leukemic mice treated with G-CSF-PE40 survived for as long as 29–45 days (Fig. 5E). We are also planning to conduct further therapeutic experiments using this murine leukemia model to obtain longer survival periods and to assess the potential adverse effects of this chimeric molecule.

Discussion

We constructed a fusion protein between human G-CSF and PE40, and demonstrated its potent cytotoxic effects against murine and human myeloid leukemia cells. Furthermore, we observed the in vivo activity of this chimeric toxin in normal and leukemic mice.

In native PE, the receptor-binding domain (domain Ia) is located at the 5′-end of the gene and the amino-terminus of the protein [4]. G-CSF was placed at the amino-terminus of PE40, so that G-CSF occupied the same position as domain Ia. Another group constructed a chimeric toxin, DAB486-G-CSF, in which human G-CSF replaced the carboxy-terminus of diphtheria toxin [33]. DAB486-G-CSF was reported to bind to the G-CSF receptor with 200-fold less affinity than wild-type G-CSF, suggesting that fusion of a macromolecule to the amino-terminus of G-CSF may interfere with its tertiary structure responsible for receptor binding. The tertiary structure of G-CSF is composed of four major α-helix bundles [34]. In this context, the residues of helix A as well as the vacant space around the amino-terminus of G-CSF appear to play essential roles in interaction with its receptor [35–40]. In contrast, our previous study indicated that the bioactivity of G-CSF is not affected by addition of extra amino acid residues at the carboxy-terminus [41]. Together with the results of the present study, these observations indicate that genetic modification of the carboxy-terminus of G-CSF is a rational approach to target the G-CSF receptor.

Although recent advances in leukemia therapy have improved the overall prognosis of patients, some patients still have refractory leukemia, with high relapse rates even after stem cell transplantation (SCT). Treatment of these leukemias has two major problems. First, leukemia cells are naturally resistant or acquire resistance to chemotherapeutic agents. Second, a significant portion of leukemia cells are non-dividing or dormant cells [42], which are insensitive to chemotherapy. Toxins have different mechanisms of action from conventional chemotherapeutic drugs and can kill non-dividing cells [1,2]. These common properties of the toxins make them attractive for use in leukemia therapy.

Although the correlation between the abundance of G-CSF receptor expression and the proliferative response to G-CSF is controversial, G-CSF stimulates clonogenic growth of myeloid leukemia cells. We reported previously that blast cells expressed G-CSF receptors and showed mitogenic responses to G-CSF in approximately 75% of patients with acute myeloid leukemia [28]. Targeting of PE to G-CSF receptors naturally results in elimination of myeloid leukemia cells as well as normal myeloid progenitor cells. In addition, physiological expression of G-CSF receptor is also observed in the placenta, endothelial cells, and platelets [11,12,43–45]. However, in the present study, pharmacological doses of G-CSF-PE40 did not induce severe adverse events except for transient neutropenia in normal mice. More extensive dose-escalation studies and repetitive treatment are required to confirm the safety and efficacy of this toxin in animal models.

G-CSF-PE40 is an attractive agent in conditioning regimens for SCT, in which normal progenitor cells are transplanted after total eradication of recipients' normal hemopoietic cells as well as leukemia cells. Neutralizing antibody production is a major problem in clinical application of chimeric toxins. The immunosuppressive agent deoxyspergualin was reported to completely suppress anti-PE40 neutralizing antibody production [46]. Almost all recipients in allogeneic SCT routinely receive immunosuppressive agents including cyclosporin A and tacrolimus. Generation of neutralizing anti-PE antibodies, which is evoked after a period of at least 2–3 weeks, usually by week 16 after the first administration of CD22-PE38 [47], is negligible in such cases.

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我々はゲフィチニブの副作用出現を予測する方法を開発するため、大規模なSNP解析を行った。対象は16週間以上にわたりゲフィチニブの投与が行われた肺非小細胞癌患者42名である。副作用の有無は7日ごとに確認しNCI-CTCグレードによって評価した。加えて治療28日目にゲフィチニブの血中濃度を測定した。また、これらの患者の末梢血よりDNAを採取し、512個の遺伝子をカバーする3,717 個のSNPのジェノタイピングを行った。治療期間中、42名中23名がグレード1以上の下痢を示した。SNP解析の結果、7個のSNPsが下痢の出現と極めて高い相関を示した(p=0.000025)。これらのSNPは同じ遺伝子（GENE1）内に存在し、3個はプロモーター領域、残りはイントロン内に存在していた。また、別の遺伝子(GENE2)内に存在する2個のSNPはゲフィチニブの血中濃度との相関を認めた(p=0.00056)。これら2つの遺伝子は同じ遺伝子ファミリーに属しており、ゲフィチニブの代謝にこれらのファミリーが関与することが示唆された。これらの結果を用いれば高い精度で副作用出現を予測することが可能となる。

Keyword: gefitinib, SNP

P-1354 *CYP2C8*遺伝子プロモーター領域の多型解析

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Genetic polymorphisms in the *CYP2C8* promoter region

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CYP2C8はタキサン系抗癌剤のなかでPaclitaxel（TXL）に特徴的な代謝酵素とされ、同剤に対する個体の応答を考える上で極めて重要な因子である。しかしながら，その遺伝子多型の存在や意義に関してはいまだ不明な点が多い。TXL応答予測への展開を念頭に，ヒト培養腫瘍細胞36株の*CYP2C8*遺伝子プロモーター領域の塩基配列を解析した。その結果，既知の-271，-370 position（ATGを+1として）に加え，-411 positionに新たな多型を見出した。頻度は-271C/C: 86.1%，C/A: 11.1，A/A: 2.8，-370T/T: 51.5，T/G: 21.2，G/G: 27.3，-411T/T: 51.6，T/C: 25.8，C/C: 22.6であった。遺伝子型と遺伝子発現量とを比較すると，-370Gアリルを持つ細胞は発現量が低く，-411Cアリルを持つ細胞は発現量が高い傾向にあり，*CYP2C8*プロモーターレポーター実験では，-411Tに比し-411Cでは有意にプロモーター活性が高い可能性が示された。現在、臨床検体におけるこれら遺伝子多型の意義について検討を進めている。

Keyword: CYP, SNP

P-1355 緑膿菌体外毒素による細胞毒性の遺伝子発現解析

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Gene expression profiles in the cellular response to recombinant *Pseudomonas* exotoxin A

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Pseudomonas exotoxin A (PE) is a single-chain toxin secreted by *Pseudomonas* aeruginosa. It kills cells by catalyzing the irreversible ADP-ribosylation and subsequent inactivation of elongation factor2. PE consists of three major domains termed Ia, II, and III. Domain Ib is a minor domain. Domain Ia has been replaced by a single-chain antigen binding protein (including growth factors or single chain antibodies) to produce targeting toxin for cancer therapy. Some of PE derived toxins are now tested under the clinical trials for the targeted cancer therapy. The PE-related toxicities are capillary leak syndrome, renal and hepatic injury mediated by Kupffer cell-secreted TNF-α. In this study we examined gene expression profiles in primarily cultured human renal tubular cells after an exposure to PE using HG-U133 set (Affymetrix) to clarify molecular mechanisms of renal damage. We found significant changes in transcription of enzymes related to amino acid synthesis and RNA binding proteins.

Keyword: Toxin, Immunotoxin

P-1356 選定された指標遺伝子群の発現データを用いる食道癌化学療法効果予測モデル

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Chemosensitivity prediction models in esophageal cancer using expression data of selected marker genes.

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【目的】食道癌における抗癌剤治療の定量的効果予測モデルを確立する。【方法】ヒト培養食道癌細胞20株を用い、1)cDNA microarray解析とMTT assayにより遺伝子発現量とIC_{50}値を求め、その順位相関解析から5-FU、CDDPなど8種類の抗癌剤の効果に関連する遺伝子を抽出、2)その中から機能の証明された遺伝子を選定、3)それらの発現量を多変量解析によりIC_{50}値に変換する効果予測式を作成した。【結果】食道癌細胞において抽出された効果関連遺伝子は他の腫瘍細胞系のそれらと著明に異なり、他の細胞系で設定された効果予測式を食道癌へ直接転用することは困難と考えられた。食道癌における有力な効果予測指標として13遺伝子が抽出されたが、そのうち*E2F1*、*ERCC2*、*GCLC*など8遺伝子は食道癌に特異的であった。これら13遺伝子に関し、その発現量と抗癌剤効果との関連性がreal-time RT-PCR定量的発現解析でも追認されたことから、多変量回帰分析によって、それら13遺伝子の発現データの代入により各抗癌剤の効果(IC_{50}値)を予測し得る計算モデルを試作した。現在その有用性評価を行っている。

Keyword: Personalized medicine, Chemosensitivity prediction

P-1357 日本人大腸癌におけるTS遺伝子3' UTRの6bp deletion polymorphismとTS発現

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Polymorphisms within 3UTR of the thymidylate synthase gene and TS expression in Japanese colorectal cancer patients

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【目的】TS遺伝子の3'-untranslateed region (3'UTR)には－6bp/1494 deletion polymorphismが存在し（Cancer Epidemiol Biomarkers Prev 2000）、－6bp alleleではmRNA instabilityをきたすことが報告されている（Pharmacogenetics 2004）。今回、日本人大腸癌における－6bp/1494 deletion polymorphismの頻度と腫瘍内TS発現との関係、補助化学療法におけるその意義を検討した。【対象と方法】大腸癌255症例（stage 1-3）。正常組織DNAにて6bp deletion多型をPCR-RFLPで解析した。原発巣のTS発現はELISAで測定した(ng/mg protein)。生存解析は5-FU系補助化学療法が試行された128例で行った。【結果】－6bp deletion多型の頻度は－6/－6(115例 45%)、－6/＋6(110例 43%)、＋6/＋6(30例 12%)。TS発現は－6/－6: 49.6±41.2、－6/＋6: 56.4±48.2、＋6/＋6: 62.2±61.3 (P=0.76, Kruskal-Wallis test)。補助化学療法群での5年健存率は－6/－6 (56例): 81%、－6/＋6および＋6/＋6(67例): 79% (P=0.90)。【総括】TS 3' UTRの6bp deletion polymorphismと腫瘍内TS発現に有意な関連は認めない。また、5-FU系補助化学療法のefficacious markerではない。

Keyword: Pharmacogenomics, Thymidylate synthase

P-1358 Cancer Cell Informaticsによる毒性物質の評価

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Evaluation of toxic substances by Cancer Cell Informatics

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Cancer Cell Informaticsとは、39種ヒト癌細胞株パネル（JFCR-39）による感受性試験とインフォーマティクスとをリンクしたメカニズムオリエンテドな化合物評価法である。我々は、本法を確立し、抗癌物質の

response/>control mean+1SE) was 63.6% (7/11) and the negative predictive value (the number of patients who failed to obtain the response/<control mean+1SE) was 70.6% (12/17). The sIL2R levels on both day 7 and 60 did not show any difference between the groups. Overall, these findings demonstrate that serum sIL2R levels increase in patients with aplastic anemia. Pretreatment serum levels of sIL2R might be closely related to clinical outcome following IS therapy, reflecting the immunological abnormalities in individual patients.

Abstract# 4220

CML Developed in a Japanese Family Transmitting a Novel Point Mutation in the Thrombopoietin Gene(TPO). Yasuo Oshima,[1,2] Norio Komatsu,[2] Keiya Ozawa,[2] Akio Fujimura*.[1] [1]*Clin Pharmacol, Jichi Med Schl, Minamikawach, Kawach, Japan;* [2]*Hematol, Jichi Med Schl, Minamikawach, Kawach, Japan.*

Introduction: Four families are reported to have hereditary thrombocythemia (HT) with a mutation in TPO. Their clinical manifestation is essentially thrombocytosis without leukemia. CML is one of myeloproliferative disorders, and shows leukocytosis and thrombocytosis associated with a proliferation of malignant clone originated from a hematopoietic stem cell (HSC). The incidence of CML is about 5 per 100,000 in Japan. Mutations of cytokine receptor including c-kit, flt-3 and G-CSF receptor are reported as a cause of AML. Especially flt-3 abnormalities are found in about 20% of AML. However, abnormality of c-mpl or TPO is not reported as a cause of leukemia. In this paper, we analyzed a CML case with novel point mutation in the TPO who still had thrombocytosis after cytogenetic complete response.

Case: Japanese, 35 y.o., male, complained leukocytosis. He had a family history of thrombocytosis in 4 individuals over 3 generations. A physical examination revealed a moderate splenomegaly. Laboratory tests at the time of diagnosis were as follows; WBC 141,000/μl (blast 1.8%, promyelo 2.4%, myelo 20.0%, meta 8.2%, stab 24.2%, seg 22.2%, immature eosinophils 1.8%, eosinophil 3.6%, immature basophils 0.4%, basophils 10.4%, mono 1.0%, lymphocytes 4.0%, erythroblast 3%), PLT 641,000/μl and NAP score 53 (nl; 156-271). Bone marrow showed hypercellularity with the increased megakaryocytes(Meg), bcr-abl fusion mRNA positive, Ph1 chromosome positive. After 5 months treatment with STI571, most of clinical findings including karyotype and fusion mRNA turned to be normal, but thrombocyte(PLT) still showed more than 1,000,000/μl. At this time, serum TPO concentration was 8.14 f mole/ml (nl; 0.40 +/- 0.28 f mole/ml, mean +/- SD). Genetic analysis of TPO revealed novel point mutation at splicing donor site of 3'-end of the exon3. A point mutation at splicing donor site is reported to cause an exon-skipping and intron-retention, which induce a malfunction of a suppressive post-transcriptional and translational regulation, and consequent high-level expression of functional TPO protein.

Discussion: TPO was cloned as a c-mpl ligand, which leads to the production of PLTs. Its receptor is a c-mpl proto-oncogene product, which is expressed not only in Meg, but also in HSC. Thus, TPO can stimulate HSC. The c-mpl transgenic mice are reported to have the increased Meg, its committed progenitor and PLT. Knockout mice of TPO presented not only the decreased Meg, but also multi-lineage committed progenitors. Thus, a modulation of c-mpl or its ligand function affects on both Meg and HSC. The c-mpl was cloned as a cellular homolog of a viral oncogene, v-mpl of myeloproliferative leukemia virus (MPL). The MPL causes myeloproliferative leukemia syndrome through v-mpl function in mice. Since v-mpl and c-mpl indicate high homology, it is possible that abnormal c-mpl function causes v-mpl like response. Through continuous stimulation of c-mpl signal, high TPO concentration may have induced a malignant transformation of HSC or supported a survival of an immature malignant clone in the present case. Improvement of thrombocytosis in CML is one of hematological responses to an anti-CML treatment such as STI571. In such a case who had good response other than thrombocytosis, an existence of HT might be considered. On the other hand, during following up HT family, occurrence of CML should be noted.

Abstract# 4221

Erythropoietin Did Not Activate the JAK/STAT or the ERK1/2 Pathway in the Human Colorectal Cell Line, HT-29. Jennifer Tullai*, Carmelata Chitikila*, Ze'ev Gechtman*, Xiwei Wang*, Linda Jolliffe*, Francis Farrell*. (Intr. by Linda Mulcahy) *Drug Discovery, Johnson & Johnson Pharmaceutical Research and Development, LLC, Raritan, NJ, USA.*

Erythropoietin (EPO) is the cytokine essential for erythropoiesis; however, the expression of EPO and EPO receptor (EPOR) is not limited to cells of erythroid lineage. Erythropoietin and EPOR expression has been localized to numerous non-hematopoietic cells and tissues including endothelial, neuronal and ovarian. Moreover, several tumor types have been shown to express both EPO and EPOR and display increased expression upon hypoxia. To shed light on the potential biological role of EPOR and tumor cells we characterized the response of human colon carcinoma HT-29 cells to EPO. Functionality of the erythropoietin receptor was assessed by radio-labeled ligand binding, cellular proliferation/signaling, and gene expression using DNA microarrays. Receptor binding experiments using [^{125}I]-EPO did not reveal measurable EPO binding activity present on the surface of HT-29 cells under both normoxic and hypoxic conditions. Moreover, EPO failed to induce cellular proliferation or an increase in the phosphorylation state of STAT5, EPOR or ERK1/2 under normoxic or hypoxic conditions at supra-pharmacological levels (25 IU/ml). Gene expression analysis revealed no significant change in gene expression in response to EPO (5 IU/ml) under normoxic conditions. On the other hand, over 347 genes exhibited greater than a 1.5 fold change in gene expression when cells were cultured under hypoxic conditions (1 % O_2). When EPO was administered to cells in the hypoxic state, 36 additional genes were observed (9 and 27 up-regulated or down-regulated, respectively). That HT-29 cells exhibit minor transcriptional changes in response to EPO raises the possibility that EPO may signal in HT-29 cells. However, the mechanism for this response is not through the previously described EPO/EPOR signal transduction pathway. This conclusion is supported by the apparent lack of EPO receptor expression on the cell surface. These results suggest that tumor microenvironment, e.g., hypoxia, exerts a greater effect than that seen by exposure to erythropoietin.

Abstract# 4222

Early Results Suggest That Epoetin Alfa 60,000 U Every 2 Weeks Improves Hemoglobin in Patients with Cancer Not Receiving Chemotherapy or Radiotherapy. Daniel Shasha*, David H. Henry*, Denise Williams. *Phillips Ambulatory Care Center, Beth Israel Medical Center, New York, NY, USA; Joan Karnell Cancer Center, Pennsylvania Hospital, Philadelphia, PA, USA; Therapeutic Area Head, Oncology, Ortho Biotech Clinical Affairs, LLC, Bridgewater, NJ, USA.*

Epoetin alfa has been shown to increase hemoglobin (Hb), decrease transfusion requirements, and improve quality of life (QOL) in patients receiving chemotherapy (CT) and/or radiotherapy (RT). However, the efficacy of epoetin alfa in cancer patients receiving neither CT nor RT is not as well characterized, particularly at doses less frequent than 3 times weekly. The objective of this open-label, nonrandomized, multicenter pilot study was to evaluate epoetin alfa 60,000 U subcutaneously (SC) every 2 weeks (Q2W) in 50 anemic patients with cancer who were not receiving CT or RT. The primary efficacy endpoint is the proportion of patients achieving a hematopoietic response (HR; Hb increase ≥2 g/dL from baseline and/or Hb increase to ≥12 g/dL at any scheduled visit) independent of transfusion within 28 days. Patients with histologically confirmed nonmyeloid malignancy, Hb ≤11 g/dL, and who had not received CT in the previous 8 weeks or RT in the previous 4 weeks were enrolled. Patients were permitted to receive hormonal therapy, androgen deprivation therapy, and/or immunotherapy. Patients were to receive epoetin alfa 60,000 U SC Q2W, with escalation to 80,000 U Q2W after 4 weeks if Hb increased ≤1 g/dL. Dose will be titrated to maintain Hb ≤13 g/dL; all patients were to receive oral ferrous sulfate 325 mg daily. Patients will be treated for up to 12 weeks, with a 4-week follow-up after last dose of study drug. As of July 2004, 18 patients are evaluable for efficacy (modified intent-to-treat, ie, all enrolled patients who received ≥1 dose of study drug and had ≥1 postbaseline Hb or transfusion evaluation) and 18 for safety (all enrolled patients who received ≥1 dose of study drug). Mean age was 74.4 ± 8.7 yrs, mean baseline Hb was 10.0 ± 1.0 g/dL (n=18), and 7/18 were men. Of these patients, 7 completed 4 weeks, 3 completed 8 weeks, and 1 completed 12 weeks on study. The HR of these patients was assessed regardless of how many weeks of study they completed at the time of this interim analysis; HR was 44.4%. Hb increased 1.4 ± 0.7 g/dL (n=11) after 4 weeks and 1.2 ± 1.4 g/dL (n=4) after 8 weeks of treatment. Adverse events were limited to 1 patient, who experienced back pain and epistaxis. No patients died during the study, and no thrombotic vascular events were reported. Early results of this study suggest that epoetin alfa 60,000–80,000 U SC given every other week is well tolerated and appears to effectively increase Hb in anemic cancer patients not receiving CT or RT. The study is currently ongoing.

Abstract# 4223

Epoetin Alfa 40,000 U QW Increases Hb and Is Safe in Anemic Patients with Cancer Not Receiving Chemotherapy or Radiation Therapy. Daniel Shasha*, Denise Williams. *Phillips Ambulatory Care Center, Beth Israel Medical Center, New York, NY, USA; Therapeutic Area Head, Oncology, Ortho Biotech Clinical Affairs, LLC, Bridgewater, NJ, USA.*

Patients (pts) with cancer often become anemic as a result of the disease and its treatment. Epoetin alfa (EPO) administered 150 U/kg SC 3 times a week in pts with cancer-related anemia not receiving chemotherapy (CT) or radiation therapy (RT) significantly reduces transfusion requirements, increases hemoglobin (Hb), and improves quality of life (QOL). EPO 40,000 U SC QW is effective in pts with cancer-related anemia receiving CT +/- RT and may be a treatment option for pts not receiving CT or RT. This open-label, multicenter, pilot study investigated clinical outcomes and safety of EPO 40,000 U SC QW in anemic (Hb <11 g/dL) pts with cancer not receiving CT or RT. Treatment duration was up to 12 wks with a 4-wk posttreatment observation period. If Hb increased <1 g/dL after 4 wks, dose was increased to 60,000 U SC QW. EPO dose was reduced for Hb >15 g/dL; dose reduction was considered for Hb increase >1.3 g/dL over 2 wks in the original protocol. Primary endpoint was proportion of pts achieving a ≥1-g/dL or ≥2-g/dL increase in Hb from baseline (BL; independent of transfusion within the previous 28 days) at any time during the study. Secondary endpoints included transfusion requirements and QOL (measured with the Linear Analog Scale Assessment; LASA). The study was temporarily suspended due to concerns of a potential increased risk of thrombotic events if Hb >13 g/dL, and restarted with an upper Hb limit of 13 g/dL and rate of rise of Hb of 1 g/dL over any consecutive 2-wk period, independent of transfusion. Due to the interruption in therapy, 3 efficacy populations were evaluated: pts who had a post BL Hb value or transfusion (modified intent-to-treat [MITT], n=91); pts who completed the study prior to suspension (presuspension, n=37); and pts who entered the study, had treatment suspended, and completed after the study was restarted (suspension, n=33). Ninety-five pts were evaluable for safety: mean age, 69 years; 45% women; 85% ECOG 0-1; mean BL Hb 10.4 ± 0.73 g/dL. For the 3 populations analyzed, 73/91 (80%), 33/37 (89%), and 29/33 (88%) pts had an Hb increase ≥2 g/dL for the MITT, presuspension, and suspension populations, respectively. 13/91 (14%), 2/37 (5%), and 3/33 (9%) pts had at best an Hb increase ≥1 g/dL for the MITT, presuspension, and suspension populations, respectively. For the MITT population, mean change in Hb from BL after 12 wks was 2.9 ± 1.54 g/dL. Mean Hb decreased 1.4 ± 1.10 g/dL during the posttreatment observation period. One pt was transfused on study. Both wk 9 and wk 17 LASA scores increased significantly from BL in all categories (Energy Level, Daily Activities, and Overall QOL; $P<0.001$). EPO dose was increased in 15 (16%) pts and decreased or held in 61 (64%) pts during the study. Mean time to first dose reduction or hold was 38 days. Most commonly reported AEs were fatigue (19%) and nausea (13%). Twenty-four (25%) pts had ≥1 serious AE. Four (4%) pts discontinued due to an AE. One pt died on study due to disease

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Primary Cell Preparation of Human Renal Tubular Cells for Transcriptome Analysis

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We initiated a toxicogenomics project using Affymetrix GeneChip® HG-U133A and HG-U133B arrays harboring 45,000 probe sets representing more than 39,000 transcripts to analyze gene expression in primary cultures of human cells after exposure to chemicals that cause tissue toxicity. In order to assess the quality of the samples studied, we prepared primary human renal cortical cell cultures from surgically resected human kidney and evaluated the origin of the cells and the effects of cryopreservation. We analyzed the primary cultures using GeneChip and compared their expression patterns with those in the Novartis Research Foundation (GNF) Gene Expression Database. The comparison with the GNF database revealed that the gene expression pattern of the cultured cells was compatible with kidney cells, indicating that we had purified human renal cortical cells. Due to the purification procedure, the primary cultured cells could be a mixture of renal components; however, we identified the major population as renal proximal tubule cells by assessing gamma-GTP activity and Glut2 antigen expression. We compared gene expression in the cells before and after cryopreservation. The expression of 567 selected housekeeping genes was unchanged by cryopreservation (Pearson's correlation coefficient $r = 0.980$; $p < 0.0001$). The analysis of more than 39,000 transcripts after normalization revealed no significant changes in expression. These results indicate that our method is satisfactory for obtaining adequate primary cell cultures of renal origin and that gene expression was not significantly changed by cryopreservation.

Keywords Cryopreservation, GeneChip, Genomics, Primary Culture, Renal Tubular Cell, Transcriptome

Our current assessments of toxicity in the development and regulation of newly created medicines and chemicals rely on simplified assays and models that may underestimate the biological complexity underlying toxic effects. Interfacing genomic technologies with toxicology provides a more profound way to investigate biological complexity and to create a more systematic toxicology. One purpose of our toxicogenomics project is to identify potential (surrogate) biomarkers that can distinguish safe agents from toxic ones in vitro; another purpose is to establish a systematic toxicology that includes more systematic mechanisms, pathways, and hypotheses of toxic events. Our goals are to reduce the time and money required to develop safe, new medicines and chemicals in the future and to improve drug risk assessment.

Since the technology is new, there are no standard technologies, no standard experimental protocols, no robust tools for data analysis, and no knowledge of how transcription products relate to toxicity. Therefore, in late 1999, governments in the US, Europe, and Japan, more than 30 pharmaceutical, chemical, agricultural, and consumer product companies, and several academic institutes (University of Surrey in the UK, Michigan

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State University, and Medical College of Wisconsin) initiated toxicogenomic projects. The program consists of hepatotoxicity, nephrotoxicity, genotoxicity, and database working groups. According to the interim findings of the nephrotoxicity working group, which were presented at an ILSI-HESI meeting on toxicogenomics in risk assessment (June 6, 2003, Fairfax, VA), transcriptional analysis yielded strong topographic specificity and some mechanistic information; the frequency of individual animal transcript changes was reduced in nonresponders and increased in cases of severe toxicity; the use of pooled RNA samples may have a dilution/skewing effect on the interpretation of genetic responses; and microarray technology has a sensitivity at least equal to traditional toxicology endpoints. Their data indicated that responder and non-responder animals exist within the same species; therefore, there must be differences in expression profiles between species. This makes analyses of human gene expression profiles essential for toxicogenomic projects. In that context, we are performing genome-wide gene expression analyses using primary human tissues after exposure to therapeutic agents or other environmental chemicals that may influence human health.

Since the kidney filters and concentrates extrinsic compounds and intrinsic waste metabolites, renal tissues are always exposed to harsh conditions and are often damaged by medicines such as aminoglycosides and platinum compounds, including cisplatin and carboplatin. Therefore, we placed a priority on the kidney as a target organ in our project. There are conventional methods for purifying human renal tubular cells for biomedical research (Detrisac et al. 1983, 1984; Nagineni et al. 1984; Horster and Stopp 1986), and since our study depends on mRNA expression profiling, it is essential to confirm cell characteristics using mRNA expression.

One obstacle to the study of primary cultured human cells is the limited availability of freshly isolated human tissues. Commercial suppliers of primary cultured human tissues usually provide cells cryopreserved in a deep freezer or liquid nitrogen. Even if freshly isolated tissues are available, cryopreserved cells that can generate experimental outcomes that represent meaningful biological responses should improve handling and allow experiments to be repeated to confirm results. There are no published reports on systematic gene expression analyses of the effects of cryopreservation on human primary cultured renal tissues; there are only a few reports on the effects of cryopreservation on animal renal tubular cells (Jung et al. 2001; Kouwenhoven et al. 2001) and human whole kidneys (Salahudeen et al. 2000), and these look at the expression of only a few genes. In this study, we confirmed the origin of cultured renal cortical cells and analyzed the effects of cryopreservation on systematic gene expression in primary cultured renal cells.

MATERIALS AND METHODS

Samples and Purification of Renal Cortical Cells

Patients 18 years of age or older admitted to the Jichi Medical School Hospital for renal resection due to a confirmed solitary tumor in one kidney, the renal pelvis, or the ureter were eligible for the study. Patients with renal dysfunction (i.e., serum creatinine levels greater than 2.0 mg/ml or abnormalities in serum sodium, potassium, or chloride) were disqualified. The patient characteristics are summarized in Table 1. The study protocol was approved by the institutional review boards, including the Bioethics Committee of Jichi Medical School and the Bioethics Committee for Human Gene Analysis. All patients or their legally authorized representatives gave written informed consent before enrollment. The study was designed, conducted, and analyzed independent of any pharmaceutical companies.

When a kidney was resected, the largest part of the tissue, including the tumor, was used for pathological diagnosis; the remaining tissue was regarded as normal cortex and was placed in cold EuroCollins solution (Kobayashi Pharmaceuticals Co., Ltd., Tokyo, Japan), which is routinely used to preserve whole human kidneys for organ transplantation in our institute. A few grams of the tissue were chopped up and washed with EuroCollins solution (Collins et al. 1969). After 60 min of continuous agitation in an intracellular-like solution (Nagineni et al. 1984) with 1500 U/ml dispase (Godo Shusei Co., Ltd., Tokyo, Japan) and 1 mM calcium chloride, cells were incubated in Hank's Balanced Salt Solution (HBSS) (Invitrogen, Carlsbad, CA) with 0.05% trypsin and 0.53 mM sodium ethylenediamine tetraacetate (EDTA) at room temperature until tubule-like debris was seen under a low-power microscope; this usually took 30 to 60 min. The cells were washed and incubated overnight in the Nutrient Mixture F-12 (D-MEM:F-12, 1:1) (Invitrogen) and 10% fetal bovine serum (FBS). Subsequently, the cells were cultured in Williams' Media E (Invitrogen) with 10% FBS at 37°C in 5% CO_2 until used for assays.

Cryopreservation

When the cells reached confluence, they were harvested and mixed with Cell Banker (Juji Field, Inc., Tokyo, Japan). Cells were aliquoted into 2.0-ml cryotubes (Asahi Techno Glass Co. Ltd., Chiba, Japan) and stored in a Nalgene Cryo Container (Nalgene Nunc International K.K., Tokyo, Japan) at −80°C overnight; the frozen cells were then removed from the Cryo Container and stored at −80°C until assayed. The primary cultured cells were grown to confluence, harvested, and used for RNA purification before and after cryopreservation. We compared RNA expression before and after cryopreservation and analyzed the changes.

Gene Expression Analysis

Total RNA was extracted using an RNeasy kit (Qiagen) according to the manufacturer's instructions. Purified RNA products were quantified using a RiboGreen® RNA Quantitation Kit (Molecular Probes, Inc., Eugene, OR) with RNA standards. When the OD_{260}/OD_{280} ratio of RNA was between 1.8 and 2.1, the transcripts were labeled as follows. One microgram of purified RNA was converted to double-stranded complementary DNA (cDNA), which was used to prepare

TABLE 1

Patient characteristics. The characteristics of patients participating in the research project are shown. All laboratory data are within normal range, except the urine blood value in case 5

Patient	Case5	Case6	Case8	Case9	Case10
Age [years old]	57	65	47	76	35
Gender	Male	Female	Male	Male	Male
Warm ischemic time [min]	12	58	38	68	50
Serum urea nitrogen [mg/dl]	13	15	13	18	14
Serum creatinine [mg/dl]	0.86	0.91	0.83	0.78	0.8
Serum sodium [mEq/L]	144	139	140	140	140
Seryn potassium [mEq/L]	4.1	3.9	4.1	4.1	4.1
Serum cloride [mEq/L]	106	102	102	105	101
Urine protein	—	—	—	—	—
Urine glucose	—	—	—	—	—
Urine blood	±	—	—	—	—
Clinical diagnosis	Left renal tumor	Right renal tumor	Left renal tumor	Right renal tumor	Right renal tumor
Histopathology	Nodular carcinoma	Clear cell carcinoma	Clear cell carcinoma	Clear cell carcinoma	Clear cell carcinoma

biotin-labeled complementary RNA (cRNA) for hybridization with the GeneChip test3, HG-U133A, and HG-U133B chips (Affymetrix, Santa Clara, CA), which harbor 45,000 oligonucleotide probe sets corresponding to approximately 39,000 transcripts. Hybridization, washing, and detection of the signals on the arrays were performed with the GeneChip system (Affymetrix) (Oshima et al. 2003). As quality control measures, we analyzed B2-oligo performance, the existence of hybridization control, background noise values, and the 3′/5′ ratio of GAPDH and actin transcripts. If one or more quality tests gave values outside of the acceptable quality range, the data were excluded from the analysis.

Selection of Kidney-Specific Genes and Housekeeping Genes

We used the Gene Expression Database of the Genomic Institute of the Novartis Research Foundation (GNF) to find kidney-specific genes (URL http://web.gnf.org) (Su et al. 2002). First, we calculated the average expression of each gene in all 47 human tissues and human cell lines. Then, we selected genes that are expressed in the kidney at levels at least 10 times the average levels. We excluded genes expressed at levels greater than 3 times the average in any tissue (or tissues) other than kidney. This stringent screening identified eight genes as kidney-specific: KL (205978_at), SLC17A3 (207298_at), AQP2 (206672_at), SLC22A2 (207429_at), KCNJ1 (210403_s_at), TP53BP1 (203050_at), NKCC2 (220281_at), and XPNPEP2 (206484_s_at). NKCC2 expression showed a large variation in the GNF database (data not shown), so we excluded it from the analysis. Since no previous publications support the kidney-specific expression of TP53BP1 or XPNPEP2, we did not use these two genes as kidney-specific positive controls.

We selected 451 housekeeping genes reported earlier (Butte et al. 2001; Hsiao et al. 2001). We used an improved GeneChip (HG-U133) compared with the HG-FL array used by Butte et al. (2001) and consequently converted the data sets from the HG-FL array format into the HG-U133 array format, resulting in 630 probe sets instead of the earlier 451 data sets. This discrepancy is partly derived from the differences in the databases used for the array design (Technical Note, Affymetrix). Since the scanner reads the absence of gene expression with vast variation and minimal reproducibility, we removed approximately 10% of the 630-probe set based on negative expression.

Statistical Analysis

Data analysis was performed using GeneSpring® version 6 software (Silicon Genetics, Redwood, CA) and included normalization, Cross Gene Error Modeling, and parametric tests. For normalization, values less than 0.01 were set to 0.01. All genes in each sample were divided by the median of a list of housekeeping genes. Data were refined using Cross Gene Error Modeling (Rocke and Durbin 2001). First, we calculated the modeled error based on 61 scanned data treated identically. We removed genes with control signals greater than the calculated base/proportion value. The statistical analysis between pre- and post-cryopreserved gene expressions was performed with a nonparametric test and the Benjamini and Hochberg False Discovery Rate for multiple testing.

Cell Staining for Flow Cytometric Analysis

Anti-rabbit goat immunoglobulin labeled with FITC (Immunotech, Cedex, France) was dissolved at 1 mg per 750 μl and mixed with 750 μl glycerol to give a final concentration of 666 μg/ml, the solution was stored at −20°C until used for assays. Cultured cells were harvested after incubation with 0.5% EDTA in phosphate buffered saline at 37°C for 5 min. After the cells were washed with MACS-FACS buffer (2mM EDTA and 3% FBS in phosphate buffered saline), 200 μl MACS-FACS buffer containing 2–5 $\times 10^6$ cells/ml were incubated with 1 μl anti-human Glut2 antibody (H-67, Santa Cruz, Santa Cruz, CA) for 30 min on ice with intermittent agitation. After washing with MACS-FACS buffer, the samples were incubated with 200 μl MACS-FACS buffer containing 2 μl anti-rabbit FITC-conjugated immunoglobulin for 30 min on ice. The cells were washed with ice-cold MACS-FACS buffer, and the cell-bound anti-human Glut2 and FITC-conjugated secondary antibody was determined by BD LSR™ (Becton Dickinson, San Jose, CA) as the fluorescence intensity of FITC. Data collection and statistical analyses were performed with CellQuest software (Becton Dickinson) according to the manufacturer's instructions.

Cell Staining for Gamma-Glutamyl Transpeptidase (gamma-GTP) Activity

We tested gamma-GTP activity because it is a known marker of proximal tubular cells. Cells were cultured on collagen-coated sterile glass slides (Asahi Techno Glass Co. Ltd., Chiba, Japan) at 37°C in a CO_2-incubator for a few days until subconfluent. For gamma-GTP staining, the cells on the chamber glass were fixed with acetone for 2–3 h. After dehydration, the cells were incubated for 5–30 min with staining buffer, which included gamma-glutamyl-4-methoxy-2-naphthylamide and dimethyl sulfoxide, and then with 0.1 N copper sulfate for 2 min. The cells were washed with normal saline and then with water. The stained cells were observed under a microscope.

RESULTS AND DISCUSSION

Purified Renal Cortical Cells

We obtained primary cells from the kidneys of patients in five cases. We tested several isolation methods, (e.g., collagenase or mechanical homogenization) in combination with HBSS containing trypsin and EDTA or HBSS containing trypsin and EDTA alone, but the protocol described in the Materials and Methods section above yielded the best results. Warm ischemia is a concern in studies using viable human samples obtained by surgical operation. To the extent that we tested, we could successfully purify viable cells after up to 68 min of warm ischemia (Table 1).

Next, we identified the origin(s) and characterized the cells. After a few weeks, the cells showed the morphology of uniform epithelial cells, which suggested that the purified cells might have a uniform origin and characters (Fig. 1a). Expression analysis revealed that five kidney-specific genes were present in these cells (Table 2). One of these genes was KL (205978_at). Kuro-o et al. (1997) cloned the mouse KL (*klotho*) gene from a transgenic mouse model with several age-related disorders; the *klotho* gene is named for the Fate in Greek mythology who spins the thread of life. Human KL cDNA clones were isolated by screening human kidney cDNA libraries with a mouse KL cDNA fragment at a low stringency (Matsumura et al. 1998). Interestingly, patients with chronic renal failure (CRF) develop multiple complications reminiscent of the phenotype observed in KL mutant mice. RNase protection, immunoblots, and immunohistochemical analyses demonstrated that KL mRNA expression and protein production were severely reduced in the kidneys of CRF patients (Koh et al. 2001). Koh et al. (2001) proposed that decreased KL expression might be one of the factors underlying the degenerative processes (e.g., arteriosclerosis, osteoporosis, and skin atrophy) observed in patients with CRF.

The function and localization of another kidney-specific gene found in these cells, *SLC17A3*, have not been determined. However, NPT1, a sodium phosphate cotransporter, has 43.5% amino acid sequence identity with SLC17A3 and is the central molecule in the control of renal phosphate excretion (Shibui et al. 1999a, 1999b). Therefore, it is possible that SLC17A3 has a function similar to that of NPT1 and is expressed in kidney.

AQP2 (AQUAPORIN 2) was identified in the cultured cells and encodes a water channel in the apical membrane of the proximal renal tubule and collecting tubule in rats (Fushimi et al. 1993). Its expression in *Xenopus* oocytes markedly increases osmotic water permeability. The function and limited localization

TABLE 2

Expression of kidney-specific genes. Averages and standard deviations of the five selected kidney-specific genes are shown. Since the Affymetrix GeneChip analyzes expression based on the ratio between perfectly matched and mismatched probe readouts, there is no simple cutoff for the expression level. These five kidney-specific genes were calculated as positive

Gene	Expr.	Average	Stndrd Dev.
KL	Pos.	7.8	0.7
SLC17A3	Pos.	11.8	1.9
AQP2	Pos.	12.0	5.9
SLC22A2	Pos.	14.7	3.0
KCNJ1	Pos.	10.4	1.0

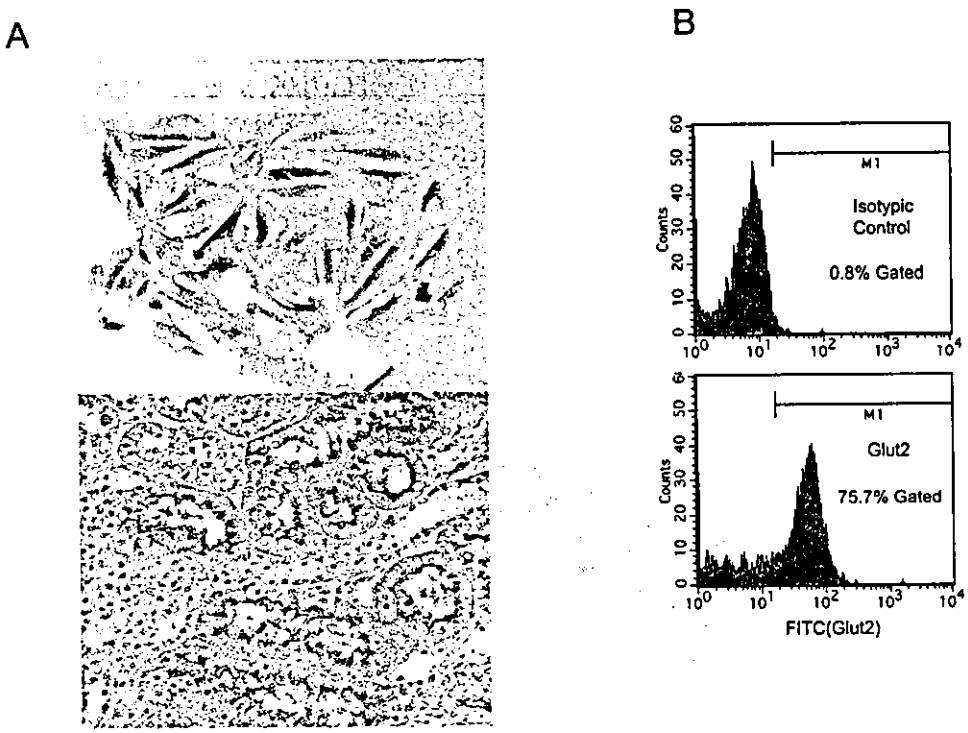

FIG. 1. The gamma-GTP and Glut2 staining of purified cells. In order to determine the origin of the purified cells, we examined two conventional renal tubular markers: gamma-GTP and Glut2. A: The primary cultured cells were positive for gamma-GTP enzymatic activity when stained as described in the Materials and Methods. The lower panel in the figure shows rat renal tubule tissue stained simultaneously to monitor the staining procedure. Since gamma-GTP is generally concentrated along the brush border, the interior of the duct structure shows high enzymatic activity. Conversely, cultured cells did not form a duct under our experimental conditions and moderate enzymatic activity was detected throughout the cells. B: Glut2 expression was analyzed using flow cytometry. As shown in the figure, 75.7% of the cells were positive for Glut2. The average and standard deviation in all cases were 62.6 and 13.3%, respectively.

of AQP2 suggest that it is a vasopressin-regulated water channel. The human homolog and the rat AQP2 share 91% amino acid identity (Sasaki et al. 1994). A defect in this gene is believed to be the basis of the autosomal dominant form of nephrogenic diabetes insipidus (Deen et al. 1994). AQP2 is detectable as both soluble and membrane-bound forms in the urine. In normal subjects, an infusion of desmopressin increases the urinary excretion of AQP2. The administration of vasopressin increased the urinary excretion by AQP2 in five patients with central diabetes insipidus, but not in four patients with X-linked or autosomal nephrogenic diabetes insipidus (Kanno et al. 1995).

SLC22A2 (Solute carrier family 22, member 2), also called *OCT2* (organic cation transporter2), was present in the cultured cells. Polyspecific organic cation transporters are critical for the elimination of many endogenous amines, exogenous drugs, and environmental toxins. Using PCR with primers derived from rat OCT1, human *OCT1* and *OCT2* were cloned from human kidney cortex cDNA (Gorboulev et al. 1997). While human *OCT2* expression was detected by RT-PCR in a few tissues, Northern blots revealed 2.5- and 4.0-kb OCT2 mRNAs mainly in the kidney (Gorboulev et al. 1997). In situ hybridization and immunohistochemistry revealed OCT2 localized at the luminal membrane of the kidney distal tubule (Gorboulev et al. 1997). *Xenopus* oocytes expressing *OCT2* showed increased cation uptake.

The fifth kidney-specific gene we found in the cultured cells was *KCNJ1* (an inwardly rectifying potassium channel,

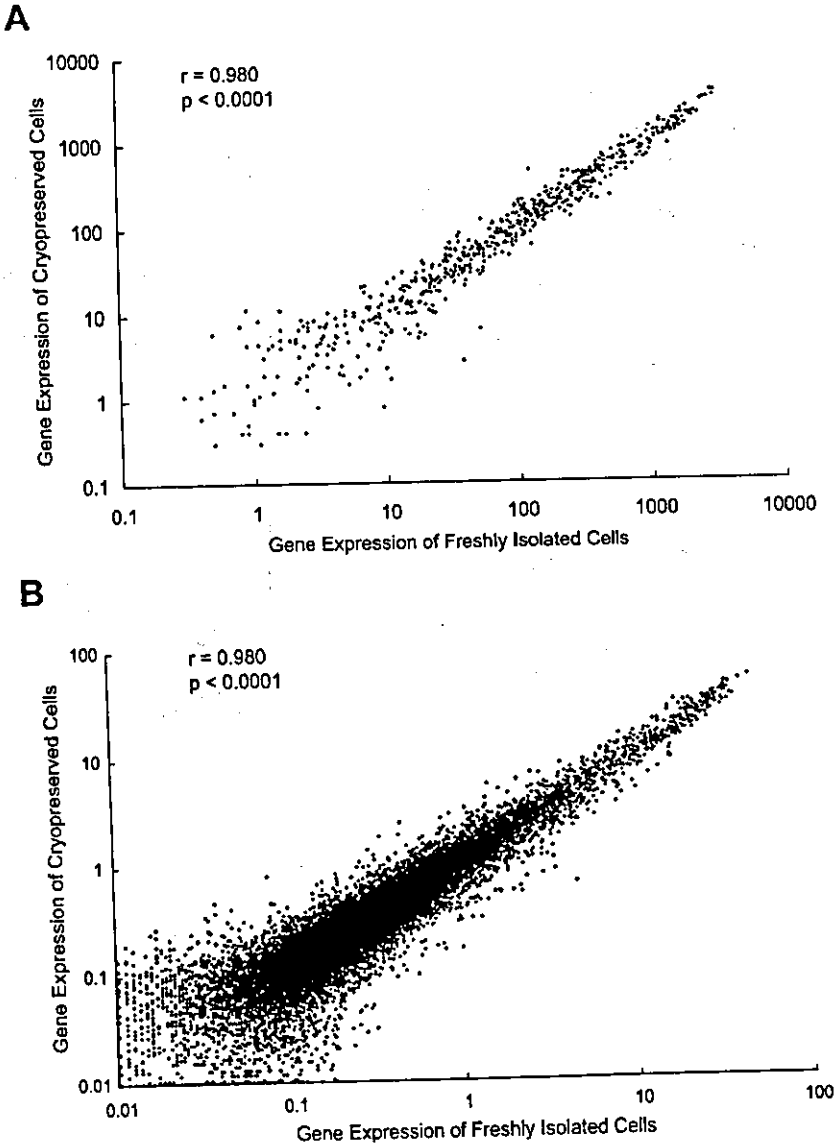

FIG. 2. The effects of cryopreservation. A: The gene expression data for 567 selected genes in cells with and without cryopreservation were compared without normalization ($r = 0.980$; $p < 0.0001$). B: Expression data for more than 39,000 genes were analyzed after normalization, as described in Materials and Methods. The removal of genes with low control signals as determined by the Cross Gene Error Model resulted in 16,147 genes; the expression data are shown here. There were no significant ($p < 0.05$) changes in the expression of any of the 16,147 genes according to nonparametric tests and the Benjamini and Hochberg False Discovery Rate for multiple testing.

subfamily J, member 1), also called *ROMK* (renal outer-medullary potassium channel), *ROMK1*, or *KIR1.1*. Inwardly rectifying potassium channels are important regulators of resting membrane potential and cell excitability. Using targeted mutations in *KCNJ1*, residues important for phosphatidylinositol 4,5-bisphosphate (PIP2) interaction were identified. Mutations in these residues associated with Andersen syndrome and Bartter's syndrome decreased channel-PIP2 interactions (Lopes et al. 2002).

Since the cells were purified from resected kidneys, the expression of kidney-specific genes is not surprising. However, these results indicate that the cells retained the characteristics of renal components after a few weeks in culture and the major population of cells in the cultures was not leukocytes or other passengers that happened to be harvested and concentrated by the artificial purification conditions.

In addition to mRNA expression, we tested two cell type-specific markers: gamma-GTP enzymatic activity and Glut2 antigen expression. At least two commercial suppliers of human proximal renal tubular cells (Cambrex Corporation, Rockland, ME, and TaKaRa Bio Inc., Ohtsu, Japan) use gamma-GTP staining for quality control. Therefore, gamma-GTP activity is regarded as a standard marker of human proximal renal tubular cells among renal tissues; it is also known as a bile duct marker. As shown in Figure 1a, the primary cultured cells were positive for gamma-GTP. Conversely, Glut2 was originally reported to be expressed in human liver, pancreatic islet cells, and the kidney (Fukumoto et al. 1988); its expression is considered to reflect renal tubular origin in kidney (Vestri et al. 2001). Flow cytometric analysis indicated that the major population of cultured cells (75.7%) expressed Glut2 on their cell surface membranes (Fig. 1b). The average and standard deviation of Glut2 expression in all cases were 62.6 and 13.3%, respectively. Based on these findings, the majority of the purified and cultured cells originated from the proximal renal tubule and retained some of the characteristics of the original tissue.

Effects of Cryopreservation

Cells in an initial primary culture can grow to cover the culture dish surface and then be removed and plated at a lower density to form a secondary culture. This process can be repeated several times, but after five to six weeks in culture, the cell morphology changes, and the viability is reduced. Therefore, primary cultures demand timely experiments. However, given the limited number of surgical operations and the limited space and time for experiments, studies with cultured cells would be more feasible if cryopreserved cells could be used. To assess the effect of cryopreservation, we compared freshly prepared primary cultures of renal tubular cells with cells that had been cryopreserved, thawed, and then cultured. First, we compared the expression of housekeeping genes before and after cryopreservation and analyzed the readout from the scanner without normalization. Pearson's correlation coefficient (r) for the 567 housekeeping genes was 0.980, and the p-value was less than 0.0001 (Fig 2). Housekeeping genes are defined as those constitutively expressed to maintain cellular functions and are presumed to be expressed at the same levels as long as cells maintain their essential functions. A comparison of all 39,000 scanned transcripts before and after cryopreservation by nonparametric statistical analysis after normalization showed that no gene expression was changed significantly ($p < 0.05$). Due to the nature of the detection system, there was some variability, especially in weakly expressed genes; this variation was determined to be insignificant using the Cross Gene Error Model. Therefore, essential cell functions appear unchanged by cryopreservation; we will use cryopreserved cells for future experiments.

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We also examined RTKN2 expression in haemopoietic malignancies. It is low or absent in myeloid leukaemias and low in most B cell malignancies, with the exception of high expression of Variant-3 in a case of mantle cell lymphoma. It is also low in CD8+ T-cell malignancies, but extremely high in two cases of CD4+CD8+ T-lymphoblastic lymphoma and in Jurkat cells.

Rho/Rac GTPases are critical in lymphocyte development and function, though relatively little is known about the function and role of effector proteins. We hypothesize that RTKN2 may play an important role in mediating the effects of the Rho GTPases in specific subsets of lymphoid cells, and perhaps in some lymphoproliferative disorders. As RTKN2 was first identified in a cell line resistant to apoptosis, its role in modulating apoptotic signals in lymphoid cells may be of particular importance.

14

EXPRESSION PROFILING OF GENE WITH UPSTREAM AML1 RECOGNITION SEQUENCE IN HEMATOPOIETIC STEM CELL-LIKE FRACTIONS FROM INDIVIDUALS WITH THE M2 SUBTYPE OF HUMAN ACUTE MYELOID LEUKEMIA

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AML1 gene is one of the most frequent targets for chromosomal translocations associated with acute myeloid leukemia. While AML1 is a transcription factor, which is critical for definitive hematopoiesis and leukemogenesis, little has been known about genes under the control of AML1 or the fusion proteins, and mechanism of their regulation.

In this study, we searched whole genome sequence and obtained a table of upstream sequences related to the gene identifiers. Thereafter, we scanned the table to generate a list of genes which have an AML1 recognition sequence in their upstream. We analyzed expression profiles of the listed genes of hematopoietic stem cell-like fractions from individuals with the M2 subtype of human acute myeloid leukemia with (n=5) and without (n=15) the distinct chromosomal translocation, t(8;21). We examined genes whose expression level differed significantly between two groups (Welch ANOVA test, $P<0.01$). A total of 276 genes were identified (151 and 125 genes were upregulated and down-regulated in the t(8;21) positive group, respectively). In this study, most of known genes, which have AML1 recognition sequence at their upstream, including M-CSF receptor, IL-3, T-cell receptor alpha chain, immunoglobulin mu heavy chain, defensin NP-3, neutrophil elastase and myeloperoxidase, were upregulated in t(8;21) positive group, and rest of them including T-cell receptor beta chain and GM-CSF produced controversial results between probe sets within each transcript. These results suggest that most genes previously reported as a target for AML1 transcription factor are upregulated by the existence of the translocation, t(8;21). There are some other well known translocations or fusion genes including t(15;17) translocation, bcr-abl fusion gene, translocation at 11q23, etc. Our strategy to analyze a relationship between these expression data and genes with upstream transcription factor recognition sequences may be useful to compile new knowledge in these fields.

15

IDENTIFICATION OF NEIGHBORHOODS OF GENES EXPRESSED BY HEMATOPOIETIC STEM CELLS

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The gene expression pattern of hematopoietic stem cells (HSC) was examined in baboons. In order to deplete committed hematopoietic progenitors animals received sublethal total body irradiation (250 cGy). All baboons (n=4) became severely leukopenic and thrombocytopenic from day 7-21, however, blood counts returned to normal by day 35-49 indicating the presence of radioresistant HSCs. Marrow CD34+ cells were reduced by 75% from day 7-35 but approached normal numbers by day 85. Colony forming cells (CFC) and cobblestone area-forming cells (CAFC) remained reduced by 95% until day 49. The day 28 CD34+ cells were used for transcriptional profiling using Affymetrix oligonucleotide arrays and an activity center algorithm which permits identification of functionally related families of genes. These CD34+ cells represent a primitive subpopulation of HSC since they were largely incapable of forming hematopoietic colonies or cobblestone areas yet they were responsible for hematological reconstitution. There was downregulation of genes involved with mRNA splicing, protein folding (chaperones), ribosomal function and translational initiation (day 28) which recovered by day 49. Seven families of genes that were overexpressed to a statistically significant degree in day 28 CD34+ cells were: (1) secreted factors and receptors such as IL-11, bone morphogenetic proteins (BMP), BMP receptors, smoothened, ciliary neurotrophic factor (CNTF), stromal derived factor-1 (SDF-1) (2) the complement system proteins (C1qRp); (3) cell adhesion molecules (CD44, ICAM, matrix metalloproteinases); (4) anti-apoptotic genes (bcl-2); (5) DNA damage and repair genes; (6) PIP2 activated K+ channels; (7) adenylate cyclases. In addition, these CD34+ cells expressed genes that are characteristic of a broad variety of non-hematopoietic tissues such as liver (C1qRp, albumin, fibrinogen), brain (CNTF, GABA/Glycine receptor) and marrow stroma (SDF-1, fibronectin, collagen etc). These studies provide insight into the genetic profile of radioresistant HSC and their ability to express genes characteristic of several non-hematopoietic tissues.

16

PERCENT CO-EXPRESSION OF CD52 AND CD20 ANTIGENS AND MEAN FLUORESCENCE INTENSITIES (MFI) ARE IMPORTANT FACTORS IN THE ADDITIVE EFFECT OF ANTIBODY-INDUCED APOPTOSIS IN CHRONIC LYMPHOCYTIC (CLL) AND NON-HODGKIN'S LYMPHOMA (NHL) CELLS: IMPLICATIONS TO ANTIBODY-BASED THERAPY

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CD20 (Rituximab) and CD52 (Campath1-H) antibodies (AB) have been employed in the treatment of NHL and CLL patients (pts), as single agents or in combination with chemotherapy. Rituximab has been effective in NHL pts, whereas Campath 1-H has been effective in CLL pts, however, only limited response was observed for Campath 1-H. We hypothesized that responsiveness to a single or combined AB therapy depends on the extent of expression of the relevant AB for each disease. We therefore determined the extent of apoptosis by CD20, CD52 and CD52+CD20 AB of blood mononuclear cells (MNC) from 10 healthy donors (HD); 17 CLL patients; and 35 bone marrow MNC from NHL pts. Apoptosis (by Annexin V) was correlated to % expression of CD20, CD52, or CD20/CD52 and to the MFI.

CD52 was highly expressed in all pts' samples. However, significant differences were observed in MFI between the 3 groups. MFI were 449 ±48 fluorescence units (FU); 70±22 and 158±81 FU, in HD, NHL and CLL pts, respectively. Mean %CD20 expression was 32±4%; 51.4±15% and 28±8%, respectively and MFI was 62±14 FU;19±8 and 35±14 FU, respectively. Mean co-expression of CD20+CD52 was 26±3%; 31±13%; and 48±21%, respectively.

Mean apoptosis by CD52, CD20 and CD52+CD20 AB for HD ...s 25,000+18,000; 23,000±21,000 and 47,000±23,000 apoptotic cells/ug antibody/24h treatment, respectively. For CLL was 17,000±16,000; 20,000±20,000 and 25,000±22,000, respectively. For NHL was 11,000±7,200; 16,000±7,000 and 13,000±19,000 cells, respectively.

Significant correlation was observed between MFI and apoptosis for CD 52 ($R=0.5$; $p=0.02$) in CLL pts and for CD20 in NHL pts ($R=0.6$; $p=0.004$). The combination of CD20+CD52 significantly increased apoptosis for CLL and NHL pts.

Conclusions: 1. Pretreatment screening for CD52 and CD20 is required for CLL and NHL pts. 2. The combination of CD52 and CD20 is effective in pts with high expression of both antigens.

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RECOMBINANT RETROVIRUSES AS TOOLS FOR IDENTIFYING NEW ONCOGENES ASSOCIATED WITH LYMPHOMA

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The recombinant retrovirus, MoFe2-MuLV, was constructed by replacing the U3 region of Moloney murine leukemia virus (MoMuLV) with homologous sequences from the feline leukemia virus (FeLV-945) LTR. Like other gammaretroviruses, MoMuLV and FeLV induce lymphoma in the natural host through insertional activation of host

Screening of genes specifically activated in the pancreatic juice ductal cells from the patients with pancreatic ductal carcinoma

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Pancreatic ductal carcinoma (PDC) is one of the most intractable human malignancies. Surgical resection of PDC at curable stages is hampered by a lack of sensitive and reliable detection methods. Given that DNA microarray analysis allows the expression of thousands of genes to be monitored simultaneously, it offers a potentially suitable approach to the identification of molecular markers for the clinical diagnosis of PDC. However, a simple comparison between the transcriptomes of normal and cancerous pancreatic tissue is likely to yield misleading pseudopositive data that reflect mainly the different cellular compositions of the specimens. Indeed, a microarray comparison of normal and cancerous tissue identified the *INSULIN* gene as one of the genes whose expression was most specific to normal tissue. To eliminate such a "population-shift" effect, the pancreatic ductal epithelial cells were purified by MUC1-based affinity chromatography from pancreatic juice isolated from both healthy individuals and PDC patients. Analysis of these background-matched samples with DNA microarrays representing 3456 human genes resulted in the identification of candidate genes for PDC-specific markers, including those for *AC133* and carcinoembryonic antigen-related cell adhesion molecule 7 (*CEACAM7*). Specific expression of these genes in the ductal cells of the patients with PDC was confirmed by quantitative real-time polymerase chain reaction analysis. Microarray analysis with purified pancreatic ductal cells has thus provided a basis for the development of a sensitive method for the detection of PDC that relies on pancreatic juice, which is routinely obtained in the clinical setting. (Cancer Sci 2003; 94: 263–270)

Pancreatic carcinoma remains the most intractable disorder among gastroenterological malignancies, with a 5-year survival rate of <5%.[1,2] More than 90% of pancreatic carcinomas are adenocarcinomas of ductal cell origin. In part because of the lack of disease-specific symptoms, individuals at an early stage of pancreatic carcinoma are rarely detected, and the probability of tumors being suitable for surgical resection at the time of discovery is low (10 to 20%). Several improvements in imaging analysis of pancreatic structure have recently been achieved, including endoscopic retrograde cholangiopancreatography (ERCP), magnetic resonance cholangiopancreatography (MRCP), and endoscopic ultrasound examination.[3] However, even with these procedures, it often remains difficult to distinguish pancreatic carcinoma from other disorders such as chronic pancreatitis. Furthermore, these methods usually detect only those pancreatic tumors with a diameter of >5 mm. Given the low 5-year survival rate (20 to 30%) even of individuals with small, resectable tumors, the sensitivity of current technologies is not sufficient to allow detection of pancreatic carcinoma at curable early stages. A cure for this disorder will thus depend on development of an approach that is able to detect tumors at an early stage of carcinogenesis.

Pancreatic ductal carcinoma (PDC) arises from epithelial cells of the pancreatic duct. Carcinoma cells of individuals with this condition are thus shed into pancreatic juice. Analysis of these cells appears a promising approach to the development of a sensitive method for the diagnosis of pancreatic carcinoma. Indeed, molecular biological analysis of these tumor cells has revealed a variety of genetic alterations associated with the pathogenesis of pancreatic carcinoma. Activating point mutations of the K-*RAS* proto-oncogene have thus been identified in >80% of individuals with pancreatic carcinoma,[4] and inactivation of the *TP53* tumor suppressor gene has been detected at a similar frequency.[5] Other mutations have been identified in the genes for p16, DPC4, and DCC.[6–8] However, K-*RAS* mutations are also evident at a relatively high frequency in nonmalignant pancreatic disorders.[9] To date, no molecular markers proven to be specific to carcinoma cells of pancreatic ductal origin have been identified.

DNA microarray analysis allows the simultaneous monitoring of the expression of thousands of genes[10,11] and is therefore a potentially suitable approach to identify PDC-specific genes. The high throughput of this methodology also may be disadvantageous, however. Without careful selection of samples for analysis or data normalization procedures, DNA microarray experiments yield large numbers of pseudopositive and pseudonegative results. In the case of PDC, a simple comparison of pancreatic tissue obtained from individuals with nonmalignant or cancerous conditions would likely not prove informative. Most normal pancreatic tissue comprises exocrine and endocrine cells, with ductal structures constituting only a small proportion of the total volume of the normal pancreas. In contrast, cancerous pancreatic tissue consists mostly of tumor cells that arise from ductal epithelial cells. A comparison between nonmalignant and cancerous tissue would thus likely identify differences between the gene expression profiles of exocrine and endocrine cells and that of tumor cells of ductal cell origin, rather than differences between those of normal and transformed cells of the same origin.

We now show that such a tissue comparison for PDC is indeed uninformative with regard to the identification of tumor-specific genes. To avoid this pitfall, we therefore adopted the strategy of "background-matched population (BAMP) screening,"[12] in which the sample characteristics are matched as closely as possible, with the exception of the feature of interest (in this case, transformation), before microarray analysis. To achieve this goal, we purified pancreatic carcinoma cells and normal ductal cells from pancreatic juice with the use of affinity chromatography based on the shared surface marker MUC1.

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Comparison of these two cell preparations by DNA microarray analysis revealed a group of genes that are potential molecular markers specific to PDC.

Materials and Methods

Preparation of pancreatic ductal cells. The study subjects comprised individuals who were subjected to ERCP and to the collection of pancreatic juice for cytological examination and who gave informed consent. The study was approved by the institutional review boards of Jichi Medical School, Aizu Central Hospital and Hiroshima University. Diagnosis of patients was confirmed on the basis both of the combination of results obtained by ERCP, cytological examination of pancreatic juice, abdominal computed tomography, and measurement of the serum concentration of CA19-9, as well as of follow-up observations. About one-third of each pancreatic juice specimen was used to purify MUC1$^+$ ductal cells. Cells were collected from the pancreatic juice by centrifugation and resuspended in 1 ml of MACS binding buffer [150 m*M* NaCl, 20 m*M* sodium phosphate (pH 7.4), 3% fetal bovine serum, 2 m*M* EDTA]. The cells were then incubated for 30 min at 4°C with 0.5 μg of mouse monoclonal antibodies to MUC1 (Novocastra Laboratories, Newcastle upon Tyne, UK), washed with MACS binding buffer, and mixed with MACS MicroBeads conjugated with antibodies to mouse immunoglobulin G (Miltenyi Biotec, Auburn, CA). The resulting mixture was subjected to chromatography on miniMACS magnetic cell separation columns (Miltenyi Biotec). The eluted MUC1$^+$ cells were divided into aliquots and stored at −80°C. Portions of the unfractionated cells as well as of the isolated MUC1$^+$ cells of each individual were stained with Wright-Giemsa solution to examine the purity of the ductal cell-enriched fractions.

Isolation of RNA and microarray analysis. Total RNA was extracted from the MUC1$^+$ cell preparations with the use of RNAzol B (Tel-Test, Friendswood, TX), and portions (20 μg) of the resulting preparations were subjected to amplification of mRNA with T7 RNA polymerase as described.[13] Biotin-labeled cRNA was synthesized from the amplified RNA (2 μg) with the use of the ExpressChip labeling system (Mergen, San Leandro, CA) and was then subjected to hybridization with microarrays (HO-1 to -3, Mergen) that contain oligonucleotides corresponding to a total of 3456 human genes (for a list of the genes, see http://www.mergen-ltd.com). The microarrays were then incubated consecutively with streptavidin, antibodies to streptavidin, and Cy3-conjugated secondary antibodies (Mergen). Detection and digitization of hybridization signals were performed with a GMS 418 array scanner (Affymetrix, Santa Clara, CA). The fluorescence intensity for each gene was normalized relative to the median fluorescence value for all genes in each array hybridization. Statistical analysis of the data was performed with GeneSpring 5.0 software (Silicon Genetics, Redwood, CA).

Real-time polymerase chain reaction (PCR) analysis. Portions of unamplified cDNA were subjected to the PCR with SYBR Green PCR Core Reagents (PE Applied Biosystems, Foster City, CA). Incorporation of the SYBR Green dye into the PCR products was monitored in real time with an ABI PRISM 7700 sequence detection system (PE Applied Biosystems), thereby allowing determination of the threshold cycle (C_T) at which exponential amplification of PCR products begins. The C_T values for cDNAs corresponding to the *β-actin* gene and target genes were used to calculate the abundance of the target transcripts relative to that of β-actin mRNA. The oligonucleotide primers for PCR were as follows: 5′-CCATCATGAAGTGTGACGTGG-3′ and 5′-GTCCGCCTAGAAGCATTTGCG-3′ for β-actin cDNA, 5′-TCCTGGGACTGTGACTTTCA-3′ and 5′-CTTTTGGTCCAGACCCTCAA-3′ for small ubiquitin-like modifier (SUMO) 1 cDNA, 5′-CCATCATGAAGTGTGACGTGG-3′ and 5′-GTCCGCCTAGAAGCATTTGCG-3′ for carcinoembryonic antigen-related cell adhesion molecule (CEACAM) 7 cDNA, and 5′-GAGACTCAGAACACAACCTACCTG-3′ and 5′-AGCCAGTACTCCAATCATGATGCT-3′ for AC133 cDNA.

Results

Purification of ductal cells from pancreatic juice. Pancreatic juice contains various types of cells, including pancreatic ductal cells, erythrocytes, neutrophils, and lymphocytes (Fig. 1A). Given that the proportions of these cellular components of pancreatic juice vary markedly among individuals, the purification of ductal cells is required for reliable comparison of gene expression profiles. Normal and cancerous pancreatic ductal cells express various mucins. Among those, MUC1 is known to be expressed in both normal and cancerous ductal cells, whereas others, such as MUC3 and MUC5, are differentially expressed in a disease-dependent manner.[14,15] We therefore developed an affinity purification approach for pancreatic ductal cells based on MUC1 as a common surface marker. Cells specifically eluted from a magnetic bead separation column exhibited an epithelial cell-like morphology (Fig. 1B).

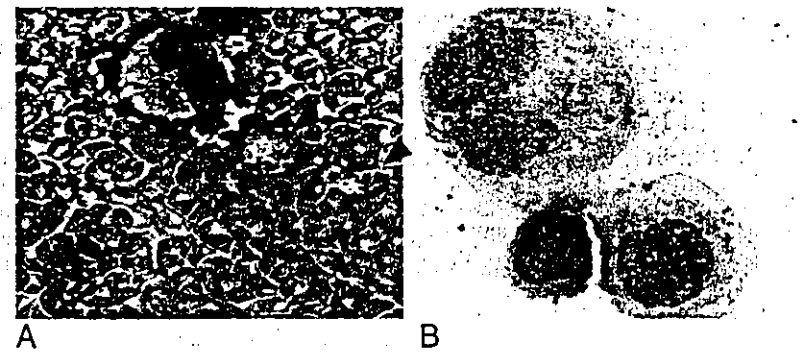
Previous attempts to identify genes whose expression is specific to PDC have often compared the gene expression profiles of normal and cancerous pancreatic tissues.[16] However, such an approach may result in the identification of genes that are differentially expressed between exocrine-endocrine cells and ductal cells. To directly examine if this is the case, we first compared the transcriptomes of surgically resected normal (n=1) and cancerous (n=2) pancreatic tissues by oligonucleotide microarray analysis. The digitized expression intensities for the 3456 human genes examined were normalized relative to the median expression level of all genes in each hybridization; in the case of the cancer tissue, the average expression value for each gene in the two specimens was further calculated. The expression level of every gene was then compared between the normal and cancerous tissues. One of the genes whose expression was most specific for the normal pancreatic tissue was that for insulin; its expression level in normal tissue was 6.869 arbitrary units (U) whereas the averaged value in the cancerous tissues was 1.22 U. Given that insulin is expressed only in islets of Langerhans, this result likely reflects the difference in the proportion of endocrine cells between the samples, not a difference in the number of *INSULIN* gene transcripts per cell between normal and cancer cells.

We next prepared MUC1$^+$ ductal cells from two individuals who were diagnosed as negative for PDC. Microarray analysis of these cells and comparison of the resulting data with those obtained with normal pancreatic tissue also identified the *INSULIN* gene as one of the most differentially expressed genes between the two types of sample; the averaged *INSULIN* expression level in the ductal specimens was 0.495 U, while that in the normal tissue section was 6.869 U.

Given that the proportion of cells of ductal origin would be expected to be markedly increased in cancerous pancreatic tissue compared with that in normal pancreatic tissue, these data support our expectation that a simple comparison of surgically resected specimens of normal and cancerous tissues from the pancreas is not a suitable approach to identify transformation-related genes of the ductal cell lineage.

Gene expression profiles of ductal cells obtained from pancreatic juice. An ideal strategy to identify potential molecular markers specific to PDC would be to compare the transcriptomes of ductal cells isolated from the pancreatic juice of healthy individuals and cancer patients. Any difference identified between the transcriptomes by such screening would thus likely reflect the transformation process, given that both of the samples would be of the same cellular origin. Furthermore, from the

Fig. 1. Purification of pancreatic duct cells from pancreatic juice. (A) Cells isolated by centrifugation from the pancreatic juice of an individual with PDC were subjected to Wright-Giemsa staining (magnification, 100×). In addition to cells of epithelial origin, both red blood cells and neutrophils (arrowheads) are apparent. (B) Cells separated from the pancreatic juice of the same individual with PDC were subjected to chromatography on a MUC1-based affinity column. Cells specifically eluted from the column were then subjected to Wright-Giemsa staining (magnification, 200×). Some of the eluted cells exhibited a cancer-specific aberrant phenotype (large nuclei with fine chromatin structure).



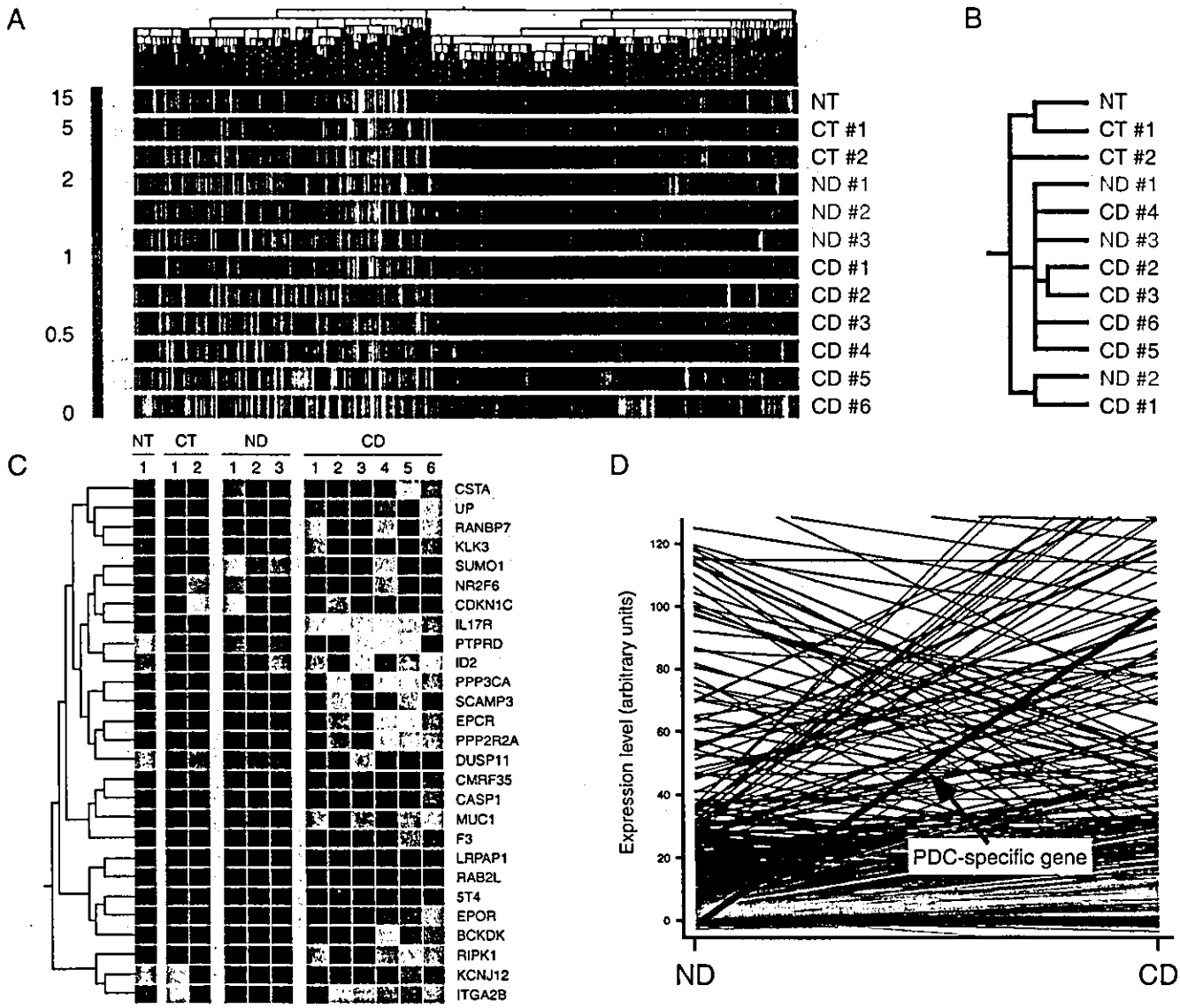


Fig. 2. (A) Hierarchical clustering of 3456 genes based on their expression profiles in pancreatic tissue specimens from one normal individual (NT) and two PDC patients (CT #1 and #2) as well as in MUC1+ ductal cells obtained from three normal individuals (ND #1–3) and six cancer patients (CD #1–6). Each column represents a single gene on the microarray, and each row corresponds to a different subject. The normalized fluorescence intensity for each gene is shown color-coded as indicated at the left. (B) Two-way clustering analysis of the transcriptomes shown in (A) was performed to assess statistically the similarity among the samples from the different subjects and to generate a subject dendrogram. (C) Hierarchical clustering of the "disease-dependent" genes. Expression intensities are shown color-coded according to the scale in (A). Gene symbols are indicated at the right. (D) Comparison of the expression levels of 3456 human genes between normal and cancerous ductal cells. The normalized value for the expression level of each gene was averaged for three normal ductal cell specimens and was compared with the corresponding value obtained with six cancerous ductal cell samples. Each line corresponds to a single gene on the array and is presented color-coded according to the expression level in the normal tissue according to the scale shown in (A). The line for a hypothetical "PDC-specific gene" is indicated in blue.

point of view of clinical application, this BAMP screening approach also appears desirable. The identification of *bona fide* cancer-specific genes would thus allow development of a sensitive method for the diagnosis of PDC based on reverse transcription and PCR (RT-PCR) analysis of cells isolated from pancreatic juice, which can be obtained during the ERCP procedure.

In an attempt to realize this goal, we compared the expression profiles of 3456 genes among one specimen of normal pancreatic tissue (NT), two specimens of cancerous pancreatic tissue (CT #1 and #2), three normal ductal cell preparations (ND #1 to #3), and six ductal cell preparations obtained from PDC patients (CD #1 to #6). The clinical information is summarized in Table 1 for the PDC patients who provided pancreatic juice. All of the ductal cell preparations of the CD patients were cytologically diagnosed to contain "class IV" cells, the proportion of which is also shown in the table. Since all CD patients already had tumor invasion into either the splenic artery or the portal vein as judged by angiography, none of them was suitable for surgical operation. Therefore, we do not have any pathological data of pancreatic tissues for any of the PDC patients in Table 1. All CD patients died within 12 months after diagnostic procedures.

Table 1. Clinical characteristics of the patients with PDC

Patient ID	Sex	Age (yr)	Liver metastasis	SA or PV invasion	Proportion of class IV cells (%)
CD #1	M	71	–	+	6.4
CD #2	F	61	–	+	45.3
CD #3	F	82	–	+	4.6
CD #4	F	68	+	+	4.2
CD #5	F	73	+	+	12.6
CD #6	F	71	–	+	33.4

M, male; F, female; yr, year; SA, splenic artery; PV, portal vein.

The ND #1–3 individuals were subjected to ERCP procedure due to a slight elevation in blood amylase level or to the echographic finding of dilation of the pancreatic duct. However, ERCP examination could detect no anomaly in their ductal structure. These individuals were also negative for PDC in cytological analysis of pancreatic juice, and are still healthy after >12 months of observation.

The gene expression profiles of each sample were subjected to clustering analysis in order to generate a dendrogram, or "gene tree," in which genes with similar expression profiles are clustered together (Fig. 2A). Such analysis revealed that the patterns of gene expression of ND #1 and #3 were similar to those of CD #2 to #6. However, despite this overall similarity, significant differences between these two types of sample were apparent, some of which might reflect the carcinogenic process.

To statistically analyze the similarity of transcriptomes among the samples, we performed two-way clustering analysis[17] to generate a "subject tree," in which samples with similar transcriptomes are grouped together (Fig. 2B). All ductal cell samples (ND and CD) were clustered in two major branches, separated from the tissue samples, which indicates that the transcriptomes of the cancerous ductal cells were more similar to those of the normal ductal cells than they were to those of the cancer tissue specimens. The transcriptomes of ductal cell samples from cancer patients #2 and #3 exhibited the greatest similarity.

Potential molecular markers for PDC. Our data suggest that a direct comparison between normal and cancerous ductal cells would be a suitable means to efficiently identify the PDC-specific

Table 2. Expression level of the disease-dependent genes

Gene symbol	GenBank #	NT	CT #1	CT #2	ND #1	ND #2	ND #3	CD #1	CD #2	CD #3	CD #4	CD #5	CD #6
DUSP11	AF023917	3.833	0.924	1.387	0.358	1.056	5.102	−0.056	22.841	3.826	22.211	30.331	15.227
KCNJ12	L36069	4.157	2.096	10.149	−0.166	−0.422	−0.284	0.356	0.169	0.087	−0.164	1.106	0.168
ITGA2B	J02764	7.498	3.165	9.311	0.322	0.261	0.773	0.730	2.072	1.668	1.284	1.000	1.340
CSTA	X05978	−0.962	−0.905	0.638	1.100	−1.487	−0.366	12.865	7.687	5.928	−0.164	2.528	0.809
UP	X90858	0.637	−0.182	−0.378	−0.447	−0.092	0.062	0.977	0.021	0.209	1.000	0.056	2.205
SUMO1	U61397	−1.043	0.557	−0.933	2.703	0.841	1.618	0.196	10.385	11.219	1.952	16.324	8.469
PPP3CA	M29550	−0.511	−0.926	−0.938	−0.747	−1.191	−0.214	−1.587	2.656	−0.427	2.267	3.231	1.701
PTPRD	L38929	2.149	0.333	0.694	1.310	0.149	1.140	−0.104	7.369	3.223	2.997	2.793	6.908
LRPAP1	M63959	−0.387	−0.790	−0.541	−0.805	−0.595	−0.265	−0.339	0.399	−0.352	−0.291	0.062	0.759
RANBP7	AF098799	0.351	0.256	−0.741	−0.389	−0.303	0.018	3.466	0.142	−0.395	1.834	0.673	1.968
EPCR	L35545	0.440	−0.558	−0.393	−0.483	0.581	−0.050	0.194	1.277	0.125	3.373	2.891	1.384
RAB2L	U68142	−0.638	−1.144	−0.829	−0.856	−1.214	−1.341	−1.507	0.226	−0.848	−0.014	0.803	−0.100
5T4	Z29083	−0.412	−0.913	−0.338	−0.710	−0.724	−0.104	0.221	−0.136	−0.492	0.897	0.102	1.044
PPP2R2A	M64929	0.501	−0.858	−0.442	−0.501	−0.376	−0.194	−0.448	1.272	−0.380	2.474	3.584	1.677
EPOR	M34986	−0.008	−1.112	−0.744	−0.692	−0.671	−0.143	−0.093	−0.246	−0.438	0.857	0.898	1.727
BCKDK	AF026548	−0.202	−1.266	−0.713	−1.002	−0.900	−0.260	−0.876	0.129	−0.256	2.188	0.852	1.089
ID2	M97796	1.247	−0.076	0.389	−0.531	0.572	1.695	1.721	6.419	3.258	0.659	4.139	2.867
NR2F6	X12794	−1.279	−0.502	1.768	1.270	−0.713	5.963	−0.921	20.658	30.415	1.672	28.746	23.043
RIPK1	U50062	0.095	8.471	−0.001	0.422	0.367	0.190	1.819	0.450	0.032	1.404	2.050	1.607
KLK3	M26663	0.384	0.192	0.780	−0.038	−0.095	−0.066	1.653	0.276	0.041	0.556	0.277	0.937
CMRF35	X66171	−1.406	−0.541	−0.824	−0.171	−0.327	−0.129	0.521	0.076	−0.024	−0.190	0.076	0.579
SCAMP3	AF005039	−0.362	−1.002	−0.628	−0.493	−1.060	−0.270	−0.734	1.936	0.099	0.781	1.827	0.410
CASP1	U13698	−0.808	−0.132	−1.027	0.025	−0.113	−0.070	0.300	−0.009	0.123	0.279	0.187	0.726
F3	J02931	−1.096	−0.562	−0.286	−0.261	−0.607	−0.325	0.364	0.349	0.038	−0.276	1.530	0.388
MUC1	J05581	−2.454	0.398	−0.759	0.771	0.330	0.070	1.775	0.534	1.387	0.863	1.642	1.925
CDKN1C	U22398	0.057	−0.434	3.034	2.572	−1.003	−0.375	−0.138	4.131	10.992	9.788	8.131	11.543
IL17R	U58917	0.707	0.647	0.432	0.765	−0.138	0.263	2.322	2.692	3.360	2.452	3.319	5.122

Expression level of the "disease-dependent" genes is shown in arbitrary units (U). Gene symbol as well as GenBank accession number (#) is indicated for each gene.

transcriptome changes while keeping pseudo-positive data minimum. To identify *bona fide* PDC-specific genes from the array data, we here took two approaches.

First, expression levels of 3456 genes were compared between ND and CD sample types by Welch ANOVA test. Twenty-seven genes were thus identified, whose expression levels were statistically significantly different in the two types ($P<0.05$). A dendrogram of such disease-dependent genes is shown in Fig. 2C. Many genes in the list, including those for *SUMO1* (GenBank accession no. U61397) and dual specificity phosphatase (*DUSP*) *11* (GenBank accession no. AF023917), were inducibly expressed in PDC cells. Like ubiquitin, SUMO1 functions as a protein "tag," transfer of which is mediated by a SUMO E_3 ligase. In contrast to ubiquitin, however, modification with SUMO1 not only drives the substrates into a proteasome pathway, but has a pleiotropic effect on the substrates, such as protection against proteolysis, induction of apoptosis, and regulation of substrate function.[18, 19] The *in vivo* role of SUMO1 is thus likely to be context-dependent, and it is an interesting question whether increased SUMO-tagging has a transforming or anti-apoptotic activity in PDC cells. The array data for these "disease-dependent" genes are shown in detail in Table 2. These genes would be good candidates to be included in custom-made DNA microarrays specialized for the diagnosis of PDC.

However, there is a caveat that this type of comparison may isolate genes whose absolute expression levels may be negligibly low. Actually, fifteen out of twenty-seven genes in Table 2 did not have expression levels of more than 3.0 U in any ductal cell preparation.

Therefore, we also tried another approach to select PDC-specific genes. The mean expression value of each gene was calculated for the ND or CD sample type, and the differences in the resulting values are represented in Fig. 2D. To identify genes whose mean expression values were induced only in the cancerous ductal cells, with the use of GeneSpring software, we searched for genes whose expression profiles were statistically similar, with a minimum correlation of 0.99, to that of a hypothetical "PDC-specific gene" (blue line in Fig. 2D) that exhibits a mean expression level of 0.0 U in the ND group and 100.0 U in the CD group. Taking the 188 genes thus identified, we then applied the criteria that the gene expression value should be (1) <3.0 U in all NT/ND samples and (2) ≥19.0 U in at least one of the CD samples. Thirty-one genes were finally identified to be "PDC-specific" (Table 3). Through this approach, we tried to extract genes whose expression levels were negligible in all normal pancreatic specimens, but significantly high in at least a part of the cancerous ones. They may be good candidates for molecular markers to develop PCR-based diagnostic tests for PDC.

These potential PDC-specific markers include the genes for FYN protein tyrosine kinase (*FYN*; GenBank accession no. M14676/M14333); insulin-like growth factor binding protein 1 (*IGFBP1*; Y00856); collagen, type I, alpha 1 (*COL1A1*; Z74615); calpain, large polypeptide L2 (*CAPN2*; M23254); eukaryotic translation elongation factor 1 beta 2 (*EEF1B2*; X60489); *AC133* (AF027208) and *CEACAM7* (X98311).

Table 3. Expression level of the PDC-specific genes

Gene symbol	GenBank #	NT	CT #1	CT #2	ND #1	ND #2	ND #3	CD #1	CD #2	CD #3	CD #4	CD #5	CD #6
FYN	M14676	−0.317	−0.982	−0.671	2.198	−1.203	−0.470	3.000	1.327	−0.029	1.435	27.266	13.246
FYN	M14333	−0.642	−0.874	−1.010	1.131	−1.229	0.721	2.771	1.340	−0.032	1.796	28.936	12.357
RGR	U14910	0.174	−1.073	−0.249	−0.795	−0.439	2.719	6.824	1.531	2.929	2.021	1.863	27.514
IGFBP1	Y00856	−0.504	−0.880	−0.671	−0.486	−1.098	−0.461	−1.244	77.812	−0.820	−0.772	52.414	8.442
DUSP1	X68277	0.062	1.488	0.701	1.412	−0.682	1.444	0.782	15.259	0.723	2.374	21.301	2.082
IL1RN	X52015	−0.288	−0.879	−0.489	2.410	−0.874	3.110	−1.102	75.070	1.968	4.507	10.914	5.436
HSJ2	L08069	−0.882	−1.352	−0.752	0.909	−1.269	2.420	−0.992	−0.165	−0.712	0.438	27.059	2.441
APCS	X04608	−0.740	−0.471	−0.212	1.062	−1.140	0.038	−1.127	22.176	6.088	19.942	0.012	−0.482
GTF2A1	U21242	−0.181	−0.754	−0.090	2.395	−1.085	2.376	−0.930	12.423	1.016	−0.140	28.966	7.717
GTF2F2	X16901	−0.697	−1.348	−0.356	−0.392	−0.231	0.523	2.121	3.955	0.329	0.655	20.209	3.319
IRF4	U52682	0.269	−1.213	−0.509	−0.835	0.141	−0.073	−0.561	0.202	−0.103	−0.236	25.817	0.570
POU2AF1	Z49194	−0.698	−1.264	−0.461	−1.069	−0.643	0.090	−0.642	2.758	−0.109	−0.623	47.368	1.189
SNRPG	X85373	−0.374	−1.027	−0.827	2.082	−1.095	3.342	−0.053	11.652	−0.214	−0.355	33.614	7.384
SLC16A3	U81800	0.463	−0.588	0.296	0.712	−0.913	0.332	−0.841	4.999	0.359	−0.222	21.756	0.495
H1F5	X83509	−0.092	−0.239	0.118	1.197	−1.118	0.418	−0.886	1.573	0.166	−0.593	23.560	0.481
GTF2B	M76766	−0.893	−0.670	−0.816	0.824	−1.126	0.467	−1.093	32.156	0.339	4.622	34.587	23.964
SNRPC	M18465	−0.149	−1.184	−0.542	−0.835	−0.403	0.547	−0.610	11.491	0.819	1.282	22.521	1.158
ECM1	U68186	−1.969	−0.882	−0.971	0.048	−0.921	−0.454	−1.218	15.501	0.389	−0.425	50.772	0.072
KLK6	AF013988	−4.069	−1.028	−3.372	−0.121	−1.441	−0.372	−1.324	26.647	0.122	−0.715	60.203	3.603
COL1A1	Z74615	−2.193	0.018	98.459	1.133	−1.197	−0.466	−1.134	10.098	13.086	3.584	131.260	10.451
CAPN2	M23254	−0.996	−1.063	0.483	1.178	−1.387	−0.030	−1.320	12.394	6.932	−0.419	20.623	0.570
RGS5	AB008109	0.026	−0.950	−0.386	−0.837	−1.315	−0.458	−1.093	0.814	0.140	−0.675	0.000	52.133
EEF1B2	X60489	−0.287	−0.713	0.037	1.154	−1.509	2.050	−1.269	9.485	20.314	−0.133	30.121	0.971
F7	M13232	−1.686	−1.055	−0.909	−0.500	−1.476	−0.512	−1.363	−0.254	−0.331	−0.770	−0.353	22.485
CEACAM7	X98311	−0.065	−0.802	−0.728	−0.247	−1.036	−0.085	−0.900	10.468	22.096	0.021	−0.244	−0.011
CAMLG	U18242	−0.703	−0.916	0.285	0.582	−1.435	0.092	−1.238	1.829	1.801	−0.431	22.154	0.461
APOA4	X13629	−3.473	−1.048	−0.974	0.105	−0.835	−0.514	−1.278	−0.240	−0.357	−0.704	−0.324	37.780
GAPDH	M33197	0.142	−0.765	1.590	2.756	−0.920	2.505	−0.854	3.372	3.908	2.241	4.070	21.745
MYBPC3	X84075	−1.825	−1.043	−1.217	−0.475	−1.472	−0.496	−1.283	−0.102	−0.307	−0.772	−0.355	521.712
AC133	AF027208	−0.741	−0.970	−0.666	0.071	−1.527	−0.264	−1.398	19.820	8.152	−0.460	0.546	−0.364
APOBEC1	L25877	−0.740	1.771	−1.215	2.591	1.060	0.982	0.979	9.583	2.584	2.151	12.211	24.971
EIF3S6	U62962	−0.703	0.244	−0.123	2.402	0.053	2.350	−0.626	11.403	28.308	0.607	11.966	6.648

Expression intensities of the "PDC-specific" genes are shown in arbitrary units (U). Gene symbol as well as GenBank accession number (#) is indicated for each gene. Two distinct oligonucleotides were spotted on the array for the *FYN* gene.

Quantitation of mRNA for potential PDC marker genes. Finally, we confirmed the expression of three of the potential PDC marker genes by real-time PCR. Unamplified cDNA was prepared from MUC1$^+$ ductal cells obtained from 8 normal individuals and 10 patients with PDC and was subjected to PCR with primers specific for *β-actin*, *SUMO1*, *AC133*, or *CEACAM7* genes. The amount of each PCR product was monitored in real time, thereby allowing determination of the corresponding C_T values. The abundance of SUMO1, AC133 and CEACAM7 mRNAs was then calculated relative to that of β-actin mRNA.

Consistent with the microarray data, expression of *SUMO1*, *AC133* and *CEACAM7* genes was highly specific to PDC; in particular, the latter two genes were almost silent in normal ductal cells (Fig. 3). These genes are thus candidates for PDC-specific markers. The expression levels of the *SUMO1*, *AC133* and *CEACAM7* genes varied among the cancer specimens, as might be expected from nonuniformity of the transformation process in pancreatic ductal cells.

Discussion

We have demonstrated that a simple comparison of transcriptomes between normal and cancerous tissue of the pancreas is not a suitable approach for characterization of the transformation process. In contrast, through screening with isolated ductal cells derived from normal and carcinoma tissue, we were able to identify a group of genes that may prove helpful in the diagnosis of PDC.

In addition to the purification of PDC cells from pancreatic juice, there is another way to isolate PDC cells, i.e., the laser capture microdissection (LCM) method.[20] Although, with LCM, it is theoretically possible to purify any cell type in a given tissue, fixation and staining procedures of the specimens prior to LCM may severely impair the quality of mRNA in the samples. Furthermore, it would be a demanding task to pick up 10^5–10^6 cells by LCM. Small number of cells obtained by LCM often requires multiple rounds of mRNA amplification before microarray experiments, making the data evaluation more difficult. Therefore, purification of intact and live PDC cells through pancreatic juice would be advantageous for obtaining high-quality mRNA and good reproducibility in transcriptome analysis.

Moreover, as with our CD cases (see Table 1), it is rare to find patients with PDC at early stages competent for surgical resection. Therefore, it may be difficult to complete a large-scale clinical screening of PDC tissue sections. In contrast, screening of hundreds of "pancreatic juice" samples is a realistic project.

For the improvement of PDC treatment, it is essential to detect PDC at the stage of curable carcinoma *in situ*. We assume that the direct analysis of PDC cell-containing specimens would be the most sensitive way to detect PDC, and, in a routine clinical setting, pancreatic juice is the only source to obtain PDC cells. These are the reasons why we attempted to develop a novel PDC diagnosis procedure based on pancreatic juice.

As expected, pancreatic juice contained various amounts of non-ductal cells (mainly blood cells). Therefore, we had first to enrich pancreatic ductal cells from the juice by means of an affinity column directed toward MUC1. It was interesting to find *MUC1* in the "disease-dependent" gene list (Fig. 2C and Table 2). In our analysis, *MUC1* expression was induced in cancerous ductal cells (1.35 U±0.547; mean value±SD) compared to normal ductal cells (0.390 U±0.354). An increase in mRNA[21] or protein[22] level of MUC1 in PDC cells has been also reported. Low yet significant expression of MUC1 in our ductal cell specimens also argues that the MUC1-column eluents did contain pancreatic ductal cells, since MUC1 is expressed only by epithelial cells, not by blood cells.

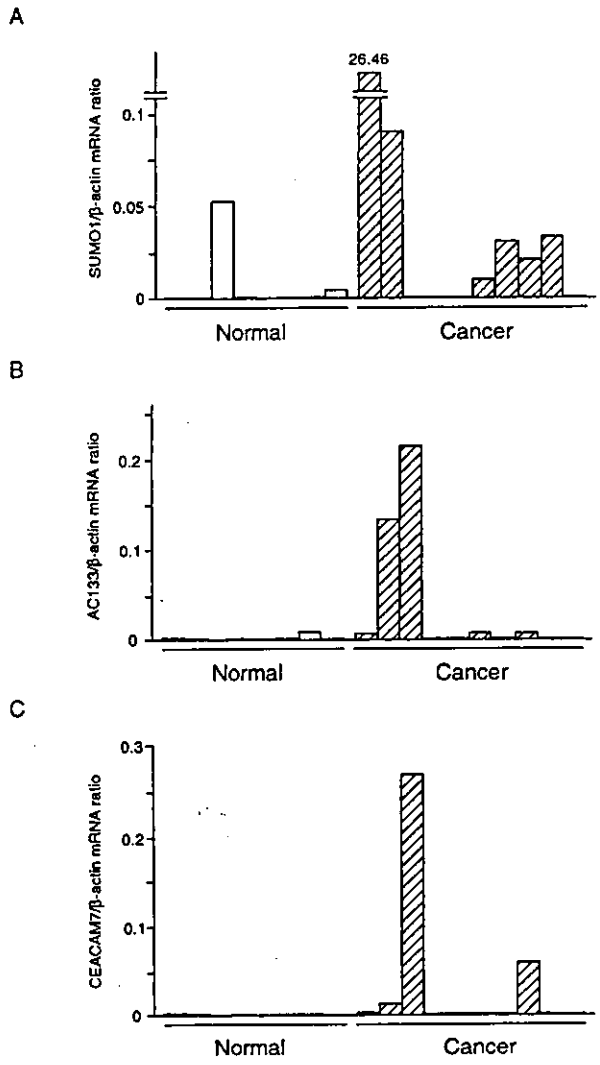


Fig. 3. Quantitation of *SUMO1*, *AC133* and *CEACAM7* gene transcripts in MUC1$^+$ ductal cells. Complementary DNA prepared from pancreatic ductal cells of 8 normal individuals and 10 PDC patients was subjected to real-time PCR with primers specific for *SUMO1* (A), *AC133* (B), *CEACAM7* (C), or *β-actin* genes. The ratio of the abundance of the target transcripts to that of β-actin mRNA was calculated as 2^n, where n is the C_T value for β-actin cDNA minus the C_T value of the target cDNA.

Our MUC1-based purification system does not discriminate normal ductal cells from malignant ones. Therefore, ductal cells isolated from PDC patients (such as CD #1–6) should be a mixture of normal ductal cells and PDC ones. Since there are no cell membrane proteins known to be specifically expressed in PDC, it is currently impossible to directly purify PDC cells from pancreatic juice. Rather, we here aimed to develop a sensitive method to detect a trace amounts of PDC cells shed into pancreatic juice.

For this purpose, there may be two distinct types of molecular markers. One type is useful in statistically distinguishing normal and cancerous ductal cell types. Such analyses choose genes whose expression level has a small deviation, and, therefore, may be suitable to construct custom-made DNA microarrays. Genes of the other type would be active only in cancerous ductal cells, but strictly absent in normal ones. These genes would be good candidates for the target transcripts used in RT-PCR-based detection systems. Expression levels of such genes in cancerous cells may have a relatively large SD, and such genes may not be expressed in all cancerous cells. However, if