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# Sp1 transcriptionally regulates *BRK1* expression in non-small cell lung cancer cells

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ABSTRACT

Following a previous study reporting that *BRK1* is upregulated in non-small cell lung cancer (NSCLC), the present study sought to clarify the role of specificity protein 1 (Sp1) in the transcriptional regulation of the *BRK1* gene. Therefore, a construct, named F8, consisting of the −1341 to −1 nt sequence upstream of the start codon of the *BRK1* gene inserted into pGL4.26 was made. A series of truncated fragments was then constructed based on F8. Segment S831, which contained the −84 to −1 nt region, displayed the highest transcriptional activity in the A549, H1299 and H520 NSCLC cell lines. Bioinformatic analysis showed a potential Sp1-binding element at −73 to −64 nt, and a mutation in this region suppressed the transcriptional activity of S831. Then the RNAi assays of Sp1 and its coworkers Sp3 and Sp4 were performed, and suppression of Sp1 by siRNA inhibited the mRNA expression of *BRK1*. Both an electrophoretic mobility shift assay (EMSA) and a chromatin immunoprecipitation (ChIP) assay demonstrated that Sp1 bound to the promoter area of the *BRK1* gene. Our data identified a functional and positive Sp1 regulatory element from −73 to −64 nt in the *BRK1* promoter, which may likely explain the overexpression of *BRK1* in NSCLC.



## 1. Introduction

BRK1 protein, also known as BRICK1, C3orf10 and HSPC300, is a subunit of the WAVE/SCAR complex, which activates the Arp2/3 complex. BRK1 combines with WAVE/SCAR, PIR121/Sra-1, Nap125 and Abi subunits to form the WAVE/SCAR complex (Gautreau et al., 2004), a consensus structure in plants and animals. When activated by the Rac1 signaling pathway, the WAVE complex releases active WAVE-BRK1, leading to the assembly of actin filaments (Eden et al., 2002; Stradal and Scita, 2006). Although little is known about the mechanism of BRK1 function, it plays a key role in cellular processes that rely on actin filaments, such as cell attachment, stretching, endocytosis, division and mobility.

Cell migration is an essential step in the metastasis of various tumor types and is mediated by the dynamic polymerization of actin filaments (Vasioukhin et al., 2000). In our previous study on NSCLC, gene expression profile data showed that *BRK1* is more highly expressed in tumor tissues than in adjacent normal tissues (Liu et al., 2007; Sun et al., 2004). Immunohistochemistry (IHC) performed on lung squamous cell carcinomas showed that the high level of BRK1 in tumor tissue is associated with lymph node metastasis, pathological grade and poor differentiation (Cai et al., 2009). Silencing of the *BRK1* gene in a NSCLC cell line causes the reorganization of actin filaments, inhibits the formation of pseudopodia and blocks the migration of cells (Cai et al., 2009). The association between *BRK1* expression and NSCLC metastasis prompted us to investigate the exact mechanism of *BRK1* upregulation.

Specificity protein 1 (Sp1) is a transcription factor that is ubiquitously expressed in various cells and tissues. Sp1 recognizes GC-rich regions and binds to DNA through three $C_2H_2$-type zinc fingers in the C-terminal domain (Kadonaga et al., 1988; Suske, 1999; Philipsen and Suske, 1999). Each zinc finger of Sp1 recognizes three bases in one strand and a single base in the complementary strand, constituting a consensus binding sequence of 5′-(G/T)GGGCGG(G/A)(G/A)(C/T)-3′ (Narayan et al., 1997; Pavletich and Pabo, 1991). Sp protein family regulates the expression of genes required for cell growth, apoptosis and angiogenesis in cancer (Black et al., 2001; Bouwman and Philipsen, 2002; Safe and Abdelrahim, 2005). The relative expression of Sp1 in cancer cells has been shown to be higher than that of adjacent normal cells in several tumor models, including gastric tumors, breast cancers thyroid tumors and lung cancers (Chiefari et al., 2002; Colon et al., 2011; Wang et al., 2003; Zannetti et al., 2000). Sixty-five percent of lung cancer patients have been shown to have a higher level of Sp1 in tumor tissues (Lin et al., 2010). Additionally, Sp3 and Sp4, two other members of the Sp family, combine with Sp1 to regulate several genes, such as *VEGF*, *VEGFR1*, *EGFR*, *PTTG1* and *c-MET* (Abdelrahim et al., 2004; Abdelrahim et al., 2007; Chintharlapalli et al., 2011; Colon et al., 2011; Pathi et al., 2011), in multiple cancer cell lines.

*Abbreviations:* NSCLC, non-small cell lung cancer; EMSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation; IHC, immunohistochemistry; RLU, relative luciferase units; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; ECL, enhanced chemiluminescence.

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In this study, we found an Sp1-binding site in the promoter region of the *BRK1* gene. Both silencing the Sp1 gene and mutating the binding site downregulated *BRK1* transcription. An EMSA and a ChIP assay directly confirmed the association between the Sp1 protein and sequence upstream of the *BRK1* gene. To the best of our knowledge, this is the first report to describe an association between the Sp1 protein and the high level of *BRK1* expression in NSCLC cell lines.

## 2. Materials and methods

### 2.1. Cell culture

The A549, H1299 and H520 NSCLC cell lines (American Type Culture Collection, Manassas, VA, USA) were used in this study. All cells were maintained in RPMI 1640 medium with 10% fetal calf serum at 37 °C in a 5% $CO_2$ atmosphere. Medium for the H1299 cells was supplemented with 1 mM HEPES, 1 mM glucose, and 1 mM sodium pyruvate.

### 2.2. Generation of BRK1 promoter-luciferase constructs

The −1341 to −1 nt sequence upstream of the ATG start codon of the *BRK1* gene was cloned and inserted into pGL4.26 to generate the luciferase-reporter construct named F8. A series of truncated fragments was then constructed from F8. Human male genomic DNA (Novagen, Schwalbach, Germany) was used as the template for PCR amplifications using Phusion DNA polymerase (Promega, Madison, WI, USA). The PCR primers used in this study are listed in Table 1. KpnI (GAGGTACC) and XhoI (GTCGACTCGAG) restriction sites were added to the 5′ end of the forward and reverse primers, respectively. The PCR was performed as follows: 94 °C for 5 min; 30 cycles of 94 °C for 30 s (denaturation), 60 °C for 30 s (annealing), and 72 °C for 90 s (extension); and an elongation step at 72 °C for 10 min. The amplified PCR products were purified with a gel extraction kit (Qiagen, Hilden, Germany) followed by digestion with KpnI and XhoI. The digested DNA fragments were inserted into pGL4.26, a firefly luciferase expression vector (Promega, USA), and constructs were confirmed by DNA sequencing.

### 2.3. Transient transfection and dual-luciferase assay

On the day before transfection, cells were plated in 96-well plates at a density of 10,000 cells/well for A549, 5000 cells/well for H1299, and 7500 cells/well for H520. When the cell culture reached 70% confluence on the next day, the growth medium was replaced with fresh medium. The cells were then transfected using 15 μl Vigofect (Vigorous Biotechnology, Beijing, China), according to the manufacturer's instructions, with 50 ng of the pRL-TK vector and 500 ng of the pGL constructs. Twenty-four hours later, the cells were harvested with passive lysis buffer and frozen at −80 °C for 2 h. Dual-luciferase activities were measured using a GENios Pro Reader (Tecan, Männedorf, Switzerland) according to the manufacturer's protocol (Promega). The promoter activity was presented as relative luciferase units (RLU) as follows: RLU = value of firefly luciferase unit / value of renilla luciferase unit (Guo et al., 2010). All experiments were performed at least three times, and the average RLU of triplicate treatments is shown for each experiment.

**Table 1**
Primers used to generate *BRK1* promoter-luciferase constructs.

| Name | Sequence | Products |
|---|---|---|
| F8 | Forward ATAGCCAGGTGTGGTAG<br>Reverse GGCCGCCGCCTGAGG | −1341 to −1 nt |
| F81 | Forward ATTAGCCAGGTGTGGTGACGTGAGC<br>Reverse GGCCGCCGCCTGAGG | −911 to −1 nt |
| F82 | Forward TAAAAATACAAAAAAATTAGCCGGG<br>Reverse GGCCGCCGCCTGAGG | −654 to −1 nt |
| F83 | Forward GCCTGGAGCAGTTGAGGGAGACGGC<br>Reverse GGCCGCCGCCTGAGG | −277 to −1 nt |
| S831 | Forward ATGGGTGTGGCCTGGCAGCGCAGGC<br>Reverse GGCCGCCGCCTGAGG | −84 to −1 nt |
| S832 | Forward ACGCCGGCGAGGACGTGACGTTGC<br>Reverse GCCTGCGCTGCCAGGCCACACCCAT | −152 to −60 nt |
| S833 | Forward GCCTGGAGCAGTTGAGGGAGACGGC<br>Reverse GCAACGTCACGTCCTCGCCGGCGT | −277 to −128 nt |

### 2.4. Mutagenesis analysis of the Sp1-binding site

Using the protocol of the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA), two mutations, Sp1-mut1 and Sp1-mut2, were generated on the predictive Sp1-binding site of the S831 construct. Double-stranded mutation PCR primers, TGGGTGTGGCTTTTTAGCGCAGG CG and TGTGGCCTGGCTTTTTAGGCGCA, were used to replace the CTGGC and AGCGC sequences, respectively, with TTTTT. The mutated constructs were confirmed by DNA sequencing.

### 2.5. RNA interference (siRNA)

ON-TARGET plus SMARTpool Sp1, Sp3 and Sp4 siRNA (Thermo Scientific, Lafayette, CO, USA) were used for gene silencing, and each contained a mixture of 4 siRNAs: GCCAAUAGCUACUCAACUA, GAAG GGAGGCCCAGGUGUA, GGGCAGACCUUUACAACUC and CUACAGAGGC ACAAACGUA for Sp1; GGUAUUCACUCUAGCAGUA, GAAAUUUGUUUG UCCAGAA, GAUAGGAACUGUUAAUACU and GCGAGAUGAUACUUUG AUU for Sp3; GGUAUUCACUCUAGCAGUA, GAAAUUUGUUUGUCCA GAA, GAUAGGAACUGUUAAUACU and GCGAGAUGAUACUUUGAUU for Sp4. The ON-TARGET plus Non-targeting Control Pool (Thermo Scientific) was used as the non-specific control. The cells were transfected with 50 nM siRNA pool using the DharmaFECT2 reagent (Thermo Scientific) for 48 h in accordance with the manufacturer's instructions.

### 2.6. Reverse transcription quantitative PCR (qRT-PCR)

Total RNA was extracted from cells using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and was then reverse-transcribed into cDNA by SuperScript II Reverse Transcriptase (Invitrogen). Real-time PCR was performed using SYBR Premix Ex Taq™ (Takara, Kusatsu, Japan) and the Mx3005 QPCR System (Stratagene). The primers used were as follows: *Sp1* forward, ATCCCACAGTTCCAGACCGT; *Sp1* reverse, ATGTTGCCTCCACTTCCTCG; *Sp3* forward, TGAAGAGTGGCAGCTCAGTG; *Sp3* reverse, TGGTACCTCTTCCACCACCT; *Sp4* forward, GCGGGATGAG CGATCAGAAG; *Sp4* reverse, CAGAGGAGAGGGCTGAGAGT; *BRK1* forward, CTGGGCTAACCGGGAGTACA; *BRK1* reverse, TTGTCACCCGAGCT TCAATGT; *18S* forward, GAAACGGCTACCACATCC; and *18S* reverse, ACCAGACTTGCCCTCCA. The real-time PCR assays were performed as follows: 95 °C for 10 s followed by 40 cycles of 95 °C for 15 s (denaturation) and 60 °C for 30 s (annealing and extension). Finally, the dissociation curve was measured for each sample. Relative expression levels of mRNA were calculated against the *18S* internal control by the $\Delta\Delta C_T$ method.

### 2.7. Western blotting

Total cellular protein was extracted with RIPA lysis buffer (Appygen, Beijing, China) followed by concentration measurement with the BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Protein lysates (30 μg) were run on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. Proteins were then transferred onto polyvinylidene difluoride (PVDF) membranes using a Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad, Hercules, CA, USA) at 75 mA for 45 min. The membranes were then blocked in 5% skim milk in PBST containing 10 mM Tris–HCl buffer saline (pH 7.6) plus 0.05% Tween-20. The resulting proteins were detected with primary anti-human Sp1, Sp3, Sp4 antibodies (Abcam, Cambridge, UK) and an anti-human β-actin antibody (Sigma, St. Louis, MO, USA) followed by an HRP-conjugated secondary antibody. The