Yes is a central mediator of cell growth in malignant mesothelioma cells

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Abstract. The constitutive activation of the Src family kinases (SFKs) has been established as a poor prognostic factor in malignant mesothelioma (MM), however, the family member(s) which contribute to the malignancy have not been defined. This study aimed to identify the SFK member(s) contributing to cell growth using RNA interference in various MM cell lines. Silencing of Yes but not of c-Src or Fyn in MM cells leads to cell growth suppression. This suppressive effect caused by Yes silencing mainly depends on G1 cell cycle arrest and partly the induction of apoptosis. Also, the knockout of Yes induces the inactivation of β-catenin signaling and subsequently decreases the levels of cyclin D necessary for G1-S transition in the cell cycle. In addition, Yes knockout has less effect on cell growth suppression in β-catenin-deficient H28 MM cells compared to other MM cells which express the catenin. Overall, we conclude that Yes is a central mediator for MM cell growth that is not shared with other SFKs such as c-Src.

Introduction

Malignant mesothelioma (MM) from the serosal membranes of the body cavities, is a particularly aggressive cancer which is characterised by rapid progression, late metastases, and poor prognosis (1). Although surgery, radiotherapy, chemotherapy, and/or their combinations have been used as therapeutic modalities, median patient survival is 8-18 months (2). MM cells exhibit resistance to many chemotherapeutic agents, including doxorubicin and cisplatin, which are nevertheless widely used to treat MM (3). A recent report of a phase III study showed that the combination of pemetrexed and cisplatin is more effective than cisplatin alone with differences in response rate of 41.3 versus 16.3% (4). However, most of the patients relapsed within a year after starting the treatment. Therefore, new therapeutic approaches are urgently needed for MM patients. In addition to conventional chemotherapy, there have been many advances in targeted therapies for several cancers, such as epidermal growth factor receptor (5). The Src family of kinases (SFK), which is a family of intracellular non-receptor tyrosine kinases, is one candidate molecule that could hold promise in the treatment of cancer patients, including MM (6).

SFK constitutes a family of 11 non-receptor tyrosine kinases; Src, Fyn, Yes, Blk, Yrk, Frk, Fgr, Hck, Lck, Lyn and Rgr that share similar structural and biochemical properties (7). Of the members, c-Src, Fyn, and Yes are widely expressed in tissues and appear to play an important role in the regulation of cell adhesion, cell growth, and differentiation (8). The activated forms of SFK, particularly c-Src, are capable of transforming many different cell types (9), and the activation or overexpression of human SFK has been observed in a range of human cancers (10). A member of SFK, Yes is the cellular counterpart of the viral v-Yes protein encoded by the Yamaguchi avian sarcoma virus (11). Amongst SFK, Yes exhibits the highest homology with 70% identity outside the N-terminus with c-Src. In v-Yes a C-terminal truncation, as in v-Src, allows the kinase to be constitutively active and highly oncogenic due to the removal of the negative regulatory Tyr. Such an activating mechanism has not been reported in human cancer, however, Yes is found frequently activated in colorectal cancer (CRC). Nonetheless, Yes activation in CRC correlates more closely with poor prognosis than does c-Src activation (12,13). It was clearly demonstrated that Yes regulates specific oncogenic signaling pathways important for CRC progression that is not shared with c-Src (13). In our preliminary experiment, we observed that some MM cells showed overexpression of Yes compared to c-Src. Based on this observation, we hypothesized that Yes also played an important role in the appearance of malignancy in MM. In this context, the present study was undertaken to confirm this hypothesis.

Materials and methods

Reagents. All culture reagents were purchased from Invitrogen (Carlsbad, CA, USA). VBL was obtained from Wako Pure Chemicals (Osaka, Japan). Non-specific (NS) small interfering RNA (siRNA), HP validated siRNAs for c-Src (cat no. SI02664151), Yes (cat no. SI00302218), and Fyn (cat no.

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Abbreviations: CRC, colorectal cancer; MM, malignant mesothelioma; RT-real-time PCR, reverse transcription-real-time polymerase chain reaction; siRNAs, short interfering RNAs; SFK, the Src family of kinases

Key words: mesothelioma, Yes, β-catenin, G1 arrest, apoptosis

SI00605451) and HiPerfect transfection reagent were obtained from Qiagen Japan (Tokyo, Japan). PCR primers were also purchased from Qiagen. Other chemicals were purchased from Sigma (St. Louis, MO, USA), unless otherwise noted. All antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA).

Cell culture. Human non-malignant transformed mesothelial cell (Met5A) and MM cells (H28, H2052, H2452 and MSTO-211H) obtained from ATCC (Manassas, VA, USA), were routinely maintained in RPMI-1620 medium supplemented with 10% fetal bovine serum and penicillin-streptomycin at 37˚C in an atmosphere of 5% CO_2.

Cell growth analysis. The cells were cultured on microtiter plates ($3x10^4$ cells/well) and treated with siRNA treatment as described in *Transfection of short interfering RNA (siRNA)*. Cell viability was then determined using the Cell Proliferation Assay kit with WST-1 reagent (Sigma), according to the manufacturer's instructions.

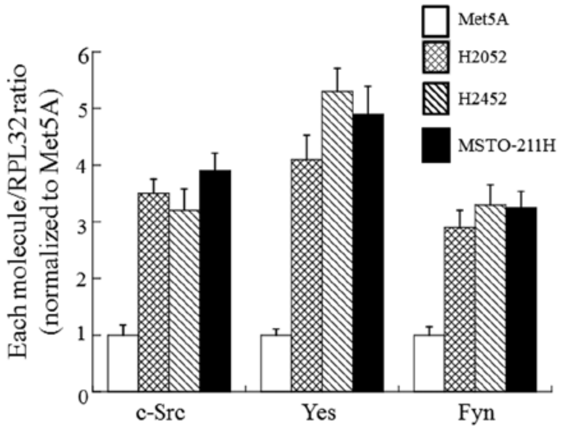

Figure 1. Each SFK family member mRNA level in non-tumorigenic mesothelial and three MM cell types. Each mRNA level was determined by RT-realtime PCR as described in Materials and methods. Each column indicates the mean from three samples; vertical lines indicate SD. Each SFK member mRNA level in MM cells has significant difference compared to that in non-tumorigenic mesothelial cells.

Cell cycle and apoptosis analysis. After the siRNA treatment the cells were harvested by trypsinization, washed with PBS, re-suspended in 70% ethanol in PBS, and kept at 4˚C for ≤30 min. Before analysis, cells were washed again with PBS and resuspended and incubated for 30 min in PBS containing 0.05 mg/ml propidium iodide, 1 mM EDTA, 0.1% Triton X-100, and 1 mg/ml RNase A. The suspension was then passed through a nylon mesh filter, and the ratio of each fraction in cell cycle was analyzed on a Becton-Dickinson FACScan (Franklin Lakes, NJ, USA), and the ratio of subG1 population was estimated to confirm the induction of apoptosis.

Transfection of short interfering RNA (siRNA). Each molecule was downregulated by short interfering RNAs (siRNAs) targeting each molecule. For transfection, the cells were seeded in each plate and transfected with HiPerfect transfection reagent according to the manufacturer's instructions. Then the cells were treated with the siRNA for 48 h, and subsequently, knockdown of each by siRNA was confirmed by RT-real-time PCR. As a negative control, NSsiRNA was used. Also, after the siRNA treatment for 48 h, WST-1 and immunoblot analysis were performed.

Gene expression analysis. Total RNA was isolated by using SV Total RNA Isolation System (Promega, Madison, WI, USA) and cDNA was synthesized as previously described (14). Real-time PCR was performed by using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems Japan Ltd. Tokyo, Japan) and SYBR Premix Ex Taq™ (Takara Bio Inc., Shiga, Japan) according to the manufacturer's instructions. The primers used were from Qiagen, and each product number was as follows: ribosomal protein CL32 (PRL32), QT01668198; c-Src, QT00039326; Yes, QT00037940; Fyn, QT00054005.

Immunoblot analysis. Immunoblot analysis was performed as previously described (14). Briefly, cell lysate was prepared in Cell Lysis/Extraction Reagent (Sigma) including phosphatase inhibitor cocktail 1, phosphatase inhibitor cocktail 2, and protease inhibitor cocktail, and 10 μg total protein extract from each sample was loaded onto a 10% SDS-polyacrylamide gel. After electrophoresis, proteins were transferred to nitrocellulose membranes. The blots were incubated with each antibody. Each immunoreactive band was detected using the ECL system (Amersham) and a cooled CCD camera-linked Cool Saver System (Atto, Osaka Japan). Molecular sizing was done using Rainbow MW marker (Amersham). Protein concentrations were determined using DC Protein Assay System (Bio-Rad, Hercules, CA, USA). Also, membrane/cytoplasm separations were done using Subcellular Protein Fractionation kit according to the manufacturer's instructions (Pierce, ThermoScientific, Tokyo, Japan).

Statistical analysis. Data were analyzed by one-way ANOVA followed by Student's t-test or Dunnett's multiple-range test. $P<0.05$ was considered significant.

Results

Expression patterns of SFK in MM cell lines. Similarly to other tumors, SFK was commonly activated in MM cells and primary MM specimens (15). In order to determine which molecule of SFK is expressed in MM cells, we compared expression patterns of SFK in non-tumorigenic mesothelial cells (Met5A) and three different types of MM cells (H2052, H2452 and MSTO-211H). As shown in Fig. 1, at least three different members of SFK, c-Src, Yes and Fyn were expressed in all cell lines tested. Compared to Met5A cells, the three MM cells showed significantly higher expression levels in the three members of SFK. Of these members of SFK, the level of Yes was the highest. Besides c-Src, Yes, and Fyn, another SFK member, Lyn, was also detected, but the level was lower than other molecules (data not shown).

Contribution of Yes to cell growth in MM cells. Recent reports showed that SFK members played different roles in the appearance of malignant phenotypes on tumor cells (9,10), so we estimated which molecule of the three SFKs examined could contribute to cell growth in MM cells. As shown in Fig. 2,

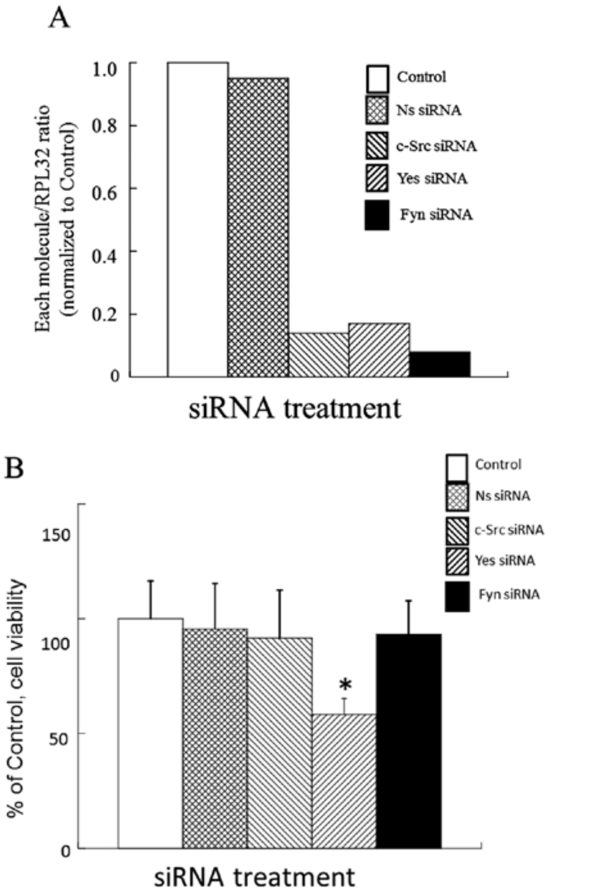

Figure 2. Silencing of each SFK member mRNA by siRNA treatment (A) and effect of each SFK member siRNA treatment on cell viability (B) in H2452 cells. (A) After siRNA treatment for 48 h, each mRNA level was determined as described in Materials and methods. Each value indicates the mean from two samples. (B) After siRNA treatment for 48 h, cell viability was determined by WST-1. Each column indicates the mean from five samples; vertical lines indicate SD. *Significant difference from other treatment groups.

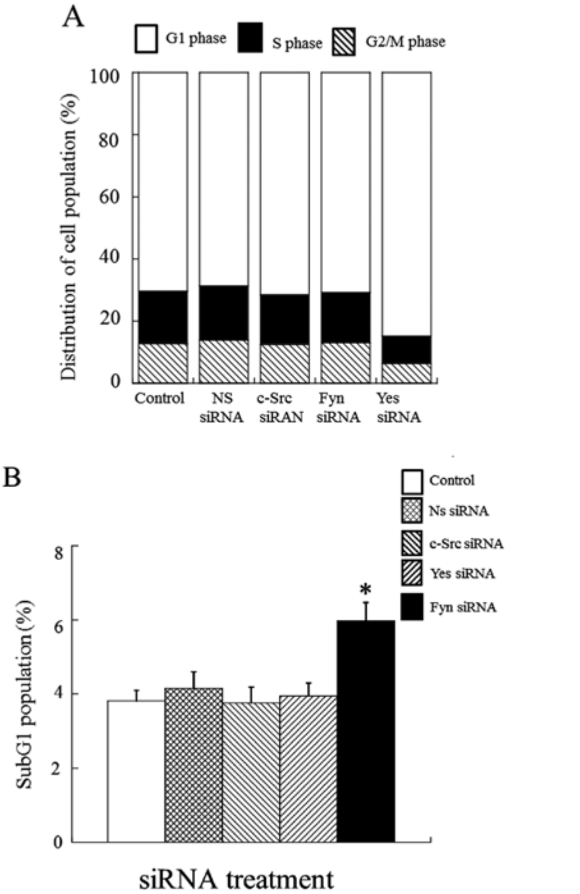

Figure 3. Effects of Yes siRNA treatment on cell cycle progression (A) and apoptosis induction (B) in H2452 cells. The cells were treated with each siRNA treatment for 48 h for FACS analysis. Each value in cell cycle is the mean from three samples, and values in subG1 are expressed as the percentage of cell numbers in subG1 to the total cell numbers in the cell cycle and means from three samples; vertical lines indicate SD. *Significant difference from other treatment groups.

only knock down of Yes by siRNA significantly reduced cell growth (-42%) in H2452 cells under almost the same silencing condition of the SFK members. Also, we observed the same effect on cell growth in H2052 and MSTO-211H cells (data not shown). These results suggest that Yes plays an important role in cell growth control of MM cells. With respect to cell cycle regulation under knockdown of Yes, G1 arrest was induced in H2452 cells (Fig. 3A). The silencing of Yes induced by siRNA significantly increased SubG1 population in H2452 cells by ~45% (Fig. 3B). H2052 and MSTO-211H cells showed similar results (data not shown). Overall, it seems that the knockdown of Yes-mediated cell growth control mainly depends on G1 arrest in the cell cycle.

Effect of Yes knockdown on β-catenin localization and signaling. In a colon carcinoma cell study (16), Yes knockdown induced β-catenin accumulation in membrane and induced the inactivation of β-catenin signaling. Thus, we next determined whether Yes silencing could affect β-catenin localization and signaling in H2452 cells. As shown in Fig. 4A, biochemical analysis of β-catenin cytosolic and membrane fractions showed that Yes knockdown restored the localization of the catenin to the membrane fraction. The knockdown of Yes

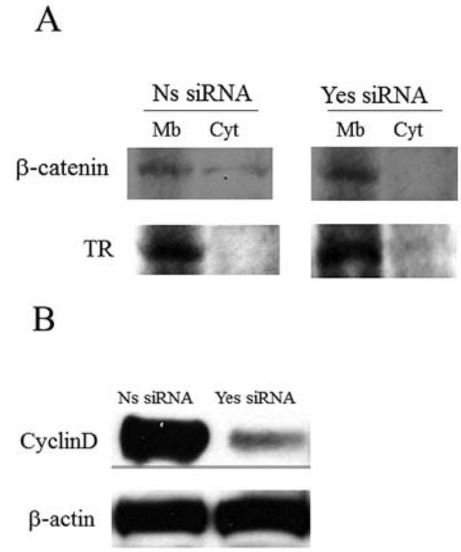

Figure 4. Effect of Yes siRNA treatment on localization of β-catenin (A) and cyclin D level (B) in H2452 cells. After the siRNA treatment for 48 h, β-catenin level in membrane (Mb) and cytosolic (Cyt) fractions from H2452 cells in each group was determined by immunoblot analysis. Transferin receptor (TR) was used as a control of membrane fraction. Also, cyclin D level was determined by immunoblot analysis, and β-actin was used as a standard to show equal loading in each group. Each result is representative one of three samples.

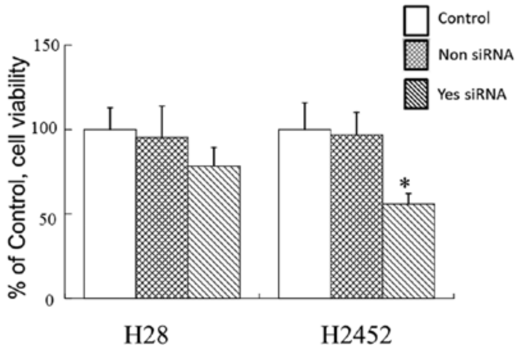

Figure 5. Differential effects of Yes siRNA treatment on cell viability in H2452 and H28 cells. After siRNA treatment for 48 h, cell viability was determined by WST-1. Each column indicates the mean from five samples; vertical lines indicate SD. *Significant difference from other treatment groups.

reduced the level of cyclin D, which is necessary for transition of G1 to S phase in cell cycle and a target molecule of β-catenin signaling (Fig. 4B) (17). Furthermore, a reduction in EphB3 (a target molecule of β-catenin signaling) mRNA level was observed upon Yes depletion (data not shown). These results suggest that Yes knockdown affects β-catenin localization and signaling in H2452 cells. Also, we confirmed similar results in H2052 and MSTO-211H cells (data not shown). Finally, in order to confirm this effect of Yes depletion, we estimated if the knockdown of Yes could influence cell growth in H28 cells which are deficient in β-catenin (18). As a result, Yes silencing had less effect on cell growth in H28 cells compared to H2452 cells which expressed β-catenin (Fig. 5). We confirmed that the knockdown level was almost the same between the two cell types (data not shown). These observations completely support the above speculation.

Discussion

MM is an aggressive malignancy, the incidence of which is expected to increase due to its association with asbestos exposure. A number of chemotherapeutic agents have been used, either alone or in combination, to treat MM with the latter multi-agent regimen generally having the highest response rates (19). Nonetheless, despite the current therapies, the prognosis for many MM patients is very poor. Several signal molecules related to growth and survival are constitutively activated in MM cells (20) and simultaneous suppression of multi-target molecules is required for an effective therapeutic agent against MM. In a recent study, it was found that SFK is a promising molecular target to perform an effective treatment in MM (21). However, at present, which member of SFK is absolutely required for effective MM treatment is unresolved. The aim of the present study was to address this issue.

It has been demonstrated that, of members in SFK, c-Src, Yes and Fyn were constantly activated in MM, through phospho-protein proteomic screen analysis (22). Actually, we observed that overexpression of three subtypes of SFK occurred in two histologically different types of MM cells compared to non-tumorigenic mesothelial cells. In a previous study, it has been reported that the contribution of some SFK members to oncogenic activity in each tissue is redundant (16). In order to clearly address this issue in MM, we utilized siRNA knockout technology. As a result, only Yes silencing was found to be associated with suppression of cell growth in MM cells, indicating that Yes is a central mediator of cell growth in MM cells.

In other studies, inhibition of SFK activation by a specific inhibitor suppresses cell growth of most of the examined MM cell lines, mainly due to G1 arrest in cell cycle (15). Reinforcing this, we have obtained similar results in our study (23). Similarly, our present study showed that the silencing of Yes contributed to G1 arrest in the cell cycle. These results suggest that, of SFK members, Yes is the main molecule to drive cell cycle progression in MM cells. With respect to a mechanism on Yes-mediated cell growth in MM cells, we can speculate that Yes stimulates cell growth via the activation of β-catenin signaling (14). In that study it was clearly demonstrated that the localization of β-catenin is changed from cytoplasm and nucleus to cell membrane by the knockdown of Yes in colon carcinoma cells and that the alteration of the localization is closely associated with loss of several malignant phenotypes such as invasion in the carcinoma cells. It is well known that β-catenin localized in the nucleus acts as a transactivator targeting for genes stimulating cell growth, that is, nuclear β-catenin forms a complex with the transcription factor TCF and induces the expression of downstream target genes including c-myc and cyclin D1, together with other transcriptional co-factors, such as CREB binding protein (CBP) (24). Of the target genes, cyclin D1 is a positive regulator of the cell cycle and promotes G1 to S phase transition in cell cycle (17). Amplification of the gene encoding cyclin D1 and overexpression of cyclin D1 protein have frequently been found in several types of human malignant neoplasms (25). In this study, we observed that the silencing of Yes caused G1 arrest in the cell cycle, possibly due to the reduction of cyclin D level. Since we also observed that Yes silencing induced a reduction in EphB3 (a target molecule of β-catenin signaling) mRNA level, the decrease of cyclin D level might partly depend on the inactivation of β-catenin signaling by Yes siRNA treatment. This speculation can be completely supported by the present data in which Yes knockdown has less effect on cell growth in H28 cells, being deficient of β-catenin signaling, than on H2452 cells in which β-catenin signaling is present.

The reason why Yes has a specific effect on cell growth in MM cells is still unclear at present. As a possible mechanism, it has been proposed that specific subcellular localization of SFK family members leads to phosphorylation of specific substrates and subsequent outcome of specific cellular events. Actually, a recent report has shown that the difference of localization among SFK family members regulates SFK signaling specificity leading to, for example, mitogenesis or neoplastic transformation (26). Also the possibility of interaction between substrates and the unique SH3 or SH2 domains of these SFK may give rise to an additional mechanism for selective signaling. Similarly it was demonstrated in a previous study with colon cancer that one mechanism by which Yes regulates its oncogenic activity is by modulation of β-catenin subcellular localization counteracting its nuclear transcriptional activity, where this cellular process was regulated by tyrosine phosphorylation (16). In order to further clarify the specific transforming activities of Yes, additional signaling pathways regulated by Yes should be elucidated. Finally, this determination may lead to establishment of a new effective treatment for MM.

Acknowledgements

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