大学　理事長　清水由洋が実施した平成２６年度「未治療原発不明癌に対する次世代シークエンスを用いた原発巣推定に基づく治療効果の意義を問う第Ⅱ相試験」の成果を取りまとめたものです。

別添１

厚生労働科学研究委託費委託業務成果報告書表紙

別添２

厚生労働科学研究委託費委託業務成果報告書目次

別添３

厚生労働科学研究委託費委託業務成果報告（総括）

別紙１

厚生労働科学研究委託費

革新的がん医療実用化研究研究事業

未治療原発不明癌に対する次世代シークエンスを用いた原発巣推定に基づく治療効果の意義を問う第Ⅱ相試験

平成２６年度　委託業務成果報告書

業務主任者　中川　和彦

平成２７（２０１５）年　３月

別紙２

委託業務成果報告書目次

目　　次

別紙３

厚生労働科学研究委託費（革新的がん医療実用化研究事業）
委託業務成果報告（総括）

未治療原発不明癌に対する次世代シークエンスを用いた原発巣推定に基づく治療効果の意義を問う第II相試験

業務主任者　中川　和彦
近畿大学医学部内科学腫瘍内科部門　教授

研究要旨　原発不明がん(Cancer of Unknown Primary: CUP)を対象とした次世代シークエンシング技術（Next generation sequenicing: NGS)を用いた遺伝子発現解析により原発巣の推定を行う新しい治療戦略を臨床第II相試験にて評価する。同時にNGSを用いて特定のがん種において既に使用されている遺伝子変異/増幅を解析し、特定の分子を標的とした分子標的治療薬がCUPの治療戦略に応用可能であるかを評価する。CUPにおける遺伝子発現プロファイルを用い、より精度の高いCUP診断薬およびCUP特異的分子標的薬の創生を目指す。

倉田　宝保　（関西医科大学内科学第一講座　教授）
鶴谷　純司　（近畿大学医学部内科学腫瘍内科部門　准教授）
西尾　和人　（近畿大学医学部ゲノム生物学教室　教授）
藤田　至彦　（近畿大学医学部ゲノム生物学教室　講師）
冨田　秀太　（近畿大学医学部ゲノム生物学教室　講師）
竹内　文乃　（慶應義塾大学医学部　助教）
掛谷　秀明　（京都大学大学院・薬学研究科　教授）

Ａ．研究目的

原発不明がん(Cancer of Unknown Primary: CUP)を対象とした次世代シークエンシング技術（Next generation sequenicing: NGS)を用いた遺伝子発現解析により原発巣の推定を行う新しい治療戦略を臨床第II相試験にて評価する。同時にNGSを用いて特定のがん種において既に使用されている遺伝子変異/増幅を解析し、特定の分子を標的とした分子標的治療薬がCUPの治療戦略に応用可能であるかを評価する。CUPにおける遺伝子発現プロファイルを用い、より精度の高いCUP診断薬およびCUP特異的分子標的薬の創生を目指す。

Ｂ．研究方法

研究期間は4年（臨床試験計画0.5年、登録2年、追跡1年、結果解析0.5年）とする。

（概要）

1. 対象となりうる患者に対して、適格性確認、同意説明を行い、同意が得られればデータセンターに登録、同時に採取済みのFFPE組織検体を近畿大学ゲノム生物学教室に送付する。

2. A. 抽出したRNAから得られた遺伝子発現データをもとに原発巣の推定を行う。具体的には、CUPSignatureの発現データは正規化処理後、発現量に基づいて算出された重みが加算された各癌腫の得点（Weighted-voting）が算出される。もっとも高い得点を示した臓器(18癌種)が推定原発部位として診断される。B. DNAの解析から治療方針に重大な影響を与える癌種特異的な遺伝子変異および遺伝子増幅を検索する。具体的には肺癌における活性型EGFR遺伝子変異、GISTにおける活性型c-KIT遺伝子変異、乳癌、胃癌におけるHER2遺伝子の増幅および大腸癌におけるRAS遺伝子変異を検索する。これらの遺伝子変異・増幅情報をAのアルゴリズムにおける癌種推定に組み込み、また主治医にその情報を提示する。これらの遺伝子変異、増幅が存在したときにはそれぞれEGFR遺伝子変異陽性肺癌（ゲフィチニブ、エルロチニブ、アファチニブのいずれかによる治療）、消化管間質腫瘍（イマチニブによる治療）、HER陽性乳癌もしくは胃癌（トラスツズマブの併用治療）と推定し保険診療に応じた治療を行う事を許容する。

3. 2によって推定された癌種に対する、もしくは遺伝子変異に対する治療を各参加施設にて施行する。

4. 目標症例数は110例、登録期間2年、追跡期間1年とし、主要評価項目は試験参加から1年後の生存率とする。追跡期間終了時にデータセンターは最終解析を行い、「最終解析報告書」を作成する。試験期間中はWJOGデータセンターにてモニタリング、監査などを行い、また、有害事象情報等を適宜参加施設に知らせる。これは前述の中川班によるCUPを対象とした前臨床試験と同様の体制であり、既に実績を有する。

5. 本試験で得られた遺伝子発現や変異プロフィールを用いた付随研究として、CUP特異的分子標的薬の早期探索研究を実施する。我々は高転移性細胞株をもちい、マウス足底投与による膝下リンパ節への転移能を評価する方法を見出した（平成２５年度がん臨床研究成果発表）。現在、CUP特異的遺伝子群のうち、我々はMIFに対する阻害薬としてレスベラトロール誘導体の有効性を示しており（Invest New Drugs

30:1878, 2012）、さらにdruggableな標的分子については、掛谷らが新規阻害剤を創製する予定である。これらの化合物のCUPに対する効果を含むPOCを上記in vivo転移モデルにて評価し、最適化を計ってゆく。
6. 全体責任者を近畿大学医学部腫瘍内科 中川和彦、研究事務局として市立岸和田市民病院 林秀敏を、また遺伝子発現解析責任者として近畿大学医学部ゲノム生物学　西尾和人、臨床・生物統計解析責任者を国立環境研究所　竹内文乃とする。
参加施設代表者は研究協力者とした。

（倫理面への配慮）
本研究では、抗癌剤感受性の高い予後良好な原発不明がん患者が本研究から最大限除外されるよう配慮する。さらに、ヘルシンキ宣言およびわが国の「臨床研究に関する倫理指針」に従い、以下の事項を厳守する。
①研究実施計画書をWJOGプロトコール審査委員会で審査し、各施設のIRB承認の得られた施設のみ症例登録を可能とする。
②全ての患者に説明文書を用いて十分な説明を行い、考慮の時間を設けた後に患者自身の自由意志による同意を文書で取得する。
③データの取り扱いに関して、直接個人を識別できる情報を用いず、データベースのセキュリティを確保し、個人情報の保護を厳守する。
④プロトコール審査委員会、効果・安全性評価委員会を組織し、研究の第三者的監視を行う。
解析でおこなうマイクロアレイによる遺伝子発現解析はヒトゲノム・遺伝子解析研究に関する倫理指針の対象ではないが、指針の趣旨を尊重し、準じた管理を行うことにより個人情報等倫理的に十分に配慮する。

C．研究結果
本年度は原発推定アルゴリズムを作成、Validationを行い確定した。
試験の遂行に際しスタートアップミーティングを行い、近畿大学医学部付属病院における倫理委員会で承認され試験が開始、第1例目が登録された。

D．考察
臨床試験が開始された。進捗状況としては予定通り遂行されている。18施設が本試験に参加し、2015～2016年度にかけて110例の登録が予定されている。

E．結論
NGSを用いたCUPの原発巣推定の治療戦略を策定した。臨床試験が開始された。

F．研究発表

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G．知的財産権の出願・登録状況

1. 特許取得　なし
2. 実用新案登録　なし
3. その他　　なし

様式第１９

学 会 等 発 表 実 績

委託業務題目「未治療原発不明癌に対する次世代シークエンスを用いた原発巣推定に基づく治療効果の意義を問う第Ⅱ相試験
機関名　学校法人　近畿大学

１．学会等における口頭・ポスター発表

発表した成果（発表題目、口頭・ポスター発表の別）	発表者氏名	発表した場所（学会等名）	発表した時期	国内・外の別
A microarray-based gene expression analysis identified diagnostic biomarkers for unknown primary cancer.	Y Fujita	2014年米国癌学会議（AACR）	2014年4月	国外
創薬シーズ開発を指向した天然物ケミカルバイオロジー -表現型スクリーニングと標的探索・同定-（招待講演）	掛谷秀昭	第20回天然薬物の開発と応用シンポジウム	2014年11月	国内
Chemical Biology for modulators targeting cancer microenvironment and cell membrane signaling （ポスター）	Kakeya, H., Yoshimura, A., Sugiyama, R., Kishimoto, S., Nishimura, S., Hattori, A., Ishikawa, F., Yomoda, Y., Lu, S., Katagiri, N., Shimada, S., Seto, E., Hayashi, H., Takahashi, N., Harada	Natural Products 2015; Natural product discovery & development in the post genomic era	2015年1月	国外

２．学会誌・雑誌等における論文掲載

掲載した論文（発表題目）	発表者氏名	発表した場所（学会誌・雑誌等名）	発表した時期	国内・外の別
Melanoma transition is frequently accompanied by a loss of cytoglobin expression in melanocytes: a novel expression site of cytoglobin.	Fujita Y, Koinuma S, Develasco MA, Bolz J, Togashi Y, Terashima M, Hayashi H, Matsuo T, Nishio K.	PLOS ONE	2014年4月	国外
Biomarkers of reactive resistance and early disease progression during chemotherapy plus bevacizumab treatment for colorectal carcinoma.	Hayashi H, Arao T, Matsumoto K, Kimura H, Togashi Y, Hirashima Y, Horita Y, Iwasa S, Okita NT, Honma Y, Takashima A, Kato K, Hamaguchi T, Shimada Y, Nakagawa K, Nishio K, Yamada Y.	Oncotarget	2014年5月	国外

位置情報を用いた疫学研究とその統計的方法	高橋 邦彦、和泉 志津恵、竹内 文乃	統計数理	2014年7月	国内
観察研究におけるバイアスの感度解析	竹内 文乃、野間 久史	統計数理	2014年7月	国内
Erlotinib alone or with bevacizumab as first-line therapy in patients with advanced non-squamous non-small-cell lung cancer harbouring EGFR mutations (JO25567): an open-label, randomised, multicentre, phase 2 study.	Seto T, Kato T, Nishio M, Goto K, Atagi S, Hosomi Y, Yamamoto N, Hida T, Maemondo M, Nakagawa K, Nagase S, Okamoto I, Yamanaka T, Tajima K, Harada R, Fukuoka M, Yamamoto N.	Lancet Oncol	2014月8月	国外
Development of On-Chip Multi-Imaging Flow Cytometry for Identification of Imaging Biomarkers of Clustered Circulating Tumor Cells.	Kim H, Terazono H, Nakamura Y, Sakai K, Hattori A, Odaka M, Girault MA, Arao T, Nishio K, Miyagi K, Yasuda K	PLOS ONE	2014年8月	国外
Tyrosine phosphoproteomics identifies both codrivers and cotargeting strategies for T790M-related EGFR-TKI resistance in non-small cell lung cancer.	Yoshida T, Zhang G, Smith MA, Lopez AS, Bai Y, Li J, Fang B, Koomen J, Rawal B, Fisher KJ, Chen AY, Kitano M, Morita Y, Yamaguchi H, Shibata K, Okabe T, Okamoto I, Nakagawa K, Haura EB.	Clin Cancer Res	2014年8月	国外
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Melanoma Transition Is Frequently Accompanied by a Loss of Cytoglobin Expression in Melanocytes: A Novel Expression Site of Cytoglobin

Yoshihiko Fujita[1]*, Satoshi Koinuma[2], Marco A. De Velasco[1], Jan Bolz[1], Yosuke Togashi[1], Masato Terashima[1], Hidetoshi Hayashi[1], Takuya Matsuo[2], Kazuto Nishio[1]

1 Department of Genome Biology, Faculty of Medicine, Kinki University, Osaka-Sayama, Osaka, Japan, **2** Department of Anatomy and Neurobiology, Faculty of Medicine, Kinki University, Osaka-Sayama, Osaka, Japan

Abstract

The tissue distribution and function of hemoglobin or myoglobin are well known; however, a newly found cytoglobin (CYGB), which also belongs to the globin family, remains to be characterized. To assess its expression in human malignancies, we sought to screen a number of cell lines originated from many tissues using northern blotting and real time PCR techniques. Unexpectedly, we found that several, but not all, melanoma cell lines expressed CYGB mRNA and protein at much higher levels than cells of other origins. Melanocytes, the primary origin of melanoma, also expressed CYGB at a high level. To verify these observations, immunostaining and immunoblotting using anti-CYGB antibody were also performed. Bisulfite-modified genomic sequencing revealed that several melanoma cell lines that abrogated CYGB expression were found to be epigenetically regulated by hypermethylation in the promoter region of *CYGB* gene. The RNA interference-mediated knockdown of the CYGB transcript in CYGB expression-positive melanoma cell lines resulted in increased proliferation *in vitro* and *in vivo*. Flow cytometric analysis using 2′-, 7′-dichlorofluorescein diacetate (DCFH-DA), an indicator of reactive oxygen species (ROS), revealed that the cellular ROS level may be involved in the proliferative effect of CYGB. Thus, CYGB appears to play a tumor suppressive role as a ROS regulator, and its epigenetic silencing, as observed in CYGB expression-negative melanoma cell lines, might function as an alternative pathway in the melanocyte-to-melanoma transition.

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* E-mail: fujita@med.kindai.ac.jp

Introduction

Hemoglobin and myoglobin are among the best studied and understood of all proteins. These globins are known to be capable of transporting and storing oxygen, thereby sustaining oxidative metabolism in cells. Cytoglobin (CYGB), a new member of the globin family that was identified together with neuroglobin (NGB), is a hexa-coordinated heme protein [1,2]. Although CYGB is known to exhibit a high intrinsic affinity to oxygen, similar to myoglobin, its physiological function remains to be clarified [3]. CYGB was originally characterized as a 21-kDa heme protein with an enhanced expression level in stellate cells in fibrotic liver and was initially named "stellate cell activation-associated protein" [4]. A role in the cellular response to tissue fibrosis has been suggested by a study in which the overexpression of CYGB provided protection against chemically induced liver fibrosis [5]. A potential role in reactive oxygen species (ROS) detoxification has also been suggested [6–8]. A human neuroblastoma cell line transfected with a plasmid DNA containing CYGB cDNA showed enhanced survival after exposure to H_2O_2 [6] and significant protection from oxidative DNA damage induced by a singlet oxygen generator [7]. Furthermore, CYGB has been shown to protect rat kidney fibroblasts against oxidative stress under ischemic conditions *in vivo* [8]. However, most functional analyses of CYGB, including the above-mentioned characterizations, have so far been performed using cells with ectopically expressed CYGB.

Cells endogenously enriched in CYGB, if found, would facilitate the functional characterization of CYGB at an endogenous level, rather than an ectopically induced level that could result in an overestimation of function. The distribution of CYGB in normal tissues has been analyzed in detail. In some studies, CYGB appears to be ubiquitously expressed in whole tissue [1,9], while the other studies have revealed some cell-types that specifically express CYGB [4,10]. Compared to normal tissues, tumor tissues or cell lines have not been extensively investigated for the presence of CYGB [11]. This lack of study can be partly explained by the absence of the chromosome region 17q25 (which contains the *CYGB* gene) in multiple malignancies [12]. The transcriptional inactivation of promoters of *CYGB* by DNA hypermethylation has also been shown in lung, esophageal and head and neck cancers [13–15]. Such transcriptional suppressions, which are frequently observed in many cancer types, suggest that *CYGB* might function as a tumor suppressor gene, making it difficult to discover cancer

cell types overexpressing CYGB. Nevertheless, 1) based on the assumption that, similar to other globins, CYGB could have a specific function in limited tissues or cell types, and 2) according to our initial aim to assess the correlation between CYGB loss and the resultant tumor malignancy, we sought to perform an extensive screening for CYGB expression in several cancer cell lines and found that several, but not all, melanoma cell lines highly expressed CYGB.

Results

CYGB Is Expressed at High Levels in Some Melanoma Cell Lines

To explore the possibility of whether some types of cancer are enriched in CYGB, we screened for CYGB using several cancer cell lines of diverse origins (Table S1). To our surprise, a TaqMan probe-based real-time quantitative PCR revealed that 3 melanoma cell lines (G361, p22, and C32TG) expressed *CYGB* mRNA several hundred-fold more abundantly than the other cell lines that were tested (Fig. 1A). In 2 melanoma cell lines (A375, MEWO), on the other hand, *CYGB* mRNA was detected at much lower levels. To confirm the expression discrepancy among the cell lines, we subjected RNA preparations from each cell to a northern blot analysis (Fig. S1A). The results were in good accordance with those of quantitative PCR analysis, with *CYGB* mRNA being abundantly expressed in the G361, p22, and C32TG cell lines but not detected in the A375 and MEWO (melanomas), A549 (lung cancer), and T47D (breast cancer) cell lines (Fig. S1B). Hypoxic (1% O_2) or anoxic (0.1%–0.2% O_2) conditions can significantly up-regulate *CYGB* mRNA in several cell lines as previously reported [11]. Among the non-melanoma cells, T98G cells (glioblastoma) alone produced a slight mRNA signal in response to anoxia for 6 hours (Fig. S1). We next searched a publicly available database for gene expression profiles. The Gene Expression Omnibus database (GEO, http://www.ncbi.nlm.nih.gov/geo) provided microarray datasets for various cancer cell lines. The relative amounts of *CYGB* mRNA calculated for the representative cell lines, including 15 melanoma cell lines, are listed in Table S2. Of these cell lines, a high amount of *CYGB* mRNA was expressed exclusively in melanoma cells, including G361 and C32. As expected, some melanoma cells including A375, SKMEL28 and HS294T formed a group that expressed *CYGB* mRNA levels that were as low as those of non-melanoma cell lines (Tables S2 and S3).

CYGB Is Overexpressed in Melanocytes

The unexpected identification of CYGB in melanoma cells prompted us to examine the presence of CYGB in melanocytes, the precursor of melanoma cells. A real-time quantitative PCR assay showed that the expression level of *CYGB* mRNA in melanocytes surpassed those observed in skin and various other normal tissues (Fig. 1B), revealing melanocyte as a prominent cell type that overexpressed CYGB. The level of protein expression in melanocytes was comparable to, or even higher than, the four CYGB expression-positive melanoma cell lines (Fig. 2A). The expression of CYGB in keratinocytes, the main cell type in the epidermis, as well as normal human dermal fibroblasts (NHDF) was only detectable in immunoblot with an increased exposure time (Fig. S2A), suggesting the predominant distribution of CYGB in melanocytes within the skin. Paraffin-embedded sections of normal human skin were then used to examine CYGB expression. Immunoreactivity using an antibody against CYGB showed the same localization at the epidermal basement membrane as that for PNL2 protein, which is often used as a marker for melanocytes,

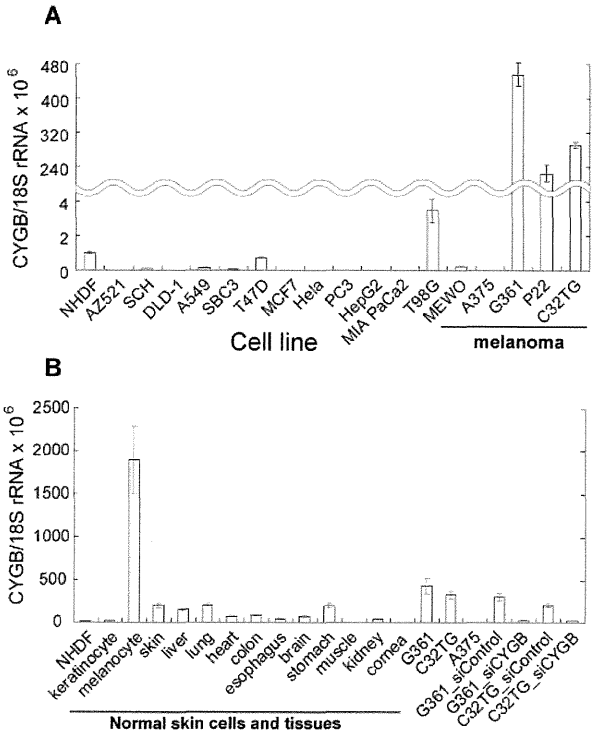

Figure 1. Some melanoma cells and melanocytes express high level of CYGB. Realtime quantitative PCR was performed using a TaqMan probe to detect the *CYGB* mRNA level in **(A)** different human cancer cell lines shown in Table S1 including 5 melanoma cells (A375, Mewo, G361, P22, C32TG) and **(B)** 3 normal cells in skin (NHDF, keratinocytes, melanocytes) and 11 normal tissues (skin to cornea) together with 2 melanoma cell lines (G361, C32TG) and their transfectants with siRNAs (si_Control and si_CYGB). In both **(A)** and **(B)**, the expression of the CYGB transcript was normalized by each 18S rRNA level (n = 3, mean ± SEM) and the normalized CYGB/18S rRNA expression ratio for normal human dermal fibroblasts (NHDF) was set equal to 10^{-6}.
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demonstrating that CYGB is highly enriched in melanocytes (Fig. 2B).

The intracellular localization of CYGB has been estimated using immunohistochemistry at the tissue level [4,10,16] or using a fluorescent signal from GFP-fused CYGB forcibly expressed in cells [7,10]. These studies have revealed that CYGB is localized in the cytoplasm of fibroblasts and their derivatives [10], while it is also detected in the nucleus in neurons, various epithelial cells, hepatocytes and connective tissue cells [16,17]. Melanocytes or G361 cells that endogenously express high amounts of CYGB enabled direct immunostaining for localization (Fig. S2B). These cells expressed CYGB in both the cytoplasm and nuclei, but the expression was rather concentrated in the nuclei of the G361 cells, while A375 cells gave only weak signals.

Epigenetic Silencing of *CYGB* Gene Occurs in Some Melanoma Cell Lines

We searched the database (GSE29359) to determine whether fluctuations in cytoglobin expression levels, as observed between the G361 and A375 cell lines, are common among melanoma patients (Table S4 and Fig. S3). A relatively high expression level of *CYGB* mRNA was apparent in 7 out of 8 normal melanocyte

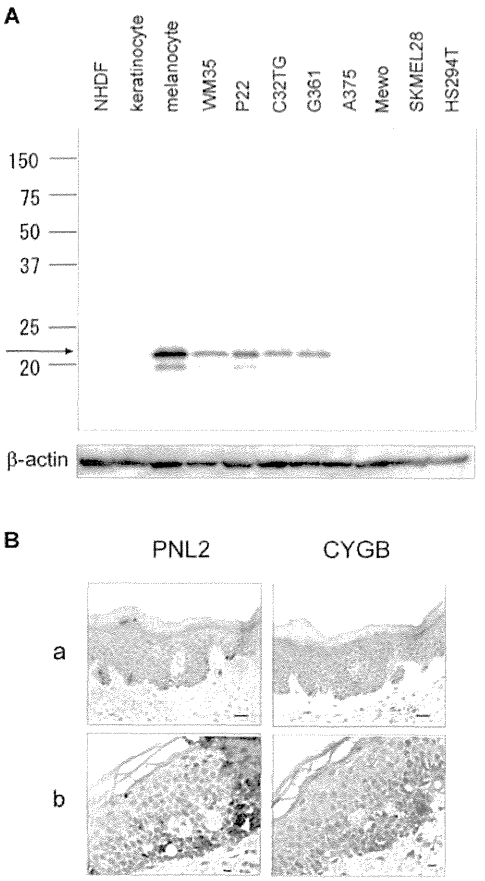

Figure 2. CYGB protein is overexpressed in melanocytes and some of its malignant offspring. (A) Immunoblot analysis of CYGB protein (indicated by an arrow) in NHDF, keratinocytes and melanocytes from skin and 8 melanoma cell lines (WM35 to HS294T). The image was obtained using ImageQuant LAS 3000 with an exposure time of 15 sec. The minor band, possibly a degradation product, is observed below the major band, which is prominent in melanocytes. The molecular mass marker (kDa) is given on the left side. β-actin was used as a loading control. **(B)** Immunohistochemical analysis of formalin-fixed, paraffin-embedded human normal skin using PNL2 (melanocyte marker) and CYGB antibodies. Two different regions (a) and (b) stained using each antibody are shown: (a) 4 × magnification, scale bar = 100 μm. (b) 20× magnification, scale bar = 10 μm.
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cell lines, but only 14 melanoma tumor tissues out of specimens from 79 melanoma patients (17.7%) reached the same level. These results suggest that most melanomas lose their CYGB expression during the melanocyte-to-melanoma transition.

Recent methylation-specific PCR assays have provided evidence of higher levels of *CYGB* promoter methylation in lung and esophageal tumors compared with adjacent nonmalignant tissues [13]. We sequenced the promoter region of the *CYGB* gene from melanocytes, G361 cells and A375 cells after bisulfite-modification followed by PCR. Twenty-four CpG sites are known to reside within the *CYGB* promoter region [18], of which 9 are shown in Fig. 3. The results revealed that all 9 CpG sites were methylated in A375 but were totally unmethylated in melanocytes and G361, demonstrating that the transcriptional inactivation of the promoter by DNA hypermethylation occurred in A375 (Fig. 3) and in other

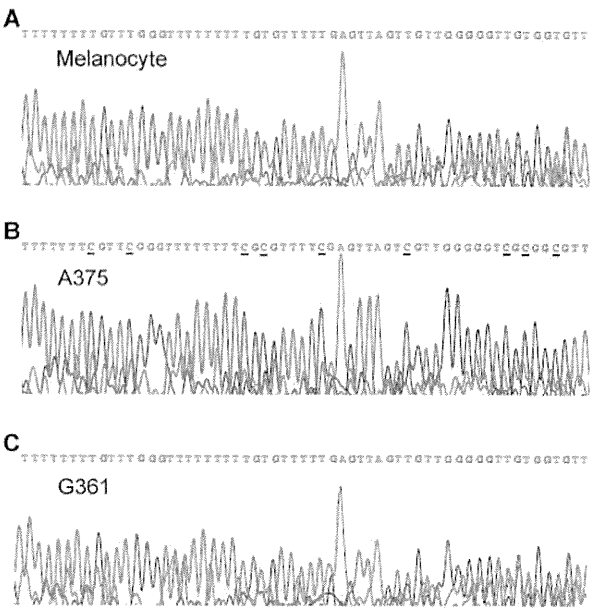

Figure 3. Sequencing histograms for the CpG island of the CYGB promoter region. Of the 24 CpG sites known to be methylated in the CYGB promoter region, 9 sites analyzed for methylation are shown. Cytosines methylated in A375 **(B)** are underlined. The corresponding cytosines are entirely unmethylated in melanocytes **(A)** and G361 **(C)**, resulting in the sequence "TpG" after bisulfite treatment.
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melanoma cell lines in which the expression of the *CYGB* gene is down-regulated (Fig. S4).

CYGB Functions as a Tumor Suppressor Protein in Melanoma Cells

The epigenetic gene promoter methylation has been well documented for many tumor suppressor genes [19]. *CYGB* has been recently suggested to function as a tumor suppressor gene in non-small cell lung cancer [18] and head and neck squamous cell carcinoma [15]. To clarify whether *CYGB* also functions as a tumor suppressor gene in CYGB-positive melanoma cells, we silenced the *CYGB* gene in G361 and C32TG cells using specific siRNA. Both a quantitative real-time RT-PCR and an immunoblot analyses demonstrated that the CYGB siRNA successfully reduced expression of CYGB in both cell lines but the control siRNA did not (Figs. 1B and 4A). We observed a significantly increased proliferation rate as a result of CYGB knockdown in the CYGB siRNA-treated cells (Fig. 4B), providing strong evidence for the tumor suppressor properties of the *CYGB* gene in melanoma.

To check the validity of these findings *in vivo*, we performed xenograft experiments. In order to maintain a long-term knockdown effect, we first established G361 cells stably expressing short hairpin RNA (shRNA) for CYGB or a nonsilencing control. As expected, a reduced expression of CYGB protein (Fig. S5A) and an increased cell proliferation rate (Fig. S5B) were apparent in cells expressing the CYGB shRNA. Equal numbers of CYGB shRNA- and control shRNA-expressing cells were injected subcutaneously into nude mice and allowed to grow, the tumor sizes were then monitored over time. Both cell lines formed tumors, but the CYGB shRNA xenografts grew significantly faster (Fig. 4C) and had less apoptotic signals (Fig. S5C and D) compared

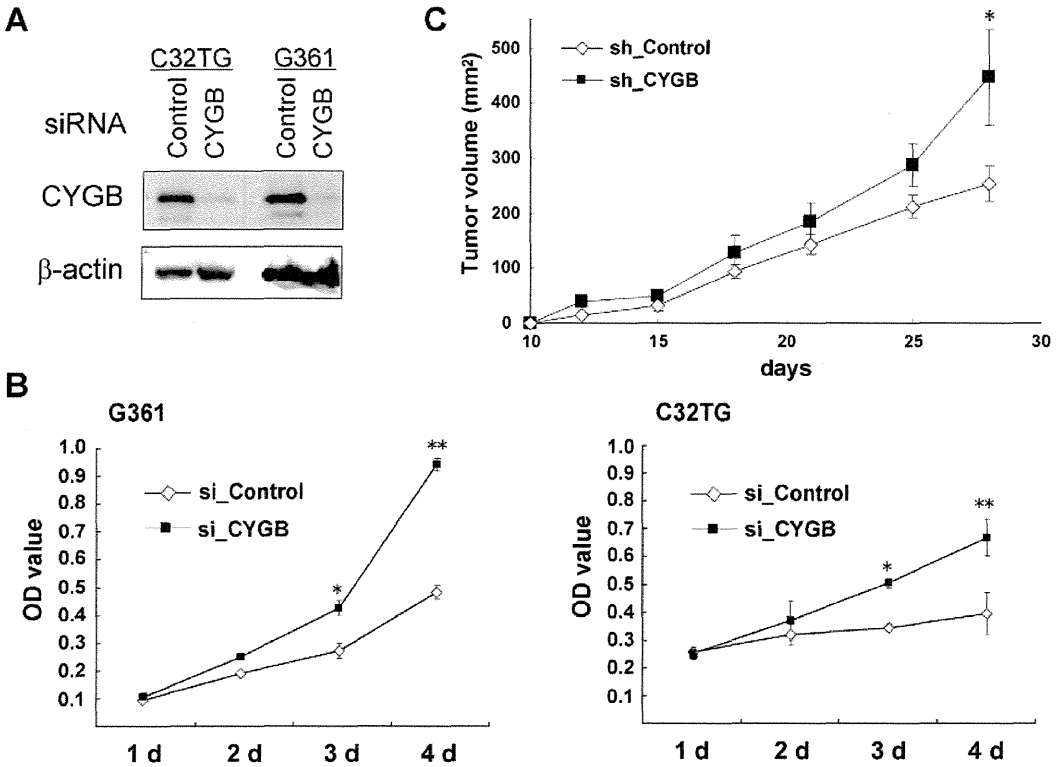

Figure 4. *CYGB*-knocked down melanoma cells increase proliferation. (A) Immunoblot data for C32TG and G361 cells transfected with CYGB siRNA or control siRNA. β-actin was used as a loading control. **(B)** Cellular proliferation pattern for G361 and C32TG cells transfected with CYGB siRNA (si_CYGB) and control siRNA (si_Control). The MTT analysis was performed daily (1d to 4d) post-transfection. The value represents the mean from three independent experiments; OD value, 570 nm. bars, SEM. * $P<0.05$, ** $P<0.01$. **(C)** Growth analysis of xenografted G361 tumors in nude mice. G361 cells expressing shRNA against CYGB or control shRNA were subcutaneously implanted into the interscapular region of five female mice. Tumor size was measured at the indicated time points. Bars, SEM. * $P<0.05$.
doi:10.1371/journal.pone.0094772.g004

with the control xenografts, again confirming the role of *CYGB* as a tumor suppressor gene.

CYGB Knockdown Causes an Increase in ROS Level

As CYGB has been reported to scavenge ROS when overexpressed in tumor cells [6,7], CYGB knockdown may raise the cellular ROS level and confer vulnerability that induces cell death. We determined the effect of CYGB knockdown on cellular ROS levels in G361 cells using flow cytometry and the redox-sensitive fluorescent probe 2′-, 7′-dichlorofluorescein diacetate (DCF-DA). CYGB knockdown for 24 hours caused a marked increase in the cellular ROS level. Co-treatment with N-acetyl-L-cystein (NAC) fully reversed the CYGB knockdown-induced increase in ROS (Fig. 5A). Exposure to 100 μM H_2O_2 for 24 hours caused a much higher ratio of early and late apoptosis in the CYGB siRNA-transfected G361 cells (61%) compared with that observed for non-treated cells (5.5%) and control cells treated with 100 μM of H_2O_2 for 24 h (8.8%) (Fig. 5B).

Discussion

In the present study, we found that melanocytes are a major cell type that is rich in cytoglobin, similar to how erythrocytes are rich in hemoglobin and myocytes are rich in myoglobin, although the function of CYGB is likely to differ from the latter two globins. Several melanoma cells that escaped from epigenetic regulation were shown to have considerable expression levels of CYGB, retaining their melanocytic character. Their high expression level of CYGB might be attributable to the gene amplification, a process by which the subchromosomal portions of the genome increase in copy number, which has been frequently observed in many human cancers [20] but not in normal cells [21]. The high expression level of cytoglobin in melanocytes is, therefore, unlikely to be due to gene amplification. Rather, some cell-type-specific factors may activate CYGB expression in melanocytes and a part of its malignant offspring, including G361. Microphthalmia-associated transcription factor (MITF) is a protein known to be responsible for the transcription of melanocyte–specific genes. MITF binds to the "CATGTG" consensus sequence found in the promoter region and activates the transcription of melanocyte–specific proteins such as tyrosinase, a melanin-synthesizing enzyme [22]. This motif was not present in the *CYGB* promoter region. Some unknown motifs, through which melanocyte-specific gene promoters are alternatively transactivated, may exist.

In early studies, the distribution of CYGB has been analyzed in normal tissues and CYGB has been found to be uniformly expressed in broad range of tissues [1,9]. Since these studies analyzed the expression in whole tissue levels, cell-type specific expression within tissues may have been underestimated in whole tissue extracts. In an independent study, Kawada et al [4] had shown enhanced expression of the rat homologue of CYGB in the stellate cells of the fibrotic liver, thus describing it as "stellate cell

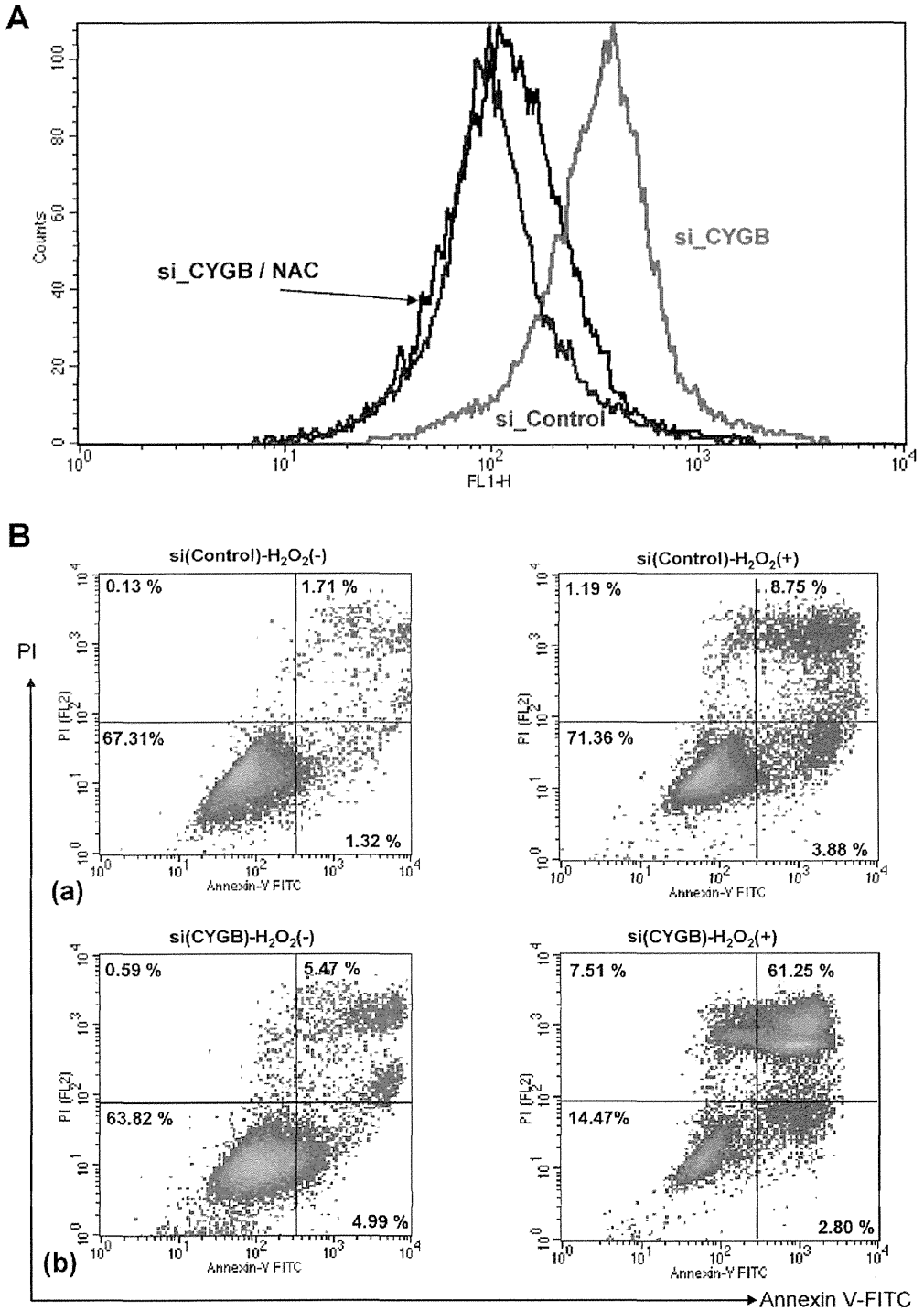

Figure 5. CYGB protects G361 cells from H_2O_2-induced cell death. A flow cytometric analysis was performed to determine the ROS level **(A)** and apoptosis **(B)** in G361 cells transfected with a CYGB-siRNA or a control-siRNA. In **(A)**, 2′-, 7′-dichlorofluorescein diacetate (DCFH-DA) was used. CYGB-knocked down G361 cells treated with 3 mM N-acetyl-L-cystein (NAC) were also compared. **(B)** Annexin V and PI staining was done after exposure of the cells to 100 μM H_2O_2 for 0 h (−) or 24 h (+). At an early stage of apoptosis the cells bind to Annexin V while still excluding PI. At a late stage of apoptosis they bind to Annexin V and stain brightly with PI. (a) Control-siRNA-transfected, and (b) CYGB-siRNA-transfected G361 cells.
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activation-associated protein" or STAP. CYGB has been also found in distinct cell populations in several tissues such as fibroblasts in connective tissue, chondroblasts in cartilage, osteoblasts in bone and neurons in colon (myenteric plexus) and in brain [10]. The CYGB levels in these cells may be as high as in melanocytes. Indeed, the overexpression of CYGB is detected in some cell lines of neuronal origin (neuroblastomas) (Table S3). Melanocytes are similar to neurons in that they are derived from pluripotent neural crest cells that differentiate into numerous cell lineages [23]. The development of melanocytes and neurons is thought to be controlled by common signaling molecules. The same signaling molecules may also promote the overexpression of CYGB in both cell types.

The nuclear localization of CYGB appears to be rather specific to melanoma cells, compared with melanocytes (Fig. S2B). CYGB has been speculated to play a role in the protection of genomic DNA from oxidative DNA damage; however, as CYGB contains no known nuclear localization signals, the mechanism of nuclear transport and its function in the nucleus remain to be determined.

Within melanocytes, melanins are formed from the successive oxidation of tyrosine, which results in the generation of hydrogen peroxide [24]. This oxidative byproduct, also generated by UV irradiation, is efficiently scavenged within the melanosomes by melanin, which in turn acts as an anti-oxidant [25]. CYGB has also been suggested to play a defensive role against oxidative stress. Human neuroblastoma cells with overexpressed CYGB showed significant protection from oxidative damage induced by H_2O_2 [6] or a singlet oxygen generator [7]. Treatment with CYGB siRNA enhanced the cellular ROS levels in fibroblasts from *CYGB* transgenic rat kidney [8]. In melanocytes, highly enriched CYGB may act as a ROS scavenger, similar to melanin.

On the other hand, melanosomes in melanoma cells not only show a dramatically reduced ability to neutralize ROS, but also actively produce excessive amounts of ROS [26]. Thus, the function of the melanosome changes from a ROS scavenger (anti-oxidant) in melanocytes to a ROS producer (pro-oxidant) in melanomas. Melanoma cells produce larger amounts of ROS and exhibit significantly higher levels of oxidative stress, compared with squamous cell carcinoma and basal cell carcinoma in the skin [27] as well as colon, pancreatic, and breast cancer cells [28]. In view of these unique melanoma properties, the elevated production of ROS seems to be a melanoma-specific defect [29], which could be caused by the heavy oxidation of melanin [24] and possibly by CYGB down-regulation for some cell types, as has been shown in the present study.

The amount of ROS produced by melanoma cells, which is within the cellular antioxidant capacity, is rather important for cellular-signaling pathways that induce apoptosis-resistance and cell proliferation [30]. ROS are thought to constitutively activate nuclear factor-kappa B (NF-κB), a transcription factor that is critically involved in cell survival. The activation of NF-κB has been proposed as an event that promotes melanoma tumor progression [31]. On the other hand, high levels of ROS exceeding the cellular antioxidant capacity may have a damaging impact on cells. If CYGB acts as a ROS scavenger in melanoma cells, it may alleviate the high levels of oxidative stress. Several cellular defense mechanisms have also evolved to protect cells from ROS. These mechanisms include repair systems, detoxifying enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) and small molecule scavengers such as glutathione (GSH). However, these antioxidant systems appear to be weakened in melanoma patients, leading to the accumulation of ROS, which may promote the cancer process [32]. Recently, Yamaura et al. [33] reported that treatment with siRNA or an inhibitor of NADPH oxidase 4 (Nox 4) decreased ROS production, thereby blocking melanoma cell proliferation. Nox 4 is known to produce superoxide anion (O_2^-), which is readily converted into hydrogen peroxide (H_2O_2). Yamaura et al. also showed that the overexpression of CAT, a scavenger of ROS (H_2O_2), caused a similar effect on melanoma cells. More recently, another group has shown that the scavenging of O_2^- by a specific compound inhibited cell growth, reduced viability, and induced apoptosis in melanoma cells, indicating that O_2^- is important for melanoma survival [34]. CYGB, like other hexacoordinated globins, may scavenge ROS utilizing heme and thiol residues [35] and could well be a target for gene silencing in melanoma. Whether or not CYGB is lost during the melanocyte-to-melanoma transition may affect tumor malignancy. Indeed, tumors without CYGB were more proliferative (Fig. 4B) under oxidative stress (Fig. 5A), a state that is vulnerable to ROS (Fig. 5B).

In conclusion, we have identified melanocyte as the prominent site of CYGB expression that greatly expands our understanding about the evolutionary diversity of the globin family. In addition, present study indicates that CYGB could be a possible candidate biomarker to predict the malignant potential of melanomas. Knowledge of the role of ROS in melanomagenesis and the mechanisms by which CYGB regulates oxidative stress can aid in the development of better antimelanoma therapies. For example, pro-oxidant compounds that target the cellular antioxidant capacity are expected to selectively kill melanoma cells.

Materials and Methods

Cell Lines

All cell lines established from human cancers as listed in Table S1 were purchased from ATCC (Manassas, VA) or Japanese Collection of Research Bioresources (Osaka, Japan). These cell lines, together with normal human dermal fibroblasts (NHDF) (Promocell, Heidelberg, Germany) were cultured in DMEM or RPMI medium (Sigma, St Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA), and the cell lines were maintained in a 5% CO_2-humidified atmosphere at 37°C. Human epidermal melanocytes (HEMa-LP) isolated from lightly pigment adult skin were purchased from Invitrogen. The cells were cultured in Medium 254 (Invitrogen) and were maintained in a 5% CO_2-humidified atmosphere at 37°C.

RNA Isolation and Quantitative Real-Time RT-PCR

Cells were washed once in ice-cold PBS and RNA was extracted from each sample using the Trizol method (Invitrogen, Carlsbad, CA). Normal human epidermal keratinocytes (NHEK) purchased as a pellet from Promocell (Heidelberg, Germany) were used to isolate the total RNA and protein. Total RNAs from normal tissues were the product of Takara (Ohtsu, Japan), except for the tongue, throat, esophagus and skin tissues, which were obtained from Biochain (San Francisco, CA). One microgram of the total RNA was reverse-transcribed with AMV reverse transcriptase using random primers and oligo (dT) primer. Real-time quantitative PCR was performed using the fluorescent TaqMan methodology and the ABI PRISM 7700 Sequence Detection System (Applied Biosystem, Foster City, CA). Ready to use, predesigned primer and probe sets (Applied Biosystems) for human CYGB (Hs00370478_m1) and housekeeping gene 18S rRNA (Hs99999901_s1) were used according to the manufacturer's guidelines. The relative expression of mRNA was calculated using the comparative Ct method.