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HER-2/neu raises SHP-2, stops IFN-γ anti-proliferation in bladder cancer

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Abstract

Gene amplification or HER-2/neu protein overexpression signals a poor outcome for bladder cancer patients. We investigated the anti-proliferative effect of IFN-γ in HER-2/neu-transfected human bladder cancer cells (TCC-N5 and TCC-N10). The cells continued growing after IFN-γ stimulation but did not activate the Janus kinase (Jak)/Stat pathway. We found Jak/Stat protein phosphatase in TCC-N5 and TCC-N10 cells with upregulated Src homology 2-containing protein tyrosine phosphatase-2 (SHP-2). After the cells had been treated with AG825, a HER-2/neu-specific inhibitor, SHP-2 expression declined, and Jak2/Stat1 reactivated. Similar results were reported in a mouse bladder cancer cell line, MBT2, with constitutive HER-2/neu overexpression. Further, AG825 pretreatment restored the anti-proliferation activity of IFN-γ in TCC-N5 and TCC-N10 cells. Therefore, the suppression of IFN-γ signaling in HER-2/neu-overexpressing bladder cancer cells might be due to SHP-2 upregulation. The regulation of SHP-2 by HER-2/neu provides a new target for blocking the HER-2/neu oncogenic pathway.

Keywords: HER-2/neu; IFN-γ; SHP-2; Jak/Stat; Cell growth

The oncogene HER-2/neu, also known as *c-erb*B-2, is on chromosome 17q21; it encodes a transmembrane glycoprotein [1]. HER-2/neu protein, associated with tyrosine kinase activity [2,3], stimulates cell growth [4]. Gene amplification or protein overexpression of the HER-2/neu gene was found in approximately 30% of human breast carcinomas [5], and is closely associated with a poor prognosis and response to therapy [5,6]. In bladder cancer, the clinical significance of HER-2/neu has remained controversial [7,8], perhaps because of different methods of assessing HER-2/neu status (detecting amplification versus detecting overexpression using polymerase chain reaction, fluorescence in situ hybridization, or immunohistochemistry) and the definition of HER-2/neu positivity [9,10]. Some well-designed studies, however, have offered strong evidence that gene amplification [11] and protein overexpression of HER-2/neu [12] are related to a poor outcome.

Interferons (IFNs), a family of cytokines, suppressed cellular proliferation in human bladder cancer cells in vitro [13,14] and in vivo [15]. Clinically, intravesical IFN-γ instillation inhibited bladder cancer growth and prevented bladder cancer recurrence [16,17]. IFN-γ activates signal transducer and activator of transcription 1 (Stat1) after it attaches to IFN-γ receptor 1 (IFNGR1) [18], and then it upregulates cell-cycle inhibitor P21/WAF1 [19]. IFN-γ also sensitizes tumor cells to the cytotoxic effect of Fas and the Fas-ligand complex, and then it initiates a caspase-associated apoptotic pathway [20,21]. IFN-γ inhibited

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the expression of HER-2/neu in cancer cells [22] by phosphorylating Stat1 [23]. Conversely, HER-2/neu overexpression downregulated MHC class I products in tumor cells by blockading IFN-γ signaling [24,25].

Two mammalian Src homology 2 (SH2)-containing protein tyrosine phosphatases, SHP-1 and SHP-2, have been implicated in the regulation of IFN signaling, and both participate in negative control in IFN-stimulated Janus kinase (Jak)/Stat pathways [26,27]. SHP-1 is expressed only in hematopoietic cells [26,28], but SHP-2 is expressed ubiquitously [27,28]. A dysfunction in SHP-2 regulation can cause abnormal cell growth and induce different kinds of cancers. Somatic SHP-2 mutations are associated with sporadic juvenile myelomonocytic leukemia [29] and the pathogenesis of *Helicobacter pylori*, the major cause of gastric carcinoma [30]. Because the SHP-1 and SHP-2 association with HER-2/neu receptors has been previously demonstrated [31,32], SHP-2 may be involved in HER-2/neu overexpression that leads to the suppression of the IFN signaling pathway in non-hematological malignancies.

To explore the influence of HER-2/neu overexpression in bladder cancer, we transfected a plasmid containing HER-2/neu genes into a human bladder cancer cell line, TCC-SUP, in which HER-2/neu protein was scarcely expressed. We found that HER-2/neu overexpression suppressed IFN-γ-mediated anti-proliferation activity and IFN-γ-induced Jak/Stat activation. Upregulation of SHP-2 by HER-2/neu overexpression might mediate the suppression. Similar results were reported in a mouse bladder cancer cell line (MBT2) with constitutive HER-2/neu overexpression [33]. These findings reveal a new mechanism by which HER-2/neu oncoprotein facilitates tumor progression.

Materials and methods

Cell culture and DNA transfections. MBT2 cells (American Type Culture Collection, Manassas, VA) overexpressing HER-2/neu and TCC-SUP cells (American Type Culture Collection, Rockville, MD) with very low HER-2/neu protein expression were grown in Dulbecco's modified Eagle's medium (Gibco-BRL, Grand Island, NY) containing 10% fetal bovine serum. To generate stable TCC-SUP cell lines overexpressing HER-2/neu protein, we cloned wild-type rat HER-2/neu complementary DNA (4.4 kb) into pSV40 vector. The generated cell lines were then co-transfected, using the calcium phosphate method, with a pMam-neo-vector (Clontech, BD Biosciences, Palo Alto, CA), which confers resistance to neomycin. We selected the TCC-N5 and TCC-N10 cell lines as the HER-2/neu-overexpressing cell lines. The TCC-C1 cell line, generated by transfecting a pMam-neo vector into TCC-SUP cells, was a control. We selected all cell lines from medium containing 400 μg/ml of neomycin.

Antibodies and reagents. Commercial sources of the antibodies used were as follows: HER-2/neu (Oncogene Science Inc., Manhasset, NY); Stat1 (N-terminus) (Transduction Laboratories Inc., Lexington, KY); phosphotyrosine-Stat1 (Cell Signaling Inc., Beverley, MA); SHP2, interferon regulator factor-1 (IRF-1), and IFNGR1 (Santa Cruz Biotechnology, Santa Cruz, CA); Jak2 (Upstate Inc., Lake Placid, NY); phosphor (Y1007/1008)-Jak2 (Biosource Inc., Worcester, MA); and β-actin (Chemicon Inc., Pittsburgh, PA). IFN-γ was from Genzyme Diagnostics (Cambridge, MA), AG825-HER-2/neu-specific inhibitor from Biomol Research Laboratories Inc. (Plymouth Meeting, PA), and sodium orthovanadate from Sigma Chemical Co. (St. Louis, MO). All of these were used according to the manufacturers' instructions.

Inhibiting cell growth using IFN-γ. TCC-SUP, -C1, -N5, and -N10 cells were plated in 6-well tissue-culture plates and incubated overnight. IFN-γ (100 ng/ml) was added to the treatment groups the next day. The culture medium of the treatment groups was supplemented with IFN-γ 72 h after plating. Cell viability was assessed using the Trypan blue dye (0.2%) exclusion method [34] 24, 72, and 120 h after plating the cells. We determined the anti-proliferation effects of IFN-γ on TCC-SUP, -C1, -N5, and -N10 cells by counting the total number of viable cells grown with or without 100 ng/ml of IFN-γ at the indicated times.

Western blotting. Fifty micrograms of protein from cell lysates was resolved using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), and was transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA); the membranes were probed with antibodies specific for the proteins of interest. Blots were developed using enhanced chemiluminescence (Amersham, Arlington Heights, IL).

Statistical analysis. Data were analyzed using Prism 4 (GraphPad Software for Science Inc., San Diego, CA). Differences between two independent groups were determined using the Mann–Whitney *U*-test. Statistical significance was set at $p < 0.05$.

Results

HER-2/neu expression increased in TCC-N5 and TCC-N10 cells

To study the interaction between IFN-γ and HER-2/neu overexpression, we created the TCC-C1 cell line from TCC-SUP cells, which were transfected with pMam-neo plasmid containing the neomycin resistance selection marker gene. TCC-N5 and TCC-N10 cell lines were created by co-transfecting pMam-neo plasmid and pSV40-neu plasmid, which contained rat HER-2/neu complementary DNA, into TCC-SUP cells. TCC-N5 and TCC-N10 cells had higher HER-2/neu protein expression (Fig. 1A).

IFN-γ did not inhibit TCC-N5 or TCC-N10 cell proliferation

TCC-SUP, -C1, -N5, and -N10 cells were grown overnight in culture plates, and then IFN-γ (100 ng/ml) or solvent only were added to each treatment group. Using the Trypan blue dye exclusion method, we determined the anti-proliferative effects of IFN-γ on these tumor cells. IFN-γ did not significantly suppress TCC-N5 or TCC-N10 cell growth (Fig. 1B). After 120 h, IFN-γ had time-dependently inhibited TCC-SUP and TCC-C1 cell growth by 63% and 60%, respectively.

HER-2/neu overexpression in TCC-N5 and TCC-N10 cells suppressed IFN-γ-induced phosphorylation of Stat1, Jak2, and downstream signaling

IFN-γ treatment induced tyrosine phosphorylation of Stat1 in TCC-SUP and TCC-C1 cells. Stat1 activation in both cell-types peaked at 30 min (lane 2) and lasted for 24 h (lane 5) (Fig. 1C). In TCC-N5 and TCC-N10 cells, however, phosphorylated Stat1 was barely evident after IFN-γ stimulation (Fig. 1C). In addition, Stat1 protein increased 24 h after IFN-γ stimulation in TCC-SUP and

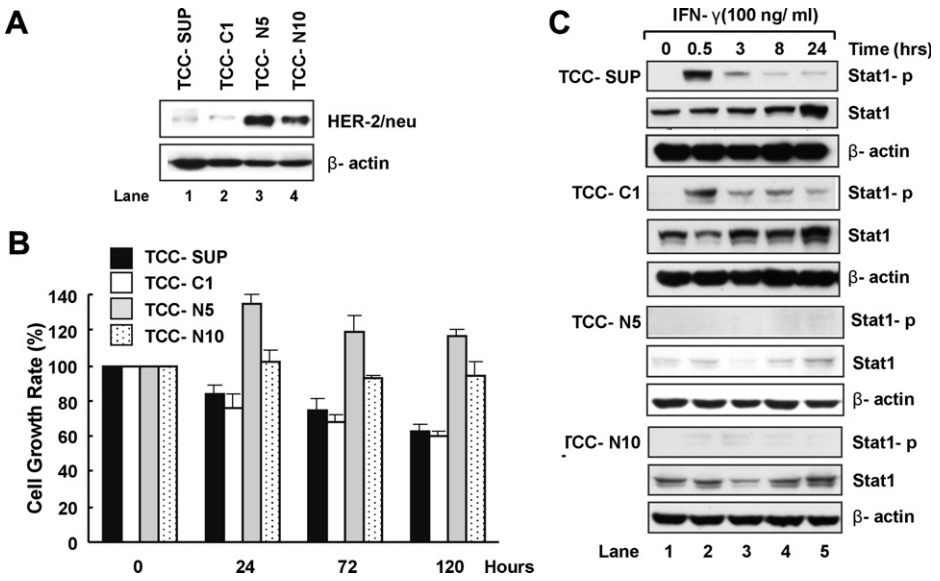

Fig. 1. Western blotting for HER-2/neu expression and treatment responses to IFN-γ. (A) TCC-N5 and TCC-N10 cells had the higher HER-2/neu expression (lanes 3 and 4). (B) IFN-γ did not inhibit growth in TCC-N5 or TCC-N10 cells. (C) Changes in phosphorylated Stat1 protein and the total amount of Stat1 protein in TCC-SUP, -C1, -N5, and -N10 cells after IFN-γ treatment. Tyrosine phosphorylation on Stat1 in TCC-SUP and TCC-C1 cells peaked at 30 min (lane 2) and lasted for 24 h (lane 5). We found few Stat1 phosphotyrosines in TCC-N5 and TCC-N10 cells after IFN-γ treatment, and total Stat1 protein did not markedly increase.

TCC-C1 cells, which might have resulted from an autoregulatory mechanism [35]. Total Stat1 protein in TCC-N5 and TCC-N10 cells remained low and was not induced by IFN-γ treatment (Fig. 1C).

To determine how HER-2/neu overexpression inhibited IFN-γ-induced Stat1 activation, we analyzed IFNGR1 and Jak2, two upstream signaling proteins in TCC-C1, -N5, and -N10 cells. Both proteins had similar expression levels (Fig. 2A). Although Jak2 protein levels were comparable in TCC-C1, -N5, and -N10 cells, IFN-γ activated Jak2 only in TCC-C1 cells (Fig. 2B). We then examined IRF-1, the downstream signal transducer in the IFN-γ/Jak/Stat pathway. IFN-γ induced IRF-1 protein expression in TCC-C1 cells, but not in TCC-N5 or TCC-N10 cells (Fig. 2C).

Twenty-four hours after TCC-N5 and TCC-N10 cells had been treated with AG825, a HER-2/neu-specific inhibitor, the phosphorylated Stat1 became evident (Fig. 3A, lane 4), and AG825 restored Stat1 phosphorylation after IFN-γ treatment (Fig. 3A, lane 5). These results (Figs. 2 and 3A) indicated that HER-2/neu overexpression did not reduce IFNGR1 or Jak2 expression but that it suppressed IFN-γ-induced phosphorylation of Jak2 and Stat1. Conclusively, HER-2/neu overexpression blocked the IFN-γ signaling pathway.

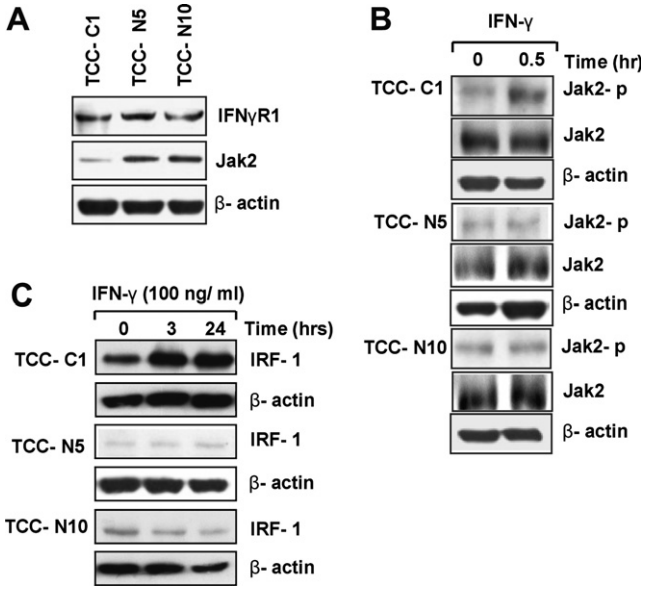

Fig. 2. IFN-γ treatment downregulated the Stat1 signaling pathways in TCC-N5 and TCC-N10 cells. (A) IFN-γR1 and Jak2 levels did not change compared to those in TCC-C1 cells (controls). (B) IFN-γ inhibited Jak2 activation in TCC-N5 and TCC-N10 cells, but not in TCC-C1 cells. (C) In TCC-C1 but not in TCC-N5 and TCC-N10 cells, IFN-γ (100 ng/ml) increased the expression of IRF-1, the downstream signal transducer of Stat1, after 3 and 24 h of treatment.

Upregulated SHP-2 in HER-2/neu-overexpressing TCC-N5, TCC-N10, and MBT2 cells suppressed IFN-γ signaling

To study whether the HER-2/neu overexpression that suppressed IFN-γ signaling is mediated by phosphatase, we added a phosphatase inhibitor to the cell culture medium. Five minutes after treatment with the non-specific phosphatase inhibitor sodium orthovanadate (1 mM), phosphotyrosine-Stat1 was induced in TCC-C1, -N5, and -N10 cells (Fig. 3B), suggesting that phosphatase induction is related to HER-2/neu-mediated suppression of IFN-γ signaling.

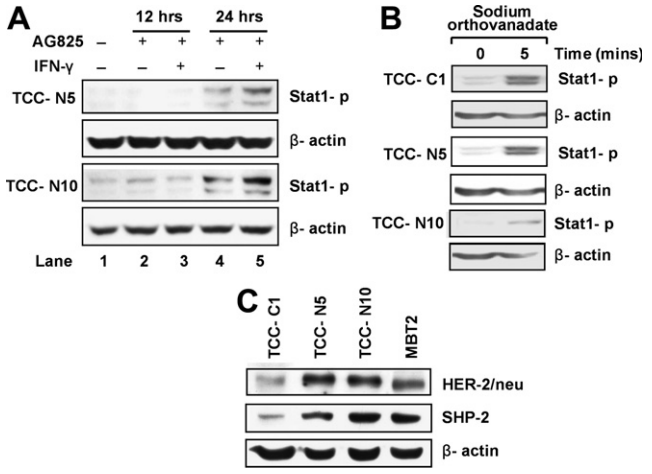

Fig. 3. AG825 and sodium orthovanadate restored Stat1 phosphorylation in TCC-N5 and TCC-N10 cells, and differential baseline SHP-2 levels in TCC-C1, -N5, and -N10 cells and MBT2 cells. (A) AG825 (40 μM) increased Stat1 phosphorylation in TCC-N5 and TCC-N10 cells 24 h after treatment (lane 4). Then IFN-γ (100 ng/ml) treatment for 30 minutes AG825 further increased Stat1 phosphorylation (lane 5). (B) Sodium orthovanadate (1 mM) induced Stat1 phosphorylation in TCC-C1, -N5, and -N10 cells 5 min after treatment. (C) The baseline SHP-2 level was higher in HER-2/neu overexpressing TCC-N5, TCC-N10, and MBT2 cells.

The cellular phosphatases SHP-1 and SHP-2 have been implicated in downregulating the interferon-stimulating Jak/Stat signaling pathway [26,27]. Our system barely detected SHP-1 expression in TCC-C1, -N5, and -N10 and MBT2 cells (data not shown); however, SHP-2 protein was highly expressed in HER-2/neu-overexpressing TCC-N5, TCC-N10, and MBT2 cells (Fig. 3C).

To define the regulatory role of HER-2/neu overexpression in SHP-2 expression, AG825 was added to TCC-N5 and TCC-N10 cells with or without IFN-γ treatment (Fig. 4A). AG825 reactivated Stat1 and IFN-γ treatment increased phospho-Stat1 (lanes 4 and 5). The SHP-2 protein level gradually declined after AG825 treatment, and IFN-γ treatment increased the decline (lanes 4 and 5). Therefore, HER-2/neu regulated the expression of SHP-2 protein in TCC-N5 and TCC-N10 cells and then suppressed IFN-γ signaling. Similar results were found in MBT2 cells (Fig. 4B). IFN-γ also downregulated HER-2/neu expression in AG825-treated MBT2 cells.

AG825 pretreatment restored IFN-γ anti-proliferation activity in TCC-N5 and TCC-N10 cells

Because we found a similar growth-rate inhibition in IFN-γ-treated TCC-SUP and TCC-C1 cells (Fig. 1B), TCC-C1 cells were controls in our study of whether AG825 reverses IFN-γ-induced growth inhibition. TCC-C1, -N5, and -N10 cells were grown for 72 h in medium containing IFN-γ (100 ng/ml), AG825 (40 μM), or both,

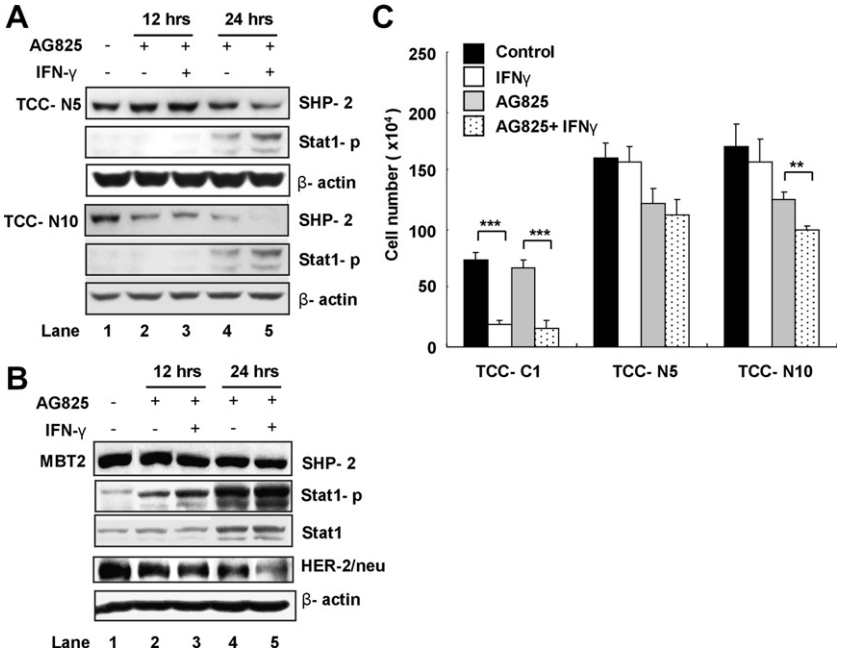

Fig. 4. AG825 downregulated SHP-2 in MBT2 cells and reactivated Stat1 in TCC-N5 and TCC-N10 cells. AG825 inhibited the HER-2 pathway by partially counteracting the IFN-γ-induced growth inhibition. (A) Treatment with AG825 (40 μM) for 12 or 24 h, and then 30 min with or without IFN-γ (100 ng/ml) treatment, downregulated SHP-2 and reactivated Stat1 in TCC-N5 and TCC-N10 cells. IFN-γ augmented the AG825 effect (lanes 4 and 5). (B) MBT2 cells received the same treatment as in (A), which reduced SHP-2 levels, and reactivated Stat1, and also which increased Stat1 protein by autoregulation (as Fig. 1C). IFN-γ also downregulated HER-2/neu expression in AG825-treated MBT2 cells (lanes 4 and 5). (C) TCC-C1, -N5, and -N10 cells (5×10^4) were incubated overnight. One group of each type was then left untreated (Control), one was treated with of IFN-γ (100 ng/ml), one was treated with AG825 (40 μM), and one was co-treated with AG825 and IFN-γ. After 72 h, IFN-γ, with or without AG825 pretreatment, strongly inhibited TCC-C1 cell growth. In TCC-N5 and TCC-N10 cells, IFN-γ alone did not affect cell growth; however, AG825 inhibited cell proliferation. Further, AG825 significantly restored the anti-proliferation effect of IFN-γ only in TCC-N10 cells ($^{**}p < 0.005$, $^{***}p < 0.0005$).

or only solvent. IFN-γ inhibited TCC-C1 (Fig. 4C) cell growth markedly with or without AG825. IFN-γ did not significantly suppress TCC-N5 or TCC-N10 cell growth. AG825 treatment alone reduced the number of viable TCC-N5 and TCC-N10 cells. IFN-γ added to AG825-pretreated TCC-N5 and TCC-N10 cells further reduced the number of viable cells, but only TCC-N10 cells significantly ($p < 0.005$). These data suggested that TCC-N5 and TCC-N10 cells were sensitive to AG825 treatment, and that HER-2/neu inhibitor restored the anti-proliferative activity of IFN-γ in TCC-N5 and TCC-N10 cells.

Discussion

In the present study, we found that HER-2/neu-overexpressing human bladder cancer cells (TCC-N5 and TCC-N10) blocked IFN-γ signaling by inducing SHP-2. Treating TCC-N5 and TCC-N10 cells with AG825 downregulated SHP-2 and reactivated Stat1. Similar results were observed in MBT2 cells with naturally constitutive HER-2/neu overexpression. Finally in TCC-N5 and TCC-N10, AG825 restored the anti-proliferation activity of IFN-γ.

Although IFN-γ has downregulated HER-2/neu expression in prostate and ovarian cancer cells [22,23] and MBT2 cells (Fig. 4B), we found no such downregulation in TCC-N5 and TCC-N10 bladder cancer cells (data not shown). The reason is likely that the HER-2/neu gene in the transfected plasmid is driven by SV40 instead of the native promoter. Because of the consistently high expression of HER-2/neu in TCC-N5 and TCC-N10 cells, the model was appropriate for studying the influences of HER-2/neu on the IFN-γ signaling pathway.

HER-2/neu abolished IFN-γ downstream signaling in TCC-N5 and TCC-N10 cells not by downregulating IFN-γR1 and Jak2 protein, but by blocking the phosphorylation of Stat1 and Jak2. That IFN-γ did not induce IRF-1 in TCC-N5 and TCC-N10 cells confirmed that Jak/Stat signaling was blocked, because IRF-1 is a major downstream product of Stat1 in mediating the anti-proliferative activity of IFN-γ [36]. Stat1 expression in cells is autoregulated by Stat1 signaling [35]; therefore, Stat1 protein levels in TCC-N5 and TCC-N10 cells were low and not induced by IFN-γ, in contrast to the normal expression in TCC-SUP and TCC-C1 cells (Fig. 1C, lane 5). These findings further supported that Stat1 downstream signaling was blocked in TCC-N5 and TCC-N10 but not in TCC-SUP and TCC-C1 cells.

In TCC-N5 and TCC-N10 cells, sodium orthovanadate, a phosphatase inhibitor, reactivated Stat1. Therefore, we suspected that the inhibition of Stat1 activation in these cells might be caused by cellular phosphatases. We then found that SHP-2 expression was upregulated by HER-2/neu overexpression and downregulated by HER-2/neu inhibitor-AG825 in the two cell lines. In addition, MBT-2 cells show high similarity to human bladder cancer biologically and they overexpress HER-2/neu constitutively [33]. So AG825 treatment increased Stat1 phosphorylation and decreased SHP-2 expression in MBT2 cells. These results were observed both in artificial and natural HER-2/neu overexpression systems, which support our hypothesis.

SHP-2 upregulated MAPK and PI3K/Akt activation through growth factors and cytokines [28,37,38], which led to cell growth. Our findings suggested that SHP-2 is induced by HER-2/neu receptors and suppresses IFN-γ signaling, which facilitates cell proliferation. Therefore, HER-2/neu-induced upregulation of SHP-2 increases cell proliferation and prevents cell death by activating MAPK and PI3K.

Although it is well known that HER-2/neu promotes cell proliferation by generating several mitogenic and transforming signals [4], little is known about how HER-2/neu suppresses MHC class I molecule expression; because the IFN-γ signaling pathway regulates this expression [39,40], SHP-2 may mediate the suppressive effect of HER-2/neu. This assumption requires verification, however.

The findings that SHP-2 is a downstream molecule of the HER-2/neu oncoprotein and that it may mediate the growth-promoting and immune-evading activities of HER-2/neu are novel. This knowledge provides new concepts for targeting HER-2/neu carcinogenic pathways.

Acknowledgments

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