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Persistent GP130/STAT3 Signaling Contributes to the Resistance of Doxorubicin, Cisplatin, and MEK Inhibitor in Human Rhabdomyosarcoma Cells

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Abstract

To test the role of STAT3 in human rhabdomyosarcoma cells, genetic approaches were used to either knockdown the expression of STAT3 and GP130, an upstream activator of STAT3 using short hairpin RNA (shRNA) or express persistently active STAT3 protein. Knockdown expression of GP130 or STAT3 sensitized cells to anti-cancer drugs doxorubicin, cisplatin, and MEK inhibitor AZD6244. On the other hand, expression of the constitutively active STAT3 protein reduced the sensitivity of rhabdomyosarcoma cells to those drugs.

In addition, we tested a small molecule STAT3 inhibitor LY5 and a GP130 inhibitor bazedoxifene in rhabdomyosarcoma cells. Our data demonstrated that the combination of LY5 or bazedoxifene with doxorubicin, cisplatin, and AZD6244 showed stronger inhibitory effects than single agent alone. In summary, our results demonstrated that GP130/STAT3 signaling contributes to the resistance of these drugs in rhabdomyosarcoma cells. They also suggested a potentially novel cancer therapeutic strategy using the combination of inhibitors of GP130/STAT3 signaling with doxorubicin, cisplatin, or AZD6244 for rhabdomyosarcoma treatments.

Keywords

STAT3; GP130; rhabdomyosarcoma; cisplatin; doxorubicin; MEK inhibitor

INTRODUCTION

Rhabdomyosarcoma is the most common soft tissue sarcoma in pediatric patients [1]. A 5-year survival rate >70% has been achieved in recent trials for patients with localized

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CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

rhabdomyosarcoma. However, the outcome for patients who present with metastatic disease remains poor [1]. Attempts to improve survival time utilizing dose intensified chemotherapy regimens were not successful, and as a consequence, survival rates have remained unchanged for over 15 years [2]. Therefore, there is an urgent need for new therapeutic strategies for the pediatric rhabdomyosarcoma.

STAT3 is frequently activated in many human solid and blood cancer types and contributes to cancer progression [3]. *Stat3* can function as an oncogene product because persistent STAT3 signaling can mediate oncogenic transformation of normal cells into malignant cells and mediate tumor formation in animal models [4]. In contrast, resistance to oncogenic transformation by multiple oncogenes is observed in STAT3 deficient fibroblasts [5]. Persistent activation of STAT3 signaling plays a crucial role in oncogenesis by stimulating cell proliferation and decreasing apoptosis induced by traditional cancer therapies [3, 6]. STAT3 promotes oncogenesis through up-regulation of its downstream targets, Cyclin D1, Bcl-2, survivin, DNMT1, VEGF, MMP-2, Bcl-xL, miR-21, miR-181B-1 among others [4, 7]. Blocking STAT3 activity through STAT3 small interfering RNAs, dominant negative STAT3 mutants, or antisense STAT3 oligonucleotides inhibits cell proliferation, suggesting that persistent STAT3 signaling is important to the survival and proliferation of many tumor cells [7, 8]. In contrast, studies using normal mouse fibroblasts demonstrated that disrupting STAT3 signaling has a much less essential effect in normal cells suggesting that blocking STAT3 functions may not induce substantial toxicity to normal cells *in vivo* [9]. More recent work investigating the potential role of STAT3 activation in sarcomas demonstrated that persistent phosphorylated STAT3 protein (P-STAT3) frequently occurs in rhabdomyosarcoma, osteosarcoma, and Ewing sarcoma [10–12]. Moreover, STAT3 inhibition using small molecule STAT3 inhibitors (STA-21, LLL3, LLL12), a dominant negative form of STAT3, or STAT3 siRNAs inhibited proliferation and induced apoptosis in rhabdomyosarcoma cell lines expressing high levels of P-STAT3 [11, 13]. Therefore, STAT3 appears to represent a valid target for therapy in childhood rhabdomyosarcomas. In this study, we further demonstrated that constitutively active STAT3 contributes to resistance to anti-cancer drugs and inhibition of STAT3 or its upstream regulator GP130 sensitizes rhabdomyosarcoma cells to these drugs.

MATERIALS AND METHODS

Rhabdomyosarcoma Cell Culture

The human rhabdomyosarcoma cell lines RH4, RH5, and RH30 were gifts from Dr. Peter Houghton. These cells were maintained in RPMI 1640 (Hyclone, USA) supplemented with 10% fetal bovine serum (FBS,) and 1% penicillin / streptomycin. The cells were cultured at 37 °C with 5% CO₂.

Western Blotting Analysis

The human rhabdomyosarcoma cells were lysed in cold radioimmunoprecipitation (RIPA) assay buffer containing proteasome inhibitors cocktail and phosphatase inhibitor cocktail. Primary antibodies including phosphorylated-specific STAT3 antibody that recognizes at tyrosine residue 705 (p-STAT3^{Y705}) and antibodies for STAT3, GP130, DYKDDDDK Tag

(anti-Flag), GAPDH, as well as secondary antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). These proteins were detected by western blotting analysis as described in our previous study [14].

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

RT-PCR was analyzed as described in the article [14, 15]. The following primers were used: Cyclin D1, annealing at 52°C, (For): 5'-GCTGGAGCCCGTGAAAAAGA-3', (Rev): 5'-CTCCGCCTCTGGCATTTTG-3'; Bcl-X1, annealing at 48 °C, (For): 5'-TTGGACAATGGACTGGTTGA-3', (Rev): 5'-GTAGAGTGGATGGTCAGTG-3'; DNMT1, annealing at 52 °C, (For): 5'- GTGGGGGACTGTGTCTCTGT -3', (Rev): 5'-TGAAAGCTGCATGTCCTCAC -3'; GAPDH, annealing at 52 °C, (For): 5'-TGATGACATCAAGAAGGTGGTGAAG-3', (Rev): 5'-TCCTTGGAGGCCATGTGGGCAT-3' (integrated DNA Technologies, USA).

Quantitative Reverse Transcriptase-PCR

RH30 cells were treated with LY5 and bazedoxifene for 24 hours. Total DNA was isolated and purified from cells using DNeasy Blood & Tissue Kit (Qiagen). Real-Time PCR amplification was performed according to the manufacturer's protocol with Applied Biosystems 7900 HT Fast Real-Time PCR System. Primer of ND2, (For): 5'-GCCCTAGAAAT AAACATGCTA-3', (Rev): 5'-GGGCTATTCCTAGTTTT ATT-3'; COI, (For): 5'-CGATGCATACACCACATGAA-3', (Rev): 5'-AGCGAAGGCTTCTCAAATCA-3'; 18S, (For): 5'-TAGAGGGACAAGTGGCGTTC -3', (Rev): 5'-CGCTG AGCCAGTCAGTGT-3'; The relative gene expression level between treatments was calculated using the following equation: relative gene expression = $2^{-(Ct\ sample - Ct\ control)}$.

Elisa Assay for Cytosol Cytochrome C

Cytochrome c release in the cytosol occurs prior to the activation of caspases which is considered the hallmark of apoptosis. To examine cytochrome c release, RH30 cells were treated by LY5 or bazedoxifene for 24 hours. The Cell Extraction Buffer (Invitrogen) was used to prepare cytosol cytochrome c. Cytochrome c was detected using Cytochrome c ELISA kit (Invitrogen).

Stable Transfection with STAT3-C, STAT3 ShRNA, and GP130 ShRNA

The STAT3-C (a murine STAT3) was a constitutively active form of STAT3, [4] which was cloned into vector with a Flag epitope. The STAT3 and GP130 short hairpin RNA (shRNA) plasmid was cloned into pSIH1-H1-puro vector (Addgene). All of them were transfected into cells by Lipofectamine 2000 (Invitrogen). Cells stable transfected with STAT3-C were selected by G418 (400 µg/ml), and cells transfected with STAT3 and GP130 shRNA were selected by puromycin (2 µg/ml), respectively. Selected colonies were confirmed by western blot analysis for the expression of STAT3-C and GP130.

STAT3-Dependent Transcriptional Luciferase Activity

The pGL4.47[luc2P/SIE/Hygro] vector (Promega, USA) is a STAT3 reporter which is a mixture of a luciferase construct under the control of sis-inducible element (SIE) including STAT3 DNA binding site. The vector was transfected to RH30 cells which could be selected by G418. The RH30-cloned cells that stably integrated the STAT3-dependent luciferase reporter construct were confirmed by Luciferase Assay System (Promega, USA). The cells were treated with different concentrations of LY5 for 24 hours. The STAT3 luciferase activity of the LY5-treated cells are compared to DMSO-treated cells which were set at 100%.

Wound Healing/Cell Migration Assay

When RH30 cells were 100% confluent, the monolayer was scratched in same width using a pipette tip. After washing, RH30 cells were then treated with 0.5 μ M and 1 μ M LY5 or DMSO. After 48 hours culture, when the wound in the DMSO control was closed, images were captured by Leica Microsystems.

MTT Cell Viability Assay

Cells were seeded in 96-well plates at a density of 3000 cells per well and cultured for 24 hours. For the sensitivity to anti-cancer drugs, cells were treated with different concentrations of doxorubicin, cisplatin, or AZD6244 in triplicate for 48 hours. The absorbance was read at 595 nm.

Small Molecular Compound and Anti-cancer Drugs

The novel small molecule STAT3 inhibitor, LY5 was designed and synthesized in Dr. Chenglong Li laboratory [16]. Bazedoxifene was demonstrated by our group to exhibit a novel function as a GP130 inhibitor [17] and was purchased from Acesys Pharmatech (USA). Cisplatin and doxorubicin was purchased from Selleck Chemicals (USA) and LC Laboratories (USA). AZD6244, an MEK inhibitor, was bought from Selleck Chemicals.

Statistical Analysis

Data were analyzed using GraphPad Prism software. Unpaired t tests were used for analyses assuming Gaussian populations with a 95% confidence interval. Data are presented as mean $\pm$ SE. Differences were analyzed with the Student t test, and significance was set at $P < 0.05$. *, ** and *** indicates $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

RESULTS

Expression of p-STAT3^{Y705} in Rhabdomyosarcoma Cells

We examined p-STAT3^{Y705} of RH30, RH4 and RH5 in medium without FBS for 24 hours to exclude the influence from growth factors in FBS by western blot. We found STAT3 phosphorylation was persistently elevated significantly in RH30 cells, but p-STAT3^{Y705} was much lower in RH5 cells (Fig. 1A). We could not detect p-STAT3^{Y705} in RH4 cells. Because STAT3 phosphorylation was higher in RH30 cells, we knockdown STAT3 in this cell line using shRNA to investigate effect of cell viability after treatment with anti-cancer drugs.

Knockdown of STAT3 Decreased Cell Viability and Sensitized Cells to Anti-Cancer Drugs

To examine the role of persistent STAT3 signaling in rhabdomyosarcoma cells, genetic approaches were used to knockdown the expression of STAT3 in RH30 cells by using shRNA. We examined the cloned cells which were selected by puromycin and found p-STAT3 and STAT3 were decreased, suggesting STAT3 was knockdown (Fig. 1A). To further evaluate the sensitivity of RH30-STAT3 knockdown cells to the anti-cancer drugs, we treated un-transfected RH30 and stable STAT3 shRNA transfected RH30 cells with different concentrations of doxorubicin, cisplatin, and AZD6244 for 24 hours. The results showed that cell viability of RH30-STAT3 knockdown cells was significantly decreased compared with parental RH30 cells after the drug treatment (Fig. 1C).

We then examined the role of GP130, the upstream activator of STAT3, in drug resistance in rhabdomyosarcoma cells. Using shRNA to knockdown the expression GP130, we found the expression of GP130 and p-STAT3^{Y705} were both decreased in the cloned cells (Fig. 1B). We treated RH30-GP130 knockdown cells and parental RH30 cells with different concentrations of doxorubicin, cisplatin, or AZD6244 for 24h. The results showed cell viability of RH30-GP130 knockdown cells was further reduced compared with un-transfected RH30 cells, suggesting the knockdown of GP130 sensitized RH30 cells to doxorubicin, cisplatin, and AZD6244 (Fig. 1D).

Constitutive Active STAT3 Protein Decreased the Sensitivity to Anti-Cancer Drugs

We used an alternative experimental approach to verify whether expression of constitutive active STAT3 protein in rhabdomyosarcoma cell lines lacking persistent STAT3 signaling could decrease the sensitivity of those cell lines to anti-cancer drugs. RH4 and RH5 cells were transfected with STAT3-C expression vector. After selection by G418, the stable cloned cells expressing persistent active STAT3 was detected using western blot. The results showed Flag epitope-tagged STAT3, p-STAT3^{Y705} and STAT3 were all increased in selected colony (Fig. 2A and Fig. 2C). We treated parental RH4 and RH5 cells as well as STAT3-C transfected RH4, RH5 cells with different concentrations of doxorubicin and cisplatin. The results showed less reduction of cell viability in RH4 and RH5 cells expressing constitutive active STAT3 than parental RH4 and RH5 cells after treatment of doxorubicin and cisplatin. These results indicated STAT3-C-transfected RH4 and RH5 cells were more resistant to those drugs compared to parental cells (Fig. 2B and 2D).

LY5 Inhibits STAT3 Activity in Rhabdomyosarcoma Cells

To investigate whether inhibition of STAT3 phosphorylation in rhabdomyosarcoma cells expressing constitutive phosphorylated STAT3 would reduce cell viability, we treated RH30 cells with LY5. Phosphorylated STAT3 was decreased in a LY5 dose-dependent fashion by western blot (Fig. 3A). To further explore whether the treatment of LY5 treatment might affect STAT3 downstream target genes, we examined the expression of Bcl-xL, Cyclin D1 and DNMT1 in LY5-treated cells by RT-PCR. Inhibition of STAT3 target genes by LY5 was confirmed (Fig. 3B).

In addition, we performed a STAT3 DNA binding assay in LY5-treated RH30 cells. Our result showed that the STAT3 DNA binding ability was inhibited in LY5-treated cells

compared with DMSO-treated control (Fig. 3C). PGL4.47[luc2P/SIE/Hygro] vector transfected RH30 cells expressed luciferase which was responsive to STAT3. We treated the RH30 cells stably transfected with luciferase vector with different concentrations of LY5 for 24 hours. Using a simple luciferase assay system, the STAT3 luciferase activity in treated cells was examined. As shown in Fig. 3D, LY5 treatment obviously reduced luciferase activity which is depended on the STAT3 transcriptional activity in a dose dependent manner.

STAT3 has been shown to be involved in wound healing and migration of cancer cells, which might lead to invasion and metastasis [18, 19]. We examined whether LY5 might affect rhabdomyosarcoma cell migration. We observed that LY5 treatment decreased migration ability in a dose dependent manner (Fig. 3E).

Blocking STAT3 Signal by LY5 Sensitized Sarcoma Cells to other Anti-Cancer Drugs Treatment

To investigate whether inhibition of STAT3 could sensitize RH30 cells to other anti-cancer drugs, RH30 cells were treated with LY5 (1 μ M) combined with various concentrations of doxorubicin, cisplatin and AZD6244 for 48 hours. As shown in Fig. 3G–I, a greater decrease of cell viability was observed in the combinational treatment group compared with single drug treated groups.

Bazedoxifene Suppressed STAT3 Phosphorylation and Sensitized RH30 Cells to other Anti-Cancer Drugs

Bazedoxifene was examined for its inhibitory of GP130/STAT3 signaling in RH30 cells. The results showed that bazedoxifene decreased the p-STAT3^{Y705} (Fig. 4A), reduced cell migration ability in RH30 cells (Fig. 4B). In addition, RH30 cells were treated with bazedoxifene combined with doxorubicin, cisplatin, or AZD6244. After 48 hours of treatment, cell viability was examined as shown in Fig. 4D–F. A statistically significant reduction of cell viability was seen in the drug combination group compared with single drug treated group. These results indicate that inhibition of GP130 signaling by bazedoxifene could also sensitize rhabdomyosarcoma cells to those anti-cancer drugs.

LY5 and Bazedoxifene Induced Cell Apoptosis

As one of the mitochondrial markers, cytochrome c release was determined in RH30 cells treated by LY5 (1 μ M and 2 μ M) and bazedoxifene (10 μ M and 20 μ M). The result showed that LY5 or bazedoxifene treatment increased release of cytochrome c, suggesting LY5 (Fig. 3F) and bazedoxifene (Fig. 4C) induced cell apoptosis. Furthermore, ND2 and COI, as mitochondrial genes, were measured by qPCR. As shown in Fig. 4G, the expression of ND2 and COI was decreased in LY5- and bazedoxifene-treated RH30 cells. These results suggest that the inhibition of GP130/STAT3 signaling could suppress mitochondrial activities and induce cell apoptosis.

DISCUSSION

Human rhabdomyosarcoma is one of the most common soft tissue sarcomas and is initially chemosensitive, but can acquire multidrug resistance during chemotherapy [20]. The development of resistance to chemotherapy is a major cause of failure in treating patients with rhabdomyosarcoma. Some genes are thought to be involved in resistance of chemotherapy, such as P-glycoprotein (P-gp), MDR associated protein 1 (MRP1), glutathione-S-transferase (GST), and others [21, 22]. Recently, inhibition of STAT3 pathway was proved to promote chemotherapeutic sensitivity of cancer [23]. In this study, we found persistent STAT3 signaling contributed to resistance of anti-cancer drug treatments in rhabdomyosarcoma cells.

Constitutive activation of STAT3 signaling participates in tumorigenesis, proliferation, survival, and invasion of various human cancers, promoting it a very attractive drug development target for tumor treatment [3, 6]. However, whether STAT3 participates in the survival and proliferation of rhabdomyosarcoma is not fully clear. We previously detected sarcoma tissues and cell lines and explored STAT3 phosphorylation is increased in 27% of rhabdomyosarcoma tissues. In this study, we found STAT3 phosphorylation was also elevated in RH30 cells, so we inhibited STAT3 in RH30 cells using shRNA and LY5 to investigate the role of STAT3 in cell proliferation, migration and treatment with anti-cancer drugs, cisplatin, doxorubicin, or a MEK inhibitor, AZD6244. We found knockdown of STAT3 further decreased RH30 cell viability upon the treatments of these drugs.

Interleukin (IL)-6 is the dominant IL-6 family members in terms of expression in solid tumors and correlate with poor prognosis in these tumor, including aggressiveness, relapse and metastasis [24, 25]. They bind to IL-6R α to form a binary complex and then recruit GP130 to form the IL-6/IL-6R α /GP130 heterotrimers. Dimerization of the trimers activate IL-6/GP130/STAT3 signaling which play important roles in cancer development [17, 26]. The results of GP130 knockdown experiment also showed inhibition of GP130 sensitized RH30 cells to doxorubicin, cisplatin and AZD6244 as the results similar to STAT3 inhibition. These results suggest that persistent GP130/STAT3 signaling is responsive for drug resistance and GP130/STAT3 inhibition should sensitize rhabdomyosarcoma cells to cisplatin, doxorubicin, and AZD6244.

Bazedoxifene is known as a selective estrogen modulator and commonly used as treatment for osteoporosis. Recently, Li found bazedoxifene could bind to GP130 D1 domain and show potential for inhibiting IL6/GP130/STAT3 cancer signaling pathway through disruption of the IL6/GP130 D1 interface as a novel small molecule GP130 inhibitor [17]. LY5 is a novel small molecule STAT3 inhibitor which exhibits potent activity against STAT3 signaling in cancer cells with constitutively active STAT3 [27]. In the study we found bazedoxifene and LY5 inhibited STAT3 phosphorylation and cell migration, as well as decreased STAT3 downstream gene expression and induced cell apoptosis in RH30 cells. These results suggest GP130/STAT3 signaling pathway is crucial for cancer cell development. The effects of bazedoxifene and LY5 combined with cisplatin, doxorubicin, or AZD6244 showed that bazedoxifene and LY5 could work synergistically with these drugs in RH30 cells, which were consistent with the results of knockdown of STAT3 by GP130 and

STAT3 shRNA. All these results indicate GP130/STAT3 inhibition can sensitize RH30 cells to anti-cancer drugs. The combination of GP130/STAT3 inhibitor with other anti-cancer drugs should be a more effective therapeutic strategy for rhabdomyosarcoma treatment.

In conclusion, persistent GP130/STAT3 signaling represents a viable target for therapy in childhood rhabdomyosarcoma. The results in this study implicate a potential cancer therapy strategy, which is the combination of GP130 or STAT3 inhibitor with cisplatin, doxorubicin and AZD6244 in rhabdomyosarcoma treatment. Furthermore, in an *in vivo* animal tumor models, drug combination studies should be tested to explore the future clinical potential of combination of GP130 or STAT3 inhibitor with these three anti-cancer drugs.

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Biography

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Fig. 1.

(A) P-STAT3^{Y705} was elevated significantly in RH30 cells, but much lower in RH5 cells in medium of 0% FBS by western blot. P-STAT3^{Y705} was not detected in RH4 cells. P-STAT3 and STAT3 were decreased in RH30 cells using STAT3 shRNA. (B) GP130 was knocked down in RH30 cells using GP130 shRNA, and p-STAT3^{Y705} was decreased. (C) & (D) Treatments with doxorubicin, cisplatin or AZD6244 further decreased the cell viability in RH30 cloned cells in which STAT3 or GP130 was knocked down by shRNA. (* $P < .05$, ** $P < .01$, *** $P < .001$).

Fig. 2.

RH4 (A) or RH5 (C) cells were stably transfected with STAT3-C vector. The expression of Flag-tagged STAT3 was examined in STAT3-C stably transfected RH4 and RH5 cells by western blot. (B) & (D) The inhibition of cell viability by cisplatin and doxorubicin in RH4 (B) and RH5 (D) cells was decreased in the presence of STAT3-C protein shown by MTT assay (* $P < .05$, ** $P < .01$, *** $P < .001$).

Fig. 3.

(A) P-STAT3^{Y705} was inhibited in RH30 cells treated with different concentrations of LY5 using western blot. (B) The mRNA expression of STAT3 downstream target genes, CyclinD1, Bcl-xL, DNMT1 were reduced in RH30 cells treated with different concentrations of LY5 by RT-PCR. (C) The STAT3 DNA binding activity was decreased in RH30 cells by LY5 using STAT3 DNA binding assay. (D) STAT3-dependent transcriptional activity was analyzed in a luciferase assay. Relative STAT3-dependent luciferase activity was declined in LY5-treated RH30 cells. (E) Wound healing assay demonstrated that LY5 could inhibit cell migration in RH30 cells. (F) Cytochrome c release was increased in LY5-treated RH30 cells. (G–I) The combinatorial effect of LY5 and anti-cancer drugs, doxorubicin (G), cisplatin (H) or AZD6244 (I). A statistically significant reduction of cell viability was observed in the combination treatment (* P <.05, ** P <.01, *** P <.001).

Fig. 4.

(A) P-STAT3^{Y705} was inhibited in RH30 cells treated with different concentrations of bazedoxifene using western blot. (B) Wound healing assay indicated that bazedoxifene could inhibit cell migration in RH30 cells. (C) Bazedoxifene treatment increased cytochrome c release. (D–F) The combinatorial effect of bazedoxifene and anti-cancer drugs, doxorubicin (D), cisplatin (E), or AZD6244 (F). A greater inhibition of cell viability was seen in the combination treatment. (G) ND2 and COI was reduced in RH30 cells treated with LY5 or bazedoxifene using qPCR (* $P < .05$, ** $P < .01$, *** $P < .001$).