

Imatinib Mesylate Induces Quiescence in Gastrointestinal Stromal Tumor Cells through the CDH1-SKP2-p27^{Kip1} Signaling Axis

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Abstract

Gastrointestinal stromal tumors (GIST) are caused by activating mutations in the KIT or platelet-derived growth factor receptor α receptor tyrosine kinase genes. Approximately 85% of GIST patients treated with imatinib mesylate achieve disease stabilization, however, often in the presence of residual tumor masses. Complete remissions are rare and a substantial proportion of patients develop resistance to imatinib. Our study was designed to determine whether imatinib-associated responses may account for these clinical findings. We report here that imatinib stimulates cellular quiescence in a proportion of GIST cells as evidenced by up-regulation of the CDK inhibitor p27^{Kip1}, loss of cyclin A, and reduced BrdUrd incorporation. Mechanistically, these events are associated with an imatinib-induced modulation of the APC/CDH1 signaling axis. Specifically, we provide evidence that imatinib down-regulates SKP2 and that this event is associated with increased nuclear CDH1, an activator of the APC that has been shown to regulate SKP2 stability. We also show that those GIST cells that do not undergo apoptosis in response to imatinib overexpress nuclear p27^{Kip1}, indicating that they have withdrawn from the cell cycle and are quiescent. Lastly, we provide evidence that a fraction of primary GISTs with high SKP2 expression levels may have an increased risk of disease progression. Taken together, our results support a model in which GIST cells that do not respond to imatinib by apoptosis are removed from the proliferative pool by entering quiescence through modulation of the APC/CDH1-SKP2-p27^{Kip1} signaling axis. These results encourage further studies to explore compounds that modulate this pathway as antitumor agents in GISTs. [Cancer Res 2008;68(21):9015–23]

Introduction

Gastrointestinal stromal tumors (GIST) are the most common mesenchymal tumors of the gastrointestinal tract. They are caused by activating mutations in the KIT or platelet-derived growth factor receptor α receptor tyrosine kinase genes (1–3) and can be effectively treated with the small molecule kinase inhibitor imatinib mesylate (STI571; Gleevec; ref. 4). Approximately 85% of patients with metastatic GIST benefit from

imatinib therapy; however, complete responses are rare (4–6). Remarkably, many patients treated with imatinib achieve a long-term stabilization of disease in the presence of residual tumor. These clinical findings and several experimental results (7) strongly suggest that tumor cell quiescence plays an important role in imatinib therapy of GIST.

Tumor cell quiescence is a major obstacle for successful cancer therapy because quiescent cells do not respond to anticancer agents that target proliferating cells (8). Furthermore, quiescent cells can remain viable and in particular tumor cells with impaired checkpoint control may re-enter the cell division cycle resulting in disease progression. Quiescence is defined by a reversible exit from the cell division cycle in G₀-G₁ (9). Experimentally, quiescence can be induced by growth factor withdrawal, contact inhibition and loss of adhesion (10). The major regulators of tumor cell quiescence are not known in detail but several lines of evidence suggest that the cyclin-dependent kinase inhibitor p27^{Kip1} is critically involved (11–13).

p27^{Kip1} is regulated on multiple levels including phosphorylation and proteasomal degradation (14). In cells that are in S or G₂ phase of the cell division cycle, high cyclin/CDK activity triggers phosphorylation of p27^{Kip1}, which is subsequently degraded, thereby maintaining low p27^{Kip1} expression levels (15). Degradation of p27^{Kip1} is primarily regulated by the SCF^{SKP2} E3 ubiquitin ligase (13). Several reports provide evidence for a second layer of regulation in which the F-box protein SKP2 is itself regulated by an ubiquitin ligase, the Anaphase Promoting Complex (APC)/CDH1 complex (16, 17). The APC becomes activated in prometaphase and remains active until the late G₁ phase of the cell cycle. It requires two activators, CDC20 during mitosis and CDH1 primarily after mitotic exit (18, 19). The APC/CDC20 complex mediates the degradation of securin, an inhibitor of sister chromatid separation, as well as cyclin B1, an inhibitor of mitotic exit. In addition, the APC/CDC20 mediates the degradation of several other substrates, such as cyclin A, which starts after nuclear envelope breakdown. After inactivation of cyclin B1/CDK1, APC/CDH1 functions to polyubiquitinate SKP2 as well as cyclin A, hence promoting the accumulation of p27^{Kip1} and suppression of CDK in G₀-G₁ (16, 17). Taken together, the APC/CDH1 complex is critical for reinforcing G₀-G₁ arrest and hence for keeping cells in a quiescent state.

Based on the clinical findings described above, the present study was designed to explore whether imatinib modulates signaling pathways that have been implicated in tumor cell quiescence. We show that imatinib treatment of GIST cells results in a significant down-regulation of SKP2 and an up-regulation of the CDK inhibitor p27^{Kip1}. We show that these events are preceded by a redistribution of the APC activator CDH1 to the nucleus. Conversely, over-expression of SKP2 can protect GIST cells from imatinib-induced cell cycle arrest. We show that imatinib-treated GIST cells that do

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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not undergo apoptosis overexpress p27^{Kip1}, indicating that they have withdrawn from the cell cycle. Lastly, we provide evidence that high expression of SKP2 in primary GISTs may be associated with an unfavorable prognosis. Our results provide the framework for future studies to target this signaling pathway to thwart tumor cell quiescence with the goal to achieve more complete and long-term remissions.

Materials and Methods

Cell culture, inhibitor treatments, and transfections. The human GIST cell line GIST882 was derived from an untreated metastatic GIST and maintained in RPMI1640 supplemented with 15% fetal bovine serum (Mediatech), 1% L-Glutamine, 50 U/mL penicillin (Cambrex), and 50 µg/mL streptomycin (Cambrex) as described earlier (7).

Cells were incubated in imatinib mesylate (1 µmol/L in DMSO; kindly provided by Novartis Pharma AG), the PI3K inhibitor LY294002 (25 µmol/L in DMSO; EMD Biosciences), the MAP/ERK kinase (MEK)1/MEK2 inhibitor U0126 (10 µmol/L in DMSO; EMD Biosciences), or mock treated with 0.1% DMSO for up to 72 h or as indicated.

Transient transfection of GIST882 cells was carried out by using the Amaxa nucleofection device (Amaxa). Briefly, cells were trypsinized and 3×10^6 cells per experiment were transfected with 5 µg of plasmid DNA (20). A pcDNA3-based expression plasmid encoding human SKP2 was a generous gift from Wilhelm Krek (ETH, Zürich, Switzerland; ref. 13). A plasmid encoding HA-tagged CDH1 was a generous gift from Michele Pagano (New York University School of Medicine, New York, NY; ref. 16).

For small interfering RNA (siRNA) experiments, pooled synthetic (SKP2) and single (CDH1) RNA duplexes were used to reduce SKP2 and CDH1 protein expression, respectively (Dharmacon). Briefly, GIST882 cells were trypsinized and 3×10^6 cells were transfected with 5 µL of 20 µmol/L annealed RNA duplexes using nucleofection (Amaxa). Cells were then transferred to 35-mm tissue culture dishes with 2 mL RPMI1640 free of antibiotics. Experiments were performed at the indicated time points.

Immunologic and cell staining methods. Protein lysates of cells growing as monolayer were prepared by scraping cells into radioimmunoprecipitation assay buffer [1% NP40, 50 mmol/L Tris-HCl (pH 8.0), 100 mmol/L sodium fluoride, 30 mmol/L sodium PPI, 2 mmol/L sodium molybdate, 5 mmol/L EDTA, and 2 mmol/L sodium orthovanadate] containing protease inhibitors (10 µg/mL aprotinin, 10 µg/mL leupeptin, and 1 µmol/L phenylmethylsulfonyl fluoride). Lysates were incubated for 1 h with shaking at 4°C and then cleared by centrifugation for 30 min at 14,000 rpm at 4°C. Protein concentrations were determined by the Bradford assay (Bio-Rad). Thirty micrograms of protein were loaded on a 4% to 12% Bis-Tris gel (Invitrogen) and blotted onto a nitrocellulose membrane.

For immunofluorescence analysis, cells grown on coverslips were briefly washed in PBS and fixed in 4% paraformaldehyde in PBS for 15 min at room temperature. Cells were then washed in PBS and permeabilized with 1% Triton-X 100 in PBS for 15 min at room temperature followed by washing in PBS and blocking with 10% normal donkey serum (Jackson Immunoresearch) for 15 min at room temperature. Cells were then incubated with primary antibodies overnight at 4°C in a humidified chamber and incubated for another 3 h at 37°C the next morning. After a brief wash in PBS, cells were incubated with FITC-anti-mouse secondary antibodies (Jackson Immunoresearch) for at least 2 h at 37°C, washed with PBS, and counterstained with 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories). Cells were analyzed using an Olympus AX70 epifluorescence microscope equipped with a SpotRT digital camera.

Primary antibodies used for immunoblotting and immunofluorescence were SKP2 clone 8D9 (Zymed), p27^{Kip1} (BD Biosciences Pharmingen), cyclin A (Novocastra), CUL1 (Santa Cruz), CDH1 (LabVision), phospho-KIT Y703 (Zymed), KIT (DakoCytomation), phospho-AKT S473 (Cell Signaling), total AKT (Cell Signaling), phospho-mitogen-activated protein kinase (MAPK) T202/Y204 (Cell Signaling), total MAPK (Cell Signaling), and actin (Sigma).

BrdUrd assay. To assess the proliferating cell fraction, GIST882 cells were incubated in BrdUrd labeling medium (Roche) for 2 h. Cells were then washed in PBS and fixed in ice-cold methanol for 10 min. After another wash in PBS, DNA was denatured by incubation in 2N HCl for 1 h at 37°C. The HCl was neutralized by immersion in 0.1 mol/L borate buffer (pH 8.5), before incubation with a mouse monoclonal anti-BrdUrd antibody (Roche) overnight at 4°C in a humidified chamber. Detection and analysis was performed as described above.

GIST tissue microarray and immunohistochemistry. A total of 28 cores from primary and metastatic GIST and 7 controls (liver) were collected from the archives of the Department of Pathology of the University of Pittsburgh School of Medicine (IRB#0509050). Slides were processed as previously described (21, 22), and SKP2 was detected using clone 2C8D9 (Zymed). The number of SKP2-positive cells was determined per high power field (HPF; $\times 40$).

Results

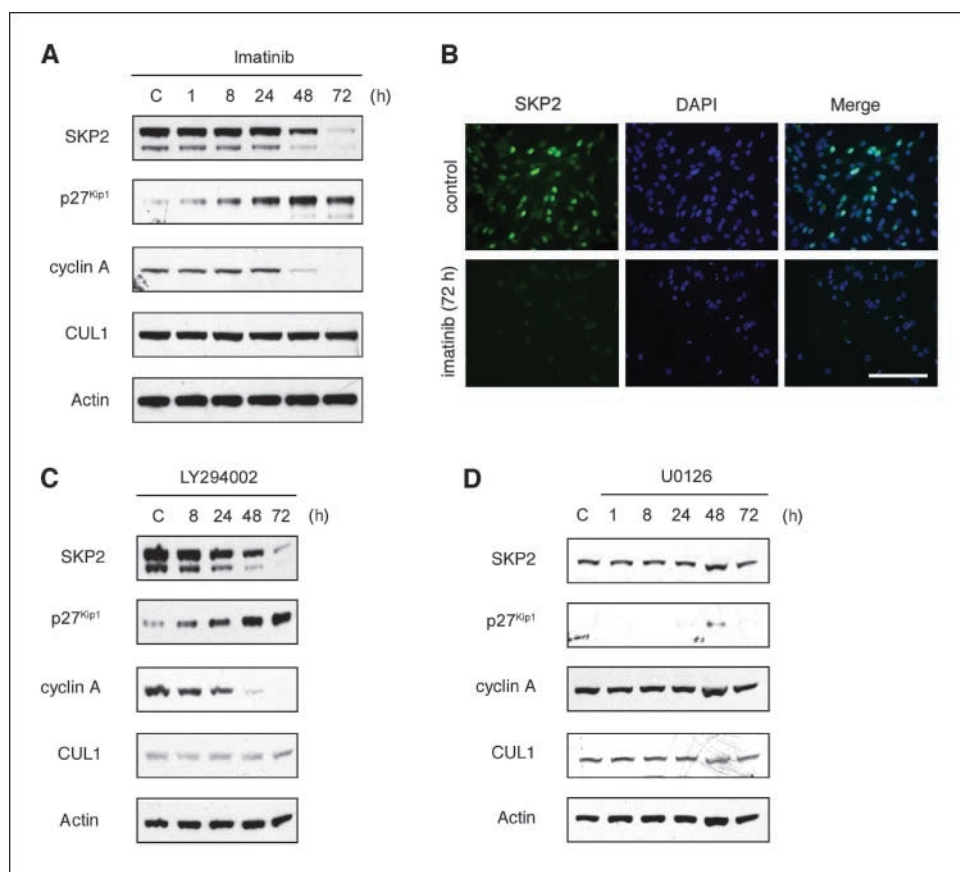
Imatinib treatment of GIST cells leads to exit from the cell cycle through down-regulation of SKP2 and increased expression of p27^{Kip1}. To ascertain whether imatinib modulates signaling pathways that have been implicated in cell cycle quiescence, we treated the GIST-derived cell line GIST882 with 1 µmol/L imatinib for up to 72 hours and analyzed cells by immunoblotting. We found that the levels of SKP2 decreased significantly after 72 hours of imatinib treatment with levels beginning to decline at 48 hours (Fig. 1A). By contrast, the levels of another component of the SCF-SKP2 complex, CUL1, did not change, indicating that the changes in SKP2 protein expression were not due to a general down-regulation of the SCF components. An up-regulation of the CDK inhibitor p27^{Kip1} was detected with maximum levels at 48 to 72 hours. Immunoblotting for expression levels of cyclin A revealed a significantly reduced expression indicating that a substantial proportion of cells had exited from S phase. In addition, these results suggest an increased activity of APC/CDH1 resulting in accelerated degradation of its substrates, SKP2 and cyclin A. As expected, imatinib inhibited the phosphorylation of KIT and its downstream targets (Supplementary Fig. S1A).

To further corroborate these results, an immunofluorescence microscopic analysis for SKP2 was performed. This experiment showed a significant loss of SKP2 expression in imatinib-treated GIST882 cells at 72 hours. These results confirm and extend previous studies (7) and indicate that imatinib treatment indeed induces quiescence as evidenced by exit from S phase in GIST cells. They furthermore suggest that imatinib can interfere with upstream regulatory pathways that modulate p27^{Kip1} stability, namely the F-box protein SKP2.

Imatinib-induced quiescence is dependent on the PI3K/AKT pathway. We next asked which pathways downstream of KIT are involved in the down-regulation of SKP2 after imatinib. Inhibition of PI3K by LY294002 led to a down-regulation of SKP2 that was similar to what was seen after imatinib treatment (Fig. 1C). Consequently, levels of p27^{Kip1} increased and the amounts of Cyclin A decreased significantly at 24 to 72 hours after treatment with this compound. No change in the levels of CUL1 protein expression was detected. As expected, LY294002 treatment inhibited AKT phosphorylation but had no effect on KIT phosphorylation or MAPK activity (Supplementary Fig. S1B).

In contrast, MEK1/MEK2 inhibition by U0126 had no effect on the levels of SKP2 and, consequently, also did not lead to changes in the amounts of p27^{Kip1} or cyclin A (Fig. 1D). U0126 treatment only inhibited MAPK phosphorylation but had no effect on KIT or PI3K/AKT activity (Supplementary Fig. S1C).

Figure 1. KIT inhibition by imatinib leads to cell cycle exit through down-regulation of SKP2, which is dependent on the PI3K/AKT pathway. *A*, immunoblot analysis of GIST882 cells treated with imatinib (1 $\mu\text{mol/L}$) for up to 72 h as indicated and probed for known regulators of the cell division cycle. Actin stain is shown to demonstrate equal loading. *B*, immunofluorescence microscopic analysis of GIST882 cells for SKP2. Cells were treated with imatinib (1 $\mu\text{mol/L}$) for 72 h. Note the significantly reduced expression of SKP2 (*bottom*). Nuclei stained with DAPI. *Scale bar*, 100 μm . *C*, immunoblot analysis of GIST882 cells treated with the PI3K inhibitor LY294002 (25 $\mu\text{mol/L}$) for up to 72 h as indicated and probed for known regulators of the cell division cycle. Actin stain is shown to demonstrate equal loading. *D*, immunoblot analysis of GIST882 cells treated with the MEK1/MEK2 inhibitor U0126 (10 $\mu\text{mol/L}$) for up to 72 h as indicated and probed for known regulators of the cell division cycle. Actin stain is shown to demonstrate equal loading.



Taken together, these results suggest that the PI3K pathway, but not the MAPK pathway, is involved in the down-regulation of SKP2 induced by imatinib treatment.

Imatinib treatment leads to nuclear redistribution of CDH1.

Our results suggest that imatinib treatment leads to cell cycle exit through down-regulation of SKP2 and subsequent stabilization of p27^{Kip1}. We analyzed next whether imatinib modulates the SKP2 degradation machinery, in particular the APC/CDH1 complex.

We did not find any significant changes in the total amount of CDH1 protein after imatinib by immunoblotting (data not shown). We hence performed an immunofluorescence microscopic analysis to determine the subcellular localization of CDH1 after imatinib treatment of GIST cells. CDH1 showed a nuclear and cytoplasmic localization in DMSO-treated control cells. In contrast, we found a rapid redistribution of CDH1 to the nucleus beginning 1 hour after start of the treatment with 1 $\mu\text{mol/L}$ imatinib (Fig. 2). Previous reports have shown that activation of the APC requires nuclear localization of CDH1 (23, 24). Our results therefore suggest that the ability of imatinib to modulate CDH1 localization may be involved in cell cycle exit and quiescence.

Down-regulation of SKP2 leads to up-regulation of p27^{Kip1} and a growth arrest in GIST cells. To corroborate these results, we sought to determine more directly whether the CDH1/SKP2 axis is involved in cell cycle regulation and quiescence in GIST882 cells. When we reduced SKP2 levels by siRNA, we detected an increase of p27^{Kip1} and a decrease of cyclin A expression (Fig. 3A, left). This was associated with a significant decrease of cells in S phase as measured by BrdUrd incorporation from 30% in cells transfected with control siRNA duplexes to 4.4%, 2.1%, and 1.8% in cells

transfected with siRNA against SKP2 for 24, 48, and 72 hours, respectively ($P \leq 0.0001$; Fig. 3A, right).

Transient overexpression of CDH1 led to a down-regulation of SKP2 protein expression, a moderate increase of p27^{Kip1} levels, and decreased expression of cyclin A (Fig. 3B, left). Likewise, cells also showed a decreased incorporation of BrdUrd from 17.6% in cells transfected with empty vector to 5.1% and 6.9% in cells transfected with CDH1 plasmids at 24 and 48 hours, respectively ($P \leq 0.0001$; Fig. 3B, right).

Conversely, siRNA-mediated down-regulation of CDH1 was associated with a moderate increase of SKP2 levels, down-regulation of p27^{Kip1} and up-regulation of cyclin A (Fig. 3C, left). Transfection of cells with siRNA targeting CDH1 caused an increase of cells in S phase as measured by BrdUrd incorporation from 15% in controls to 23.5%, 67.7%, and 39.4% after 24, 48, and 72 hours, respectively ($P \leq 0.001$; Fig. 3C, right).

Taken together, these results show that modulation of the CDH1-SKP2-p27^{Kip1} signaling pathway in GIST cells has significant effect on cell proliferation and, hence, underscore the central role of this pathway in GIST cell cycle exit and quiescence.

The imatinib-induced cell cycle exit depends on SKP2. We next wanted to test directly whether SKP2 can modulate exit from the cell division cycle in imatinib-treated GIST882 cells. Cells were transiently transfected with either empty vector or SKP2 plasmid DNA followed by treatment with either DMSO or 1 $\mu\text{mol/L}$ imatinib for a total of 48 hours starting 24 hours after transfection. The proportion of proliferating cells was assessed by BrdUrd incorporation (Fig. 4A). A 1.7-fold increase of cells in S phase was detected in DMSO-treated populations from 19% in empty

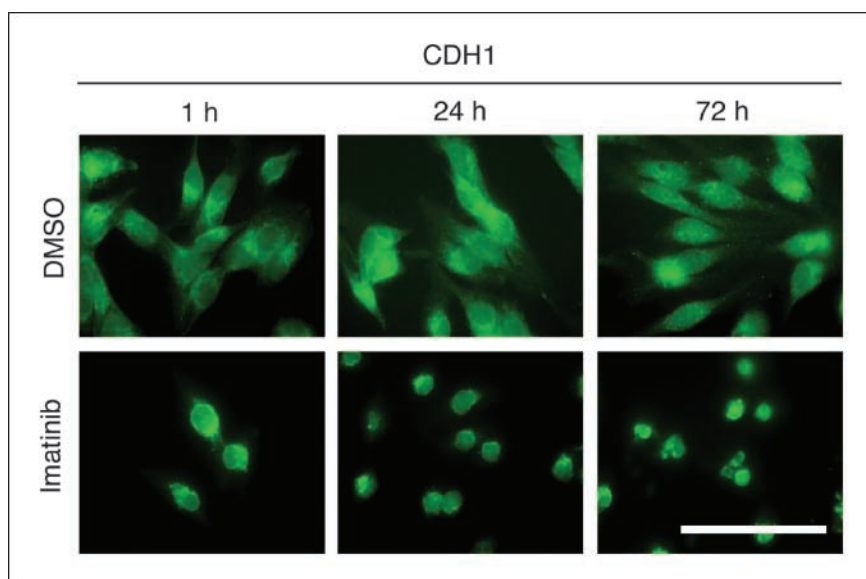


Figure 2. Imatinib treatment of GIST leads to alterations of the subcellular localization of CDH1 in a time-dependent manner. Immunofluorescence microscopic analysis of GIST882 cells for CDH1. Cells were treated with imatinib (1 $\mu\text{mol/L}$) for up to 72 h as indicated. Note the rapid redistribution of CDH1 to the nucleus already after 1 h of treatment (*bottom*). Scale bar, 50 μm .

vector-transfected cells to 32% in SKP2-transfected populations ($P \leq 0.01$; Fig. 4B). Imatinib treatment caused a significant decrease of cells in S phase from 19% to 2.4% in empty vector-transfected populations ($P \leq 0.0001$). However, overexpression of SKP2 in imatinib-treated populations was found to impede cell cycle exit and a significant proportion of cells remained in S phase (17.7%) with no significant differences between empty vector- and SKP2-transfected controls (19% and 32%, respectively; $P > 0.05$; Fig. 4A and B).

These results indicate that overexpression of SKP2 significantly attenuates the cell cycle exit induced by imatinib and underscore the role of the CDH1-SKP2-p27^{Kip1} axis in GIST cell cycle exit and quiescence.

Cells that survive imatinib treatment overexpress nuclear p27^{Kip1}. Based on our results, we speculated that cells that do not undergo apoptosis in response to imatinib should be driven into quiescence and express high levels of nuclear p27^{Kip1}. To test this idea, we treated GIST882 cells for up to 72 h with imatinib compared with DMSO used as control. Cells were then analyzed for nuclear p27^{Kip1} expression by immunofluorescence microscopy (Fig. 5). We found a significant increase of cells displaying nuclear p27^{Kip1} in imatinib-treated GIST882 populations from 12% in DMSO-treated controls to 54.6% after 24 hours, 73.1% after 48 hours, and 86.4% after 72 hours of imatinib treatment (all $P \leq 0.0001$; Fig. 5B). Cells with an apoptotic phenotype typically had no nuclear p27^{Kip1} expression and our results hence indicate that GIST cells that do not undergo apoptosis in response to imatinib instead up-regulate p27^{Kip1} and exit the cell cycle. Cells undergoing apoptosis after imatinib treatment increased from 0.7% in DMSO-treated controls to 0.8% after 24 hours, 5.7% after 48 hours, and 14.2% after 72 hours of imatinib treatment. These numbers, however, do not reflect the percentage of ~70% to 80% of cells undergoing apoptosis after imatinib that is usually seen in this cell type (20). The main reason for this are the experimental conditions used here where many of the loosely attached apoptotic cells were lost during the harsh staining process.

SKP2 is expressed in primary GISTs. Our *in vitro* results suggest that SKP2 is a critical regulator of cell cycle exit

in imatinib-treated GIST882 cells. We therefore sought to determine SKP2 expression in a series of primary GISTs. An immunohistochemical analysis of 28 primary GISTs and seven normal control tissue specimens was performed. Nineteen of 28 cases (68%) contained cells expressing detectable levels of SKP2 with some GISTs showing an expression that was similar to highly proliferative tissues such as crypts of the small bowel (Fig. 6). The average number of SKP2-positive cells per core was determined by counting 2 HPF ($\times 40$) from 2 independent experiments. The overall average number of SKP2-positive cells per HPF was 4.6 with a range from 0 to 56 SKP2-positive cells per HPF. Twenty-three GISTs showed 0 to 5 SKP2-positive cells/HPF, 2 GISTs had 6 to 10 SKP2-positive cells/HPF, and 3 showed >10 SKP2-positive cells/HPF.

We obtained the National Comprehensive Cancer Network (NCCN) risk score for all patients (25). This scoring system stratifies GIST patients into very low, low, intermediate, high, or no risk of progressive disease based on tumor size, organ site, and mitotic index. Two cases could not be stratified into risk groups because of insufficient data for their tumor localization at time of publishing the NCCN risk score (25). We found a significant correlation between high risk of progressive disease and >5 SKP2-positive cells per HPF (χ^2 test $P \leq 0.0006$; Fig. 6B). These cases also had a significantly higher mitotic count (>5 mitoses/50 HPF; $P \leq 0.0001$; Fig. 6B). Conversely, most patients with no to intermediate risk for progression showed ≤ 5 SKP2-positive cells per HPF and a low mitotic count of ≤ 5 mitoses per 50 HPF.

Discussion

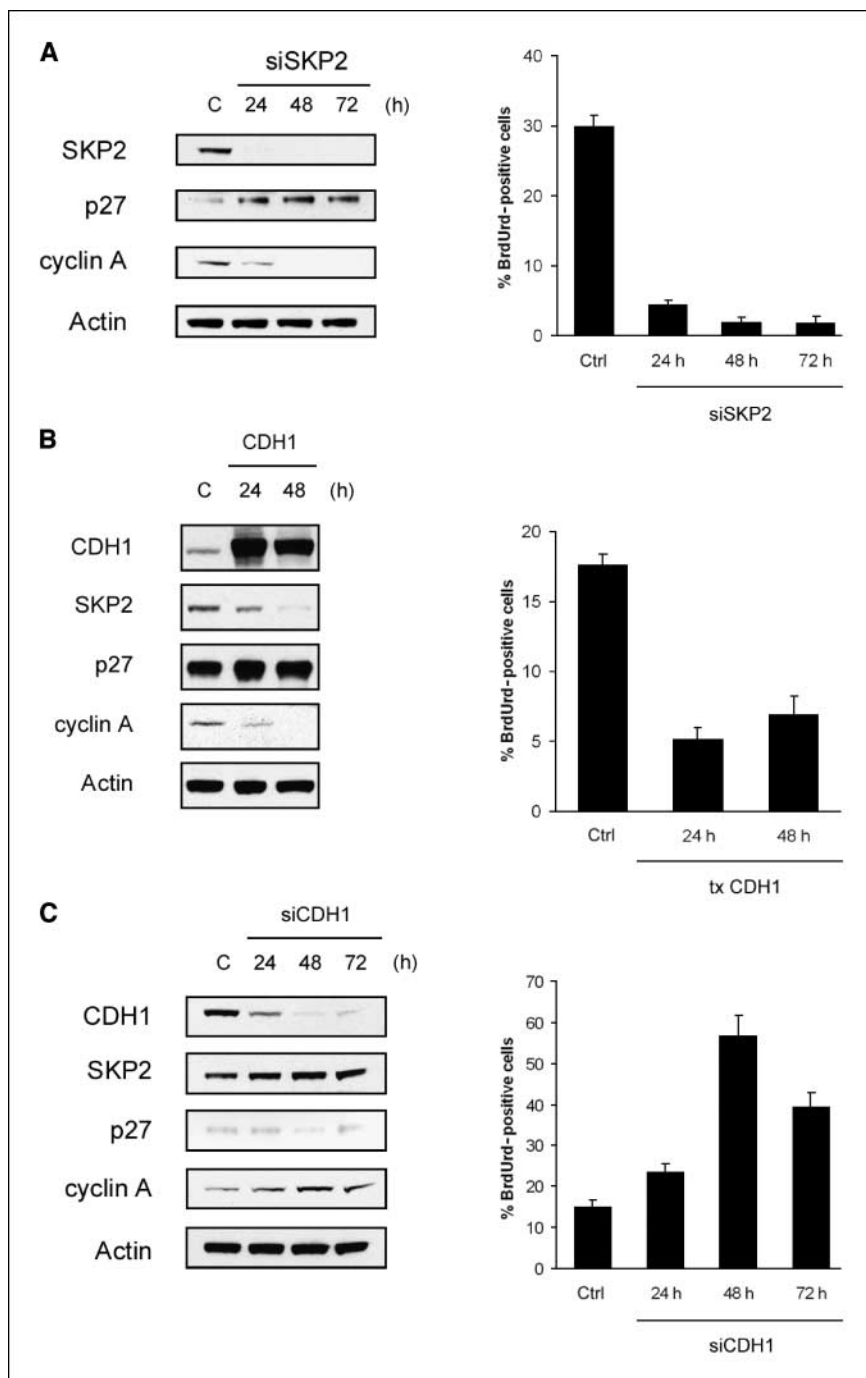
Approximately 85% of GIST patients treated with imatinib achieve disease stabilization, however, often in the presence of residual tumor masses. Complete remissions are rare and a substantial proportion of patients develop resistance to imatinib over time (4–6).

The present study was designed to determine whether imatinib-associated responses may account for these clinical findings. We report here that imatinib stimulates quiescence in a

proportion of GIST cells that is associated with an imatinib-induced modulation of the CDH1-SKP2 signaling axis in GIST cells. We provide evidence that imatinib down-regulates SKP2 and that this event is associated with increased nuclear CDH1, an activator of the APC that has previously been shown to regulate SKP2 stability. We also show that GIST cells, which do not undergo apoptosis in response to imatinib express nuclear p27^{Kip1}, suggesting that they have withdrawn from the cell cycle. Lastly, we provide evidence that a fraction of primary GISTs with high SKP2 expression levels may have an increased risk of disease progression.

Some of our findings raise important questions that warrant further investigation. First, it is worth pointing out that the cell line used in this study, GIST882, possesses a homozygous missense mutation affecting one of the kinase domains of the KIT gene (exon 13). This is a very uncommon mutation in GISTs that is seen only in ~1% of cases. Because studies on patient response suggest that tumors with these mutations do not respond as well to imatinib as GISTs harboring the more common mutations in exons 9 and 11, the antiproliferative effect of imatinib *in vitro* may also be different in this cell line. It will therefore be imperative to verify our results in other GIST cell lines harboring activating mutations

Figure 3. Modulation of the CDH1/SKP2 axis leads to changes in cell cycle behavior in GIST. *A to C*, immunoblot analysis of GIST882 cells transiently transfected with either siRNA to reduce expression levels of SKP2 (*A*), an expression plasmid encoding CDH1 (*B*), or siRNA to reduce expression levels of CDH1 (*C*). Immunoblots (*left*) were probed for SKP2, CDH1 (where applicable), p27^{Kip1}, and cyclin A. Actin stain is shown to demonstrate equal loading. Quantification of the percentage of GIST882 cells showing BrdUrd incorporation after knockdown of SKP2 protein levels by siRNA (*A*), transient overexpression of CDH1 (*B*), or knockdown of CDH1 protein levels by siRNA (*C*; *right*). *Ctrl*, control. *Columns*, mean of at least triple quantification of at least 100 cells; *bars*, SE.



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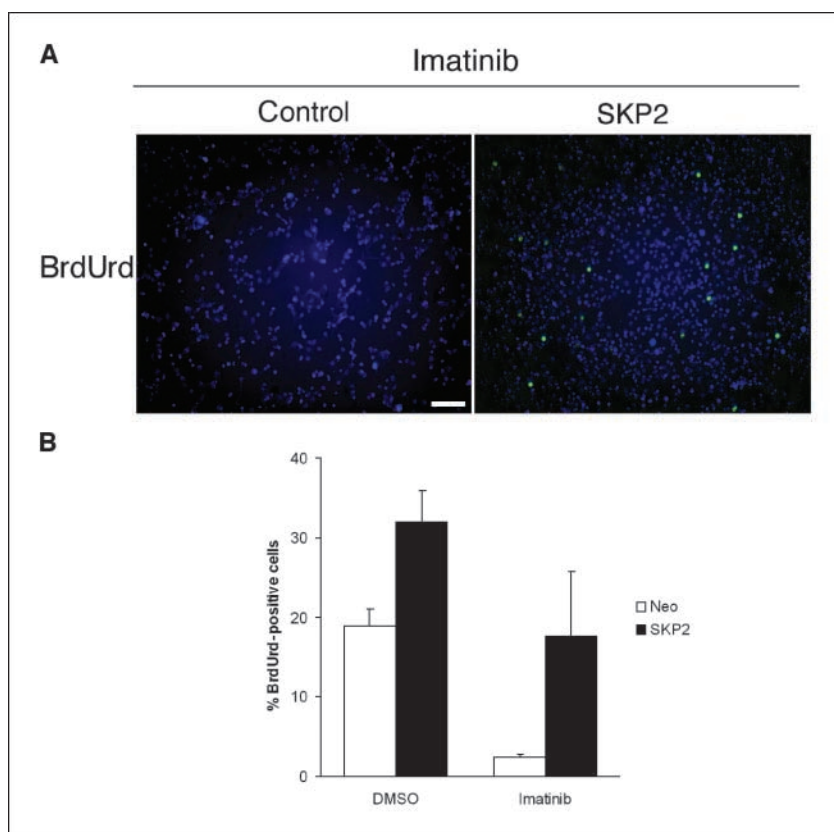


Figure 4. SKP2 has a direct effect on cell cycle regulation in imatinib-treated GIST. *A*, immunofluorescence microscopic analysis of BrdUrd incorporation in GIST882 cells transiently transfected with empty vector or SKP2. Cells were treated with imatinib (1 $\mu\text{mol/L}$) for a total of 48 h starting 24 h after transfection. *B*, quantification of the percentage of GIST882 cells showing BrdUrd incorporation after transient transfection with either empty vector (*Neo*) or SKP2 and treatment with either DMSO or imatinib (1 $\mu\text{mol/L}$) for a total of 48 h starting 24 h after transfection. *Columns*, mean of at least triple quantification of at least 100 cells; *bars*, SE.

outside of the kinase domain. Furthermore, it will be worthwhile to compare SKP2 and p27^{Kip1} immunohistochemistry in matched preimatinib and postimatinib samples, such as GIST xenograft models. These experiments are currently under way in our laboratory.

Second, all of our experiments have been performed within a 72-hour treatment interval and we do not know how long cells are able to remain in a quiescent state. It is possible that a prolonged cell cycle arrest requires continued inhibition of KIT, which is in line with the clinical observation that patients that discontinue imatinib treatment are more likely to develop disease progression. Quiescence is defined as reversible cell cycle arrest in G₀ and our findings that p27^{Kip1} is increased, whereas cyclin A expression is diminished are in line with this notion and furthermore underscore the important role of APC/CDH1 in maintaining cellular quiescence. Although quiescent cells are viable, prolonged quiescence may lead to apoptosis. Whether some of these cells also acquire markers of senescence and hence irreversibly withdraw from the cell cycle remains to be determined. Moreover, modulation of the CDH1-SKP2-p27^{Kip1} axis might not be the only pathway leading to and maintaining quiescence in GIST cells treated with imatinib. Recent reports, for example, show that constitutive, CUL3-mediated degradation of cyclin E is required for quiescence in hepatocytes (26). Further research will therefore be necessary to dissect the exact mechanisms of quiescence in imatinib-treated GIST.

Third, the precise mechanism of imatinib-induced redistribution of CDH1 into the nuclear compartment remains to be determined. Previous reports have shown that active APC/CDH1 complexes localize to the nucleus and that relocalization of CDH1 to the

cytoplasm inactivates the APC (24). In yeast, CDH1 is nuclear during G₁ and is exported into the cytoplasm in S phase until the end of mitosis, which is regulated by phosphorylation (23). Other studies have shown that PI3K can up-regulate SKP2 levels (27). Our results suggest that KIT-PI3K signaling may be important for preventing CDH1 localization to the nucleus thereby increasing SKP2 expression, but more detailed studies to address this question are necessary.

Our study confirms and extends previous reports analyzing the cellular response to imatinib in a different malignancy, chronic myeloid leukemia (CML). Treatment of CML cells with imatinib led to a relocalization of p27^{Kip1} to the nucleus in a PI3K-dependent manner. Remarkably, erlotinib treatment of non-small cell lung cancer cells expressing mutated epidermal growth factor receptor likewise induced the translocation of p27^{Kip1} to the nucleus and increased the half-life of p27^{Kip1} by down-regulation of SKP2 expression (28). These results suggest that certain similarities in the mode of action of small molecule kinase inhibitors may exist.

A large number of studies have examined expression of members of the SKP2-p27^{Kip1} axis in human cancer of all types. In fact, the majority of malignant tumors showed a reduction of p27^{Kip1} protein levels (29). More recently, it has become clear that deregulation of SKP2 in human cancer is involved in the down-regulation of p27^{Kip1} (30–32). Both, reduced levels of p27^{Kip1} and overexpression of SKP2, have been shown to correlate with poor prognosis in breast cancer and other tumors (30, 33–37). The relevance of SKP2 as prognostic marker in GISTs needs to be validated in a larger cohort of patients and in a prospective manner. Whether SKP2 is a more accurate

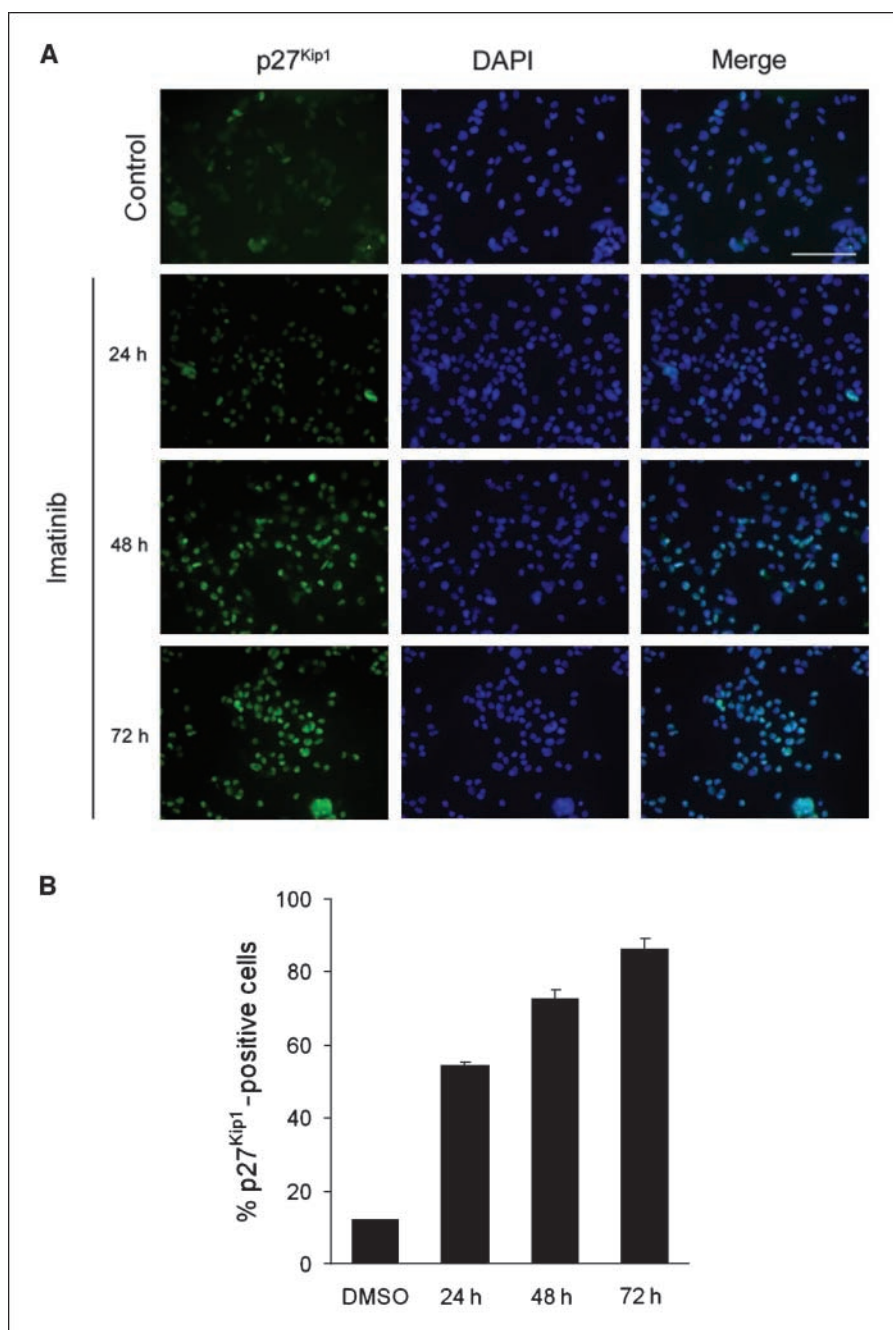
prognostic marker than decreased levels of the CDK inhibitor p27^{Kip1} in GIST (38, 39) remains likewise to be determined.

There is mounting evidence that imatinib affects GIST cell proliferation and apoptosis through multiple pathways. We have recently described a novel proapoptotic pathway in GIST cells that involves soluble histone H2AX (20). In the present study, we show that imatinib can also drive GIST cells into cell cycle arrest and quiescence. In fact, the pathway leading to tumor cell quiescence may even be more relevant for the response to imatinib as judged by certain clinical findings and results from GIST mouse models that show a relatively moderate increase of apoptosis in response to imatinib (40). Our results

suggest that apoptosis and quiescence are, at least in the time frame of our experiments, mutually exclusive, and future studies are warranted to determine why tumor cells respond differently to imatinib therapy. Our results would suggest a model in which GIST cells that are not rapidly eliminated by imatinib-induced apoptosis exit the cell cycle and enter quiescence. These results help to explain the clinical observations that many patients develop stable disease in the presence of residual tumor and that complete remissions are overall relatively rare.

Future studies will focus on the question whether it is possible to revert quiescent GIST cells into a state in which they can

Figure 5. Cells that survive after imatinib treatment express high levels of nuclear p27^{Kip1}. *A*, immunofluorescence microscopic analysis of p27^{Kip1} in GIST882 cells treated with either DMSO or imatinib (1 μmol/L) for up to 72 h as indicated. Nuclei stained with DAPI. *Scale bar*, 100 μm. *B*, quantification of the percentage of GIST882 cells showing nuclear p27^{Kip1} after treatment with either DMSO or imatinib (1 μmol/L) for up to 72 h as indicated. *Columns*, mean of at least triple quantification of at least 100 cells; *bars*, SE.



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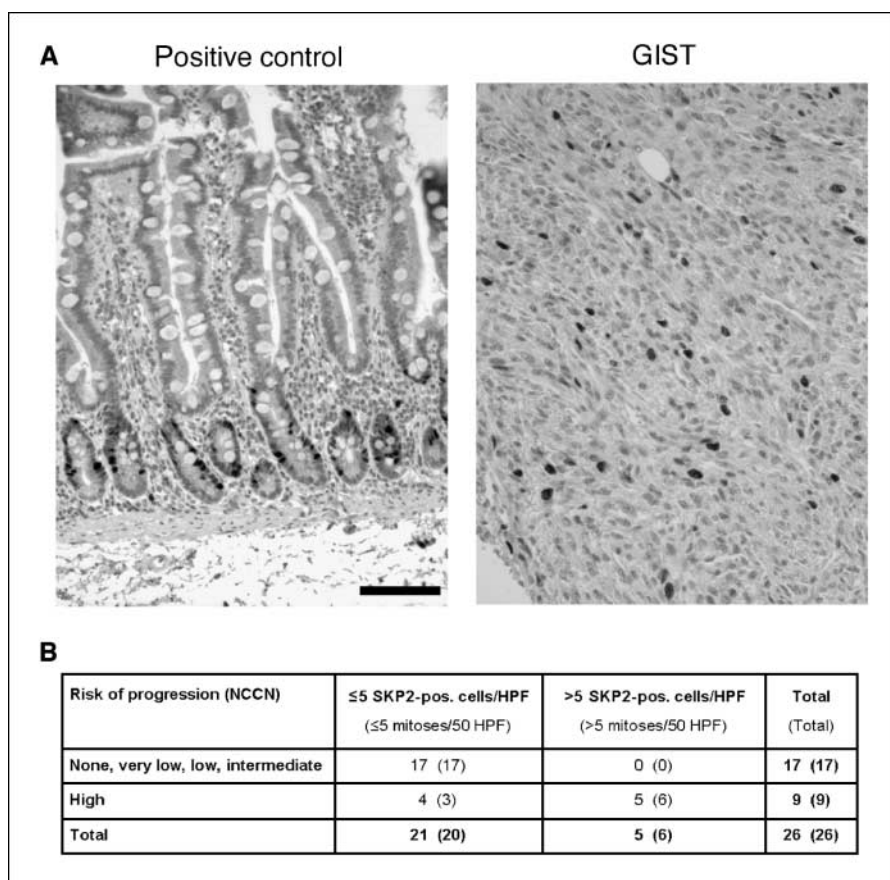


Figure 6. SKP2 expression in primary GISTs correlates with increased risk of progression. A, immunohistochemical analysis of SKP2 expression in GIST and adjacent normal small bowel. Note that the expression level of SKP2 in a primary GIST (*right*) is similar to the highly proliferating crypts of the jejunum (*left*). Scale bar, 100 μ m. B, SKP2 expression, mitotic index and risk for progression in a series of 26 GIST. *SKP2-pos*, SKP2-positive.

more easily be eliminated by apoptosis. Moreover, novel approaches to shift the cellular responses to imatinib toward apoptosis instead of cell cycle arrest are necessary to make imatinib-based therapies more effective with a goal toward cure.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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