YES1 Is a Targetable Oncogene in Cancers Harboring YES1 Gene Amplification

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Abstract

Targeting genetic alterations of oncogenes by molecular-targeted agents (MTA) is an effective approach for treating cancer. However, there are still no clinical MTA options for many cancers, including esophageal cancer. We used a short hairpin RNA library to screen for a new oncogene in the esophageal cancer cell line KYSE70 and identified YES proto-oncogene 1 (YES1) as having a significant impact on tumor growth. An analysis of clinical samples showed that YES1 gene amplification existed not only in esophageal cancer but also in lung, head and neck, bladder, and other cancers, indicating that YES1 would be an attractive target for a cancer drug. Because there is no effective YES1 inhibitor so far, we generated a YES1 kinase inhibitor, CH6953755. YES1 kinase inhibition by CH6953755 led to antitumor activity against YES1-amplified cancers in vitro and in vivo. Yes-associated protein 1 (YAP1) played a role downstream of YES1 and contributed to the growth of YES1-amplified cancers. YES1 regulated YAP1 transcription activity by controlling its nuclear translocation and serine phosphorylation. These findings indicate that the regulation of YAP1 by YES1 plays an important role in YES1-amplified cancers and that CH6953755 has therapeutic potential in such cancers.

Significance: These findings identify the SRC family kinase YES1 as a targetable oncogene in esophageal cancer and describe a new inhibitor for YES1 that has potential for clinical utility.

See related commentary by Rai, p. 5702

Introduction

Some cancers depend on a single dominant oncogene for proliferation and survival, a phenomenon called “oncogene addiction” (1). In recent years, some patients with cancers that feature oncogene addiction have greatly benefited from various molecular-targeted agents (MTA). Examples of successful MTAs include anaplastic lymphoma kinase (ALK) inhibitor alectinib for ALK fusion cancer, EGFR inhibitor erlotinib for EGFR-mutant cancer, a-HER2 antibody trastuzumab for HER2-gene-amplified cancer, and v-raf murine sarcoma viral oncogene homolog B (BRAF) inhibitor vemurafenib for BRAF-mutant cancer (1). These MTAs are dramatically effective in cancers with genetic alterations in the target gene, and their success illustrates why it is important to characterize the cancer type and match the treatment to it. Various MTAs are already approved; however, many cancers with high unmet medical needs still have no clinical MTA options. Esophageal cancer is such a cancer; the 5-year survival rate is less than 20% and clinical MTA options are few, especially for the squamous type (2).

SRC, a nonreceptor protein tyrosine kinase, has been identified as being encoded by a proto-oncogene, and the nine members of the SRC family, which includes YES1, SRC, FYN, LYN, and LCK, have various important cellular functions, such as cell growth, adhesion, survival, and differentiation (3). Reports of genetic alterations in the SRC family are few; however, gene amplification of the site containing YES1 has been reported in clinic (4, 5). Furthermore, a recent study found that YES1 gene amplification was a key mechanism of resistance to EGFR or HER2 inhibitors (6, 7). Although the transforming ability of YES1 has yet to be proved, there have been various reports of an association between YES1 and tumorigenicity (8). For example, down-regulating YES1 by short hairpin RNA (shRNA) significantly inhibited cell growth in several malignancies, including colon carcinoma, rhabdomyosarcoma, and basal-like breast cancer (9–11), and some miRNAs were shown to regulate tumor progression via YES1 regulation (12). Taken together, these reports suggest YES1 may play a key role in cancer progression.

Furthermore, there are reports of YES1 participating in numerous signaling pathways. Various mechanisms, including interactions with receptor tyrosine kinases, activate YES1, which in turn activates individual substrates (focal adhesion kinase, BCR1, and paxillin, etc.), resulting in the production of factors involved in growth, survival, invasion, and metastasis (3). In this way, YES1 signaling has been roughly explained, but not fully investigated. Some studies reported that YES1 is associated with YAP1 (13, 14). YAP1, originally isolated as a protein that binds to the SH3 domain of c-Yes (15), is known as a transcriptional coactivator...
in the Hippo pathway and is reported in many studies to be involved in tumor progression (16, 17). YAP1 is dysregulated in tumors and its functions include disruption of contact inhibition, tumor progression, suppression of apoptosis, and release from senescence. The activity of YAP1 is downregulated by phosphorylation of five HxRxS consensus motifs by the large tumor suppressor (LATS) kinase. Serine-phosphorylated YAP1 localizes to cytoplasm and is degraded by proteasomes (16, 18). Tyrosine-phosphorylated YAP1 by YES1, SRC, LCK, or Abelson murine leukemia viral oncogene homolog (c-ABL) kinases is also reported, but its function seems context-dependent and has not been fully investigated (13, 14, 19–21).

Although selective inhibitors to the SRC family kinases (SFK) have not yet made their way into clinic, dasatinib and bosutinib are well known as SFK/multikinase inhibitors. These inhibitors also inhibit ABL, which accounts for their approval for the treatment of Philadelphia chromosome-positive chronic myeloid leukemia. Although these inhibitors can kill various types of solid tumor in preclinical models, their off-target toxicity prohibits their use as therapeutics (3, 22). Therefore, no SFK inhibitors have so far been approved to treat solid tumors.

In this study, we conducted a shRNA library screening and identified YES1 kinase as an attractive target among the amplified genes in esophageal cancer. We proved the oncogenic activity of YES1, displayed the YES1 gene amplification in clinical, generated an inhibitor that was highly selective against SFK and especially against YES1, and examined its potential efficacy to inhibit YES1 kinase in YES1 gene-amplified tumors. We also evaluated the association of YES1 with YAP1 as a key factor in YES1 gene-amplified cancers and found that YES1 kinase activity regulated YAP1 activity by nuclear translocation and serine phosphorylation, and that YAP1 transcriptional activity contributed to the proliferation of YES1 gene-amplified cancers. In summary, we propose that a YES1 inhibitor has therapeutic potential for YES1 gene-amplified cancers.

Materials and Methods

Cell lines and reagents

Cell lines were obtained from the ATCC, Asterand, Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), European Collection of Authenticated Cell Cultures, Immuno-Biological Laboratories, NCI, RIKEN Bio Resource Center, Health Science Research Resources Bank, Health Protection Agency Culture Collections, Korean Cell Line Bank, DS Pharma Biomedical, and the Japanese Collection of Research Bioresources (JCRB) Cell Bank. Esophageal cancer cell lines KYSE70, KYSE140, KYSE-180, KYSE270, KYSE-410, KYSE-450, and KYSE-510 were kindly provided by Dr. Yutaka Shimada (Toyama University, Toyama, Japan) via DSMZ or JCRB (23). All cell lines were obtained more than 1 year prior to experiments and were propagated for less than 6 months after thawing. All cell lines were cultured according to the manufacturer’s instructions and confirmed as Mycoplasma negative by culture or PCR methods described elsewhere. Cellular experiments were performed within 20 passages after thawing.

Generations of Rat-2/KYSE70/OACP4 C/K562 stable transductants

Rat-2 cells (Human science) were infected with an empty lentivirus (Rat-2_mock) or lentivirus containing YES1 wild-type (Rat-2_YES1) prepared using pCDH-EF1-MCS-IRES-Puro (System Biosciences). KYSE70, OACP4 C, and K562 were infected with lentiviruses containing YES1 wild-type (YES1-WT) or mutant T348I (YES1-GK) prepared using pE2-Lv105 (GeneCopeia). Cells stably expressing the above genes were established through tolerance to puromycin following the lentivirus infection.

shRNA library screening and data analysis

We used Custom Lentiviral shRNA library DECIPHER Human Module 1, DECIPHER Human Module 2, and DECIPHER Human Module 3 (Cellecta). Each module contains approximately 27,500 shRNAs targeting approximately 5,000 genes, with 5–6 shRNAs targeting each gene. Further details of the protocol are in Supplementary Materials and Methods. NGS data were analyzed using the Redundant siRNA Activity algorithm (24), which calculates gene-centric P values. We defined P < 0.05 as a screening hit. We used four genes, PLK1, RBX1, KIF11, and EIF3A, as essential genes for positive controls, and luciferase for a negative control.

Immunoprecipitation of YES1

Cells were lysed in Cell lysis Buffer (Cell Signaling Technology) supplemented with cOomplete and PhosSTOP (Roche). To immunoprecipitate the YES1 proteins, Dynabeads Protein G (Invitrogen) conjugated to anti-YES1 antibody (Wako, 013-14261) was added to cell lysate and incubated for 2 hours at 4°C. Precipitates were washed three times in the wash buffer, followed by denaturing with the sample buffer solution containing a reducing agent for SDS-PAGE (Life Technologies). The samples were then subjected to SDS-PAGE and analyzed by Western blotting.

Western blot analysis

Western blot analysis was performed as described previously (25, 26). The details of the antibodies are in Supplementary Materials and Methods.

Protein kinase assay

The inhibitory activity against each kinase was evaluated as described previously (27). The details of the recombinant proteins are in Supplementary Materials and Methods.

Cell viability assay

Cells were seeded in 96-well plates and incubated at 37°C with inhibitors. After 4 or 7 days, cell viability was measured using Cell Counting Kit-8 Solution (Dojindo Laboratories) or CellTiter-Glo Assay (Promega).

Mouse xenograft study

The mouse xenograft study was performed as described previously (28). All in vivo studies were approved by the Chugai Institutional Animal Care and Use Committee. Female BALB/c-nu/nu mice (CAnN.Cg-Foxn1nu/Crlj) were obtained from Charles River Laboratories and kept under specified pathogen-free conditions. Cells (5 × 10⁶ to 7 × 10⁶) were suspended in 200 μl Hank’s Balanced Salt Solution and injected subcutaneously into the right flank of the mice. For the Rat-2_mock and Rat-2_YES1 models, an equal amount of Matrigel (Corning) was mixed with the cell suspension. Tumor size was measured using a gauge twice per week, and tumor volume (TV) was calculated using the following formula: TV = ab³/2, where a is the length of the tumor and b is the width. Once the tumors reached a volume of approximately 200–300 mm³, animals were randomized into groups (n = 3, 4, or 5 in each group),
could be a target molecule for esophageal cancer therapy. YES1 had no oncogenic activity (8). However, we proved YES1 gene amplification, which compared KYSE70 with NCI-H2009, the number found by using ViaFect (Promega). After 24 hours, cells were treated with the compound for another 24 hours. Cell lysates were analyzed for firefly and Renilla luciferase activities with the Dual-Glo Assay System (Promega). Firefly luciferase activity was normalized by calculating the ratio to Renilla luciferase activity. The relative luciferase activity was reported as a fold change to the compound-untreated control.

Quantitative real-time PCR
Total RNA was extracted and purified using RNAeasy 96 Kit (Qiagen) and cDNA was synthesized using Transcriptor Universal cDNA Master (Roche) according to the manufacturer’s instructions. Real-time PCR analysis was run on the LightCycler 480 System (Roche). For data analysis, counts were normalized to the housekeeping gene GAPDH at the same condition. Counts were reported as fold change relative to the untreated control. Details of the PCR primers are in Supplementary Materials and Methods.

Immunofluorescence
Cells were cultured on coverlips or 96-well plates. Cells were fixed, permeabilized, and blocked by Image-it Fixation/Permeabilization Kit (Life Technologies). Then cells were incubated with YAP1 antibody (Abcam, ab52771) at room temperature for 60 minutes. The secondary antibody was goat anti-Rabbit IgG coupled with Alexa Fluor 88 (green; Life Technologies) incubated at room temperature for 60 minutes in the dark. Nuclei were counterstained with Hoechst 33342. Images were acquired by immunofluorescence microscopy (Nikon, E-600 Eclipse or Thermo Fisher Scientific Inc., Cellomics ArrayScan VTI System).

Results
YES1 has an oncogenic role
In a shRNA library of 15,000 genes, we set out to identify an oncogene with a genetic alteration in esophageal cancer, by screening the KYSE70 esophageal cancer cell line, using HCT-116 (colorectal cancer) and NCI-H2009 (lung cancer) cell lines as controls. In the first trial, which compared KYSE70 with HCT-116, the number of genes with a significant impact on growth found only in KYSE70 was 488. In the second trial, which compared KYSE70 with NCI-H2009, the number found only in KYSE70 was 1,164. There were 128 genes common to both trials. We screened genetic alterations of the 128 genes in the Cancer Cell Line Encyclopedia (CCLE) database (https://portals.broadinstitute.org/ccle) and identified only one with gene amplification, YES1 (Fig. 1A), which suggested that YES1 could be a target molecule for esophageal cancer therapy.

According to an in vitro experiment in a previous publication, YES1 had no oncogenic activity (8). However, we proved YES1’s oncogenic activity with in vivo experiments. We lacked access to esophageal cancer cell lines with YES1 deletion, so we used the normal rat fibroblast cell line Rat-2 to generate clones that had been stably transduced with an empty lentiviral vector (Rat-2_mock) or a lentiviral vector expressing YES1 (Rat-2_YES1). The use of a normal fibroblast cell line to validate oncogenic activity is a common procedure for validating transformation of oncogenes such as ALK fusion, ROS fusion, and FGFR fusion (28–30). We used the YES1-transduced bulk cells and checked YES1 expression in Rat-2_YES1 cells in vitro and in vivo (Fig. 1B and C). We observed that YES1 overexpression promoted tumorigenicity in Rat-2_YES1 cells compared with the Rat-2_mock cells (Fig. 1D), and Rat-2_YES1 tumors were about 13 times larger than Rat-2_mock tumors at 45 days after inoculation (Fig. 1E), suggesting that YES1 has oncogenic potential in vivo.

YES1 gene amplification occurs in various cancer types
To evaluate the frequency of YES1 genetic alterations in clinical, we analyzed copy number alterations and mutations in The Cancer Genome Atlas (TCGA) data using cBioPortal (http://www.cbioportal.org/; refs. 31, 32) and found YES1 gene amplification in various tumor types (Table 1), but because the biological function of each YES1 mutation is unknown, we did not analyze them in detail. We confirmed YES1 gene amplification by FISH in a tissue microarray of esophageal cancer and lung cancer (Supplementary Fig. S1A). Next, we used the PrognoScan database (33) to analyze the correlation between YES1 expression and prognosis for these tumor types. In esophageal and lung cancer, the prognosis for patients with high YES1 expression was worse than for those with low YES1 expression at a level that is statistically significant, suggesting the contribution of YES1 in these malignancies (Supplementary Fig. S1B). Taken together, we hypothesized that YES1 gene amplification accompanied with high expression could be an anticancer target, not only for esophageal cancer but also for other cancers, and we set about generating a YES1 inhibitor.

YES1 kinase inhibitory activity of CH6953755
To obtain a YES1 inhibitor, we performed a high-throughput screening of a chemical library with more than half-a-million compounds at Chugai Pharmaceutical Co. Ltd. and identified a lead compound that inhibited a relatively broad range of kinases but had a different chemical scaffold from known SFK inhibitors. We then structurally modified and improved the pharmacokinetics profile and selectivity against SFKs, especially YES1. Finally, we generated an aminopyrazole derivative, CH6953755, as a potent and orally available YES1 inhibitor (Fig. 2A; ref. 34).

To investigate the selectivity of CH6953755 and compare it with other known SFK inhibitors, dasatinib (35) and bosutinib (36), we evaluated the inhibitory activity against 39 kinases in an enzyme assay (Fig. 2A and B; Supplementary Table S1). The IC\textsubscript{50} value of CH6953755 on the enzyme activity of YES1 was 1.8 nmol/L. Although bosutinib had similar IC\textsubscript{50} value as CH6953755 and dasatinib had about 10-fold higher activity on YES1 kinase inhibition, CH6953755 exhibited more specificity for YES1 among the tested SFKs compared with both compounds (Fig. 2B and C; Supplementary Table S1). We used dasatinib for a further comparison study of CH6953755 based on its potent YES1-inhibiting activity but low selectivity.

To evaluate the kinase selectivity of CH6953755 further, we used a KINOMEscan Panel (DiscoveRx) consisting of 456 WT and mutant kinases (Supplementary Table S2). In the panel, CH6953755 at 10 nmol/L bound (at more than 65% inhibition to an ATP analog) to only eight WT kinases, including YES1 and four other SFKs.
YES1 kinase inhibition leads to cell growth inhibition of YES1-amplified cancer cell lines

Next, we investigated whether YES1 kinase inhibition inhibited the cell growth of YES1-amplified cancer cells. First, we analyzed the YES1 expression and copy number data of 1,035 cancer cell lines in the CCLE database to determine the profile of lines harboring YES1 gene amplification. We defined YES1 gene amplification as having a copy number over four. We confirmed that
YES1 expression was upregulated in amplified cell lines (Supplementary Fig. S2A), and validated this upregulation by measuring YES1 protein levels in four YES1-amplified and four non-YES1-amplified cancer cell lines (Supplementary Fig. S2B). These results confirmed that YES1 gene amplification includes the upregulation of YES1 expression in YES1-amplified cancer cell lines both in mRNA and protein levels. Next, we assessed the antiproliferative activity of YES1 inhibitors. CH6953755, dasatinib, EGFR inhibitor erlotinib, mTOR inhibitor everolimus, and a MEK inhibitor CH4987655 (37) were tested against a panel of 66 cancer cell lines, seven of which had YES1 amplification. YES1-amplified cancer cell lines showed higher sensitivity to CH6953755 than non-YES1-amplified cancer cell lines (Fig. 3A; Supplementary Table S3). CH6953755 prevented the autophosphorylation at Tyr426 of YES1 that upregulates enzymatic activity in KYSE70 cells harboring YES1 amplification (Fig. 3B). Dasatinib also showed more potent efficacy against YES1-amplified cell lines than against nonamplified lines (Supplementary Fig. S3A), but YES1 amplification did not seem to predict the efficacy of erlotinib, everolimus, or CH4987655 (Supplementary Fig. S3B–S3D, Supplementary Table S2). Therefore, we considered that YES1 gene amplification status could predict the efficacy of a YES1 inhibitor. Next, we tried to check whether the CH6953755 cytotoxicity was the result of YES1 inhibition in YES1-amplified cancer cell lines using the commonly used chemical–genetic approach of engineering an inhibitor-resistant mutant of YES1 (38, 39). From YES1-amplified KYSE70 and OACPC4 C, and YES1 nonamplified K562 cell lines, we generated clones stably transduced with a lentiviral vector expressing YES1-WT or mutant T348I, a so-called gatekeeper mutation (YES1-GK) that had a point mutation at the binding site with CH6953755 and was expected to be resistant to CH6953755. In KYSE70 cells expressing YES1-GK (KYSE70_YES1-GK), phosphorylation at Tyr426 was not suppressed by CH6953755 treatment, unlike in KYSE70 cells expressing YES1-WT (KYSE70_YES1-WT; Fig. 3C). As expected, the two cell lines expressing YES1-GK with amplified YES1 gene became resistant to CH6953755, while this was not the case with the line without amplified YES1 (Fig. 3D). Similar results were found with dasatinib (Supplementary Fig. S4). These results indicated that YES1 kinase inhibition inhibited the cell growth of YES1-amplified cancer cell lines.

Furthermore, we analyzed whether the other SFKs had an effect on YES1-amplified cancer cell lines. We examined SFK expression profiles in YES1-amplified cancer cell lines and determined the dominance of YES1 expression (Supplementary Fig. S5), suggesting that the contributions of SFKs other than YES1 were low in YES1-amplified cancer cell lines. We focused on SRC as representative of other SFKs and confirmed this with a cell growth inhibition assay using KYSE70 clones stably transduced with an empty lentivirus or lentivirus-expressing SRC-WT or YES1-WT (KYSE70_mock, KYSE70_SRC-WT, and KYSE70_YES1-WT) treated with CH6953755 or dasatinib. Efficacy against KYSE70_mock and KYSE_YES1-WT was almost the same for treatment with CH6953755 and dasatinib, while the efficacy of CH6953755 in KYSE70_SRC-WT was less sensitive than in KYSE70_YES1-WT or KYSE70_mock, while the efficacy of dasatinib was pretty much unchanged (Supplementary Table S4). It was thought that SRC initiated activation of the same downstream signal as YES1, which contributed to cell proliferation when SRC was expressed in KYSE70, and SRC inhibitory activity of CH6953755 was about 20-times weaker than YES1 inhibitory activity, while the SRC inhibitory activity of dasatinib was almost same as YES1 (Supplementary Table S1). On the basis of the evidence from these experiments, we think that YES1 activity is dominant among SFKs, and that the cytotoxicity of CH6953755 resulted from YES1 inhibition in YES1-amplified cancer cell lines, all of which suggests the therapeutic potential of a YES1 inhibitor for YES1-amplified cancers.

### Table 1. The clinical prevalence of YES1 gene amplification in various cancer types

<table>
<thead>
<tr>
<th>Cancer type</th>
<th>Frequency</th>
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<tbody>
<tr>
<td>Esophageal squamous cell carcinoma (TCGA, provisional)</td>
<td>6.3% (6/96 cases)</td>
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<tr>
<td>Esophageal adenocarcinoma (TCGA, provisional)</td>
<td>5.7% (5/88 cases)</td>
</tr>
<tr>
<td>Head and neck squamous cell carcinoma (TCGA, provisional)</td>
<td>5.1% (27/530 cases)</td>
</tr>
<tr>
<td>Lung squamous cell carcinoma (TCGA, provisional)</td>
<td>4.5% (23/511 cases)</td>
</tr>
<tr>
<td>Bladder urothelial carcinoma (TCGA, provisional)</td>
<td>4.4% (13/301 cases)</td>
</tr>
<tr>
<td>Sarcoma (TCGA, provisional)</td>
<td>3.0% (8/265 cases)</td>
</tr>
<tr>
<td>Ovarian serous cystadenocarcinoma (TCGA, provisional)</td>
<td>3.0% (18/606 cases)</td>
</tr>
</tbody>
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NOTE: We analyzed the clinical copy number alteration data using cBioPortal (http://www.cbioportal.org/) on August 18, 2018. The results shown here are based upon data generated by the TCGA Research Network (https://cancer.gov/).
that YES1 gene amplification is a noncanonical YAP1 activation mechanism in cancer cells. The transcription factor TEAD has been reported to bind YAP1 and play a major role in YAP1 oncogenic function (41). YAP1 functions as a transcriptional coactivator for TEAD to induce gene expression, such as ANKRD1 and CYR61, thereby promoting cell growth, proliferation, and survival (16, 42). Therefore, we transfected the cell lines with TEAD luciferase reporter and tested the effect of YES1 inhibitor, CH6953755, on luciferase activity. CH6953755 suppressed TEAD luciferase reporter activity in YES1-amplified KYSE70 and RERF-LC-AI (Fig. 5A). To confirm that the suppression was derived from YES1 kinase inhibition, we utilized KYSE70_YES1-GK and found that CH6953755 could not suppress their TEAD luciferase reporter activity, even though it could suppress the activity in KYSE70_YES1-WT (Fig. 5B). Furthermore, CH6953755 suppressed the expression of YAP1 downstream genes, ANKRD1 and CYR61, in KYSE70_YES1-WT, without affecting the YAP1 expression level, and this suppression was abrogated in KYSE70_YES1-GK (Fig. 5C). These phenomena were also observed with dasatinib treatment (Supplementary Fig. S7A and S7B), suggesting that YES1 kinase activity regulates YAP1 activity.

Figure 2.
Compared with dasatinib and bosutinib, CH6953755 tends to be selective to YES1. A, Chemical structures of CH6953755, dasatinib, and bosutinib. B, Kinase inhibitory activity of CH6953755, dasatinib, and bosutinib against 39 kinases. C, Kinase inhibitory activity of CH6953755, dasatinib, and bosutinib against five SFKs.
YES1 regulates YAP1 activity by controlling its nuclear translocation and serine phosphorylation via kinase activity

Our next question was how YES1 regulated YAP1 activity. First, we looked at the YAP1 localization. Multikinase inhibitor dasatinib has been reported to inhibit nuclear localization of YAP1 (20, 21), so we hypothesized that YES1 regulated YAP1 activity by controlling YAP1 localization. We evaluated the localization of YAP1 and confirmed the phenomenon in KYSE70, YES1-WT or -GK after treatment with CH6953755. YAP1 nuclear localization was inhibited in the KYSE70 cell line expressing YES1-WT, and the inhibition was abrogated in KYSE70_YES1-WT or KYSE70_YES1-GK (Fig. 6A; Supplementary Fig. S8). Furthermore, we confirmed this phenomenon in in vivo setting (Supplementary Fig. S9). Next, we evaluated the effects of YAP1 phosphorylation sites on CH6953755 activity. Prior work has demonstrated that YES1 phosphorylation of YAP1 at the tyrosine 357 site regulated YAP1 transcriptional activity (13), so we examined the TEAD luciferase reporter activity when the following YAP1 mutations were being expressed. Y357E, which is phosphomimetic, or Y357F, which is phosphodead (19). Neither the YAP1 Y357E nor the Y357F mutation affected the suppression of YAP1 transcriptional activity by CH6953755 (Fig. 6B), which suggested that phosphorylation of YAP1 at tyrosine 357 did not have any impact on the control of YAP1 activity by YES1 in YES1-amplified cancer.

Then we assessed the effect of YAP1 serine sites, which are important for YAP1 regulation in the canonical Hippo pathway. Several groups have reported that YAP1 5SA, which is mutated in all of the HxRxxS consensus motifs, makes YAP1 constitutively active by abolishing the phosphorylation by LATS (43), so we generated two KYSE70 clones, #1 and #7, which were stably transduced with a lentiviral vector expressing YAP1 5SA. In these clones, the suppression of TEAD luciferase reporter activity after treatment with CH6953755 was abrogated (Fig. 6C), and the antiproliferative activity of CH6953755 was diminished (Fig. 6D). Taken together, the findings suggest that YES1 regulates YAP1 activity by serine phosphorylation indirectly and by nuclear localization. In conclusion, YES1-YAP1 signal inhibition by CH6953755 leads to antitumor activity in YES1-amplified cancer cells.

Discussion

In this study, first, we screened a shRNA library to identify YES1 as a fascinating oncogenic target, and we also analyzed the Project Achilles data (44). The frequency of YES1 gene amplification among cell lines in Project Achilles is 2.6% (13/502). We used four genes, PIK1, RBX1, KIF11, and EIF3A, as essential genes for positive control, but these genes showed no significance in YES1-amplified cancer cell lines based on our analysis of Achilles data. Next, we changed the criteria for positive control by using the average of the most potent three of the four individual EIF3A shRNAs to show significance (P < 0.05). Four of 13 YES1-amplified cancer cell lines met this criterion, but we did not identify either YES1 or YAP1 as hits. This result differs from
our shRNA screening result, but we think that this is due to the different experimental and analytic conditions, as well as the possibility of lower silencing efficiency of YES1 shRNAs and YES1 copy number in the analyzed cell lines with a lower copy number than KYSE70. We determined that we could confirm our shRNA screening result showing that YES1 had an important role in KYSE70 by using the chemical–genetic approach of engineering an inhibitor-resistant GK mutant of YES1 (Fig. 3D).

Figure 4.
CH6953755 shows selective antitumor activity against YES1-amplified xenograft tumors. Mice bearing Rat-2_YES1 cells were treated with CH6953755 60 mg/kg or vehicle orally once daily for 10 days. A, Tumor volume in each group was measured. Data are shown as mean ± SD (n = 3). Two-tailed Student t test: *P < 0.05, versus vehicle treatment at final day. B, Xenograft tumors were extracted 24 hours after the last dosing and analyzed by Western blotting (n = 3). YES1 proteins were immunoprecipitated (IP) from cell lysates and blotted (IB). C–E, CH6953755 at the indicated doses was given orally once daily to xenograft models bearing cells with YES1 amplification, KYSE70 (for 11 days) and RERF-LC-AI (for 14 days; C) or xenograft models bearing cells without YES1 amplification, ACHN (for 12 days) and HARA (for 11 days; D). Tumor volume and body weight change in each group were measured. Data are shown as mean ± SD (n = 4). C, Parametric Dunnett test: *P < 0.05; **P < 0.01; ***P < 0.001; n.s., no significant difference, versus vehicle treatment at final day. D, Two-tailed Student t test: n.s., no significant difference, versus vehicle treatment at final day. E, Xenograft tumors of KYSE70 (n = 4 and 3) and RERF-LC-AI (n = 4 and 2) were extracted 24 hours after the last dosing and analyzed by Western blotting. YES1 proteins were immunoprecipitated from cell lysates and blotted. CN, copy number.
We demonstrated that YES1 has oncogenic potential and is activated by gene amplification in several tumor types. Therefore, patients with the amplified YES1 gene are expected to benefit from a YES1 inhibitor. At present, the well-known SFK/multikinase inhibitors dasatinib and bosutinib are not effective against solid tumors in clinical. One often-cited reason is that there are no prognostic biomarkers related to SFK activity that can be used to select patients for clinical trials (3, 22). However, even if an appropriate biomarker was found, the dose regimen for chronic myeloid leukemia is insufficient to inhibit phosphorylation of SRC and, moreover, is close to the MTD (45, 46). Consistent with the finding in clinical, dasatinib even at the MTD achieved moderate in vivo efficacy against YES1-amplified KYSE70 xenograft model (Supplementary Fig. S6), whereas treatment with CH6953755 achieves significant efficacy at a nontoxic dose (Fig. 4C). These data suggest that the selectivity of CH6953755 will allow the dose in clinic to be increased to a level sufficient to suppress YES1 kinase and that CH6953755 could be effective in meeting the unmet medical needs of patients with YES1-amplified cancer.

The YES1 gene might also be enriched in patients insensitive to several current standards of care. One study reported that treatment with 5-fluorouracil (5-FU) upregulated YES1 and YAP1 in patients with colorectal cancer and these patients have a poor prognosis (47). Treatment with 5-FU was also reported to increase the gene copy number of the region that includes thymidylate synthetase (TYMS), a target of 5-FU, in patients with colorectal cancer, and this increase is considered to be one of the mechanisms of 5-FU resistance (48). As the chromosome locus of TYMS is so close to YES1, the YES1 gene could be amplified together with the TYMS gene. Because 5-FU is the first-line therapy in esophageal cancer, this pattern of resistance could also happen there. Although patients with esophageal cancer in general have quite a poor prognosis, the segment of patients with high YES1 expression has a worse prognosis than the segment with low expression (Supplementary Fig. S1B). Furthermore, YES1 amplification is a mechanism of resistance to some MTAs (6, 7). For example, acquired amplification of YES1 is a recurrent mechanism of resistance to EGFR inhibition in EGFR-mutant lung cancers for which YES1 inhibition is effective (49). Moreover, reports show that YAP1 is essential for the suppression of antitumor
After 24 hours, cells were treated with the indicated concentrations of CH6953755 for 24 hours, and the relative luciferase activity was determined. Data are shown as mean +/- SD (n = 3). Parametric Dunnett test: *, P < 0.05; n.s., no significant difference, versus YAP1_WT for each compound concentration.

Subsequently, cell viability was measured. Data are shown as mean +/- SD (n = 3). Parametric Dunnett test: ***, P < 0.001; n.s., no significant difference, versus DMSO control treatment for each cell line.

We revealed that YES1 regulates YAP1 activity by controlling YAP1 localization, and that the YES1-YAP1 signal is important in the proliferation of YES1-amplified cancers. In the cell growth inhibition assay, the YES1-amplified cell line KYSE-510 was insensitive to CH6953755 and dasatinib (Supplementary Table S3), and harbored not only YES1 gene amplification but also YAP1 gene amplification. This suggests that YES1 could exist upstream of YAP1, which also supports our findings. However, two points are still unclear: how precisely does YES1 regulate YAP1 activity? Are there other important YES1 downstream signals in YES1-amplified cancers? Regarding YAP1 regulation by YES1, previous research found that YES1 directly phosphorylated YAP1 at tyrosine 357 and regulated YAP1 transcriptional activity in β-catenin-driven cancers (13). In contrast to previous research, our evaluation of YES1-amplified cancer expressing phosphomimetic Y357E and phosphodead Y357F showed that tyrosine 357 phosphorylation did not contribute to YAP1 transcriptional activity (Fig. 6B). On the other hand, our evaluation in YES1-amplified cancer expressing YES1-GK proved that YES1 kinase activity is indispensable for YAP1 transcriptional activity and nuclear translocation (Figs. 5B and C and 6A), and the evaluation in YES1-amplified cancer expressing five serine-mutated YAP1 implies that YAP1 serine phosphorylation has an
effect on the YES1-YAP1 signal (Fig. 6C and D). These results suggest that YES1 may indirectly activate YAP1 via phosphorylation of some other kinase involved in the regulation of YAP1 serine phosphorylation, which in turn suggests that the mechanism by which YES1 regulates YAP1 may be dependent on the cellular context. As for other YES1 downstream signals important for proliferation, we consider that signals other than YAP1 do contribute to the proliferation of YES1-amplified cancers. In cells expressing the YAP1 constitutive active form, YAP1 5SA, suppression of YAP1 transcriptional activity by CH6953755 was abrogated completely, but the abrogation of cell growth inhibition was partial (Fig. 6C and D). Therefore, we think that known YES1 downstream signals, such as MAPK and AKT, or other signals may be involved in the proliferation of YES1-amplified cancers.

In summary, we suggest that YES1-YAP1 signal inhibition is a mode of action through which CH6953755 achieves efficacy, and that CH6953755 has therapeutic potential for patients harboring the YES1 gene amplification.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: N. Hanamana, Y. Nakanishi Development of methodology: N. Hanamana, Y. Nakanishi, K. Ogasawara Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): N. Hanamana, T. Mizuno, K. Horiguchi-Takei, N. Akiyama, H. Tanimura, M. Hasegawa Writing, review, and/or revision of the manuscript: N. Hanamana, Y. Nakanishi, K. Ogasawara, H. Ebike, H. Koyano, T. Mio

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Acknowledgments
We thank Yasue Nagata, Maiko Iizawa, Yumiko Hashimoto, Tomoaki Hayashi, Yusuke Ide, and Emie Sawamura for performing pharmacologic assays. Chiaki Senoo, Hiroshi Tanaka, Kenji Kashima, Hironori Mutoh, Yuko Aoki, Masahiro Aoki, and Takuo Tsukuda for helpful discussion; and Hideaki Mizuno for PrognoScan analysis. This study was funded by Chugai Pharmaceutical Co., Ltd.

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Received October 26, 2018; revised February 18, 2019; accepted July 23, 2019; published first August 7, 2019.

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