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Original Article

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Rare Incidence of *ROS1* Rearrangement in Cholangiocarcinoma

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Purpose

The recent discovery and characterization of an oncogenic *ROS1* gene rearrangement has raised significant interest because small molecule inhibitors are effective in these tumors. The aim of this study was to determine frequency and clinicopathological features associated with *ROS1* rearrangement in patients with cholangiocarcinoma (CCA).

Materials and Methods

A total of 261 patients who underwent surgery for CCA between October 1997 and August 2013 were identified from an international, multi-institutional database. *ROS1* rearrangement was evaluated by break-apart fluorescence *in situ* hybridization using tissue microarrays of these patients.

Results

Of 261 CCA evaluated, three cases (1.1%) showed *ROS1* rearrangement by fluorescence *in situ* hybridization (FISH), all of which were derived from intrahepatic origin. ROS1 protein expression was observed in 38 samples (19.1%). Significantly larger tumor size was observed in ROS1 immunohistochemistry (IHC)–negative patients compared with ROS1 IHC–positive patients. *ROS1* FISH–positive patients had a single tumor with a median size of 4 cm and well-to-moderate differentiation. Overall, there was no difference in terms of baseline characteristics, overall survival, and recurrence-free survival between *ROS1*-positive and -negative patients.

Conclusion

ROS1 rearrangement was detected in 1.1% of CCA patients. Although rare, conduct of clinical trials using ROS1 inhibitors in these genetically unique patients is warranted.

Key words
Cholangiocarcinoma, *ROS1*, Fluorescent *in situ* hybridizations

Introduction

Cholangiocarcinoma (CCA) is a lethal biliary tract cancer [1]. The age-adjusted incidence rate is 3.3 per 100,000 among Asians, and despite the relatively low incidence, the overall incidence of CCA is increasing worldwide [2,3]. CCA is generally regarded as a surgical disease, and surgical resection offers the only chance for long-term survival [4]. However, most patients are diagnosed with advanced-stage disease, which limits the treatment options. In addition, even after curative-intent surgery, the survival outcomes are disappointing with a 5-year survival rate of 20%-30% [5]. Combination chemotherapy with gemcitabine and a platinum agent is regarded as a standard first-line treatment [6]; however, the prognosis is still poor and overall survival (OS) remains less than 12 months. Therefore, efforts to improve outcomes for CCA by incorporating novel targeted therapeutics are urgently needed.

Contemporary research techniques have enabled identification of several markers involved in molecular pathogenesis of CCA [7]. In a recent study from Singapore, 206 somatic mutations were identified through exome sequencing of liver-fluke associated CCA [8]. *TP53* accounted for the most common cancer-related gene (44%), followed by *KRAS* (17%) and *SMAD4* (17%). Genetic alterations associated with deactivation of histone modifiers including *ARID1A* (9%) were also identified. However, none of these genetic alterations is amenable to targeted therapy. Recently, in the first phase 3 trial to assess a targeted therapy plus chemotherapy combination, improvement in OS was not observed by treatment with gemcitabine and oxaliplatin with or without erlotinib. Only a small improvement in median progression-free survival was observed in a subset of patients with CCA (5.9 months vs. 3.0 months) [9], and there was no distinct predictive biomarker for erlotinib response in this study.

ROS1 rearrangements were recently detected in CCA patient tissues, suggesting that this could play a role as a driver oncogene. The reported incidence of *ROS1* rearrangement was 8.7% of CCA patients [10]. Interestingly, fused-in-glioblastoma-c-ros-oncogene 1 (*FIG-ROS*) fusion accelerated cholangiocarcinogenesis in mouse models [11], supporting ROS1 as a novel therapeutic target in CCA. *ROS1* rearrangement was previously detected in other solid tumors including non-small-cell lung cancer (NSCLC) and glioblastoma [12-14]. To date, nine different *ROS1* fusion partners have been identified, all of which are potentially targetable due to the intact cytoplasmic portion of the ROS1 tyrosine kinase domain [15]. Due to the biological similarity of *ROS1* and anaplastic lymphoma kinase (ALK), inhibition of ROS1 by several ALK inhibitors has been demonstrated [16]. Recent data from a phase 1 trial of crizotinib in the *ROS1*-positive NSCLC expansion cohort showed an overall response rate of 72% [17]. Therefore, identification of *ROS1* rearrangement in CCA could offer a new therapeutic option in treatment of this fatal disease.

The current study uses the largest data set yet generated of CCA. The aim of this study was to determine the frequency of *ROS1* rearrangement and to evaluate clinicopathological features associated with *ROS1* rearrangement in CCA patients.

Materials and Methods

1. Patients

A total of 261 patients with CCA who underwent surgical resection with curative intent between October 1997 and August 2013 were identified at two institutions (Yonsei University College of Medicine, Seoul, Korea; National University of Singapore, Singapore). The database was reviewed retrospectively and only patients with histologically confirmed CCA and available tissue for ROS1 analysis were included. Patient records/information was anonymized and de-identified prior to analysis. This study was approved by the Institutional Review Boards of Severance Hospital and Singapore General Hospital and the requirement for consent was waived.

Out of 261 patients, 242 patients (93%) were available for ROS1 analysis. Standard demographic and clinicopathologic data were collected, including sex, age, and primary tumor characteristics. Specifically, data were collected on primary tumor location, size, and number as well as morphologic subtype and presence of vascular invasion, biliary invasion, lymph node involvement, and stage. Staging was done according to the seventh edition of the American Joint Committee on Cancer/Union for International Cancer Control staging manual. Dates related to treatment including surgery, recurrence, and last follow-up were collected on all patients. OS was measured from the date of diagnosis until the date of death and recurrence-free survival (RFS) was measured from the date of surgery until the date of recurrence.

2. Tissue microarrays

The tumor samples were fixed in 10% buffered formalin, processed, and embedded in paraffin using the standard protocol [18]. All hematoxylin and eosin–stained slides were reviewed, and representative areas were carefully selected and marked on individual paraffin blocks. Three 3.0-mm tissues cores were taken from each tumor specimen.

3. *ROS1* fluorescence *in situ* hybridization

For identification of *ROS1* rearrangement, fluorescence *in situ* hybridization (FISH) assays were performed using a break-apart probe to ROS1 (Break-Apart Rearrangement Probe, Abbott Molecular, Des Plaines, IL) according to the manufacturer's instructions using tissue microarrays. At least 100 nuclei of tumor cells per case were evaluated. FISH positivity for *ROS1* rearrangement was defined as > 15% of tumor cells with a split signal or > 15% of single green signals. FISH studies were interpreted by two experienced evaluators (J.E.Y. and Y.N.P.) who were blinded to the clinical data.

4. Immunohistochemical staining

Protein expression of ROS1 was detected by immunohistochemical staining in the Korean cohort, and it was not performed in the Singapore cohort due to lack of unstained slides. Tissue microarray sections were stained using the Ventana automated immunostainer BenchMark XT (Ventana Medical Systems, Tucson, AZ). The slides were dried at 60°C for 1 hour and deparaffinized using EZ Prep (Ventana Medical Systems) at 75°C for 4 minutes. Cell conditioning was performed using CC1 solution (Ventana Medical Systems) at 100°C for 8 minutes. ROS1 antibody (rabbit monoclonal, clone D4D6, Cell Signaling Technology, Danvers, MA) was diluted to 1:10, followed by treatment, and incubation at 37°C for 2 hours. Signals were detected using the OptiView DAB IHC Detection Kit (Ventana Medical Systems). Counterstaining was performed using Hematoxylin I (Ventana Medical Systems) for 4 minutes at room temperature.

5. Statistical analysis

Statistics were obtained using established methods and presented as percentages, mean, or median values. Survival was estimated using the Kaplan-Meier method for the median and 95% confidence interval (CI); comparison between groups was performed using a two-sided log-rank test. Fisher exact test, t test, or Mann-Whitney U test were used for comparison of differences between groups. p-values of ≤ 0.05 were considered statistically significant. Statistical analyses were performed using SPSS ver. 20.0 (IBM Co., Armonk, NY).

Table 1. Demographic and clinical characteristics of patients

Variable	No. (%) (n=261)
Age, median (range, yr)	65.6 (33-91)
Sex	
Male	148 (56)
Female	113 (44)
Tumor location	
Intrahepatic	208 (80)
Extrahepatic	53 (20)
Tumor size, median (range, cm)	4.6 (0.8-15)
Multiple tumors	51 (19)
Tumor differentiation	
Well-moderately differentiated	211 (80)
Poorly differentiated	50 (20)
Lymph node disease	67/209 (32)
CA19-9, median (range, U/mL)	71.9 (0-20,000)
Stage	
I	88 (34)
II	55 (21)
III	23 (9)
IV	95 (36)

CA19-9, carbohydrate antigen 19-9.

Results

1. Patient characteristics

The clinicopathological features of 261 patients including 208 intrahepatic CCAs and 53 extrahepatic CCAs are shown in Table 1. The median age of all patients was 65.6 years and males (56%) were predominant compared with females (44%). The majority of patients presented with intrahepatic CCAs (80%), a single tumor (81%) with a median tumor size of 4.6 cm. The tumor was well-to-moderately differentiated in 80% of patients. Lymphadenectomy was performed in 209 patients (80%), and 67 patients (32%) presented with lymph node positive disease. The median preoperative carbohydrate antigen 19-9 level was 71.9 U/mL (range, 0 to 20,000 U/mL; reference value, ≤ 37 U/mL). In comparison of samples from the Korea and Singapore cohorts (Supplementary Table 1), no significant difference in clinicopathological features was observed between the two cohorts.

2. Break-apart FISH and immunohistochemical analysis of *ROS1*

Among 261 patients screened for *ROS1* rearrangement, 245 cases were analyzed by FISH (detection rate, 93.9%), and

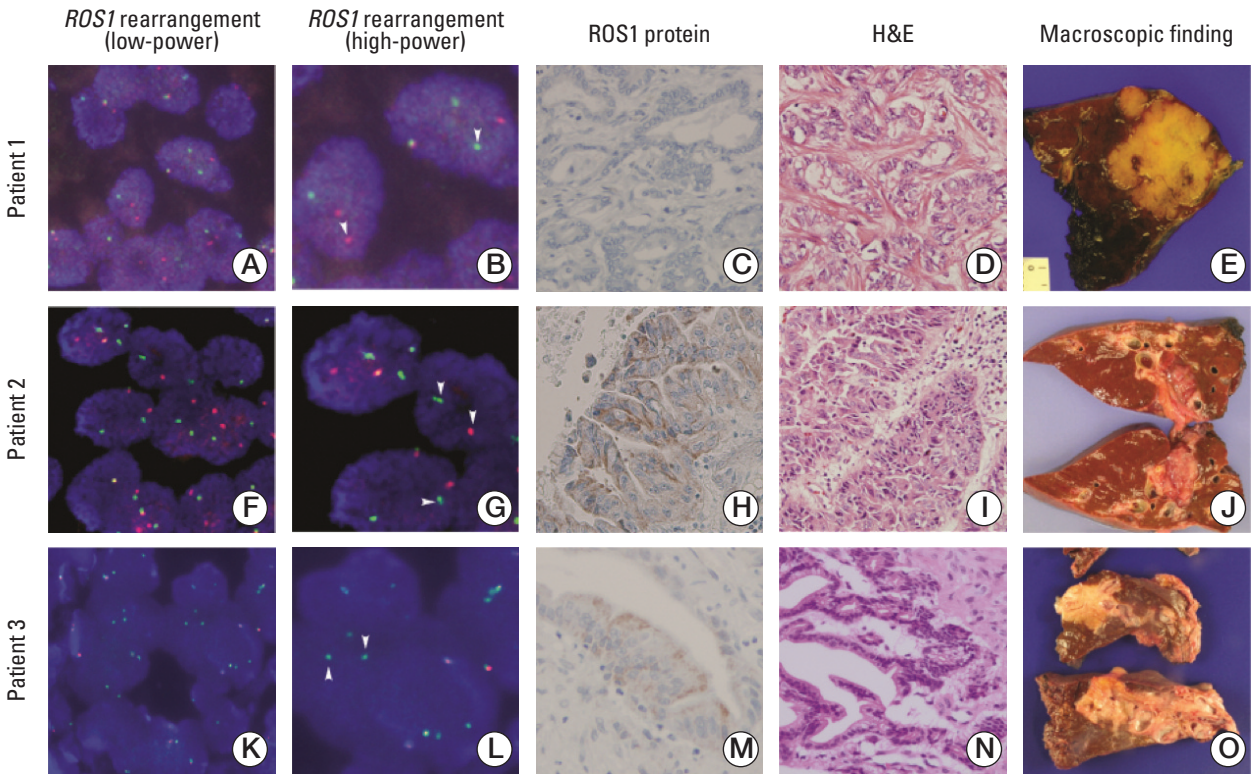

Fig. 1. Representative *ROS1* rearrangement features in cholangiocarcimomas. Fluorescence *in situ* hybridization (FISH) showing *ROS1* rearrangement with break-apart signals in patients 1 and 2 and single green signals in patient 3. FISH showing *ROS1* rearrangement with break-apart signals in low-power field (A, F, K) and high-power field (B, G, L). FISH showing *ROS1* rearrangement with single green signals (K, L). The arrows indicate *ROS1* break-apart or single green signals (A, F, K, ×400; B, G, L, ×630). Immunohistochemical stain for ROS1 in *ROS1* rearranged cholangiocarcinoma (C, H, M, ×200). Microscopic features (D, I, N, H&E staining, ×200) and photographs of surgical specimens (E, J, O) are also shown.

FISH signal was not detected in 16 cases due to sample quality. Three cases showed *ROS1*-positivity (1.2%) by FISH; patients 1 and 2 showed break-apart signals, while patient 3 showed a single signal as shown in Fig. 1. Comparison of *ROS1*-negative and -positive patients showed no significant differences in baseline demographic and clinical characteristics. For immunohistochemistry (IHC) analysis, 198 cases were available for analysis of ROS1 protein expression. ROS1 protein expression was observed in 38 samples (19.1%). Comparison of *ROS1*-negative and -positive patients showed a significant difference in tumor size, while there was no difference in other parameters. *ROS1*-negative patients had a significantly larger tumor size than *ROS1*-positive patients (p=0.025) (Supplementary Table 2). Of note, among three *ROS1* FISH–positive cases, two patients (66.7%) showed ROS1 immunoreactivity (Fig. 1). The sensitivity for the detection of *ROS1* rearranged CCA was 66.7% with 81.5% specificity.

3. Clinical outcome of patients

The clinical outcomes of all patients and *ROS1*-positive versus *ROS1*-negative patients were compared. The median OS was 36.6 months (95% CI, 26.8 to 46.3 months), and the median RFS was 9.9 months (95% CI, 7.5 to 12.4 months) for all patients (Fig. 2A and B). There were no statistical differences in terms of OS and RFS (Fig. 2C and D) between *ROS1* FISH–positive and *ROS1* FISH–negative patients, and there was no statistical difference in terms of OS and RFS between ROS1 IHC–positive and ROS1 IHC–negative patients.

4. Analysis of *ROS1* FISH–positive patients

A detailed summary of the clinicopathological data of three patients with *ROS1* rearrangement is shown in Table 2. These patients were all intrahepatic CCA, and no signs of hepatolithiasis and parasitic infection were detected.

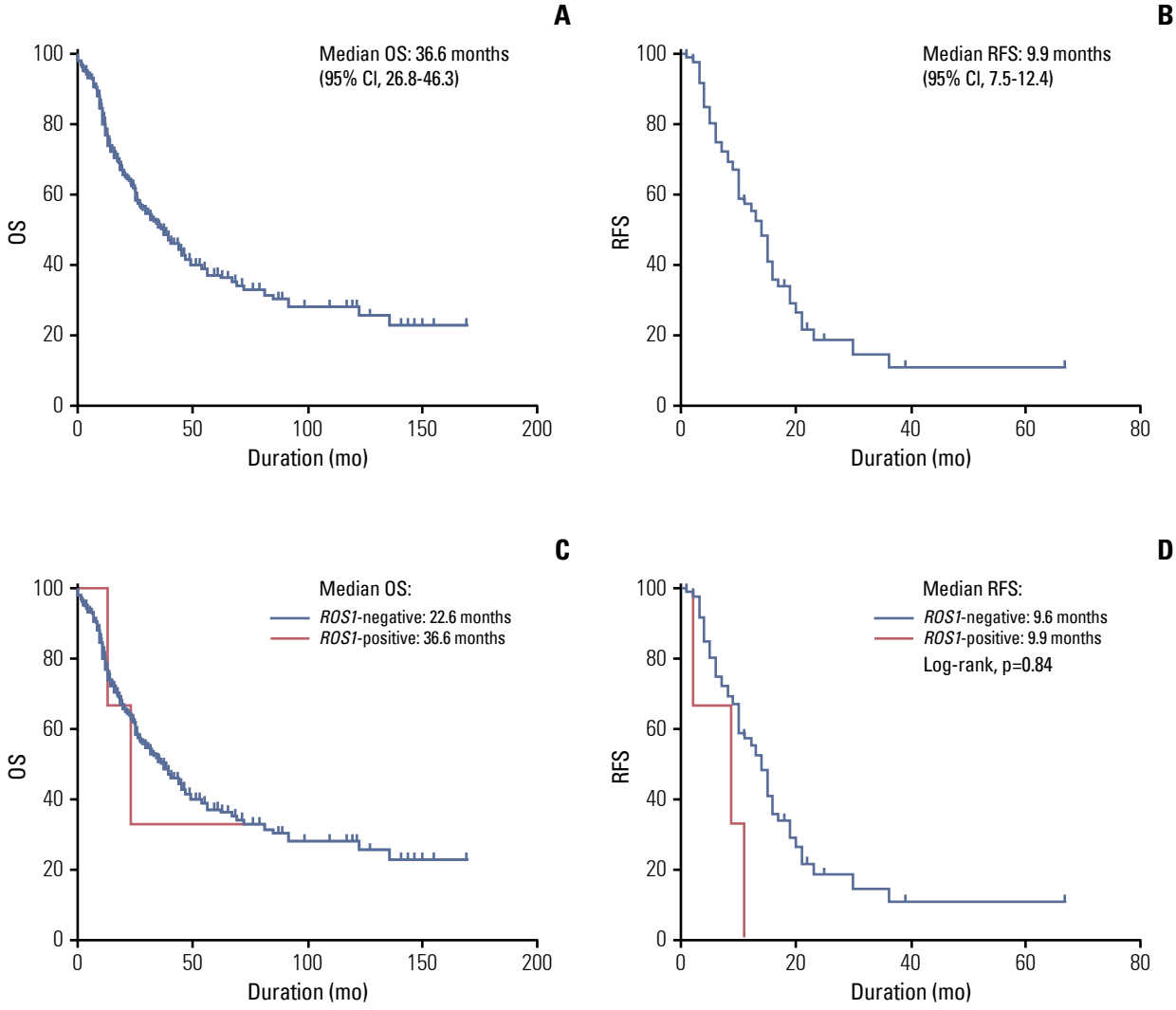

Fig. 2. Survival analysis. (A) Kaplan-Meier curve of median overall survival (OS) of all patients. (B) Kaplan-Meier curve of median recurrence-free survival (RFS) of all patients. (C) Comparison of OS between *ROS1*-positive and -negative patients. (D) Comparison of RFS between *ROS1*-positive and -negative patients. CI, confidence interval.

Patient 1 had a 6.5-cm-sized mass forming tumor with moderate differentiation. Patient 2 had a 3-cm-sized CCA with intraductal growth and well differentiation. Patient 3 had a 3-cm-sized CCA with periductal infiltrative growth and moderate differentiation. Patients 1 and 3 showed lymph node metastasis of CCA.

Discussion

In this study, we screened CCA tissue archives and found that approximately 1.1% of CCA patients harbored *ROS1* rearrangement. Of note, all *ROS1*-positive patients had intrahepatic CCA. To date, this is the first large-scale screening effort to examine the frequency of *ROS1* rearrangement in CCA patient tissue.

CCA is a fatal disease with a huge unmet need for novel therapeutics and efforts have been made to define genetic sub-classification of CCA. The potentially targetable signaling pathways in CCA include *EGFR*, *VEGF*, *HER2/neu*, *MET*,

Table 2. Clinicopathological features of *ROS1*-rearranged cholangiocarcinoma patients

No.	Age (yr)	Sex	*ROS1* FISH pattern	ROS1 protein expression	Tumor size (cm)	Location	Gross type	Histologic type	Differentiation	Non-tumor liver	Stage
1	66	F	Break-apart	Negative	6.5	Intrahepatic	Mass forming	AC	MD	Chronic hepatitis, B-viral	pT1N1M0
2	73	M	Break-apart	Positive	3.0	Intrahepatic	Intraductal growth	AC	WD	Macrovesicular steatosis, mild	pT1N0M0
3	60	M	Single-green	Positive	3.1	Intrahepatic	Periductal infiltrative growth	AC	MD	Non-specific reactive hepatitis, mild	pT4N1M0

FISH, fluorescence *in situ* hybridization; F, female; AC, adenocarcinoma; MD, moderately differentiated; M, male; WD, well-differentiated.

AKT, *PI3K/mTOR*, *MEK*, *PARP1/2*, *IDH1*, and *IDH2* [1]. However, clinical trials targeting these genetic alterations have not been successful in proving survival benefit. Combinations of doublet chemotherapy with anti-EGFR agents have shown modest results, and responses with small molecule inhibitors are limited. Use of erlotinib, lapatinib, sorafenib, cetuximab, and bevacizumab has been attempted in biliary tract cancer patients, but the response rates are between 0%-12%, median progression-free survival of 1.8 to 3.7 months, and median OS of 4.4 to 9.8 months. Therefore, greater effort is required for identification of genomically homogenous patient subsets who would benefit from specific targeted agents.

The discovery and characterization of *ROS1* rearrangement in CCA has attracted significant clinical interest because small molecule inhibitors have effective antitumor activity. Because *ALK* and *ROS1* share an approximately 49% amino acid sequence in the kinase domain, ALK inhibitors have been proved to be effective in inhibiting ROS1 activity [16]. In addition, an orthotopic allograft mouse model of intrahepatic CCA validated *FIG-ROS1* fusion as a potent oncogene where it cooperates with *KRAS* and mutant *p53* to accelerate tumor onset [11]. These results suggest that *ROS1* rearrangement in CCA may be a promising druggable target requiring further investigation.

While nine fusion partners to *ROS1* have been identified (*FIG*, *CCDC6*, *CD74*, *EZR*, *KDELR2*, *LRIG3*, *SLC34A2*, *SDC4*, and *TPM3*), all of which retain the ROS1 cytoplasmic kinase domain [15,16], only *FIG-ROS1* fusion transcript has been identified in CCA so far. The frequency of *ROS1* rearrangement in CCA has not yet been well-defined. Gu et al. [10], who screened for *ROS1* rearrangement by reverse transcriptase-polymerase chain reaction (RT-PCR) in CCA patients, reported the frequency of *FIG-ROS1* rearrangement as 8.7% (2 out of 23). However, Arai et al. [18] recently reported that the *FIG-ROS1* fusion in CCA was 0% when screened by RT-PCR in 102 patients. We report the frequency of *ROS1* rearranged tumors as 1.1% in the largest cohort ever. Despite its low incidence, we agree that *ROS1* rearrangements warrant a new molecular subtype of CCA. Indeed, the results of an ongoing clinical trial of a drug targeting *ROS1* rearrangement in CCA patients are eagerly awaited (NCT02374489).

Regarding the screening method of *ROS1* rearrangement, FISH and RT-PCR have been commonly used, although time consuming, costly, and not suitable for massive screening. Accurate identification of *ROS1*-rearranged cancers by IHC analyses using an anti-ROS1 rabbit monoclonal antibody (D4D6) showing 100% (8/8) sensitivity and 100% (138/138) specificity when compared with break-apart FISH in lung cancer was recently reported [19]. However, there are no concrete data validating the screening utility of IHC in CCA. In a recent study IHC screening of ROS1 expression in intra-

hepatic CCA showed 37.1% (72/194) positivity, but all were FISH-negative [20]. Similarly, in our study incidence of ROS1 protein expression was significantly higher than gene rearrangement (19.1% vs. 1.2%). As protein expression of ROS1 may result from amplification and epigenetic changes, we should be cautious in interpreting results of ROS1 immunohistochemistry in CCA. For now, IHC cannot be an efficient screening method, and further validation with respect to ROS1 diagnostics should be established in the future.

The limitation of this study is the lack of information on the fusion variants in *ROS1*-positive samples, which was due to insufficient tissue samples and poor quality of extracted RNA in formalin-fixed, paraffin-embedded tumors.

Conclusion

In conclusion, we screened for *ROS1* rearrangement in the largest-ever international of cohort of 261 CCAs including 208 intrahepatic CCAs and 53 extrahepatic CCAs. *ROS1* rearrangement was detected in 1.2% of total CCAs and 1.4% of intrahepatic CCAs. Our results indicate that future screening efforts may offer a new therapeutic option for advanced CCA patients. Further investigation on the impact of ROS1 inhibition on the survival of CCA patients harboring *ROS1* rearrangements is necessary.

Electronic Supplementary Material

Supplementary materials are available at Cancer Research and Treatment website (http://www.e-crt.org).

Conflicts of Interest

We deeply appreciate Abbott for providing the *ROS1* FISH probes.

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