Constitutive activation of signal transducer and activator of transcription 3 regulates expression of vascular endothelial growth factor in human meningioma differentiation

Mao Xiu Zhang · Xu Zhao · Zhi Gang Wang · Wei Ming Zhao · Yun Shan Wang

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Abstract
Purpose Janus tyrosine kinases (JAKs) and signal transducer and activator of transcription factors (STATs), especially STAT3, are constitutively activated in human cancers. The function of STAT3 in the pathogenesis of meningioma remains unknown. In this study, we investigated the role of JAK1/STAT3 regulating vascular endothelial growth factor (VEGF) expression in the occurrence and progression of human meningioma.
Methods We detected the expression of JAK1, p-JAK1, STAT3, p-STAT3, and VEGF in human meningioma and normal dura tissues by RT–PCR, Western blot analysis, and immunohistochemistry.
Results JAK1, p-JAK1, STAT3, p-STAT3, and VEGF showed high expression in grade I and grade II meningioma. The level of STAT3 activation was associated with VEGF expression; all meningioma tumors that expressed p-STAT3 also expressed VEGF. Both frequency of positivity and expression were enhanced with increasing tumor grade; high frequencies and levels were found in grade II tumors, with no expression detected in normal dura tissues (P < 0.05).
Conclusions VEGF is directly regulated by constitutive STAT3 activity and associated with meningioma differentiation. STAT3 has an important role in the occurrence and development of human meningioma by regulating VEGF expression.

Keywords Constitutive activation · Human meningioma · p-STAT3 · STAT3 · VEGF

Abbreviations
JAK1 Janus tyrosine kinase 1
p-JAK1 Phosphorylated Janus tyrosine kinase 1
p-STAT3 Phosphorylated signal transducer and activator of transcription 3
STAT3 Signal transducer and activator of transcription 3
VEGF Vascular endothelial growth factor

Introduction
Constitutive activation of the Janus tyrosine kinase/signal transducer and activator of transcription (JAK/STAT) signal pathway is frequently detected in human cancers and contributes to oncogenesis. JAK1 and STAT3 are activated in response to interleukin-6 in human fibrosarcoma cells (Guschin et al. 1995), and the levels are consistently higher in low-differentiated gliomas than in high-differentiated...
gliomas (Cattaneo et al. 1998). Dephosphorylated JAK1 and STAT3 reduce the expression of STAT3-regulated VEGF in various human tumor cell lines (Haridas et al. 2009). As well, the JAK1/STAT3 signal pathway plays a role in cell transformation and carcinogenesis (Coppo et al. 2009; Tam et al. 2007). STAT3 is a member of the STAT family of STAT1-4, STAT5a, STAT5b, and STAT6, which mediates cell survival, growth, and differentiation in many cancerous cell lines and human tumors (Bowman et al. 2000; Rubin Grandis et al. 2000). Constitutively, STAT3 in tumor cells promote tumor invasion and angiogenesis by regulating the expression of target genes and modulates fundamental cellular processes, such as proliferation and differentiation.

Tumor angiogenesis is required for tumor growth and development stimulated by angiogenic inducers. VEGF, originally isolated from tumor cells, is one of the major inducers of tumor angiogenesis and regulates vascular endothelial proliferation (Ferrara et al. 2003). STAT3alpha might play a central role in activation of autocrine VEGF in gliomas (Schaefer et al. 2002). Colon cancer cells showed inhibited angiogenesis and metastasis with blocked STAT3 and VEGF expression (Kim et al. 2008). As an intracellular regulator, STAT3 is involved in tumor vascular remodeling and tumor development by regulating VEGF expression. Most brain tumors overexpress VEGF, which leads to an abnormally permeable tumor vasculature. As a solid tumor, meningioma depends on neovascularization by angiogenesis for expansion. However, the function of the JAK1/STAT3 signal pathway through mediating VEGF expression remains unknown in the pathogenesis of human meningioma.

In this study, we explored whether VEGF expression regulated by the constitutive activation of the JAK1/STAT3 signal pathway might be involved in the occurrence and development of human meningioma.

Materials and methods

Reagents

Trizol reagent was from Sigma; the first-strand cDNA synthesis kit was from Fermentas; the human primers for JAK1 (accession number NC_000001.10), STAT3 (accession number NC_000017.10), VEGFa (accession number NC_000006.11), and Beta-actin (accession number NC_000007.13) were designed with use of sequence information in GenBank and synthesized by Invitrogen; JAK1, p-JAK1 (Tyr1022/1023), p-STAT3 (Tyr705), VEGF, and Beta-actin primary antibodies were from Santa Cruz Biotechnology; STAT3 primary antibody was from Cell Signaling Technology; horseradish peroxidase-conjugated (HRP) anti-rabbit/mouse IgG secondary antibodies were from Santa Cruz Biotechnology; the DAB kit was from Zymed; the lysis buffer was from Promega; and the enhanced chemiluminescence (ECL) kit was from Amersham.

Clinical materials

We obtained 40 meningioma specimens (26 from males; donor mean age 49.0 ± 13.0 years, range 17–72 years) from the Department of Neurosurgery, the Second Hospital of Shandong University, China. None of the patients had undergone any preoperative treatments, such as chemotherapy or radiotherapy; histological differentiation was classified according to the 2000 World Health Organization classification. As much as 30 cases were grade I meningioma (well differentiated), and 10 were grade II (moderately differentiated). We obtained 6 normal dura samples from patients with cerebral trauma. The protocol was approved by the ethics committee of the Second Hospital of Shandong University, China. The patients gave their informed consent for use of samples.

One half of each sample was immediately frozen in liquid nitrogen for RT–PCR and Western blot analysis, and the other half was fixed in 15% formalin for histological typing and immunohistochemistry.

RT–PCR

Meningioma and normal samples were homogenized with 1 ml Trizol reagent; total RNA was isolated according to the manufacturer’s instructions. RNA concentration and integrity were evaluated by measuring absorbance at 260 and 280 nm, and cDNA was prepared according to the manufacturer’s instructions for the RT–PCR kit. The sequence of primers was as follows: JAK1, sense 5’-AGTG C CCTGA GACTACTTTGGA-3’ and antisense 5’-AGGTC ACGCTCCTTACA-3’ (371 bp); STAT3, sense 5’-AACT CTTGGGACCTGGTGTG-3’ and antisense 5’-CGGACTG GATCTGGGTCTTA-3’ (371 bp); VEGFa, sense 5’-CTGC TGTCTTGGGTGCAATTG-3’ and antisense 5’-CTCATG TTGTGTTGCTGTAG-3’ (378 bp); Beta-actin, sense 5’-GTGGGGCGCAGCCACGCA-3’ and antisense 5’-CTCCTTAA TGTCA CGACAGATTTC-3’ (539 bp). A 20-μl volume reaction for PCR included 1.5 μl cDNA and 1.0 unit Taq polymerase. PCR reaction involved denaturation at 94°C for 2 min, then annealing for 32 cycles at 94°C, 55°C, and 72°C for 1 min each, with extension at 72°C for 2 min. The PCR products underwent electrophoresis on a 1% agarose gel; gene expression was normalized to that of Beta-actin by 1D Image analysis software (Eastman Kodak).
Western blot analysis

After being weighed, tumor and normal dura tissues were homogenized with liquid nitrogen, and lysis buffer was added to 200 µl; protein concentration was evaluated by measuring absorbance at 562 nm. Proteins were separated on 10% SDS–PAGE, then electroblotted on PVDF membrane; the membranes were blocked with 10% non-fat dry milk in TBS-T overnight at 4°C, then incubated with primary antibodies against JAK1, p-JAK1, STAT3, p-STAT3, VEGF, and Beta-actin (each dilution 1:1,000) for 1.5 h at room temperature. After a wash with TBS-T, membranes were incubated with HRP-conjugated secondary antibodies (dilution 1:2,000) for 1 h at room temperature. Immunoreactivity was detected by use of an ECL kit for 2–5 min according to the manufacturer’s instructions. Protein expression was normalized to that of Beta-actin by 1D Image analysis software.

Immunohistochemistry

Normal dura and tumor tissue sections (5 µm thick) were incubated at 60°C for 45 min and immediately deparaffi- nized in xylene (5 min × 2), then gradient ethanol (5 min each in 95%, 90%, 80%, 70%). Endogenous peroxidase activity was blocked with 3% H2O2 in methanol for 10 min; sections were washed with phosphate buffered saline (PBS) and treated with antigen retrieval buffer in a microwave oven to unmask the antigens for 10 min. After a PBS wash, sections were blocked with diluted normal goat serum for 20 min, then primary antibodies against JAK1, p-JAK1, STAT3, p-STAT3, and VEGF (each dilution 1:100) overnight at 4°C. After a PBS wash, the appropriate secondary antibodies were added for 30 min at 37°C, then sections were washed with PBS and underwent DAB coloration for 3–5 min. The primary antibodies were substituted with PBS for negative control tissue slides for each experiment. The tissue sections were examined by two independent investigators. Protein expression was classified as follows (Preusser et al. 2005): (1) cytoplasmic staining: with 0–15% positive tumor cells was negative; <50% was low expression, ≥50% was high expression; or nuclear staining: with no positive tumor cells was negative; <10% was low expression, ≥10% was high expression.

Statistical analysis

SPSS v11.0 software (SPSS Inc., Chicago, IL) was used for statistical analysis. Expression of genes was analyzed by independent-sample Student t test. The frequency of positivity of genes in different tissues at the mRNA and protein levels were tested by Fisher’s exact test. Spearman analysis was used to assess the correlation between gene expression and meningioma differentiation. A P < 0.05 was considered statistically significant.

Results

mRNA expression of JAK1, STAT3, and VEGFα upregulated in meningioma

Among 30 grade I meningioma cases, JAK1 expression was detected in 11; STAT3 expression was detected in all JAK1-positive cases, and VEGFα expression was detected in all cases positive for both JAK1 and STAT3 and 1 case negative for both JAK1 and STAT3. JAK1 and STAT3 were detected at the same time in 8 out of the 10 grade II cases; VEGFα expression was found in 9 out of 10 grade II cases, including 8 cases positive for both JAK1 and STAT3. The frequencies of positivity and level of genes were high in grade I and II cases; no expression was found in normal dura tissues; high levels were found in grade II tissues (Table 1, Figs. 1, 2; P < 0.05).

Correlation between meningioma differentiation and frequency of positivity and expression of genes by RT–PCR

The frequency of positivity and expression of genes were correlated with meningioma differentiation status (Table 1, Figs. 1, 2). The relative expression of JAK1 was strongly correlated with that of STAT3, the expression of STAT3 was also strongly correlated with that of VEGFα (Table 2).

Table 1: mRNA frequency of JAK1, STAT3, and VEGFα expression in normal dura and human meningioma tissue

|         | Normal dura (n = 6) Positive | Grade I tumor (n = 30) Positive | Grade II tumor (n = 10) Positive | r     | P*      |
|---------|-------------------------------|---------------------------------|---------------------------------|-------|---------|
| JAK1    | 0                             | 11 (36.7%)                      | 8 (80.0%)                       | 0.376 | 0.017   |
| STAT3   | 0                             | 11 (36.7%)                      | 8 (80.0%)                       | 0.376 | 0.017   |
| VEGFα   | 0                             | 12 (40.0%)                      | 9 (90.0%)                       | 0.434 | 0.005   |

# P < 0.05, compared with normal dura tissue or grade I tumor. The inter-class correlation coefficients assess the correlations between frequency of positivity and tumor differentiation status. * P < 0.05 was considered statistically significant.
Constitutive activation of STAT3 upregulated VEGF protein expression in meningioma

Among grade I meningioma cases, JAK1 and STAT3 expression were found in the same 11 out of 30 cases; VEGF was highly expressed in all cases expressing both JAK1 and STAT3; p-JAK1 and p-STAT3 were detected in 8 cases expressing both JAK1 and STAT3. The ratio of p-JAK1 to p-STAT3 was 72.7% (8/11). Among grade II meningioma cases, JAK1, STAT3, and VEGF were detected in the same 8 out of 10 cases; p-JAK1 and p-STAT3 expression were detected in the same 7 cases expressing JAK1, STAT3, and VEGF. The ratio of p-JAK1 to p-STAT3 was 87.5% (7/8). The relative frequency of positivity and expression of p-JAK1 and p-STAT3 were positively correlated with histological status of meningioma; high levels were found in grade II tumors (Tables 3, 4, Figs. 2, 3).

Correlation analysis of JAK1 versus STAT3, STAT3 versus VEGF, p-JAK1 versus p-STAT3, p-JAK1 versus
Table 2 Correlation analysis of JAK1 or STAT3 and VEGF expression normalized to that of Beta-actin by RT–PCR

|                      | Grade I tumor (n = 30) | Grade II tumor (n = 10) |
|----------------------|------------------------|-------------------------|
|                      | r  | P  | r  | P  |
| JAK1/STAT3           | 0.944 | 0.000 | 0.741 | 0.014 |
| STAT3/VEGFa          | 0.855 | 0.000 | 0.780 | 0.008 |

Table 3 Frequency of positivity of JAK1, p-JAK1, STAT3, p-STAT3, and VEGF in normal dura and meningioma tissue on Western blot analysis

|                      | Normal dura tissue (n = 6) | Grade I tissue (n = 30) | Grade II tissue (n = 10) | r  | P  |
|----------------------|---------------------------|------------------------|--------------------------|----|----|
| JAK1                 | Positive                  | 11 (36.7%)             | 8 (80%)                  | 0.376 | 0.017 |
| p-JAK1               | Positive                  | 8 (26.7%)              | 7 (70%)                  | 0.388 | 0.013 |
| STAT3                | Positive                  | 11 (36.7%)             | 8 (80%)                  | 0.376 | 0.017 |
| p-STAT3              | Positive                  | 8 (26.7%)              | 7 (70%)                  | 0.388 | 0.013 |
| VEGF                 | Positive                  | 11 (36.7%)             | 8 (80%)                  | 0.376 | 0.017 |

# P < 0.05 compared with normal dura tissue or grade I meningioma tissue. The inter-class correlation coefficients assess the correlations between frequency of positivity and tumor differentiation status. * P < 0.05 was considered statistically significant.

Table 4 Correlation analysis of JAK1 or STAT3 activation and VEGF expression normalized to that of Beta-actin by Western blot analysis

|                      | Grade I tumor (n = 30) | Grade II tumor (n = 10) |
|----------------------|------------------------|-------------------------|
|                      | r  | P  | r  | P  |
| JAK1/STAT3           | 0.821 | 0.000 | 0.655 | 0.040 |
| p-JAK1/p-STAT3       | 0.951 | 0.000 | 0.653 | 0.041 |
| STAT3/VEGF           | 0.832 | 0.000 | 0.654 | 0.040 |
| p-JAK1/VEGF          | 0.767 | 0.000 | 0.876 | 0.001 |
| p-STAT3/VEGF         | 0.794 | 0.000 | 0.914 | 0.000 |

Discussion

The JAK/STAT signal pathway has been reported to be involved in the oncogenesis of human cancers. STATs are selectively activated mainly by activated JAKs (including JAK1, JAK2, JAK3, and TYK2), which leads to STAT protein activation, nuclear translocation, and regulation of target gene expression. The function of the JAK1/STAT3 signal pathway is best explained in SCID mice models: JAK1−/− and STAT3−/− mice die during embryogenesis because of impaired neurological and lymphoid development (Rodic et al. 1998; Takeda et al. 1997; Akira 1999). The JAK1/STAT3 signal pathway plays a critical role in cell transformation and carcinogenesis (Coppo et al. 2006; Tian et al. 1994; Tam et al. 2007). p-STAT3 is dysregulated in various human tumors, including head and neck squamous cell cancer (Rubin Grandis et al. 2000), leukemia (Carlesso et al. 1996), multiple myeloma (Bharti et al. 2003), and lymphomas (Weber-Nordt et al. 1996). Abnormal STAT3 activity induces permanent changes in gene expression that ultimately lead to a malignant tumor phenotype. Constitutive activation of STAT3 is associated with growth stimulation and anti-apoptotic effects in malignant disease.

In our study, we found JAK1 was significantly correlated with STAT3, and they were both over-expressed in meningioma, with the highest levels in grade II tumors; no expression was found in normal dura tissues. The expression of JAK1 and STAT3 was associated with tumor differentiation status (Table 5, Fig. 4; P < 0.05). Other studies have shown JAK1 and STAT3 markedly down-regulated in the normal adult rat brain (De-Fraja et al. 2000; Gautron et al. 2006). However, JAK and STAT families of proteins are highly expressed and are important effectors in brain tumors (Magrassi et al. 1999). Our data showed varied expression of total JAK1 and STAT3 by tumor differentiation status, which suggests that the expression pattern of the JAK1/STAT3 signal pathway is associated with the development of meningioma.

VEGF, and p-STAT3 versus VEGF in grade I and grade II tumors showed strong correlation (Table 4). All meningioma tumors positive for p-STAT3 also showed VEGF expression. VEGF expression was also correlated with meningioma differentiation (Table 3; P < 0.05).

Constitutive activation of STAT3 upregulates VEGF protein expression associated with human meningioma differentiation.

Results of frequency of positivity seen on immunohistochemistry were in agreement with those seen on Western blot analysis (Table 3). Among grade I tumors, 9 cases expressed low levels of JAK1 and STAT3 and 2 high levels; low expression was found in all p-JAK1- and p-STAT3-positive cases (8/8); 9 cases expressed a low level of VEGF and 2 a high level. Among grade II cases, both JAK1 and STAT3 were highly detected in 7, with low expression in 1 case; high levels of p-JAK1 and p-STAT3 were found in 6 cases, and low levels in 1 case; a high level of VEGF was found in all VEGF-positive cases (8/8). Relative frequency of positivity and levels of p-JAK1 and p-STAT3 were higher in grade II than in grade I tumors. Tumor tissues expressing p-STAT3 also expressed VEGF; the high levels were found in grade II tumors. Co-expression of p-STAT3 and VEGF was significantly correlated with meningioma differentiation (Tables 3, 5, Fig. 4; P < 0.05).
Tyrosine phosphorylation is crucial for the function of STAT proteins regulated by the receptor-driven JAK catalytic activity (Lee et al. 1997; Turkson and Jove 2000). Tyrosine phosphorylation of STAT3 (p-STAT3) is a critical step for translocation to the nucleus and regulation of the expression of target genes. We found that the expression of p-JAK1 correlated with that of p-STAT3 in meningioma on Western blot analysis and immunohistochemistry; the relative expression differed by meningioma grade, with the high level in grade II tumors. The relative frequency of positivity and level of p-JAK1 and p-STAT3 were positively correlated with histological differentiation of meningioma (Tables 3, 5, Fig. 4; \( P < 0.05 \)), which confirms that the activated JAK1/STAT3 signal pathway is associated with progression of human meningioma. A high level of p-STAT3 reduces patient survival in hormone-refractory prostate cancer, which suggests that activation of the JAK1/STAT3 signal pathway is involved in development of this disease.

### Table 5

| Gene          | Grade I tumor | Grade II tumor | \( r \) | \( P \) |
|---------------|---------------|----------------|--------|-------|
|               | Low expression | High expression | Low expression | High expression |
| JAK1          | 9             | 2              | 1       | 7     | 0.685 | 0.001 |
| p-JAK1        | 8             | 0              | 1       | 6     | 0.873 | 0.000 |
| STAT3         | 9             | 2              | 1       | 7     | 0.685 | 0.001 |
| p-STAT3       | 8             | 0              | 1       | 6     | 0.873 | 0.000 |
| VEGF          | 9             | 2              | 0       | 8     | 0.809 | 0.000 |

Fig. 3 Western blot analysis of protein expression of JAK1, p-JAK1, STAT3, p-STAT3, and VEGF. a Protein level of JAK1, p-JAK1, STAT3, p-STAT3, and VEGF in normal dura and tumor tissue. Beta-actin was a loading control. b Relative protein expression of JAK1, p-JAK1, STAT3, p-STAT3, and VEGF normalized to that of Beta-actin. \( *P < 0.05 \) compared with normal dura tissues or grade I tumor.

Fig. 4 Immunohistochemistry of meningioma tissues (normal dura tissues not shown): a, c, e, g, i Negative control of JAK1, p-JAK1, STAT3, p-STAT3, and VEGF, respectively, in meningioma tissue. Positive protein expression of b JAK1 mainly in cytoplasm, d p-JAK1 mainly in cytoplasm, f STAT3 mainly in cytoplasm, h p-STAT3 mainly in nucleus, and j VEGF mainly in cytoplasm. Original magnification 600×.
cancer (Tam et al. 2007). So constitutive activation of JAK1/STAT3 signal pathway can be considered as an oncogenic event in the occurrence and development of tumors (Knoops et al. 2008; Zhang et al. 2000).

Protein phosphorylation has an important role in cellular processes because it regulates the functional activities of signal proteins. Activated by JAKs, phosphorylated STATs translocate from the cytoplasm to nucleus, where they regulate transcription and protein expression of target genes. Downstream targets of STAT3, such as bcl-xl, cyclin D1, and VEGF, are important in preventing apoptosis, enhancing invasion, and promoting metastasis and angiogenesis (Haridas et al. 2009; Catlett-Falcone et al. 1999; Schaefer et al. 2002; Mahboubi et al. 2001). Constitutive activation of STAT3 induces changes that lead to initiation and/or maintenance of oncogenesis. As a significant predictive factor of prognosis in patients with solid tumors, angiogenesis is essential for tumor growth, progression, and metastasis (Folkman 2001; Ohta et al. 1996). VEGF is considered as a major regulator of angiogenesis in various brain tumors (Berkman et al. 1993; Huang et al. 2005). As a solid tumor, meningioma depends on neovascularization through angiogenesis for expansion. We found high mRNA and protein levels of VEGF associated with meningioma grade. Tumor tissues expressing p-STAT3 also expressed VEGF, and p-STAT3 was significantly correlated with VEGF expression (Tables 3, 4, 5 and Figs. 2, 3, 4; P < 0.05). STAT3 can directly bind the VEGF promoter to upregulate VEGF expression in many tumors (Niu et al. 2002; Wei et al. 2003). p-STAT3 induces overexpression of VEGF, which is decreased during angiogenesis by inhibition with antagonists of VEGF–VEGFR signaling (Chen et al. 2008). So p-STAT3 is involved in angiogenesis by mediating VEGF expression, which agrees with other researches (Schaefer et al. 2002; Loeffler et al. 2005; Wang et al. 2004). The production and action of VEGF regulation by activated STAT3 is necessary in the occurrence and development of human meningioma.

**Conclusion**

VEGF expression is significantly correlated with STAT3 activity, and co-expression of p-STAT3 and VEGF is associated with meningioma differentiation status. STAT3 is the point of convergence for the meningioma angiogenic event as the JAK1/STAT3 signal pathway mediates VEGF expression. So STAT3 has an important role in the occurrence and development of human meningioma.

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**Conflict of interest statement** The authors declare that no any financial and personal relationships with other people or organisations that can inappropriately influence the work, they have full control of all primary data and agree to allow the journal to review if requested.

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