Cancer-Type OATP1B3 mRNA in Extracellular Vesicles as a Promising Candidate for a Serum-Based Colorectal Cancer Biomarker

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Cancer-type organic anion transporting polypeptide 1B3 (Ct-OATP1B3) mRNA is a variant isoform of the liver-type OATP1B3. Because Ct-OATP1B3 mRNA shows an excellent cancer-specific expression profile in colorectal cancer (CRC), and that its expression levels are associated with CRC prognosis, it holds the potential to become a useful CRC detection and diagnosis biomarker. While the potential is currently justified only at the tissue level, if existence of Ct-OATP1B3 mRNA in CRC-derived extracellular vesicles (EVs) is validated, the findings could enhance its translational potential as a CRC detection and diagnosis biomarker. Therefore, this study aims at proving that Ct-OATP1B3 mRNA exists in CRC-derived EVs, and can be detected using serum specimens. To examine the possibility of Ct-OATP1B3 mRNA being existed in extracellular milieu, we isolated EVs from the human CRC (HCT116, HT-29, and SW480) cell lines, and prepared their cDNAs. The RT-PCR results showed that Ct-OATP1B3 mRNA was clearly present in EVs derived from the human CRC cell lines. Then, in order to further explore the possibility that Ct-OATP1B3 mRNA in CRC-derived EVs can be detected in serum, we isolated serum EVs derived from human CRC xenograft mice, and then performed RT-PCR. The results showed that Ct-OATP1B3 mRNA could be found in all serum EV and CRC tissue samples of the mice examined. Collectively, our findings, which show that Ct-OATP1B3 mRNA exists in EVs and can be detected in (at least) mouse serum, strengthen the potential use of Ct-OATP1B3 mRNA as a serum-based CRC biomarker.

Key words organic anion transporting polypeptide 1B3; SLCO1B3; colorectal cancer; extracellular vesicle; cancer biomarker

Worldwide, colorectal cancer (CRC) is one of the most frequently encountered malignant tumors and is a major cause of related deaths.1) As part of efforts aimed at overcoming its high mortality and morbidity, patient-friendly and easy-to-apply CRC detection methods play crucial roles, thus making it an area where a serum-based CRC biomarker could contribute significantly. Ideally, a biomarker of this type would also be expected to carry information related to CRC pathologic conditions or a phenotypic signature, which would provide precision information for CRC treatment. However, no such CRC biomarker is clinically available at present.

As a promising candidate of such biomarkers, we previously identified cancer-type organic anion transporting polypeptide 1B3 (Ct-OATP1B3), the mRNA of which is a variant isoform of the liver-type OATP1B3 that mediates cellular uptake of various compounds at hepatocytes.2,3) Ct-OATP1B3 mRNA is predominantly associated with CRC tissues, but not with their matched normal tissues or hepatocytes, and its expression can be clearly observed, even in the early tumor stages.4–6) Furthermore, since Ct-OATP1B3 mRNA levels in CRC tissues can be plainly associated with overall survival in CRC patients,6) Ct-OATP1B3 mRNA expression has the potential to provide an indicator for cancer development as well as its progression. Currently, however, that potential can only be justified only at the tissue level.

Meanwhile, extracellular vesicles (EVs), such as exosomes and microvesicles, have gained extensive attention as a prospective source of biomarkers for cancer detection and diagnosis.7,8) EVs are small spherical and lipid-bilayered vesicles in which a variety of cellular components, including RNA, are enclosed.8,9) Since EVs are released extracellularly from various cell types, including tumor cells,10) they can be found in accessible body fluids, such as serum.7,8) To date, it has been reported that several cancer-associated RNAs in EVs secreted from CRC tissues stably exist in serum, and that their detection can help differentiate CRC victims from healthy individuals.11–13)

Based on the above-mentioned findings, we began our study by presuming that Ct-OATP1B3 mRNA could exist in CRCderived EVs, and that they might be detectable in serum. If
validated, the resulting findings could be expected to enhance the translational potential of Ct-OATP1B3 mRNA as a CRC detection and diagnosis biomarker. Based on the results obtained, we can now provide experimental evidence that the Ct-OATP1B3 mRNA is a cargo of CRC-derived EVs, and that it can be detected using serum specimens.

MATERIALS AND METHODS

Cells HCT116 cells were obtained from Dr. B. Vogelstein (Johns Hopkins University, Baltimore, MD, U.S.A.). HT-29 and SW480 cells were obtained from American Type Culture Collection (Manassas, VA, U.S.A.). HCT116 cells stably expressing the tetracycline repressor protein (HCT116/TR) were developed previously.14

Mouse colon carcinoma Colon-26 (C26) cells were obtained from Riken Cell Bank (Tsukuba, Japan). C26 cells stably expressing Ct-OATP1B3 mRNA (Ct-OATP1B3/C26) and their control cells (Mock/C26), as well as HCT116/TR cells tetracycline-inducibly expressing Ct-OATP1B3 mRNA (Ct-OATP1B3 tet-on/HCT116) and their control cells (Mock/HCT116), were established using a previously-prepared Ct-OATP1B3/pcDNA3.15 and an empty pcDNA3.1(−)Neo (Thermo Fisher Scientific, Waltham, MA, U.S.A.), respectively.

Animal Models The Animal Research Committee of Chiba University approved the study protocol used in this research. BALB/c mouse (7-week-old male, Japan SLC [Shizuoka, Japan]) was injected subcutaneously into their left flank with 5×10⁵ Ct-OATP1B3/C26 or Mock/C26 cells. Similarly, to develop human CRC xenograft model mouse, BALB/c nude mouse (7-week-old male, CLEA Japan [Tokyo, Japan]) were injected subcutaneously into their left flank with 2×10⁶ HCT116 cells or 3×10⁶ SW480 cells.

Isolating EVs from the Culture Medium or Mouse Serum The culture medium of human CRC cell lines was collected and filtered with a 0.22-µm polycarbonate membrane filter. EVs were isolated from the medium using a differential centrifugation method.

When the CRC model mouse tumor reached 1000 mm³, the serum was collected and filtered with a 0.22-µm PVDF filter. Serum EVs were isolated using a Total Exosome Isolation kit (from serum) (Thermo Fisher Scientific) according to the manufacturer’s protocol.

mRNA Expression Analysis Total RNA was collected from human CRC cells, EVs, and CRC tissues and then their cDNA was synthesized. Ct-OATP1B3 mRNA expression was examined by RT-PCR or quantitative real-time PCR (qPCR). Human or mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was used as an internal control.

Others Please see the figure legends and the supplementary materials for additional information regarding materials and methods.

RESULTS

As a first step to examine the possibility that Ct-OATP1B3 mRNA could be detected in extracellular milieu, EVs were isolated from the human CRC (HCT116, HT-29, and SW480) cell lines. The results of transmission electron microscopy (TEM) and Western blotting analyses confirmed that the isolated EVs were round-shaped vesicles expressing CD81, which is an EV marker protein (Figs. 1A, B). Importantly, an endoplasmic reticulum marker, calnexin, was not detected in any of the EV samples (Fig. 1B), thereby suggesting there was little or no contamination with intracellular compartments in the EV fraction.

Next, RT-PCR was conducted to determine if Ct-OATP1B3 mRNA is detectable in the EVs. In our results, Ct-OATP1B3 mRNA was clearly present in EVs derived from HCT116, HT-29, and SW480 cells (Fig. 1C). Moreover, by taking advantage of the tetracycline-inducible Ct-OATP1B3 mRNA expression system, we performed comparison analysis between the mRNA levels of EVs and cells. From these results, we found that the Ct-OATP1B3 mRNA levels in the EVs were in apparent agreement with its intracellular level in the system (Fig. 1D).

Since our in vitro results open the prospect that Ct-OATP1B3 mRNA can be secreted from the human CRC tissue and circulate in the bloodstream, we were motivated to conduct in vivo experiments to further explore this possibility. Using the method described in supplementary materials, the serum EVs were isolated from BALB/c mice and confirmed by TEM and size-distribution analyses, where the EVs showed a round-shape with a mean diameter of 62±17 nm (Figs. 2A, B). Using the same method, we isolated serum EVs derived from BALB/c mice bearing Ct-OATP1B3/C26 or Mock/C26 cells, and then performed RT-PCR. The results showed that Ct-OATP1B3 mRNA was detected in the serum EVs derived from mice bearing Ct-OATP1B3/C26 cells, but not those extracted from mice with mock cells (Fig. 2C). Finally, we tested to determine whether Ct-OATP1B3 mRNA could also be detected in serum EVs derived from human CRC xenograft mice. The RT-PCR result showed that Ct-OATP1B3 mRNA could be found in all serum EV and CRC tissue samples of the mice examined (Fig. 2D).

DISCUSSION

Our results clearly substantiate the finding that Ct-OATP1B3 mRNA exists in EVs secreted from the human CRC cells, and that the CRC EVs containing Ct-OATP1B3 mRNA can be detected in serum (at least) in mice. Therefore, these findings unequivocally point to the feasibility of Ct-OATP1B3 mRNA being detectable in serum CRC EVs in humans as well. Furthermore, in line with the fact that Ct-OATP1B3 mRNA expression holds extremely high specificity and sensitivity in both early- and late-stage CRC tissues (the area under the receiver operating characteristic curve values are 0.91–0.93 in the study with 97 matched-pairs of CRC patients6), it can also be speculated that Ct-OATP1B3 mRNA in serum EVs could serve as a promising distinguishable biomarker between CRC victims and healthy individuals.

Furthermore, the finding that Ct-OATP1B3 mRNA expression levels in EVs reflects its expression levels in parental cells should also be highlighted. Previously, we have reported that high/low Ct-OATP1B3 mRNA expression levels in CRC tissues can be associated with better/poor prognosis in CRC patients.6 Therefore, uniquely as a CRC biomarker, Ct-OATP1B3 mRNA in EVs have the potential to not only identify CRC, but also provide related prognostic information. However, we are certainly aware that the translational potential of Ct-OATP1B3 mRNA in EVs as a CRC biomarker...
is still in an early stage and must be extensively investigated. The efforts include the validation of Ct-OATP1B3 mRNA existence in serum EVs in human CRC patients and the characterization of their diagnostic properties. In such attempts, it is important to establish methods allowing for Ct-OATP1B3 mRNA quantification in serum EVs to determine an appropriate cut-off value that maximizes its diagnostic accuracy. It will also be important to seek its combination partners because it has become evident that the usage of multiple biomarkers show preferable results when used in CRC diagnosis.\(^{16,17}\)

Furthermore, considering that only a small portion of human serum is currently available for routine CRC screening, improvement of Ct-OATP1B3 mRNA detection method is necessary. For example, development of a pre-amplification of EV mRNA or a more sensitive quantification method. In addition, although there will be some difficulties and cautionary notes,\(^{18-20}\) in order to not only make Ct-OATP1B3 mRNA detection more easily, but also to specify its origin, consideration should be given to collect and concentrate CRC-specific EVs from whole serum in future studies.

CONCLUSION

In conclusion, our results clearly demonstrate that Ct-OATP1B3 mRNA exists in EVs, and that they can be detected...
in serum in human CRC model mice. Although multiple challenges lie ahead, our present findings widen the possibility that Ct-OATP1B3 mRNA can eventually be established as a serum CRC detection and diagnosis biomarker. Since development of a practically useful serum-based CRC detection system has long been desired for overcoming the high mortality and morbidity of CRC, the translational potential held by Ct-OATP1B3 mRNA of CRC-derived EVs is definitely worth pursuing.

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Conflict of Interest The authors declare no conflict of interest. Just for transparency purpose, we declare that, while we have not received any royalties or licensing fees from any institutes, companies or individuals to date, Tomomi Furihata and Kan Chiba are listed as the inventors of the patents JP5901046, US9115405, and US2012014977[pending], which include the potential of Ct-OATP1B3 as a diagnostic/detection biomarker in cancer, and our institute Chiba University is an owner of them.

Supplementary Materials The online version of this article contains supplementary materials.

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