は認められなかった。一方、H-9細胞では抗Fas抗体によりアポトーシスは誘導されたが、培養液中Cyt.C濃度の上昇は軽微であった。これらの結果より、肝細胞死においてはMtが中心的な役割を果たすことが示唆された。一方、ヒト血清Cyt.C濃度はAST値、ALT値との間に正の相関関係を有し、線維化進行例では有意に上昇していた。さらに8-NG発現群での有意な低下、4-HNE発現群での有意な上昇を認め、8-OHdGの発現が強い症例ほど高値であった。加えて血清Cyt.C濃度は、B型慢性肝炎に比してC型慢性肝炎では高値の傾向があり、C型慢性肝炎では鉄染色陰性群に比して陽性群では有意に高値であった。

D.考察、E. 結論

ウイルス感染などに惹起される酸化ストレスはMtからのCyt.Cの放出を促すが、同時に誘導される細胞死抑制系とのバランスで肝細胞死が決定されることが示された。またヒト慢性肝疾患では、とくにC型肝炎ウイルス感染がより強い酸化ストレスを惹起することが示唆された。加えて血清Cyt.C濃度は酸化ストレスによるMt障害や種々の細胞内応答の指標となりうることが考えられた。本邦におけう肝がんの大半が肝炎ウイルス感染を基礎にしており、慢性肝疾患が肝がんの前癌状態であるが明らかとなっている。炎症は肝発がんの多段階に関与するが、とりわけ炎症にともなう酸化ストレスの発生はDNA障害や細胞障害を惹起し、肝発がんのinitiationになることが推定される。そのために、酸化ストレス障害を非侵襲的に評価することは高リスク

群を絞り込むうえで重要である。今後、酸化ストレスの強い肝疾患患者において、肝発がんが"より高頻度に認められるか"をprospectiveに解析するとともに、そのような症例において血清GPC-3測定を介して早期に肝発がんを診断し振るか否かを検討していく予定である。

F.健康危険情報

該当するものはない。

G. 研究発表

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H.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

厚生労働科学研究費補助金（肝炎等克服緊急対策研究事業）
分担研究報告書

GPC3の肝細胞癌の外科療法の効果と再発の判定、および免疫療法への応用

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研究要旨

現在GPC3ペプチドワクチンによる肝細胞癌切除、RFA治療後の再発予防の臨床試験を計画中であるが、その基礎となる肝癌治療成績を検討した。1992年7月から2001年８月までに当院で治療をおこなった968例を対象とした。初回治療は単発の40%が切除、28%がablation、20%がTAEまたはTAI、その他が12%であった。多発では27%、9%、50%、14%であった。予後規定因子を比例ハザードモデルにより検討すると、Vp、腫瘍径、腫瘍数、治療法、臨床病期、部位が有意な予後因子となった。治療成績の検討から当院での治療方針は妥当であると考えられた。

A.研究目的

肝細胞癌（以下ＨＣＣ）に対する治療法の選択基準はいまだ曖昧であるが、各治療法の効果は明らかになりつつあり、今後はどの治療をいかに行うかをevidenceに基づいて系統化することが重要である。当院では内科外科すべての患者の治療をカンファレンスで一元的に管理し治療の系統性を追究してきたが、HCCに対する手術・非手術的治療を組み合わせた最適な治療戦略を明らかにする。

B.研究方法

1992年7月から2001年8月まで当院で入院治療を受けたＨＣＣ患者は968例であった。小肝癌に対しては肝機能良好例には原則手術を施行、肝機能不良例には患者の希望を反映させ手術/Ablationを選択した．進行例では巨大，多発、主脈管の腫瘍塞栓の有無が重要な因子であるが、巨大肝癌には積極的に手術を施行、多発肝癌のうち巨大腫瘍＋肝内転移のパターン、腫瘍塞栓のある患者でも塞栓が摘除可能なら減量切除＋術後TAEを施行した。それ以外の進行例、肝機能不良例にはTAEを基本とし切除、Ablation、Radiation(EBRT、 RALS、 陽子線)の局所治療を組み合わせ行った。またSupportive careのみの成績との比較も行った。

（倫理面への配慮）

実際の治療においては治療法の選択に関する詳細な説明により患者の同意のもと治療をおこなっており問題はない。

C.研究結果

小肝癌に対する切除後の3/5年生存率は89/74%で、腫瘍数1vs3で生存率に有意差が見られた。進行例でも根治切除例では3/5年生存率は66/54%であった。Ablationの治療成績は3cm、3個以内ではほぼ手術に匹敵する成績であった。進行例に対するTAE先行治療の3/5年生存率は27/8.7%で肝機能不良例ではTAE関連の合併症も見られた。陽子線は腫瘍縮小効果が見られた。

D.考察

小肝癌と根治切除可能な進行癌、根治術不能の進行例でも肝機能良好例では手術を優先させた治療を行うべきである。3.0cm、3個以下の場合ablationも有効である。 TAE＋局所療法では予後延長効果は明らかでないが散発性に長期生存例が見られ患者を肝不全に陥らせないようにしながら積極的な治療を試みるべきである。

E.結論

肝細胞がんの治療においてEBMに基づく治療の系統化は可能であり、有効な治療戦略を構築できる。今後は根治治療後の再発予防のために有効な手段の開発が望まれるが、そのベースとなる治療成績とこれまでの治療戦略を要約した。

F.健康危険情報

特になし

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H.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

厚生労働科学研究費補助金（肝炎等克服緊急対策研究事業）
分担研究報告書

肝細胞がんに対する局所壊死療法後の再発および予後に関する研究

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研究要旨

エタノール注入(PEI)あるいはラジオ波焼灼(RFA)による経皮的局所壊死療法は肝細胞癌における標準治療として広く普及している。しかし、治療後高率に再発を認めることから、再発予防が重要な課題となっている。局所壊死療法施行例における局所制御および再発率を検討し、治療後補助療法の前向き臨床試験の妥当性の検証とhistorical controlを作成した。対象は1992年7月から2004年12月までにPEIまたはRFAを施行した初回治療例134例である。局所コントロール不能例はPEI6例(6.8%)、RFA3例(6.6%)、全体で9例(6.7%)であった。累積再発率は1年31%、3年75%、5年89%。PEIとRFAの治療法別では累積再発率に差は認めなかった。肝切除でもほぼ同様の再発率を認めており、局所壊死療法と切除を含めた包括的な補助療法の臨床試験が妥当である。

A.研究目的

エタノール注入(PEI)あるいはラジオ波焼灼(RFA)による経皮的局所壊死療法は良好な局所壊死効果が得られることから肝細胞がんにおける標準治療として位置づけられ、広く普及している。しかし一方では高率な再発などの解決すべき課題もみられる。本研究では、当院において局所壊死療法が行われた肝細胞癌症例での再発率および生存期間を検討し、本治療後補助療法の前向き臨床試験の妥当性と試験計画における参考資料としてhistorical controlを作成した。

B.研究方法

対象は1992年7月（当院開院時）から2004年12月までにPEIまたはRFAを施行した初回治療例134例である。経皮的壊死療法の主な適応基準は、1) 原則として3cm、3個以内の腫瘍、2) 治療する腫瘍が超音波で十分描出される、3) PS 0-2、4) 肝機能などから切除不能、5) 切除可能例でも十分なsafety marginが確保でき、安全に施行が可能、6) 腹水はないかコントロールが可能、7) T-Bil 3mg/dl以下、8) 出血傾向がない、9) 肝性脳症がない、10) 文書による本人の同意がある、などである。1999年5月まではPEIを中心に施行し、1999年6月RFAの導入以降はRFAを第一選択として治療を行った。1セッションの治療が終了した後dynamic CTあるいはMRIで局所壊死効果を判定し、腫瘍の残存が認められたときはPEIまたはRFAを追加した。それでも残存を認める場合には、肝動脈塞栓化学療法（TACE）あるいは切除術により完全治療を目指した。完全治療が行われた後治療部位に接した部位あるいは近傍からの再発を局所再発とし、それ以外の再発を他部位再発と定義した。また肝切除例のうち、3cm以下、3個以内の病変を有する症例を対照として比較した。累積再発率、累積無増悪生存率および累積生存率はKaplan-Meier法により算出した。

（倫理面への配慮）

本研究における治療に際しては、患者には文書を用いて十分な説明を行い、患者自身による同意を本人より文書で取得した。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、個人情報の保護を遵守した。

C.研究結果

1) 患者背景

初回治療としてPEIは88例、RFAは46例に施行された。腫瘍径は中央値20mm(7-29mm)、Child-Pugh分類による肝障害度はA72例、B44例、C8例であった。

2)局所コントロールと再発

局所への追加治療は19例(14.2%)で必要であったが、最終的な局所コントロール不能例はPEI6例(6.8%)、RFA3例(6.6%)、全体で9例(6.7%)であった。

局所および他部位を含めた再発はPEI62例(70.5%)、RFA28例(60.9%)、全体で90例(67.2%)に認められた。全134例における累積再発率は1年31%、3年75%、5年89%と高率であった。PEIとRFAの治療法別では、累積再発率は1年28%、38%、3年71%、86%、5年85%、100%であり、両群に差は認められなかった。

3)生存期間

全体の累積生存率は1年98%、3年76%、5年50%、10年27%であった。PEIとRFAの治療別では、5年生存率はそれぞれ47.2%と72.0%とRFAで良好であったが、統計学的な差は認めなかった。RFAが導入された1999年以降において、肝機能良好なChild-Pugh Aの例で肝切除とRFAの生存期間を比較すると、3年生存率は肝切除97%、RFA90%、5年生存率は肝切除92%、RFA72%であり、両群に差はみられなかった(p=0.15)。

D.考察

3cm、3個以内の比較的早期例ではPEI、RFAによる局所壊死療法で高率な局所コントロールが得られ、肝切除に匹敵する効果が期待できる。しかしながら背景に肝硬変など慢性肝疾患を有する例が多く、肝内他部位を中心とした再発が高率に認められ、長期成績の向上には再発予防が必須である。これまでインターフェロン、レチノイド、ビタミンK2など試みられているが、有効な再発予防治療は確立していない。養子免疫による肝切除後の補助療法について無作為化比較試験が行われ、有意に無再発生存期間が改善したと報告されている。今後Glypican 3を標的としたペプチドワクチン療法が肝細胞がんの根治治療後の補助療法として有効か否かを確認する臨床試験を行う意義は大きいと考えられる。

肝細胞癌においては、B型肝炎、C型肝炎の有無、肝硬変の有無、肝機能の程度、治療法など再発や予後に対する多くの関連因子が認められている。肝細胞癌における治療法の開発にはランダム化が必要となる。再発予防の確立を目的とした治療法の開発においても、最終的には無作為化比較試験が必要となるが、本研究班のGlypican 3を標的としたペプチドワクチン療法の可能性を見るためには前段階の臨床試験が必要となる。今回の検討や切除例の実績およびいくつかの根治治療後の再発の検討では、1年再発率は約30%程度と考えられる。この1年生存率30%を半減できる可能性があるか否かを主要評価項目として、臨床第II相試験を行い、この目標を達成できる可能性があるなら、多数例による無作為化比較試験に進むというのが妥当と考えられる。

E.結論

PEIおよびRFAによる局所壊死療法は、適切な施行により肝切除と同等の局所制御が可能である。今後再発抑制を目的とした有効な治療法の確立を目指す必要があり、これら局所治療を包括した臨床試験が妥当である。

F.健康危険情報

特になし

G.研究発表

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H.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

IV. 研究成果の刊行に関する一覧表

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V. 平成１７年度班会議プログラム

平成１７年度班会議プログラム

（平成１８年１月２６日　KKRホテル東京「葵の間」）

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主任研究者　西村　泰治
分担研究者　中面　哲也
研究協力者　千住　覚

Highly Sensitive Detection of Melanoma at an Early Stage Based on the Increased Serum Secreted Protein Acidic and Rich in Cysteine and Glypican-3 Levels

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Abstract

Purpose: There are no available tumor markers detecting primary melanoma at an early stage. The identification of such serum markers would be of significant benefit for an early diagnosis of melanoma. We recently identified glypican-3 (GPC3) as a novel tumor marker but could diagnose only 40% of melanomas. Thereby, we focused out attention on secreted protein acidic and rich in cysteine (SPARC) overexpressed in melanoma as another candidate for tumor marker.

Experimental Design: Secreted SPARC protein was quantified using ELISA in the sera from 109 melanoma patients, five patients with large congenital melanocytic nevus, 61 age-matched healthy donors, and 13 disease-free patients after undergoing a surgical removal. We also quantified GPC3 and 5-*S*-cysteinyldopa in the same serum samples and compared these markers for their diagnostic value.

Results: The serum SPARC concentrations in melanoma patients were greater than those in healthy donors ($P = 0.001$). When we fixed a cutoff value at the mean concentration plus 2 SD of the healthy donors, the serum SPARC was found to have increased in the sera of 36 of the 109 (33%) melanoma patients, whereas there were three (4.9%) false-positive cases of 61 healthy donors. Surprisingly, 19 of 36 patients showing increased SPARC levels were in stages 0 to II. The serum SPARC level decreased under the cutoff level in 10 of 13 patients after surgical removal. Using SPARC and GPC3 in combination thus enabled us to diagnose 47 of 75 (66.2%) melanoma patients at an early stage (0-II).

Conclusions: SPARC or its combination with GPC3 is thus considered a potentially useful tumor marker, especially for melanoma at an early stage.

The incidence rates for melanoma have steadily increased worldwide, and the mortality rates have increased as well. Several molecules have been evaluated as tumor markers to detect melanoma (1-3). Recently, several investigators reported the 5-*S*-cysteinyldopa and melanoma-inhibitory activity to be useful as a marker for melanoma progression or for monitoring metastatic melanoma (4-9). However, current methods are not sensitive enough to detect melanoma in its early stages. Thus, there is a need for new tumor markers that can detect primary melanoma in the early stages. We recently reported that glypican-3 (GPC3), which is overexpressed in melanoma, is a novel tumor marker for melanoma (10).

Secreted protein acidic and rich in cysteine (SPARC), also called osteonectin or BM-40, is a matricellular glycoprotein that modulates cellular interaction with the extracellular matrix during tissue remodeling (11). SPARC was overexpressed in primary and metastatic melanomas, and an overexpression of SPARC by melanoma cell was associated with an invasive phenotype *in vivo* (12, 13). In this study, we detected SPARC in the sera of melanoma patients at higher concentrations than in healthy donors. Indeed, SPARC was detected in the sera of 33% of all melanoma patients, irrespective of the clinical stages and even in the sera of patients with stage 0 *in situ* melanoma. Moreover, the combined use of SPARC and GPC3 will thus make it possible to diagnose melanoma, especially in the early stages (0-II).

Materials and Methods

Tissues, blood samples, and cell lines. After receiving their informed written consent, we obtained tissue, serum, and plasma samples from the patients with melanoma and large congenital melanocytic nevus treated at the Department of Dermatology, Graduate School of Medical Sciences, Kumamoto University from 1997 to 2004. We also obtained 61 serum samples and 21 plasma samples from age-matched and

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sex-matched healthy donors from Hiraki hospital (Kumamoto, Japan) after receiving their informed written consents. All samples were anonymized, numbered at random, and stored at −80°C until use. We collected the patient profiles from medical records to determine the clinical stages, according to the Unio Internationale Contra Cancrum/American Joint Committee on Cancer tumor-node-metastasis classification (14). The subjects consisted of 113 consecutive and preoperative patients with melanoma comprising 52 male and 61 female patients with an average age of 67 years (range, 22-91 years): 15 had stage 0 melanoma (*in situ*); 30 had stage I melanoma; 30 had stage II melanoma; 19 had stage III melanoma; and 19 had stage IV melanoma. Five patients with large congenital melanocytic nevus consisted of four male and one female patients with an average age of 21 years (range, 4-38 years). All patients were of Japanese nationality.

Melanoma cell lines CRL1579, G361, HMV-I, and SK-MEL-28 were kindly provided by the Cell Resource Center for Biomedical Research Institute of Development, Aging, and Cancer, Tohoku University (Sendai, Japan), and 888mel and 526mel were provided by Dr. Yutaka Kawakami (Keio University, Tokyo, Japan). The cell lines were cultured in DMEM or RPMI 1640 supplemented with 10% FCS. Human epidermal melanocytes, neonatal (HEMn), in culture medium 154S supplemented with human melanocyte growth supplements, were purchased from KURABO (Osaka, Japan).

Quantitative reverse transcription-PCR. The *SPARC* mRNA levels were analyzed using real-time reverse transcription-PCR, as described previously (15). We designed *SPARC* gene-specific PCR primers to amplify the fragments of 374 bp; *SPARC* PCR primer sequences were sense, 5′-CGAAGAGGAGGTGGTGGCGGAAAA-3′ and antisense, 5′-GGTTGTTGTCCTCATCCCTCTCATAC-3′. Reaction mixtures contained 2 μL of DNA Master SYBR green I, 1 mmol/L $MgCl_2$, 0.4 μmol/L of each primer, and 1 μL of cDNA in a total volume of 20 μL. The PCR cycles were 95°C for 10 minutes followed by 35 cycles of 95°C for 1 second, 68°C for 1 second, and 72°C for 16 seconds. For *β-actin*, we used LightCycler-Primer Set Human *β-actin* (Search LC, Heidelberg, Germany). The quantification was achieved by comparisons with an internal standard curve containing 10-fold dilutions of HEMn cDNA probe. The relative expressions of *SPARC* mRNA were calculated as the ratio of the *SPARC/β-actin* expressions from three replicate reverse transcription-PCR experiments.

Western blot analysis and immunohistochemical examination. SDS-PAGE and Western blotting were done as described previously (16). The membranes were incubated with anti-SPARC monoclonal antibody AON-5031 (Haematological Technologies, Inc., Essex Junction, VT). Immunohistochemical examination was done using the DakoCytomation EnVision+ System according to the manufacturer's instructions with minor modifications, as described previously (17). Briefly, 4-μm-thick paraffin sections were cut and stained with AON-5031 at a dilution of 1:2,000 (0.216 μg/mL). After washing, the sections were incubated for 60 minutes with polymer/horseradish peroxidase-labeled goat anti-mouse IgG at room temperature. 3,3′-Diaminobenzidine tetrahydrochloride was used as the chromogen. The intensity of staining was classified as weak; weaker than the adjacent epidermis, moderate; same as the adjacent epidermis and strong; and stronger than the adjacent epidermis. These samples were estimated independently by two observers in a blinded manner (T.K. and S.F.).

Double-determinant (sandwich) ELISA. The SPARC concentrations in the culture supernatants of melanoma cell lines, sera, and plasma were measured by ELISA in duplicated wells in each plate assay. ELISA was done as described previously (16). All samples were tested in a blinded manner. We used mouse anti-human SPARC monoclonal antibody ON1-1 (Zymed Lab, South San Francisco, CA) with 0.05 μg/well and biotinylated polyclonal goat anti-human SPARC antibody EYR01 (R&D Systems, Minneapolis, MN) with 0.01 μg/well. To obtain a serum-free culture supernatant, cells were grown to near confluence, washed twice with PBS, and kept in a serum-free medium. After 24 hours, the medium was collected and centrifuged for 10 minutes at 375 × *g* to remove debris. The samples were divided and diluted at 1:4 with 10% Block Ace (Dainippon Pharmaceutical, Osaka, Japan) to serve as samples for ELISA. The serum and plasma samples were diluted at 1:200 with 10% Block Ace as described above. In this ELISA system, human SPARC HON-3030 (Haematological Technologies) was used to estimate the standard curve to quantify the SPARC protein based on absorbance data.

Statistical analysis. We analyzed all of the data using the StatView statistical program for Macintosh (SAS, Cary, NC) and then evaluated the statistical significance using Student's *t* test, χ^2, and Fisher's exact test. Because the SPARC concentration values exhibited a normal distribution in each group, the values were analyzed using Student's *t* test. We considered $P < 0.05$ to be statistically significant.

Results

Expression of SPARC mRNA and protein in human melanoma. The expression levels of SPARC in various melanoma cell lines were determined by quantitative reverse transcription-PCR (Fig. 1A) and Western blot (Fig. 1B). SPARC was expressed in all cell lines tested, except for HMV-1, in both mRNA and protein levels. SPARC proteins in the human tissue specimens were examined by Western blotting (Fig. 1C) and an immunohistochemical analysis (Fig. 1D). The vertical growth phase of primary melanomas and lymph node metastasis expressed a large amount of SPARC, whereas large congenital melanocytic nevi and radial growth phase of primary melanomas showed a moderate expression. Normal skin, including a few melanocytes, showed a weakly positive expression (Fig. 1C). Hence, all the examined tissue samples of melanomas and large congenital melanocytic nevi were positive for SPARC protein. An immunohistochemical analysis of SPARC was made on primary melanomas (33 cases), metastatic melanomas (seven cases), and melanocytic nevus (14 cases) tissue specimens. The results obtained from primary melanoma are summarized in Table 1, and the representative strong staining of primary melanoma patient is shown in Fig. 1D. SPARC was detected immunohistochemically in all 33 independent primary melanoma lesions (weak, 7; moderate, 14; strong, 12) and in all seven metastatic lesions tested (weak, 0; moderate, 2; strong, 5). SPARC was predominantly located in the cytoplasm of malignant cells. All of the 14 melanocytic nevi lesions also showed a positive expression (weak, 4; moderate, 6; strong, 4).

Presence of soluble SPARC protein in the culture supernatants of melanoma cell lines and sera from melanoma patients. We detected soluble SPARC protein using ELISA. Soluble SPARC protein could be detected in the culture supernatants of all human melanoma cell lines tested, with the exception of HMV-1, and cultured melanocyte HEMn (Fig. 2A). The concentration of soluble SPARC secreted from each cell line into the culture supernatant did not always correlate with the expression levels of SPARC mRNA and protein (Fig. 1A and B and Fig. 2A).

Serum SPARC concentrations for 109 preoperative melanoma patients, five patients with large congenital melanocytic nevus, and 61 healthy donors are shown in Fig. 2B and Table 1. Figure 2C shows the standard curve for ELISA detection of SPARC that confirmed the linearity of the ELISA determination of SPARC concentration. According to these data, we were convinced that the range for accurate detection of serum SPARC was between 0 and 16 μg/mL by using 200-fold diluted serum samples. We could obtain reproducible results in three independent ELISA assays, and the representative results were shown. The mean ±

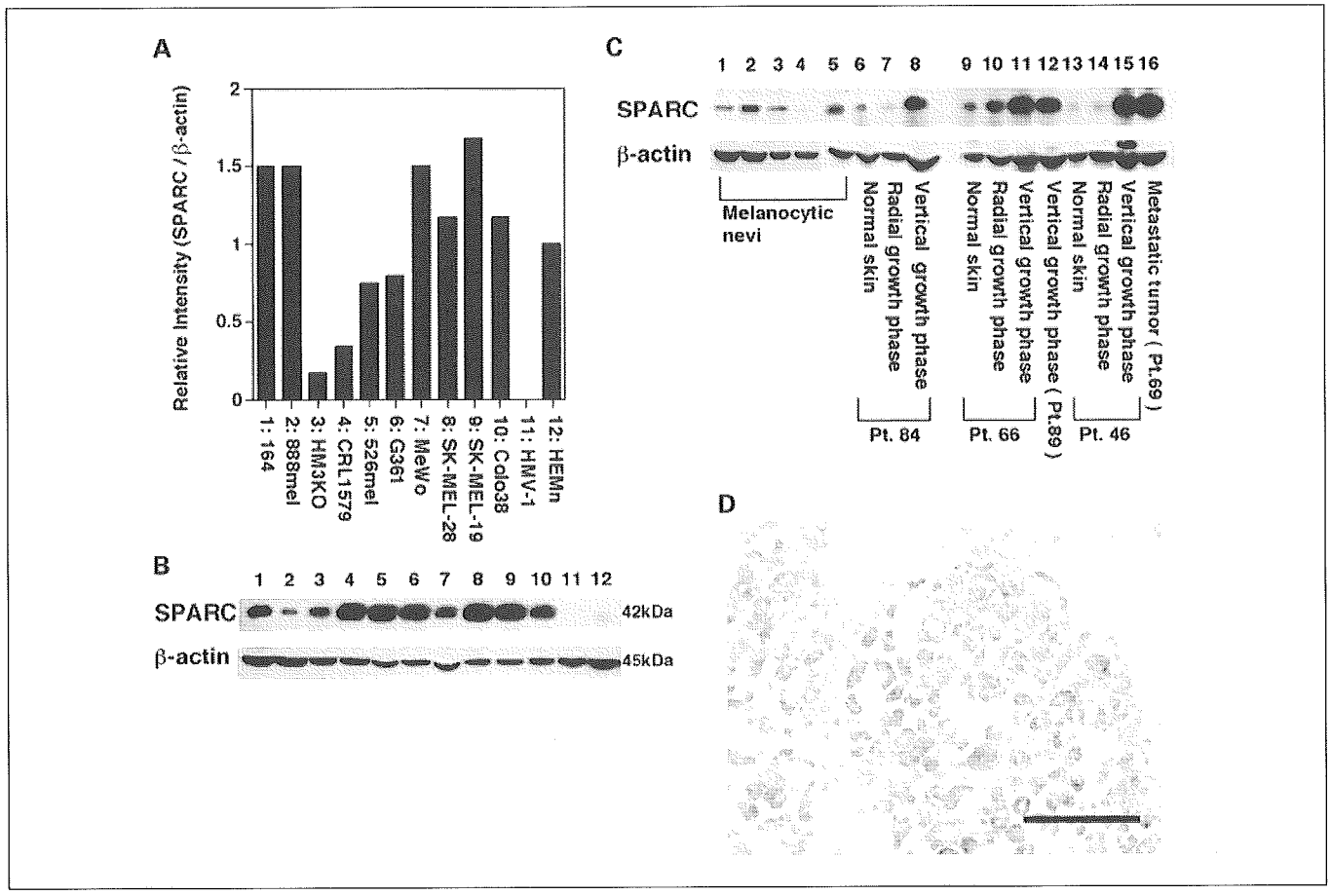

Fig. 1. Expression of SPARC mRNA and protein in human melanoma and melanocytes. *A,* differential expressions of *SPARC* mRNA in various human melanoma cell lines (*lanes 1-11*) and HEMn (*lane 12*) were analyzed using real-time reverse transcription-PCR. The relative expressions of *SPARC* mRNA were calculated as the ratio of the *SPARC/β-actin* expressions from three replicate reverse transcription-PCR experiments. 164; lane 2, 888mel; lane 3, HM3KO; lane 4, CRL1579; lane 5, 526mel; lane 6, G361; lane 7, MeWo; lane 8, SK-MEL-28; lane 9, SK-MEL-19; lane 10, Colo38; lane 11, HMV-I; lane 12, HEMn. *B,* Western blot analysis of human melanoma cell lines (*lanes 1-11*) and HEMn (*lane 12*) were done using anti-SPARC monoclonal antibody. The lanes were the same as those observed in (*A*). *C,* Western blot analyses of human tissues from melanocytic nevi (*lanes 1-5*) and melanoma patients (*lanes 6-16*) were done. Tissues originated from the following patients. Patient 84: lane 6, normal skin; lane 7, radial growth phase; lane 8, vertical growth phase. Patient 66: lane 9, normal skin; lane 10, radial growth phase; lane 11, vertical growth phase. Patient 89: lane 12, radial growth phase. Patient 46: lane 13, normal skin; lane 14, radial growth phase; lane 15, vertical growth phase. Tissue of metastasis to lymph node of patient 69: lane 16. The patient ID numbers are the same as those shown in Table 1. *D,* expression pattern of SPARC protein in primary melanoma. SPARC immunoreactivity in melanoma cells localized predominantly in the cytoplasm. Bar, 50 μm.

SD serum SPARC concentration in 109 preoperative melanoma patients (2.02 ± 1.02 μg/mL) was significantly greater than that in the 61 healthy donors (1.62 ± 0.36 μg/mL; $P = 0.001$, Student's t test). When the cutoff value was fixed at 2.34 μg/mL, which was the mean SPARC concentration plus 2 SD in the healthy donors, 36 of 109 (33.0%; 3.21 ± 0.70 μg/mL) melanoma patients were positive for increased serum SPARC. Thereby, the sensitivity of this assay was 33.0%. On the other hand, three (4.9%; 2.46 ± 0.08 μg/mL) and two (40%) positive cases were found in 61 healthy donors and five melanocytic nevi patients, respectively. Thus, the specificity of this assay was 92.4%. The prevalence of increased SPARC protein in the sera of melanoma patients was higher than that in healthy donors. The presence of a significant amount of SPARC in the sera from melanoma patients suggested that melanoma cells secrete SPARC in melanoma patients.

Among the 30 cases of melanoma patients in which both immunohistochemical staining and ELISA detection of serum SPARC were done, increased serum SPARC was detected in nine patients (30%; Table 1). In six of nine patients (patients 39, 50, 42, 76, 90, and 112), strong SPARC protein expression was immunohistochemically detected in their melanoma cells, and moderate SPARC protein expression in melanoma cells was observed in two patients (patients 44 ad 49). Patient 96 expressed weak SPARC protein in melanoma cells. However, 7 of 13 cases expressing strong SPARC protein did not secrete SPARC in the sera. Because we were not able to prepare serum and tissue samples from the same patients with congenital melanocytic nevus, we could not evaluate the correlation between the serum SPARC levels and the expression levels of SPARC in melanocytic nevi tissue in these patients.

Human platelets contain and secrete SPARC protein in the sera of healthy donors (18). Thus, we measured SPARC in plasma to eliminate the influence of the SPARC secreted from the platelets. The plasma concentrations of SPARC in 11 preoperative patients with melanoma and 21 healthy donors were shown in Fig. 2D. The mean SPARC value in 11 preoperative melanoma patients (0.61 ± 0.65 μg/mL) was significantly greater than that in the 21 healthy donors (0.14 ± 0.14 μg/mL; $P = 0.003$). When the cutoff value was fixed at

Table 1. Profiles of 113 Japanese patients with melanoma and quantification of SPARC, GPC3, and 5-*S*-cysteinyldopa in sera of patients

Patient ID	Stage*	Age	Sex	Type	Serum concentrations of tumor markers			
					SPARC staining†	SPARC (μg/mL)‡	GPC3 (units/mL)§	5-*S*-cysteinyldopa (nmol/L)‖
1	0	60	M			4.7	0	4.6
2	0	64	F			1.2	0	2.8
3	0	78	F			3.1	0	2.9
4	0	74	M			3.3	0	6.2
5	0	85	F			0.9	25	3.2
6	0	72	M			3.7	0	3.8
7	0	48	F			2.2	38	3.3
8	0	69	F			1.5	22	2.6
9	0	66	F			2.5	8	4.7
10	0	65	M			3.0	0	6.8
11	0	51	F			1.9	92	1.8
12	0	76	F			3.2	8	5.5
13	0	50	M			3.0	0	6.8
14	0	82	F		++	1.3	39	2.0
15	0	65	M			1.8	0	6.4
16	IA	33	M	Mucous		1.8	103	1.9
17	IA	82	F	LMM		NT	40	5.5
18	IA	75	F	SSM		1.8	25	3.9
19	IA	41	F	SSM		1.8	20	2.3
20	IA	70	F	Mucous		2.3	0	2.4
21	IA	78	M	ALM	++	1.2	0	11.0
22	IA	60	F	ALM		NT	0	3.6
23	IA	61	M	ALM		3.3	0	1.0
24	IA	62	F	ALM		1.2	0	5.9
25	IA	73	M	ALM		0.7	0	3.6
26	IA	70	M	LMM		1.7	20	8.0
27	IA	33	F	Mucous		2.3	10	3.6
28	IA	66	M	ALM		1.2	0	5.9
29	IA	76	M	ALM		1.3	0	5.8
30	IA	58	F	ALM		1.5	0	4.4
31	IA	89	M	LMM		0.5	0	47.6
32	IA	87	F	ALM		1.8	7.4	NT
33	IA	81	F	ALM	++	1.4	0	4.5
34	IA	68	F	LMM	++	1.5	77	1.8
35	IB	58	F	ALM		0.6	61	2.5
36	IB	58	F	Mucous		2.2	0	8.6
37	IB	66	F	Mucous		2.0	23	2.9
38	IB	56	F	SSM		2.0	10	2.3
39	IB	64	F	ALM	+++	2.8	20	7.5
40	IB	84	F	ALM		2.0	0	2.0
41	IB	79	M	ALM		2.9	0	7.3
42	IB	76	F	ALM		2.2	0	5.7
43	IB	74	F	ALM		1.6	0	2.8
44	IB	75	M	LMM	++	3.4	0	6.6
45	I?	82	F	ALM		1.6	0	2.9
46	IIA	74	F	SSM		2.3	106	17.4
47	IIA	75	M	ALM		NT	54	NT
48	IIA	74	F	ALM		1.9	16	3.4
49	IIA	64	M	ALM	++	2.9	0	4.6
50	IIA	47	F	SSM	+++	3.9	0	4.3

(*Continued on the following page*)

Table 1. Profiles of 113 Japanese patients with melanoma and quantification of SPARC, GPC3, and 5-*S*-cysteinyldopa in sera of patients (Cont'd)

Patient ID	Stage*	Age	Sex	Type	Serum concentrations of tumor markers			
					SPARC staining†	SPARC (μg/mL)‡	GPC3 (units/mL)§	5-*S*-cysteinyldopa (nmol/L)‖
51	IIA	77	F	LMM		1.3	34	3.9
52	IIA	83	F	ALM		1.9	0	4.0
53	IIA	71	F	LMM	++	2.0	82	3.4
54	IIA	73	M	ALM	+++	2.3	43	4.0
55	IIB	50	F	SSM	++	1.4	75	6.4
56	IIB	72	M	LMM		2.0	73	7.0
57	IIB	88	M	ALM		1.8	0	1.2
58	IIB	63	M	ALM		1.6	0	3.7
59	IIB	77	M	SSM		3.0	0	NT
60	IIB	69	M	ALM		1.0	15	4.6
61	IIB	57	M	ALM		3.8	0	3.4
62	IIB	69	F	ALM		0.6	0	3.3
63	IIB	71	M	ALM	++	1.7	0	4.7
64	IIB	85	F	LMM		2.6	79	4.8
65	IIB	72	M	ALM		1.8	43	6.3
66	IIB	70	M	ALM		2.6	0	0.4
67	IIB	68	F	ALM	+++	1.2	3.9	4.0
68	IIC	79	F	ALM		NT	25	3.8
69	IIC	42	M	SSM	+++	4.2	0	6.3
70	IIC	72	F	ALM	+	2.2	0	3.6
71	IIC	75	F	Mucous	+	1.8	0	8.7
72	IIC	77	M	ALM	+++	0.6	16	7.4
73	IIC	83	M	SSM		1.4	10	13.3
74	IIC	84	M	LMM		1.6	41	4.2
75	IIC	91	F	ALM		1.1	0	6.0
76	IIIA	83	M	ALM	+++	2.7	0	7.7
77	IIIA	55	M	ALM	+++	1.2	0	8.2
78	IIIA	86	F	ALM	++	1.7	0	9.7
79	IIIA	79	F	ALM	++	0.6	0	6.1
80	IIIA	70	M	ALM		2.1	0	4.0
81	IIIA	63	F	SSM		2.3	10	11.8
82	IIIA	79	M	NM		5.6	10	4.0
83	IIIA	53	F	Mucous		2.4	0	5.2
84	IIIA	53	M	ALM	+	1.6	0	1.9
85	IIIB	85	M	ALM		3.8	140	9.2
86	IIIB	56	M	LMM		2.7	0	15.5
87	IIIB	59	M	Mucous		3.2	0	1.2
88	IIIB	77	M	ALM	+	1.8	67	7.1
89	IIIC	35	F	NM	+++	1.6	132	8.4
90	IIIC	63	F	ALM	+++	2.6	18	4.9
91	IIIC	50	F	Unknown		1.2	0	5.9
92	IIIC	47	M	Mucous		3.9	0	10.3
93	IIIC	70	M	ALM		1.2	22	24.2
94	IIIC	63	M	ALM	+++	2.2	0	14.4
95	IV	47	F	SSM		3.3	35	12.7
96	IV	77	M	ALM	+	2.7	0	748
97	IV	65	M	Unknown		2.4	0	492
98	IV	78	M	Mucous		2.5	0	44.6
99	IV	60	F	SSM	+	1.4	0	32.4
100	IV	76	F	Mucous	+++	1.4	0	1.1

(*Continued on the following page*)

Table 1. Profiles of 113 Japanese patients with melanoma and quantification of SPARC, GPC3, and 5-*S*-cysteinyldopa in sera of patients (Cont'd)

Patient ID	Stage*	Age	Sex	Type	Serum concentrations of tumor markers			
					SPARC staining†	SPARC (μg/mL)‡	GPC3 (units/mL)§	5-*S*-cysteinyldopa (nmol/L)‖
101	IV	72	F	SSM		0.9	0	981
102	IV	73	F	SSM		0.3	0	56.1
103	IV	45	F	Unknown		0.8	10	5.5
104	IV	60	F	Mucous		2.2	8	8.6
105	IV	72	M	NM		0	0	225
106	IV	50	M	SSM		2.7	0	957
107	IV	47	F	NM		0	0	257
108	IV	22	M	Unknown		3.2	57	25.8
109	IV	39	M	NM		0.5	0	170
110	IV	74	F	ALM		2.7	0	395
111	IV	68	M	Unknown		2.1	34	74.2
112	IV	66	F	ALM	+++	2.8	0	246
113	IV	67	F	Unknown		3.5	53	151
Nevus1		32	M	Congenital		3.2	0	3.5
Nevus2		38	M	Congenital		2.1	0	4.1
Nevus3		24	M	Congenital		1.1	0	NT
Nevus4		9	F	Congenital		0.1	0	18
Nevus5		4	M	Congenital		2.9	0	21

NOTE: We could obtain reproducible results in three independent ELISA assays, and the representative results were shown.
Abbreviations: ALM, acral lentiginous melanoma; SSM, superficial spreading melanoma; LMM, lentigo maligna melanoma; NM, nodular melanoma; Congenital, congenital melanocytic nevus; UICC, Unio Internationale Contra Cancrum; AJCC, American Joint Committee on Cancer; TNM, tumor-node-metastasis; MIA, melanoma-inhibitory activity; NT, not tested.
*Clinical stages are according to the UICC/AJCC TNM classification (11).
†The expression of SPARC protein detected by immunohistochemical analysis. The intensity of staining was classified as +, weaker than adjacent epidermis; ++, same as adjacent epidermis; and +++, stronger than adjacent epidermis.
‡Soluble SPARC in the sera was quantified by ELISA. We fixed the cutoff value at 2.34 μg/mL in this study, and increased values are underlined.
§Soluble GPC3 in the sera was quantified by ELISA. We fixed the cutoff value at 1 unit/mL, and positive values are underlined.
‖We quantified 5-*S*-cysteinyldopa using high-performance liquid chromatography. The cutoff value was fixed at 10 nmol/L (6), and increased values are underlined.

0.43 μg/mL (a mean SPARC concentration plus 2 SD in the healthy donors), 4 of 11 melanoma patients (36.4%) were positive for an increased plasma SPARC as observed in the serum samples of the melanoma patients. In addition, one (4.8%) positive case of 21 healthy donors was observed.

Comparison of serum concentration of SPARC, GPC3, and 5-S-cysteinyldopa in patients with melanoma classified by stage. The above results clearly indicate that SPARC is a novel tumor marker for melanoma. We next compared the serum concentrations of SPARC, GPC3, and 5-*S*-cysteinyldopa in patients with melanoma classified by clinical stage (Tables 1 and 2). We fixed the cutoff level at 1 unit/mL in GPC3 and at 10 nmol/L in 5-*S*-cysteinyldopa as reported (6, 10). Figure 2E shows the serum concentrations of SPARC quantified by ELISA in 109 melanoma patients classified by stage. Although the serum concentrations of 5-*S*-cysteinyldopa increased markedly in patients at stage IV (10), the percentages of serum SPARC positive patients were almost equal among the five clinical stages as seen in GPC3 (10). To our surprise, we detected an increase of SPARC in the sera of patients with very small lesion of melanoma such as stage 0 or I. No significant correlation was observed between the patients positive for each of three markers (Table 1). More importantly, 18 of 36 SPARC-increased patients were negative for both GPC3 and 5-*S*-cysteinyldopa, and many were classified as cases of relatively early Unio Internationale Contra Cancrum stages 0, I, and II (Table 1). The positive rate of these three tumor markers in patients with melanoma, as classified by stage, is shown in Table 2. The total positive rates of increased SPARC (36 of 109, 33.0%) and GPC3 (48 of 113, 42.5%) were significantly higher than the rate for 5-*S*-cysteinyldopa (25 of 110, 22.7%). The positive rates of increased SPARC (8 of 15, 53.3%) and GPC3 (7 of 15, 46.7%) at stage 0 were significantly higher than that for 5-*S*-cysteinyldopa (0 of 15, 0.0%; $P < 0.001$). In addition, when we use SPARC and GPC3 in combination, the positive rates at stage 0 (13 of 15, 86.7%), stage I (14 of 28, 50.0%), stage II (20 of 28, 71.4%), and stage III (12 of 19, 63.2%) were all significantly higher than that of 5-*S*-cysteinyldopa ($P<0.05$). In all, the positive rate of increased SPARC or GPC3 in patients at stages 0 to II (47 of 71, 66.2%) was significantly higher than that of 5-*S*-cysteinyldopa (4 of 71, 5.6%; $P< 0.001$). On the other hand, the positive rate of 5-*S*-cysteinyldopa in stage IV patients (16 of 19, 84.2%) was significantly higher than that of SPARC or GPC3 in combination (12 of 19, 63.2 %). Finally, we were able to detect 78 of 107 (72.9%) cases of preoperative melanoma patients by the combined use of SPARC, GPC3, and 5-*S*-cysteinyldopa. This is an extremely high positive rate.

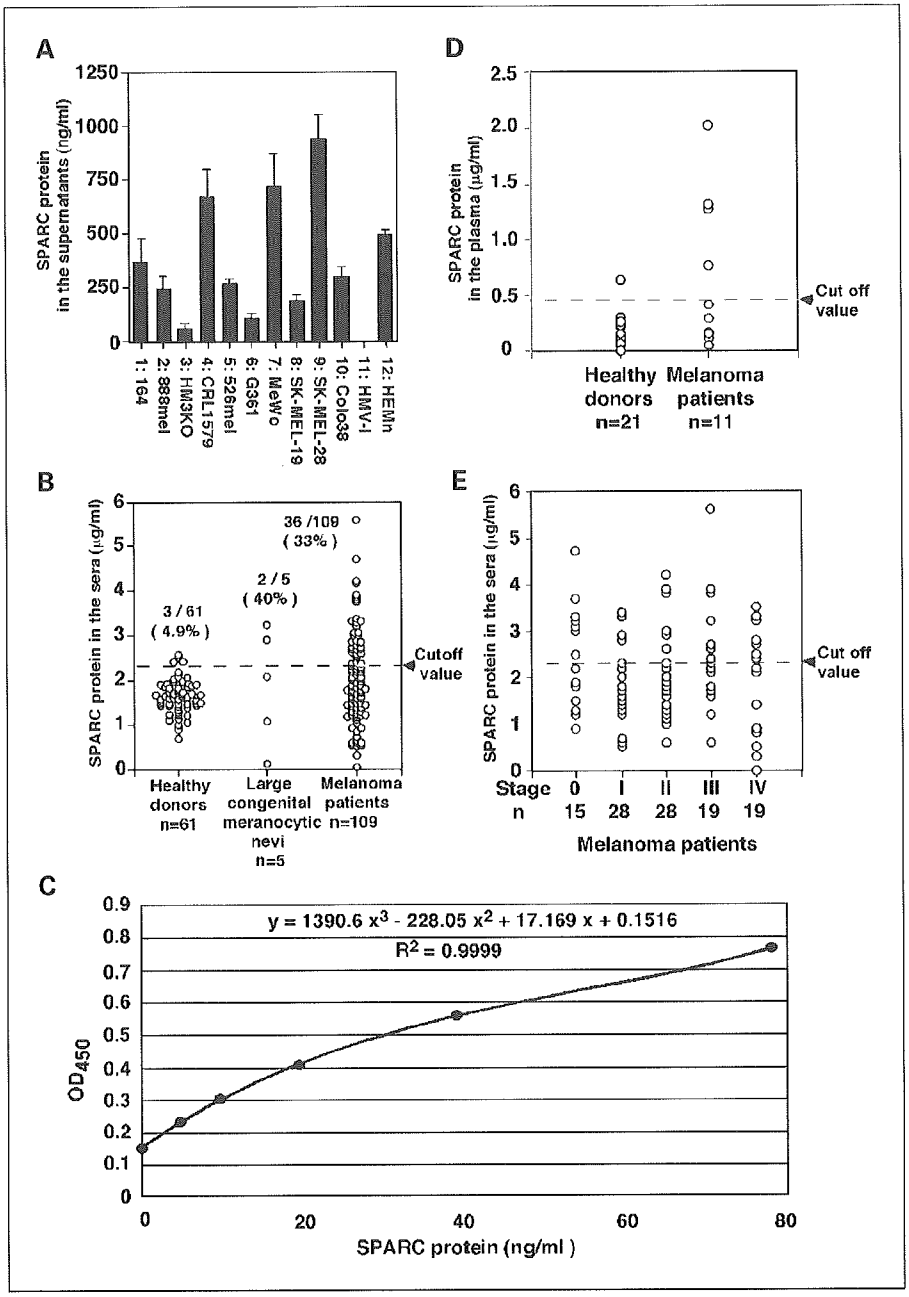

Fig. 2. Quantification of soluble SPARC protein using ELISA. *A*, quantification of SPARC protein secreted in the culture supernatant of melanoma cell lines and HEMn by ELISA. The serum-free culture supernatant was obtained as described in Materials and Methods. Representative of three independent and reproducible experiments with similar results. Columns, mean; bars, SE. *B*, distribution of SPARC protein concentrations estimated by ELISA in sera from 109 patients with melanoma, five patients with melanocytic nevus, and 61 healthy donors. When we fixed the cutoff value at 2.34 μg/mL (the mean SPARC concentration plus 2 SD of the healthy donors), SPARC protein was detected in the sera of 36 of 109 (33.0%) patients with melanoma, 2 of 5 (40%) of patients with melanocytic nevus, and 3 of 61 (4.9%) patients in healthy donors. We could obtain reproducible results thrice. Representative results. *C*, standard curve to quantify the SPARC protein based on absorbance data. Serially diluted human SPARC HON-3030 was used to estimate the standard curve. *D*, the distribution of SPARC protein in plasma from 11 patients with melanoma and 21 healthy donors by ELISA. When we fixed cutoff value at 0.43 μg/mL (mean SPARC concentrations plus 2 SD of the healthy donors), SPARC protein was detected in the plasma of 4 of 11 (36.3%) patients with melanoma and 1 of 21 (4.8%) healthy donors. *E*, comparison of the serum concentration of SPARC in patients with melanoma as classified by stage.

Table 2. Positive rates of increased serum levels of SPARC, GPC3, 5-*S*-cysteinyldopa, SPARC + GPC3, and SPARC + GPC3+5-*S*-cysteinyldopa in patients with melanoma classified by stage

Stage	SPARC (%)	GPC3 (%)	5-*S*-cysteinyldopa (%)	GPC3 + SPARC* (%)	GPC3 + SPARC + 5-*S*-cysteinyldopa† (%)
0	8/15 (53.3)	7/15 (46.7)	0/15 (0.0)	13/15 (86.7)	13/15 (86.7)
I	4/28 (14.3)	12/30 (40.0)	2/29 (6.9)	14/28 (50.0)	15/27 (55.6)
II	7/28 (25.0)	16/30 (53.3)	2/28 (7.1)	20/28 (71.4)	19/27 (70.4)
III	8/19 (42.1)	7/19 (36.8)	5/19 (26.3)	12/19 (63.2)	13/19 (68.4)
IV	9/19 (47.4)	6/19 (31.6)	16/19 (84.2)	12/19 (63.2)	18/19 (94.7)
Total	36/109 (33.0)	48/113 (42.5)	25/110 (22.7)	71/109 (65.1)	78/107 (72.9)

*GPC3 + SPARC: either or both of two markers was positive.
†GPC3 + SPARC + 5-*S*-cysteinyldopa: at least one of three markers was positive.

Decrease of serum SPARC protein in post-operative melanoma patients. The changes in the serum levels of three tumor markers (SPARC, GPC3, and 5-*S*-cysteinyldopa) before and after surgical treatments in SPARC-positive 13 patients are shown in Table 3. In 10 of 13 patients, the serum SPARC levels decreased to below cutoff levels after the surgical treatments, although GPC3 and 5-*S*-cysteinyldopa values were negative before and after the operation in the majority of these patients. It should be noted that SPARC is useful tumor marker to follow the efficacy of surgical treatments. In the case of patients 87 and 92, whose melanoma recurred, the serum SPARC values once decreased to below negative levels and then later increased again, although the serum SPARC level in patient 69 did not increase again when tumor recurrence was identified. The 5-*S*-cysteinyldopa level increased in all patients whose melanoma recurred. Further examinations are needed to elucidate whether the serum SPARC is useful for detecting recurrent tumors.

Table 3. Changes in serum levels of SPARC before and after surgical treatments in the serum SPARC-increased 13 melanoma patients

Patient ID*		SPARC (μg/mL)	GPC3 (units/mL)	5-*S*-cysteinyldopa (nmol/L)
1	Pre ope	4.7	0	4.6
	POD566	1.6	0	4.9
9	Pre ope	2.5	8	4.7
	POD1484	0.5	0	4.1
10	Pre ope	3	0	6.8
	POD15	3.4	0	3.3
12	Pre ope	3.2	7.7	5.5
	POD7	2.3	40	8.8
13	Pre ope	3	0	6.8
	POD37	3.2	0	5
23	Pre ope	3.3	0	1
	POD274	0.9	0	4
44	Pre ope	3.4	0	6.6
	POD140	2	0	3.4
	POD217	2.3	0	4.9
49	Pre ope	2.9	0	4.6
	POD1472	1.6	0	3.7
50	Pre ope	3.9	0	4.3
	POD1008	3.3	0	5.3
	POD1358	2.6	0	2.9
61	Pre ope	3.8	0	3.4
	POD1567	2.3	0	5
69	Pre ope	4.2	0	6.3
	POD329	2	0	4.3
	POD462 (Meta)	1.6	0	3.4
	POD559 (Free)	0.4	0	31
87	Pre ope	3.2	0	8.8
	POD82	2.4	0	5
	POD345	1.4	0	5.1
	POD713 (Meta)	3.5	45	46
92	Pre ope	3.9	0	10.3
	POD465	0.3	0	7.8
	POD678	2.4	0	7.3
	POD762 (Meta)	2.2	0	10

NOTE: Increased values are underlined.
Abbreviations: POD, postoperative days; Meta, metastasis; Free, disease free.
*Patient ID was the same as shown in Table 1.

Discussion

SPARC is a matricellular glycoprotein that modulates cellular interaction with the extracellular matrix during tissue remodeling. Although the specific functions of SPARC still remain unclear, it also plays an important role in wound repair, cell proliferation, cell migration, morphogenesis, cellular differentiation, and angiogenesis (11, 19–21). SPARC was first identified in 1981 as a major noncollagenous constituent of bovine bone (22) and is expressed abundantly in the bone and platelets (23). Recently, many publications have described a high expression of SPARC in a variety of human malignancies (12, 24–35). Tumor-derived SPARC was reported to stimulate tumor progression in many types of cancers. The expression levels of SPARC correlated with the histologic grade of tumor cells (25, 26, 30, 33). A higher SPARC expression was associated with local tumor invasion (24, 29–31, 33); metastasis to the lymph nodes, liver, and bone (24, 28, 32, 33); and poor prognosis and survival (24, 32, 33, 35). Conversely, the expression levels of SPARC was inversely correlated with the degree of malignancy in ovarian cancer (34). In most cancers, SPARC protein is overexpressed in the stromal cells of tumor tissue but is rarely expressed in cancer cells themselves (24, 31). In contrast, melanoma cells by themselves have been shown to express a high level of SPARC, and such increased levels are associated with an invasive phenotype *in vivo* (12, 13).

We confirmed the expression of SPARC at the mRNA and protein levels in human melanoma cell lines and melanoma tissue specimens. In addition, we proved that SPARC was secreted and detected in the culture supernatants of melanoma cell lines and the sera of melanoma patients. In this study, increased serum levels of SPARC were observed in 33% of the melanoma patients. A correlation between the serum levels of tumor markers and tumor progression has been reported (6), as is the case of 5-*S*-cysteinyldopa in their study. However, no significant correlation was observed between the serum SPARC levels and the progression levels of melanoma in this study. We wonder why only 33% of the melanoma patients showed increased levels of SPARC in the sera, and such SPARC concentrations did not correlate with tumor progression, although most melanoma tissues specimens express SPARC protein and almost all examined melanoma cell lines secreted SPARC protein. In melanoma cell lines, the expression level of SPARC did not completely correlate with the levels of soluble SPARC secreted in the culture supernatant (Fig. 1B and Fig. 2A). The same phenomena were also observed in melanoma patients in this study. Sixteen of 24 patients who expressed moderate or strong SPARC protein in melanoma cells did not show elevated SPARC protein in their sera (Table 1). Therefore, not all but some subpopulations of melanoma cells might secrete SPARC protein in the sera of melanoma patients. The concentration of soluble SPARC secreted into the culture supernatant positively correlated with the cell number in the culture dish (data not shown). However, the serum SPARC concentration was not positively associated with the tumor size