Fig. 3

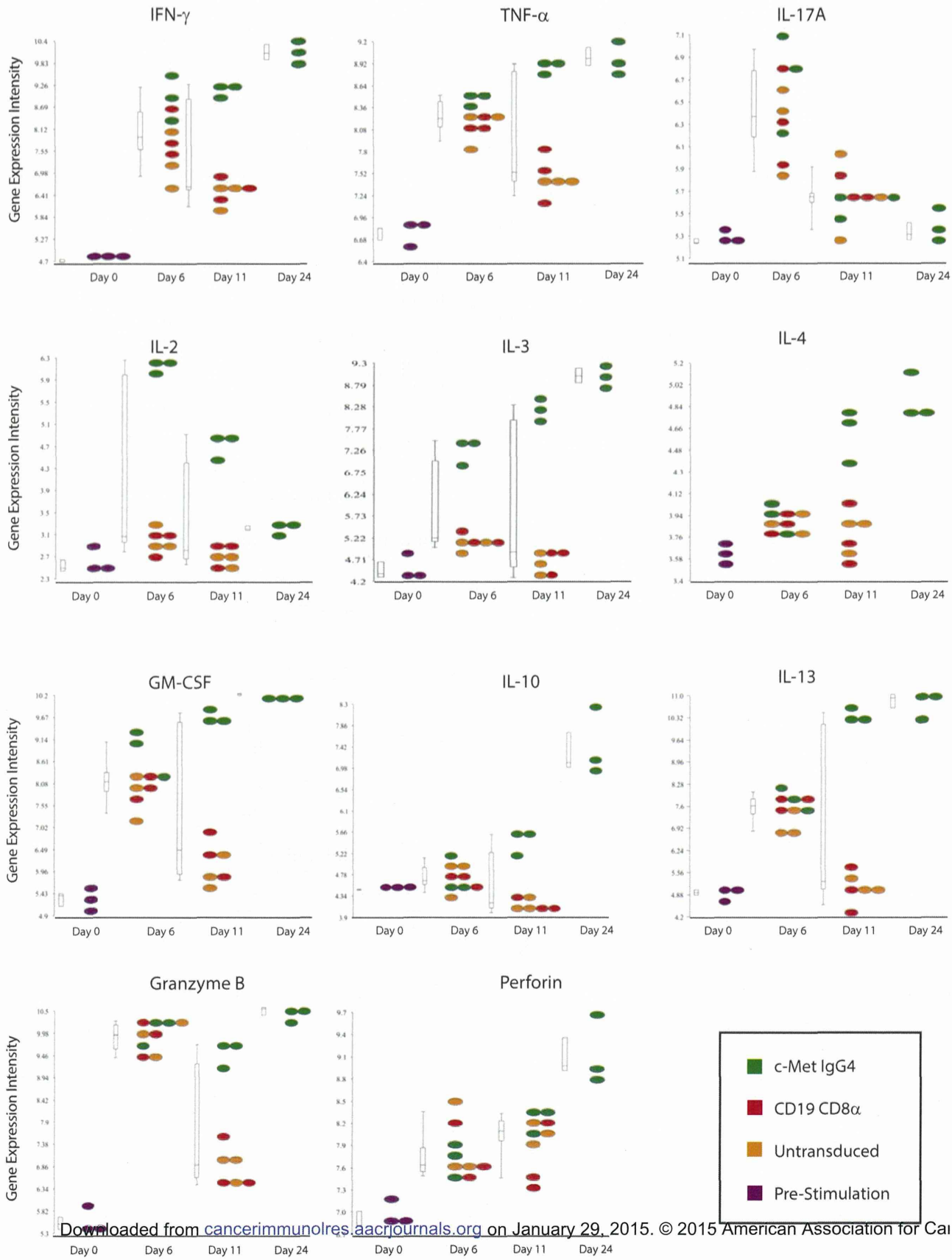


Fig. 4

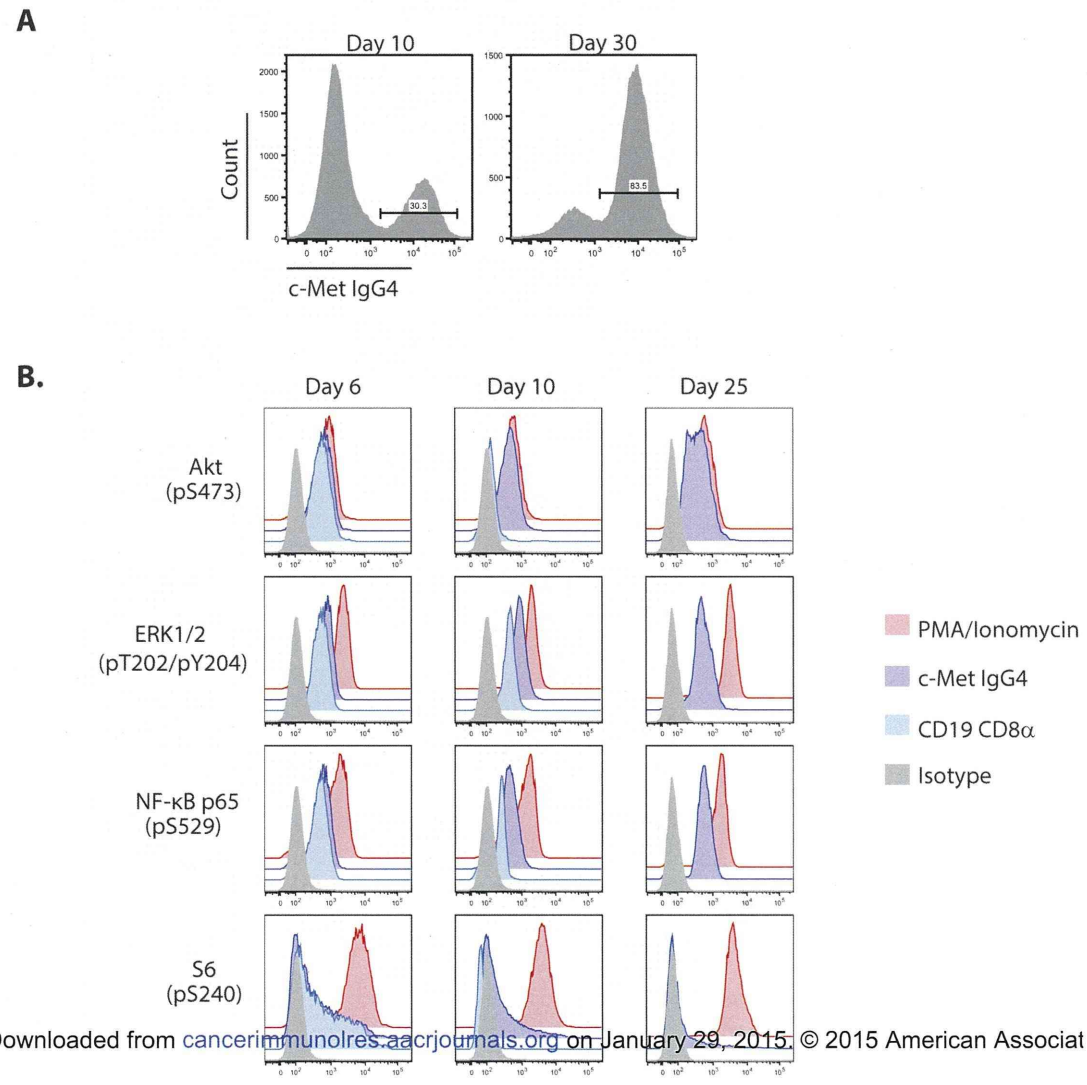


Fig. 5

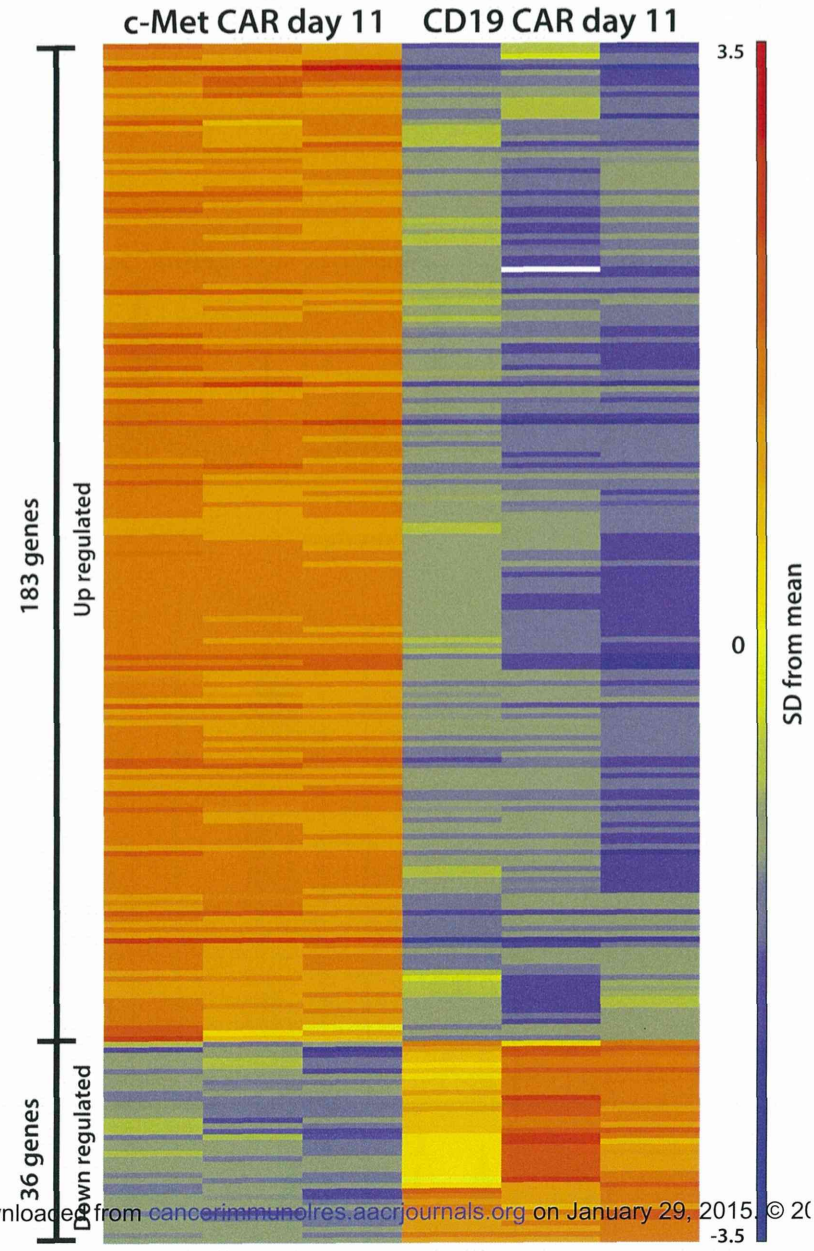
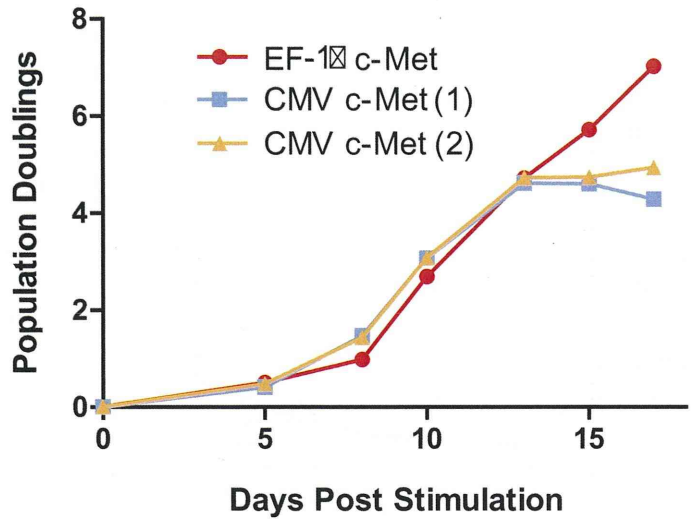
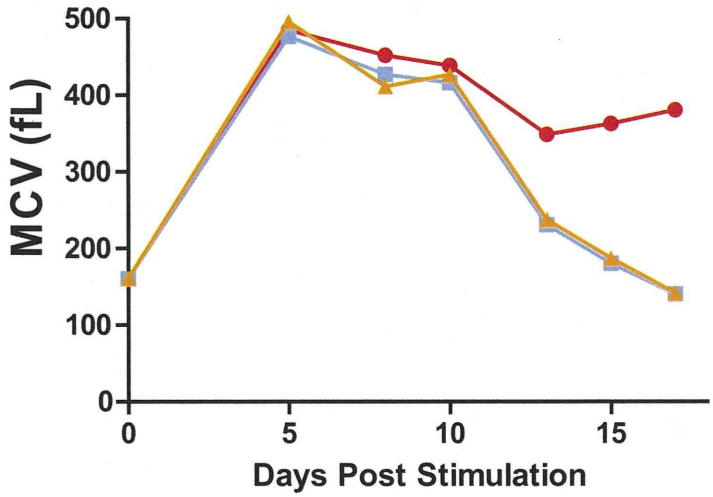


Fig. 6 A,B,C

A



B



C

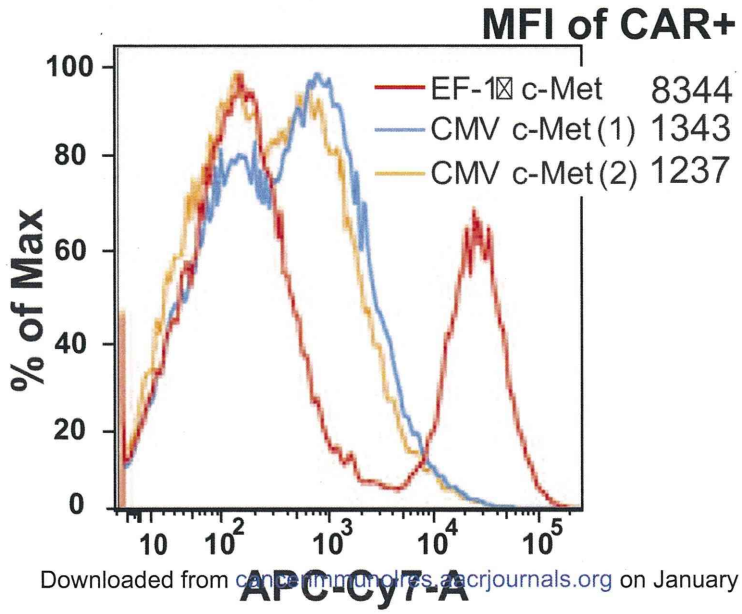
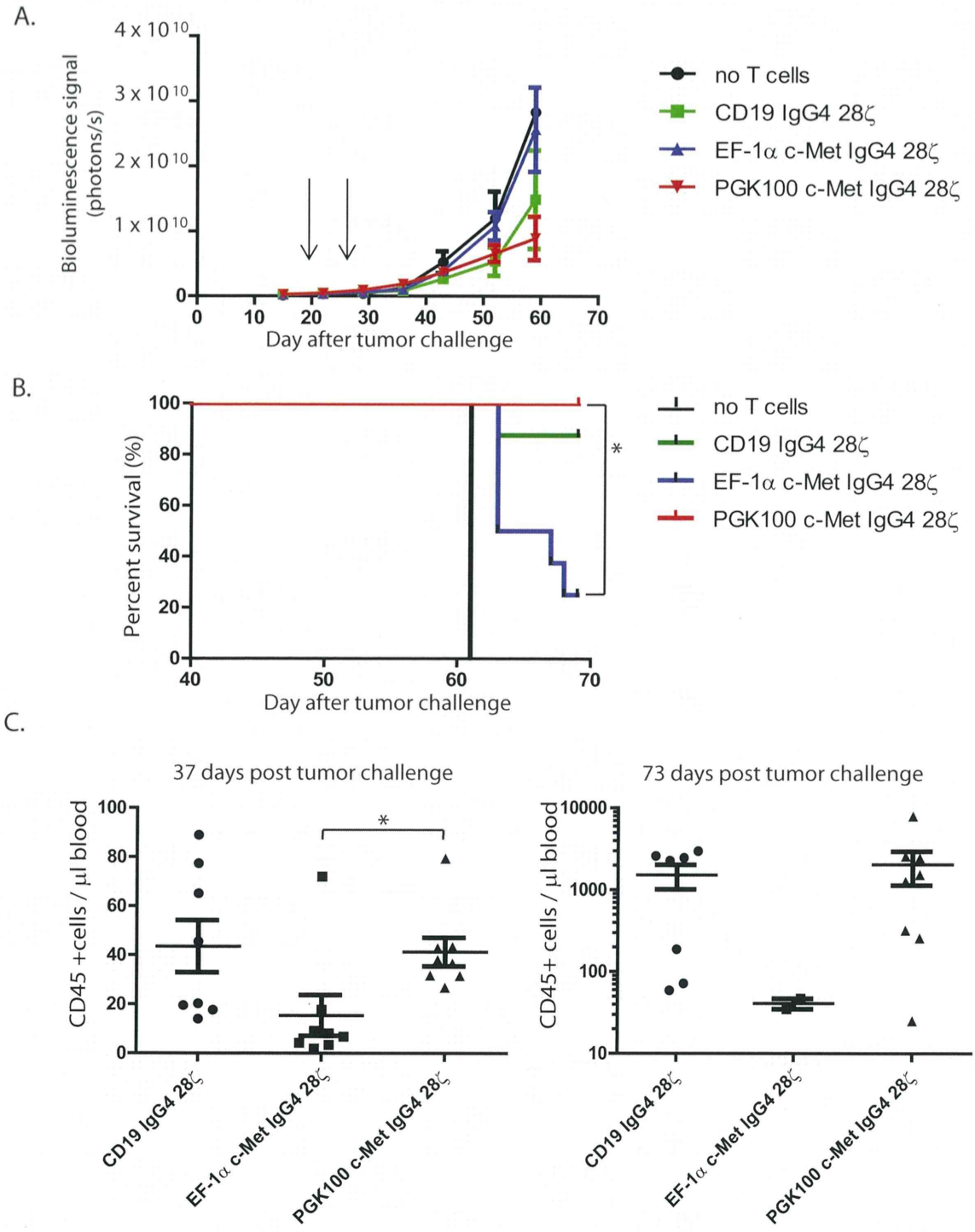


Fig. 7A-C



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Unique expression features of cancer-type organic anion transporting polypeptide 1B3 mRNA expression in human colon and lung cancers

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Abstract

Background: We have previously identified the cancer-type organic anion transporting polypeptide 1B3 (Ct-OATP1B3) mRNA in several human colon and lung cancer tissues. Ct-OATP1B3 is a variant of the liver-type OATP1B3 (Lt-OATP1B3) mRNA, which is a hepatocyte plasma membrane transporter with broad substrate specificity. However, in cancer tissues, both the detailed characteristics of Ct-OATP1B3 mRNA expression and its biological functions remain unclear. With this point in mind, we sought to characterize Ct-OATP1B3 mRNA expression in colon and lung cancer tissues. In addition, we attempted to obtain functional implication of Ct-OATP1B3 in cancer cells.

Methods: Matched pairs of cancer and normal tissues were collected from 39 colon cancer and 28 lung cancer patients. The OATP1B3 mRNA expression levels in each of these tissues were separately determined by quantitative real-time polymerase chain reaction. Mann–Whitney U test and Fisher's exact test were used in statistical analysis. The Ct-OATP1B3 functional expression in colon cancer cells was then examined by Western blotting and transport analyses.

Results: Ct-OATP1B3 mRNA, but not Lt-OATP1B3 mRNA, was abundantly expressed in colon cancer tissues at a higher detection frequency (87.2%) than that of the adjacent normal tissues (2.6%). Furthermore, it was found that Ct-OATP1B3 mRNA expression was often detected in early colon cancer stages (88.9%, n = 18), and that its expression was associated with well-differentiated colon cancer statuses. On the other hand, Ct-OATP1B3 mRNA also showed a predominant and cancer-associated expression profile in lung tissues, although at frequencies and expression levels that were lower than those obtained from colon cancer. As for attempts to clarify the Ct-OATP1B3 functions, neither protein expression nor transport activity could be observed in any of the cell lines examined.

Conclusions: Based on the unique characteristics of the Ct-OATP1B3 mRNA expression profile identified in this study, Ct-OATP1B3 mRNA can be expected to become a biomarker candidate for use in colon (and lung) cancer diagnosis. Simultaneously, our results advance the possibility that Ct-OATP1B3 might play yet unidentified roles, in addition to transporter function, in cancer cell biology.

Keywords: OATP1B3; SLCO1B3; Colon cancer; Lung cancer; Cancer biomarker; Cancer-specific expression; Transporter

Background

It has been acknowledged that early detection and appropriate treatment are essential for overcoming the high mortality and morbidity of cancer, and recent advances have provided excellent results showing that utilization of cancer-associated molecules in cancer diagnosis and cancer therapy can contribute significantly to the development of more sensitive and accurate detection methods, as well as to the improvement of treatment outcomes in various types of cancer. As part of these extensive ongoing research efforts aimed at identifying molecules closely associated with cancer, the specific aberrant mRNA products found in cancer cells have attracted significant levels of attention [1-3]. Recent findings have shown that those aberrant gene products are among the hallmarks of cancer, and that they often play a role in an oncogenic

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pathway, as exemplified by the association of the human epidermal growth factor receptor 2 splicing variant with breast cancer cell invasion and trastuzumab resistance [4]. Thus, the characterizations of cancer-associated alternative mRNA products offer clear opportunities not only for development of diagnostic, prognostic, and therapeutic methods for cancer treatment, but also for gaining new insights into cancer biology.

Recently, we have made our first report on the identification of the cancer-type organic anion transporting polypeptide 1B3 (Ct-OATP1B3, [GenBank: NM_019844 (for SLCO1B3, gene symbol) and GenBank: AB669023 (for the alternative region)] in human colon and lung cancers [5], and other research groups have subsequently confirmed its existence in colon and pancreas cancers [6,7]. Ct-OATP1B3 is an mRNA variant of the liver-specific OATP1B3 (which is hereafter referred to as the liver-type OATP1B3, Lt-OATP1B3) that mediates cellular uptake of a variety of endogenous compounds (e.g., cholecystokinin-octapeptide sulfated, CCK-8, or estradiol-17β-D-glucuronide, E_2G), as well as drugs (e.g., paclitaxel or imatinib) at the basolateral membrane of hepatocytes [8,9]. Previously, the detection of OATP1B3 (either or both Ct- and Lt-OATP1B3) has been reported in various cancer tissues, and it had been presumed that the mRNA was identical to Lt-OATP1B3 [10-15]. However, in contrast with this presumption, our investigations, along with others, have revealed that Ct-OATP1B3 mRNA expression is strongly associated with cancerous colon and lung tissues, but not with matched normal tissues or hepatocytes, and that Ct-OATP1B3 mRNA is expressed predominantly over Lt-OATP1B3 mRNA in these cancer tissues [5-7]. Therefore, those findings have shown that Ct-OATP1B3, but not Lt-OATP1B3, is a primary mRNA isoform, at least in these cancer tissues, which opens up the possibility of it being an intriguing cancer-associated molecule that can be used in the development of cancer biomarkers or therapeutic targets. Nevertheless, our current understanding of Ct-OATP1B3 mRNA expression profile in cancer tissues remains premature due to a small number of cancer tissues examined to date. Therefore, step-by-step characterization of Ct-OATP1B3 mRNA expression using a larger cohort will be required in order to validate and increase the feasibility of its clinical application.

Another important issue that needs to be addressed is clarification of the Ct-OATP1B3 function in cancer cells. Because the Ct-OATP1B3 mRNA transcription start site is located within the intron 2 of the *SLCO1B3* gene [5], Ct-OATP1B3 mRNA lacks the region that corresponds to the Lt-OATP1B3 N-terminal coding region. Therefore, the distinctive translation start codon usage is likely to occur in Ct-OATP1B3 mRNA (Figure 1). Our original research predicted a transporter-like translation product, which we termed Ct-OATP1B3-C [5]. Although, based on its deduced structure, Ct-OATP1B3-C is still expected to have a transporter function like Lt-OATP1B3, its functional expression remains to be demonstrated. Meanwhile, other studies have predicted the abilities of another product, Ct-OATP1B3-v1. However, Thakkar et al. [6] have reported that Ct-OATP1B3-v1 showed limited CCK-8 transport activity owing to its predominant localization in the cytoplasmic fraction, whereas Imai et al. [7] reported the cell surface expression of Ct-OATP1B3-v1 with transporting activities against fluvastatin and rifampicin. Therefore, additional examinations are absolutely essential to settling these controversies.

Based on all above-mentioned circumstances, this study aimed at characterizing the Ct-OATP1B3 mRNA expression profile using a larger number of colon and lung cancer tissue specimens, while simultaneously exploring its application potential as a cancer biomarker. Furthermore, we report on our attempt to identify a transporter-like entity derived from Ct-OATP1B3 mRNA.

Methods

Human-derived materials

Thirty-nine matched-pairs of human colon cancer and adjacent normal tissues were obtained from colon cancer patients who had undergone surgery (from June 2011 to June 2013) at the Chiba Cancer Center Research Institute (Chiba, Japan). Twenty-eight matched-pairs of human lung cancer and adjacent normal tissues were obtained from lung cancer patients who had undergone surgery (from January 2010 to February 2012) at the Chiba University Hospital (Chiba, Japan). The patient information is shown in Tables 1 and 2. The source of human liver tissue was described in our previous research [16]. Fifty donor-pooled human hepatocytes (Hep50) were purchased from Celsis IVT (Baltimore, MD). Use of human samples in this study was approved by the Ethics Committees of Graduate Schools of Pharmaceutical Sciences and Medicine, Chiba University, and the Ethics Committee of the Chiba Cancer Center Research Institute. Written informed consent was obtained from each patient.

The tumor stages of the colon and lung cancer patients were determined using the Union for International Cancer Control (UICC) tumor-node-metastasis (TNM) classification system. The differentiation status of colon cancer tissues was determined by pathological examination.

Cells and cell culture

LS180 and HCT116 (colon cancer) cells were obtained from DS Pharma Biomedical (Osaka, Japan) and Dr. B. Vogelstein (Johns Hopkins University, Baltimore, MD), respectively.

LS180 cells were cultured in Eagle's Minimum Essential Medium (Wako, Osaka, Japan). HCT116 cells were maintained in Dulbecco's Modified Eagle's Medium (Wako).

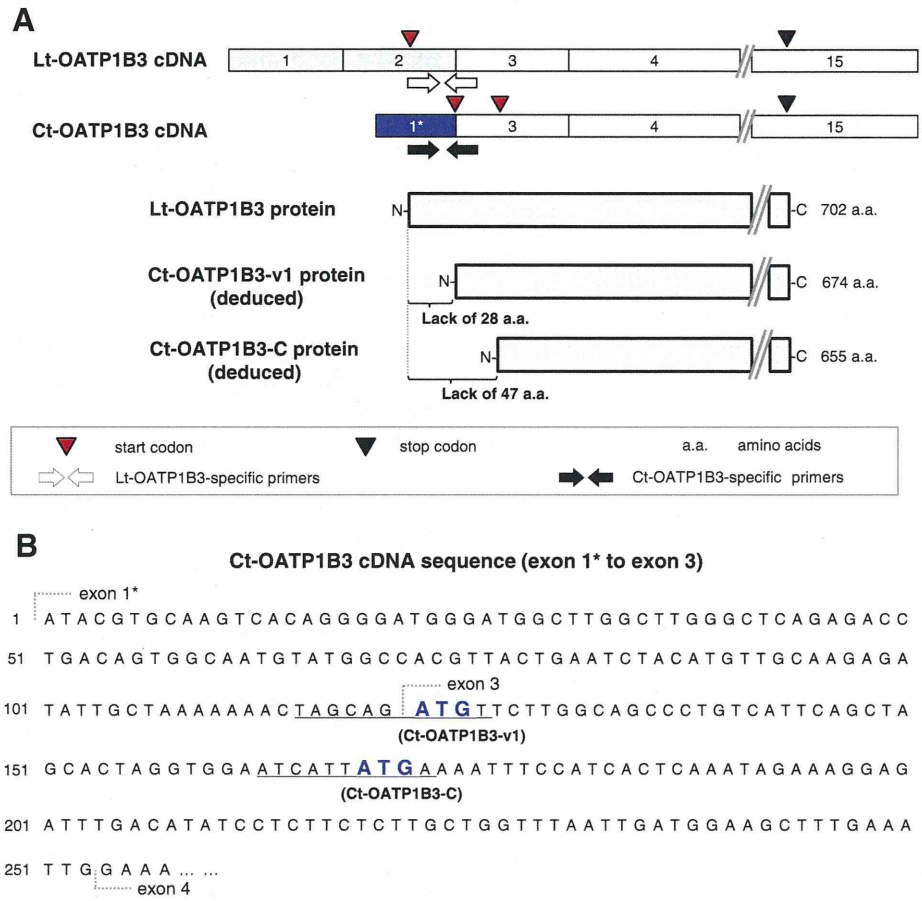

Figure 1 Schematic illustration of the Ct-OATP1B3 cDNA structure and its predicted translation products. (A) The cDNA structures of Ct- and Lt-OATP1B3 along with their predicted translation products were shown. The sky-blue boxes indicate the Lt-OATP1B3-specific exons, while the blue box indicates the Ct-OATP1B3-specific exon. The white boxes indicate the common exons. Each exon number is shown in the box. The translation product of Lt-OATP1B3 or the predicted translation products of Ct-OATP1B3 (Ct-OATP1B3-C and Ct-OATP1B3-v1) are shown below the cDNA structures. The red and black triangles indicate the translation start and stop codons, respectively. The open and closed arrows indicate an isoform-specific primer set for Lt-OATP1B3 or Ct-OATP1B3 detection, respectively. **(B)** The 5'-end cDNA sequence of Ct-OATP1B3 (exon 1* to 3) was shown. The bold blue letters indicate the predicted translation start codon (ATG) of Ct-OATP1B3-C or Ct-OATP1B3-v1. The underlines indicate the key sequences supposedly determining translation efficiency, while the consensus Kozak sequence is 5'-A/GNNatgG-3', where small "atg" is the translation start codon.

All culture mediums were supplemented with 10% (v/v) heat-inactivated fetal bovine serum and antibiotics. The cells were grown at 37°C with 5% CO_2.

Total RNA isolation and cDNA synthesis

Total RNA isolation from the human tissues and cells was performed using an ISOGEN II (NipponGene, Tokyo, Japan) according to the manufacturer's protocol. cDNA synthesis from extracted RNA (1 μg) was performed using random hexamers as described previously [5].

Quantitative real-time polymerase chain reaction (qPCR)

qPCR was performed using the methods described in our previous report [5]. Briefly, a SYBR green-based method was used for Lt-OATP1B3 mRNA expression quantification with the primer set (5'-AACAGCAGAGTCAGCATCT TCAG-3' and 5'-AACATCTTGAATCCATTGCAGC-3'). Ct-OATP1B3 mRNA expression level was determined using a fluorescent probe-based method with the primer set (5'-TTGGCTTGGGCTCAGAGA-3' and 5'-TGCCAA GAACATCTGCTAGTTT-3'), and universal probe #59 (Roche, Basel, Switzerland). The cDNA standard curve for each OATP1B3 mRNA was synthesized from each OATP1B3 mRNA at different copy numbers (from 10^3 to 10^8 copies for Ct-OATP1B3, and from 10^3 to 10^6 copies for Lt-OATP1B3). Each OATP1B3 expression level (copies/ng total RNA) in colon tissues, lung tissues, and cancer cell lines was calculated using its corresponding standard curve based on the condition that the mRNA expression level below 10^3 copies/ng total RNA, which was regarded as the level below the quantification limit (QL), the qPCR Ct-value of which was over 35. The mRNA expression levels

Table 1 Demographic and clinical characteristics of colon cancer patients

Variable		N (total = 39)
Age at surgery (year)	Median	67
	Range	41-84
Sex	Male	30
	Female	9
Location	Cecum	3
	Ascending colon	5
	Transverse colon	1
	Descending colon	2
	Sigmoid colon	11
	Rectum	17
Stage	0	1
	I	2
	II	15
	III	12
	IV	4
	Unknown	5
Differentiation	Well	10
	Moderate	21
	Unknown or unclassified	8

over QL were regarded as positive expression (or positive patients), whereas those under the QL were regarded as negative expression (or negative patients).

The total OATP1B3 mRNA (Ct- plus Lt-OATP1B3) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA levels were respectively determined using universal probe #59 with the primer set (5'-CGGCCTAACCTTG ACCTATG-'3 and 5'-TGAGTTGCAATAAGAAAGTGG TACA-3'), and probe #60 with the primer set (5'-AGCCA CATCGCTCAGACAC-'3 and 5'-GCCCAATACGACCA AATCC-3'). Data were calculated using the delta-delta-Ct method, where the GAPDH mRNA levels were used as a normalization control.

Table 2 Demographic and clinical characteristics of lung cancer patients

Variable		N (total = 28)
Age at surgery	Median	67
	Range	44-81
Sex	Male	20
	Female	8
Cancer type	Adenocarcinoma	12
	Squamous cell carcinoma	12
	Adenosquamous carcinoma	1
	Small cell carcinoma	1
	Large cell carcinoma	2
Stage	I	20
	II	6
	III	2
Brinkman index[a]	<400	5
	>400	23

[a]Brinkman index was calculated as the product of the number of cigarettes smoked per day multiplied by the number of years of smoking.

Western blot analysis

Whole cell lysates were prepared from each cancer cell line using the lysis buffer or the Tris/Sucrose/EDTA buffer. The soluble membrane fractions were prepared from the whole cell lysates. Briefly, after centrifugation at $1,000 \times g$ for 10 min, the supernatant was ultracentrifuged at $100,000 \times g$ for 45 min. Then, the soluble membrane fractions were collected by dissolving the precipitates with the Tris/Sucrose/EDTA buffer supplemented with 0.8% (v/v) Nonidet P-40, 0.4% deoxycholic acid, and 0.08% sodium dodecyl sulfate (SDS), followed by a second ultracentrifugation under the same conditions. Using the same procedure, the soluble membrane fraction of human liver tissue was prepared.

The proteins were separated by SDS-polyacrylamide gel electrophoresis, and then transferred onto a polyvinylidene difluoride membrane. The membrane was blocked with 5% skim milk for one hour at room temperature. Affinity purified rabbit polyclonal anti-OATP1B3 antibodies, which recognize the C-terminal region of Lt-OATP1B3, were used as the primary antibodies (1,000-fold dilution, HPA004943, Sigma, St. Louis, MO). Anti-Na^+/K^+ ATPase (1,000-fold dilution, Sigma) and anti-β-actin (500-fold dilution, Sigma) were also used. Immunocomplexes were detected with ECL Western blotting detection reagents (GE Healthcare, Giles, UK).

Transport assays

[^{3}H]-CCK-8 and [^{3}H]-E_2G were obtained from PerkinElmer (Boston, MA), while non-radiolabeled CCK-8 and E_2G were purchased from the Peptide Institute (Osaka, Japan) and Sigma, respectively. The transport assay was performed using LS180 and HCT116 cells based essentially on the previously described method [17]. Final concentrations of CCK-8 and E_2G were 1 μM and 0.5 μM, respectively. The transport activity for each substrate was determined within a linear range (5 min). In the inhibition analysis, bromosulfophthalein (BSP, Sigma) (100 μM) was used.

Statistical analysis

Mann–Whitney U test was used to determine differences of mRNA expression levels between two groups. Fisher's exact test was used to determine the univariate relationship of the frequency of Ct-OATP1B3 or Lt-OATP1B3 mRNA expression between normal and cancer tissues. In

all of the comparative analyses, only the positively detected expression data were used. All statistical analyses were performed using the Statcel software (OMS Publishing Inc., Tokyo, Japan).

Others

Detailed development methods for the Ct-OATP1B3-C and Ct-OATP1B3-v1 expression plasmids, development of human embryonic kidney (HEK) 293 cells transiently or stably expressing each OATP1B3 isoform, as well as anti-OATP1B3 rabbit serum preparation, are described in Additional file 8 (supplemental materials and methods). Experimental procedures for the Lt-OATP1B3 expression plasmid and immunocytochemistry were described in the previous report [18].

Results

Expression profile of each OATP1B3 mRNA isoform in human colon cancer

Ct-OATP1B3 and Lt-OATP1B3 mRNA quantification was performed using isoform-specific primer sets. The results showed that Ct-OATP1B3 mRNA expression frequencies in cancer and normal tissue specimens were 87.2% (34/39) and 2.6% (1/39), respectively (Fisher's exact test, $P = 1.24 \times 10^{-15}$) (Figure 2). In addition to this high positive frequency, the mRNA levels in cancer tissues were strikingly higher than those in normal tissues (Figure 2). Accordingly, the apparent tumor/normal expression ratio (the T/N ratio) of Ct-OATP1B3 mRNA in each tissue pair was very high (Additional file 1: Figure S1A), even though the exact T/N ratios could not be calculated due to the quantitatively undetectable Ct-OATP1B3 mRNA levels in most normal tissues.

In contrast to the cancer-specific expression of Ct-OTP1B3 mRNA, Lt-OATP1B3 mRNA expression frequencies in cancer and normal tissue specimens were 53.8% (21/39) and 51.3% (20/39), respectively (Fisher's exact test, $P = 0.5$) (Figure 2). The median values of Lt-OATP1B3 mRNA levels were similar between cancer and normal tissues (Figure 2), and were strikingly lower than those in human pooled hepatocytes (Table 3).

When the Ct- and Lt-OATP1B3 mRNA levels in cancer tissues were compared, the median value of Ct-OATP1B3 mRNA levels in cancer tissues was 12.2-fold higher than that of Lt-OATP1B3 mRNA levels (19.5×10^3 vs. 1.6×10^3 copies/ng total RNA, $P = 2.9 \times 10^{-6}$) (Figure 2). In addition, Ct-OATP1B3 mRNA levels were also higher than Lt-OATP1B3 mRNA levels in individual cancer specimens (Additional file 2: Figure S2A).

It should be noted that the similar Ct- and Lt-OATP1B3 mRNA expression results were obtained using a ΔΔCt-method with the GAPDH mRNA level used as a normalization control (data not shown).

Taken together, our data showed that Ct-OATP1B3 mRNA was expressed in a cancer tissue-specific manner, and that its mRNA expression was predominant over Lt-OATP1B3 mRNA expression in colon cancer tissues.

Associations of Ct-OATP1B3 mRNA levels in colon cancer tissues with clinico-pathological variables

Exploration of the clinico-pathological characteristics of Ct-OATP1B3 mRNA expression profiles in colon cancer

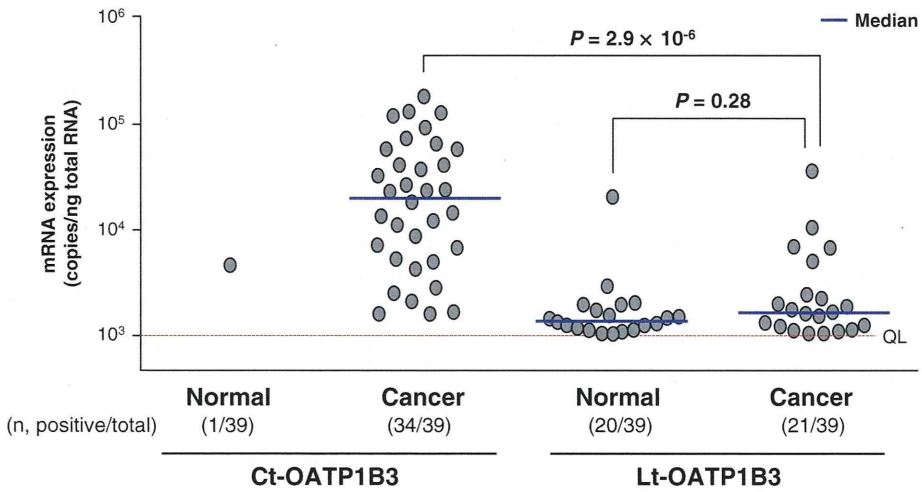

Figure 2 Expression profiles of Ct- and Lt-OATP1B3 mRNA in human colon cancer patients. The copy numbers of two OATP1B3 mRNA isoforms in each sample were separately determined by qPCR using the isoform-specific primers. Each dot represents the mean of Ct- or Lt-OATP1B3 mRNA expression levels (copies/ng total RNA), which was obtained from three independent determinations, each performed in duplicate. The red line indicates the value of the quantification limit (QL). The total number of specimens, together with the number of positive expression specimens, in each group is shown in parentheses. Horizontal solid lines denote the median value of positively detected mRNA levels in each group. The statistical significance of median difference between two groups was examined using the Mann–Whitney U test.