Abstract. Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related death worldwide. Src is involved in multiple processes of cancer metastasis; however, its significance in HCC is not well defined. In the present study, overexpression of Src phosphorylation (Y416) was observed in the highly metastatic MHCC97H cell line; additionally, through inhibition of Src kinase activation, HCC cell proliferation, migration, invasion and colony formation were significantly reduced in vitro. Tumour growth was not affected in the orthotopic xenograft HCC model, but the metastatic potential was inhibited as revealed by reduced lung metastatic foci after administration of saracatinib. Phosphorylation level of Src pathway signalling molecules, such as Src, FAK and Stat3, were also reduced in vitro and in vivo, as a result of the anti-metastatic effects caused by saracatinib treatment. In conclusion, we demonstrated the pro-metastatic role of Src in HCC, and further experiments suggest the use of the Src inhibitor in combination with cytotoxic agents and other anticancer treatments to improve HCC prognosis.

Introduction

Hepatocellular carcinoma (HCC), one of the most common malignancies, represents the third leading cause of cancer-related death worldwide (1). Although surgical resection and local ablation therapy, including percutaneous ethanol injection and radiofrequency ablation, can be considered as curative treatments for patients in the early stages, intrahepatic and distant metastases are major obstacles in obtaining long-term survival. More than 50% of patients have been reported to relapse within 5 years and more than half of these recurrences occur within 2 years after surgical resection due to intrahepatic or distant metastases (2-5). Thus, there is an urgent need for novel treatment strategies to prevent metastasis.

Src, the archetypal member of a 9-gene family (including Src, Yes, Fyn, Lyn, Lck, Hck, Fgr, Blk and Yrk), plays a key role in cell adhesion, invasion, proliferation, survival and angiogenesis during tumour development (6). To date, numerous studies have indicated that Src expression and its activity are elevated in lung (7), breast (8,9), ovarian (10), pancreatic (11), colon (12,13) and gastric cancers (14). Upon activation, it recruits several signalling molecules to control cell migration and cytoskeleton rearrangement, to increase growth rates and invasion characteristics of tumours, and to resist anoikis in tumour cells (6,15-17). Conversely, reduced Src expression may potentially induce retarded tumour growth and reduction of metastatic tendencies. Saracatinib (AZD0530), a tyrosine kinase inhibitor (TKI) selective for Src, has been associated with inhibition of cell migration and proliferation in breast cancer (18); additionally, it has been tested in a phase II trial to treat head and neck squamous cell carcinoma (19) and advanced castration-resistant prostate cancer (20). In HCC, Src overexpression has been observed in 87.5% of the specimens, and phosphorylated Src (tyr-416) (activated form) was found to be closely associated with metastatic potential, such as microvessel invasion or intrahepatic metastasis (unpublished data). Although Src plays a role in tumorigenesis and in the development of various human cancers, it remains largely unclear as to whether Src affects tumour metastasis in HCC.

In the present study, we investigated the effect of saracatinib on HCC. The results indicated that saracatinib reduced lung metastasis, rather than inhibiting primary tumour growth. The results revealed the promising effect of saracatinib in the treatment of HCC metastasis and further studies suggest the use of the Src inhibitor in combination with cytotoxic agents and other anticancer treatments to improve HCC prognosis.
Materials and methods

Cell lines and reagents. HCC cell lines (MHCC97H and Hep3B) and a human normal hepatic cell line (L02) were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). All of these cell lines were strictly maintained according to the supplier’s instructions and established procedures. Polyclonal antibodies to Src, p-FAK and NADPH were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Polyclonal antibodies to Stat3, p-Stat3 (Y705) and p-Src (Y416) were obtained from Cell Signalling Technology (Danvers, MA, USA). Polyclonal antibodies to p-FAK (Y861) were obtained from Invitrogen by Life Technologies (Carlsbad, CA, USA). Saracatinib was obtained from AstraZeneca International (Alderley Park, UK) and was dissolved in dimethyl sulfoxide (DMSO; Fermentas, Pittsburgh, PA, USA) to a stock solution of 10 mM and stored at −20°C.

Cell proliferation assay. Cell proliferation was measured using Cell Counting Kit-8 (CCK-8), according to the manufacturer’s protocols (Dojindo, Kumamoto, Japan). Briefly, cells (MHCC97H, Hep3B and L02) were triplicate plated onto 96-well plates (2.0x10^3 cells/well). Twelve hours later, saracatinib was added to the cells at doses of 0-128.0 µM (including 0, 0.125, 0.25, 0.5, 1.0, 2.0, 8.0, 16.0, 32.0, 64.0 and 128.0 µM), and were incubated for 0, 24, 48 or 72 h. The medium was then refreshed, followed by the addition of 10 µl/well of CCK-8 solution and incubated at 37°C for an additional 2 h. The absorbance at 450 nm represented the number of viable cells and was measured using a Bio-Rad 680 microplate reader (Bio-Rad, Hercules, CA, USA).

Wound healing assay. The monolayer of cells (MHCC97H, Hep3B and L02) was wounded by scraping with a 200-μl pipette tip and rinsed several times with sterile phosphate-buffered saline (PBS) to remove dislodged cells. Cells that had migrated into the wound area were stained with crystal violet and their images were captured for analysis.

Cell migration and invasion assays. Cell migration was assessed using a Transwell™ Permeable Supports system (Corning, Corning NY, USA) according to the manufacturer’s instructions. Briefly, cells (MHCC97H, Hep3B and L02) were detached from the cell culture plates, washed with PBS buffer, and resuspended to 1x10^5 cells/µl with serum-free culture medium [Dulbecco's modified Eagle's medium (DMEM), minimum essential medium (MEM) and RPMI-1640 medium, respectively]. The cells were seeded onto the upper chambers of Matrigel-coated (BD Biosciences, Franklin Lakes, NJ, USA) filter inserts (8.0-μm pore size) and mixed with 0, 0.1 or 1.0 µM saracatinib (100 µl) per well. Culture medium (600 µl) containing 10% FBS was added to the lower chambers as a chemoattractant. After being cultured for 48 h, filter inserts were removed from the wells. The cells on the upper surface of the filter were wiped with a cotton swab. The filter was fixed with 4% paraformaldehyde for 10 min and stained with Giemsa dye (Sigma, Munich, Germany) for 15 min. The cells invading the lower surface were counted using an inverted microscope. The migration assay was performed as described for the invasion assay, but without the coating of Matrigel.

Colonization formation assay. MHCC97H, Hep3B or L02 cells (1x10^3 cells/well) were mixed with 200 µl of 0.6% agarose, 150 µl of complete medium and 50 µl of a solution containing 0, 0.1 or 1.0 µM saracatinib, and then seeded into 6-well plates pre-coated with 0.6% low melting point agarose. Photomicrographs of colonies were captured 2 weeks later and quantified by counting the number of colonies formed.

Western blotting. Cells grown in 100-mm dishes were washed twice with ice-cold PBS before lysis by incubation for 20 min in 1 ml of ice-cold cell lysis RIPA buffer [10 mM Tris (pH 8.0), 150 mM NaCl, 1% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS), 1% Triton X-100, 10 mg/ml leupeptin, 10 mg/ml aprotinin and 1 mM phenylmethylsulfonyl fluoride (PMSF)]. The protein concentrations of the lysates were measured using a Bradford protein assay kit (Bio-Rad). Equivalent amounts of protein were mixed with 6X SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer, electrophoresed in a 4-20% linear gradient Tris-HCl-Ready Gel (Bio-Rad), and then transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% non-fat dry milk in Tris-buffered saline, pH 7.4, containing 0.05% Tween-20 and were incubated with specific primary antibodies and horseradish peroxidase-labelled secondary antibodies (Rockland, Gilbertsville, PA, USA) according to the manufacturer’s instructions. The protein of interest was visualized and quantitated using the LI-COR Odyssey Infrared Imaging system (LI-COR Biosciences, Lincoln, NE, USA). Cells (5x10^4) were lysed on ice with RIPA buffer [10 mM Tris (pH 8.0), 150 mM NaCl, 1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, 10 mg/ml leupeptin, 10 mg/ml aprotinin and 1 mM PMSF]. Next, 50 µg of whole cell protein extracts were separated by 10% SDS-PAGE and transferred onto a PVDF membrane. The membrane was subsequently blocked with 5% bovine serum albumin (BSA; Sigma-Aldrich, Inc.) in Tris-buffered saline plus Tween-20 (TBST) for 1 h, and then incubated overnight at 4°C with specific primary antibodies, followed by washing with TBST buffer and incubation with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. After intensive TBST washing, the membrane was subjected to chemiluminescent detection using the Bio-Rad Imaging Lab system.

HCC orthotopic xenograft model and evaluation of lung metastasis. The HCC orthotopic xenograft model with high metastatic potential was established with the MHCC-97 cell line. Four-week-old nude mice (BALB/c nu/nu) were subcutaneously injected with 1x10^7/0.2 ml of MHCC97H cells in the left upper flank region to establish subcutaneous xenograft models. Four weeks later, the tumour nodules with a diameter of 0.8 cm were removed and cut into 1 mm³ pieces and implanted into the livers of the nude mice to establish orthotopic xenograft models. The 24 mice were randomized into three groups, each comprised of eight mice. Three days later, the mice were orally administered saracatinib daily at doses of 0, 50 or 100 mg/kg/day for 35 consecutive days. Mouse weights were evaluated biweekly. On day 35, all mice were sacrificed. The livers and lungs were collected, the wet weights were determined, and then the livers and lungs were immediately fixed in 10% neutral formalin solution for further quantification.
immunohistochemistry. The liver wet weights were substituted for tumour weights for evaluation of tumour growth inhibition by saracatinib. Lung metastasis was determined by examining consecutively the serial sections of total lungs, using hematoxylin and eosin staining under a microscope. Metastasis inhibition was then evaluated determining the metastasis rate of all mice in the groups and the number of metastatic foci for the whole lung of each mouse (21). Animal care and procedures were approved by our Institutional Animal Care and Use Committee (Ethics Committee of Haikou People's Hospital; permit no. 2013-14).

Immunohistochemistry. Immunohistochemistry was performed as previously described (22). Briefly, after deparaffinization, hydration and blocking of endogenous peroxidase (0.3% H₂O₂ for 20 min); antigen retrieval was performed as previously described (22). Briefly, after deparaffinization, hydration and blocking of endogenous peroxidase (0.3% H₂O₂ for 20 min); antigen retrieval was performed using Tris-ETDA (pH 9.0) by microwave heating for 5 min. Sections were blocked with 5% BSA for 30 min at room temperature, and then the primary antibodies, including rabbit polyclonal anti-p-FAK (Y-861) (diluted 1:300), rabbit polyclonal anti-p-Src (Y-416) (diluted 1:100), rabbit polyclonal anti-p-Stat3 (Y-705) (diluted 1:100) or rabbit IgG serum (negative control), were incubated overnight at 4˚C. Sections were detected using the EnVision detection system (Dako Denmark A/S, Glostrup, Denmark) and DAB following counterstaining with hematoxylin, and then dehydrated through alcohol and xylene before mounting under coverslips. Slides were evaluated semi-quantitatively at a magnification of x100 under light microscopy in a blinded fashion on a scale of 0-4.

Statistical analysis. All statistical analysis was performed using GraphPad Prism version 5.0. Quantitative variables are expressed as the mean ± standard deviation (SD) and were analysed by ANOVA. The χ² or Fisher's exact tests were applied to compare qualitative variables. Results were deemed statistically significant at P<0.05.

Results

Src kinase activation correlates with the metastatic potential of HCC in vivo and in vitro. To investigate Src expression and its correlation with the metastatic potential in HCC, three cell lines (MHCC97H, Hep3B and L02) were used. Among the three cell lines, a high lung metastatic rate reaching 100% was found in mice injected with the MHCC97H cells, whereas no lung metastasis was observed in mice injected with Hep3B and L02 cells (data not shown). As autophosphorylation of Src at tyrosine 416 (p-Src-Y⁴¹⁶) is a marker of its activity (23), we next evaluated the expression of p-Src-Y⁴¹⁶ and total Src in the three cell lines. The results indicated that Src was expressed in all three cell lines, but there were significantly different activation levels in the three cell lines. The p-Src-Y⁴¹⁶ and Src expression in MHCC97H, a cell line with high metastatic potential, was much higher than that in the other two cell lines (Fig. 1). The results indicated that the metastatic potential of HCC was closely related to Src activation.

Saracatinib is involved in the inhibition of Src phosphorylation in the MHCC97H cells. As saracatinib has been reported to inhibit Src activation in prostate cancer cell lines (24), we determined whether saracatinib is also an inhibitor of Src in HCC cell lines with high metastatic potential. Treatment of the MHCC97H cells with saracatinib partially prevented the phosphorylation of Src in a dose- and time-dependent manner (Fig. 2). This suggests that saracatinib is involved in inhibition of Src activation in HCC cell lines with high metastatic potential.

Saracatinib inhibits the invasive potential of HCC cells. To evaluate the antiproliferative effect of saracatinib on HCC, the MHCC97H, Hep3B and L02 cell lines treated with saracatinib were used. The results showed that HCC proliferation was inhibited significantly in a dose-dependent manner in all three cell lines. The IC₅₀ values in the MHCC97H, Hep3B and L02 cell lines were 14.09, 22.75 and 31.06 µM, respectively (Fig. 3). Subsequently, the wound healing and Transwell migration assays were used to evaluate cell migration capacity. Cell migration was significantly inhibited by saracatinib in all three cell lines in a dose-dependent manner both in the wound healing assay (Fig. 4A) and in the Transwell migration assay (Fig. 4B). For the Transwell migration assay, the average numbers of migrated cells in the MHCC97H cell line treated with 0, 1.0, and 10.0 µM saracatinib were 348.0±31.4, 326.7±21.8 and 242.0±20.3, respectively. Inhibitory effects were also observed in the Hep3B and L02 cell lines. The numbers of migrated cells in the Hep3B cell line treated with 0, 1.0 and 10.0 µM saracatinib were 296.7±11.6, 237.3±15.2...
and 184.3±15.6, respectively; while in the L02 cell line they were 130.3±10.0, 83.3±13.3 and 54.7±9.0, respectively.

The Matrigel invasion chamber assay revealed that saracatinib significantly inhibited the invasion of MHCC97H, Hep3B and L02 cells. After being treated with 0, 1.0 and 10.0 µM saracatinib, the average numbers of invaded cells were 251.3±31.0, 210.0±20.0 and 167.0±16.1 in the MHCC97H cell line; 165.0±18.0, 130.3±21.0 and 50.7±10.1 in the Hep3B cell line; and 120.3±10.5, 83.3±13.3 and 47.0±16.1 in the L02 cell line, respectively (Fig. 4C).

As colony formation is a vital feature in the high metastatic potential of carcinomas, the impact of saracatinib on colony formation in the MHCC97H and Hep3B cell lines was observed. Considering L02 is a normal hepatocyte cell line, we did not use this cell line in this experiment. The results showed that colony formation was significantly inhibited with saracatinib. After treatment with 0, 1.0 or 10.0 µM saracatinib for 14 days, the numbers of formed spheres were 15.0±3.0, 9.7±2.1 and 7.3±1.5 in the MHCC97H cell line, respectively (P<0.05; Fig. 4D left); and 22.7±5.0, 10.7±2.1 and 3.7±2.1 in the Hep3B cell line, respectively (P<0.05; Fig. 4D right). These findings suggest that Src promotes migration and invasion of HCC cells in vitro, and these capacities are inhibited by saracatinib.

**Saracatinib inhibits the metastatic potential in the HCC orthotopic xenograft model.** To verify the inhibitory effect of saracatinib on the HCC metastatic potential in vivo, the effect of saracatinib on HCC metastasis in an orthotopic xenograft model was then tested.

Following administration of 50 or 100 mg/kg/day saracatinib for 5 weeks, tumour growth and lung metastasis were evaluated. The results showed that the liver weight (represented as the tumour weight) was slightly different between the groups (Fig. 5A). The results indicated that growth inhibition of tumour was not achieved at the present dose. We next compared the effect of saracatinib on the lung metastasis of the model. The results showed that 8/8, 6/8 and 5/8 nude mice developed lung metastasis in the control, 50 and 100 mg/kg/day group, respectively (Fig. 5B and C). Although no statistically significant difference was found in the lung metastasis rates among the groups, inhibitory effects were identified regarding the number of metastatic foci after saracatinib treatment. The numbers of lung metastatic foci were 15.6±4.98, 9.17±2.56 and 7.00±1.58 in the control group, 50 and 100 mg/kg/day group, respectively. The results revealed saracatinib inhibition as evaluated by the number of metastasis foci either with 50 mg/kg/day (P=0.0164) or 100 mg/kg/day saracatinib (P=0.0043; Fig. 5D).

**Saracatinib inhibits Src phosphorylation and downstream signals of FAK and Stat3 phosphorylation.** Src plays a vital role in the modulation of cellular signalling pathway components relevant to cell proliferation, migration, invasion and angiogenesis, including receptor tyrosine kinase, FAK, Stat3 and adaptor protein P130Cas (25-28). Therefore, Src activity and its downstream signals were evaluated in vitro and in vivo to further understand the underlying mechanisms involved in the anti-metastatic effect of saracatinib. Phospho-Src (Y416), which is known to activate Src, was significantly inhibited after treatment with saracatinib in the MHCC97H cells. FAK and Stat3 are known to be downstream signals of Src and their phosphorylation was also evaluated following Src inhibition. The results revealed that Y816 phosphorylation of FAK and Y705 phosphorylation of Stat3 were inhibited as well, indicating that the anti-metastasic effect of saracatinib involved inhibition of FAK and Stat3. These results support that the signalling axis Src/FAK/Stat3 plays a crucial role in promotion of metastasis of HCC (29-31). The effect of saracatinib on the inhibition of the Src/FAK/Stat3 axis in a dose- and time-dependent manner was confirmed (Fig. 6A and B). In accordance to in vivo metastasis inhibition of saracatinib in the orthotopic xenograft model, phosphorylation of Src, FAK...
Figure 3. Saracatinib inhibits cell proliferation in the MHCC97H, Hep3B and L02 cell lines. The IC_{50} values were 14.09, 22.75 and 31.06 µM in the MHCC97H, Hep3B and L02 cell lines, respectively. (A) Saracatinib inhibits cell proliferation in all three cell lines in a dose-dependent manner in the (B) MHCC97H, (C) Hep3B and (D) L02 cell lines.

Figure 4. Saracatinib inhibits the invasive potential of HCC cells. (A) The migratory capacity of the MHCC97H, L02 and Hep3B cells before and after treatment with saracatinib for 48 h was analysed by wound-healing assay. (B) MHCC-97H, L02 and Hep3B cells treated with different concentrations of saracatinib were subjected to Transwell assay. (C) The invasive capacity of the MHCC97H and Hep3B cells was analysed using Transwell filter chambers coated with Matrigel. (D) Representative images of colonies formed in the MHCC97H and Hep3B cells treated with saracatinib in soft agar. The mean ± SD for triplicate determinations is plotted; *P<0.05.
Figure 5. Effects of saracatinib on tumour growth and lung metastasis in vivo. (A) Tumour weight was observed when saracatinib was administered at 0, 50 or 100 mg/kg/day for 5 weeks after the MHCC97H cell inoculation. (B) Following administration of 0, 50 or 100 mg/kg/day saracatinib for five weeks, a representative H&E staining of the lungs is shown in the three groups. (C) Following administration of 0, 50 or 100 mg/kg/day saracatinib for five weeks, the number of lung metastatic foci are shown in the three groups. (D) Following administration of 0, 50 or 100 mg/kg/day saracatinib for five weeks, the metastasis rate in the 3 groups is shown.

Figure 6. Saracatinib inhibits phosphorylated Src (p-Src) and downstream signalling molecules, phosphorylated FAK (p-FAK) and phosphorylated Stat3 (p-Stat3). (A) Dose-dependent inhibition of p-Src, p-FAK and p-Stat3. (B) Time-dependent inhibition of pSrc, p-FAK and p-Stat3 (the mean ± SD for triplicate determinations is plotted). (C) Immunohistochemical analysis of tumour samples in the xenograft HCC model showed that saracatinib decreased expression of p-Src, p-FAK and p-Stat3 (original magnification, x400).
and Stat3 were inhibited in HCC tissues using immunohistochemistry. However, phosphorylation was not completely inhibited in vivo at doses of 50 and 100 mg/kg/day (Fig. 6C). This result may explain the limited anti-metastatic effects of saracatinib in nude mice.

Discussion

Intrahepatic and distant metastases represent a key challenge to achieve long-term survival for patients with HCC. Studies concerning anti-metastasis of HCC have attracted considerable attention in recent years. Various achievements have been obtained in studies from the exploration of possible targets to prevent metastasis, such as miR-124 (32), erythropoietic leukemic viral oncogene homolog 3 (ERBB3) (33), matrix metalloproteinase (MMP), c-Met, CD151, PDGFRα, transcription factor late SV40 factor (LSF) (34) and p38MAP kinase (35). However, promising targets are not yet available for clinical use in HCC.

The role of Src kinases has recently been well defined in cancer tumorigenesis, tumour progression and metastasis. Dysregulation of Src has been considered to lead malignant transformation and tumour progression, and to promote metastasis in various types of cancers (36-38). A small amount of reagents have been developed to inhibit Src, and also have been applied in phase I or II trials (39-41). Although phase II clinical trials for Src inhibitors have shown a modest effect against tumour growth in breast cancer, as well as head and neck squamous carcinoma, clinical significance is being overlooked for its metastatic role in cancers, including HCC (19,41,42). In HCC, 36/45 (55.4%) patients were identified with overexpressed activated Src, and its overexpression was closely associated with proliferation and metastatic potential (43-45); however, the significance of inhibition of Src in HCC metastasis has not been elucidated.

In the present study, we tested the effects of saracatinib, a specific Src inhibitor, on HCC cell proliferation, migration, invasion and colony formation in vitro. We found that Src activation was correlated with the metastasis potential of HCC cell lines. MHCC97H, a cell line with high metastatic potential, showed a higher expression level of total Src and activated Src compared with the other two cell lines. Saracatinib effectively inhibited the activation of Src and its downstream signals (FAK and Stat3), as well as the metastatic potential (including cell migration, invasion and colony formation) in the two HCC cell lines. In the orthotopic xenograft HCC model, saracatinib inhibited lung metastasis, without influencing primary tumour inhibition. These results indicated that Src is mainly involved in the process of cancer metastasis, and suggest that, for anticancer therapy, the use of saracatinib alone is not sufficient to effectively control cancer growth. Thus, saracatinib combined with other antiproliferative agents may be used to block metastasis formation, and to reduce primary tumour growth. Furthermore, although inhibition of metastasis was observed in the orthotopic xenograft model, saracatinib was not capable of completely blocking lung metastasis and activation of the Src/FAK/Stat3 axis, indicating that a more complex mechanism exists for Src activation such as cross-talk regulation or feedback regulation (46). For example, in breast cancer, Src was activated by tyrosine kinase receptor and the estrogen receptor; although, saracatinib alone did not effectively inhibit tumour growth and related signalling molecules, including phosphorylated Src (Y416) and its downstream signalling molecules (Akt and MAPK). However, saracatinib combined with anastrozole (an estrogen inhibitor) significantly inhibited tumour growth and Src (Y416), Akt (T473) and MAPK (T202/Y204) phosphorylation (41). Our present results also indicate that the anti-metastatic effect of saracatinib should not be overlooked, even if it fails to completely inhibit lung metastasis in the orthotopic model.

Notably, the Hep3B cells, with non-metastatic potential were also found to possess relatively high levels of p-Src and Src. Despite this, there is evidence of a prominent role of Src in metastasis and in other tumour events such as epithelial-mesenchymal transition (EMT) and in the development of invasion in various tumours (47,48). In the future, we may prove more evidence of the role of Src in other HCC progression-related events, such as cell adhesion, invasion, proliferation, survival and angiogenesis.

Moreover, the emerging role of Src has also been demonstrated in mediating resistant tumours (46,49). Application of saracatinib or its combination with other targeted reagents (eg. trastuzumab) is encouraged based on more detailed activation mechanisms of Src in overcoming various resistance mechanisms, which warrant further investigation, particularly in systemic chemotherapy-resistant HCC.

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References


