Breast Cancer Research

Pro-oncogene pokemon promotes breast cancer progression by upregulating survivin expression

Breast Cancer Research 2011, **13**:R26 doi:10.1186/bcr2843

Xuyu Zu (zuxuyu0108@hotmail.com)
Jun Ma (jma@siumed.edu)
Hongxia Liu (liuhx@sz.tsinghua.edu.cn)
Feng Liu (liuf@sz.tsinghua.edu.cn)
Chunyan Tan (tancy@sz.tsinghua.edu.cn)
Lingling Yu (xiaoling-0011@163.com)
Jue Wang (wangj@sz.tsinghua.edu)
Zhenhua Xie (xiezh@sz.tsinghua.edu.cn)
Deliang Cao (dcao@siumed.edu)
Yuyang Jiang (jiangyy@sz.tsinghua.edu.cn)

ISSN 1465-5411

Article type Research article

Submission date 17 September 2010

Acceptance date 10 March 2011

Publication date 10 March 2011

Article URL http://breast-cancer-research.com/content/13/2/R26

Pro-oncogene pokemon promotes breast cancer progression by upregulating survivin expression

Xuyu Zu[1,2], Jun Ma[3], Hongxia Liu[1], Feng Liu[1], Chunyan Tan[1], Lingling Yu[1], Jue Wang[1], Zhenhua Xie[1], Deliang Cao[3] and Yuyang Jiang[1,4]*

[1]The Guangdong Provincial Key Laboratory of Chemical Biology, the Graduate School at Shenzhen, Tsinghua University, Lishui Road, Shenzhen 518055, People's Republic of China

[2]Institution of Clinical Medicine, The First Affiliated Hospital of University of South China, Chuanshan Road, Hengyang 421001, People's Republic of China

[3]Department of Medical Microbiology, Immunology and Cell Biology, Simmons Cancer Institute, Southern Illinois University School of Medicine, 913 N. Rutledge Street, Springfield, IL 62794, USA

[4]School of Medicine, Tsinghua University, Beijing 100084, People's Republic of China

*Corresponding author, Yuyan Jiang, jiangyy@sz.tsinghua.edu.cn

Abstract

Introduction: Pokemon is an oncogenic transcription factor involved in cell growth, differentiation, and oncogenesis, but little is known about its role in human breast cancer. This study aimed to reveal the role of pokemon in breast cancer progression and patient survival and to understand the underlying mechanisms.

Methods: Tissue microarray analysis of breast cancer tissues from patients with complete clinicopathological data and more than 20 years' follow-ups was used to evaluate pokemon expression and its correlation with the progression and prognosis of the disease. DNA microarray analysis of MCF-7 cells that overexpress pokemon was used to identify pokemon target genes. Chromatin immunoprecipitation and site-directed mutagenesis were utilized to determine how pokemon regulates survivin expression, a target gene.

Results: Pokemon was found to be overexpressed in 158 of 182 (86.8%) breast cancer tissues and its expression was correlated with tumor size ($P = 0.0148$) and lymph node metastasis ($P = 0.0014$). Pokemon expression led to worse overall ($n = 175$, $P = 0.01$) and disease-related ($n = 79$, $P = 0.0134$) patient survival. DNA microarray analyses revealed that in breast cancer cells MCF-7, Pokemon regulates the expression of at least 121 genes involved in several signaling and metabolic pathways, including antiapoptotic survivin. In clinical specimens, pokemon and survivin expression was highly correlated ($n = 49$, $r = 0.6799$, $P < 0.0001$). Chromatin immunoprecipitation and site-directed mutagenesis indicated that pokemon induces survivin expression by binding to the GT-boxes in its promoter.

Conclusions: Pokemon promotes breast cancer progression through upregulating survivin expression and thus may be a potential target for the treatment of this malignancy.

Introduction

Pokemon, also referred to as a factor that binds to inducer of shot transcripts-1 (FBI) or leukemia/lymphoma related factor (LRF), is the product of ZBTB7 gene [1]. Pokemon is a pro-oncogenic protein overexpressed in lung cancer, diffuse large B cell lymphomas (DLBCL), non-Hodgkin's lymphoma (NHL), liver cancer, follicular lymphomas and breast cancer [1-4]. In animals, Pokemon was found to induce cell transformation via repressing the tumor suppressor ARF/p53 pathway [1]. In addition, Pokemon is also implicated in transactivation of HIV-1 Tat gene, adipogenesis, osteoclastogenesis, and fatty acid synthesis [5-8]. However, the expression and role of Pokemon in human breast cancer remains unclear.

Pokemon functions as a transcription regulator with active roles in cell growth, differentiation and oncogenesis [2, 9, 10]. Pokemon interferes with GC box recognition by Sp1 via interacting with the zinc finger DNA binding domain, resulting in the repression of ADH5/FDH transcription [11]; Pokemon also affects the transcription of NF-κB responsive genes by associating with the p65 subunit and inducing its nuclear import and stabilization [12]. The target genes of Pokemon include extracellular matrix collagen type I, II, IX, X and XI, aggrecan, fibronectin, elastin, cartilage oligomeric atrix protein (COMP), alchohol dehydrogenase ADH5/FDH, ARF and Rb tumor suppressors, and c-fos and c-myc oncoproteins [9, 11-15]. In this study, we found that in breast cancer cells, Pokemon stimulates survivin expression by binding to its promoter.

Survivin, a member of the inhibitor of apoptosis proteins (IAP) [16], plays an important role in cell apoptosis and mitotic regulation. Survivin is expressed in fetal and cancer cells but

not in normal adult cells. Survivin is highly expressed in breast, colorectal, lung, gastric, and bladder cancers, as well as melanoma, hepatocellular carcinoma, and malignant lymphoma, and its expression in these cancers is associated with poor clinical prognosis [17-25]. Much effort has been made to understand the regulatory mechanism of survivin expression. Various studies showed that survivin expression is regulated by multiple oncogenes, tumor suppressors, and growth factors, such as p53, Sp1, kruppel-like factor 5 (KLF5), and epidermal growth factor receptor (EGFR) [26-30]. In the present study, we found that survivin expression is correlated with Pokemon expression in human breast cancer cells and demonstrated that Pokemon induces its expression by binding to the GC boxes in its promoter.

Materials and methods

Tissue microarray and clinical data

Microarrays of human breast carcinomas were provided by the Yale Cancer Center Critical Technologies group. Two microarrays were used in this study: YTMA-23 (Yale Tissue Microarray-23) containing 246 breast cancer cases with complete clinical records and more than 20 years' follow-ups (Additional file 1) and YTMA-89 consisting of 54 recurrent breast cancer cases. Paraffin-embedded, formalin-fixed specimens of breast carcinoma were identified from the archives of the Yale University Department of Pathology, as available from 1961 to 1983. Complete treatment information was unavailable for the entire cohort of 246 primary breast cancer, but most patients were treated with postsurgical local radiation. None of the node-negative patients were given adjuvant systemic therapy. Among the node-positive patients,

~ 15% were given chemotherapy primarily consisting of adriamycin, cytoxan, and 5-fluorouracil, and some were given tamoxifen (post-1978) [31, 32]. Another two adjacent arrays composed of 50 breast cancers with matching normal adjacent tissues were obtained from Cybrdi Inc (CC08-1-07; Cybrdi, MD). Ethical approval was obtained from human research ethical advisory committee of Tsinghua University for this study.

Cell culture

Human breast cancer cells MCF-7 and MDA-MB-231 (American Type Culture Collection, Manassas, VA) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heated fetal bovine serum (FBS), 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37^{o}C, 5% CO_2.

Immunohistochemistry

After dewaxing and hydration, tissue microarray slides were immerged into preheated citric acid buffer (pH 6.5) at 90-95^{o}C for 20 min with microwaving. After being blocked with 5% horse serum for 30 min, slides were incubated with anti-Pokemon or anti-survivin antibodies (ABcam, MA) (1:50) at 4^{o}C in a humid box overnight. Thereafter, slides were washed 3 times and then incubated with HRP-conjugated secondary antibody (1:800; Pierce, IL) at room temperature for 1 hour. Enhanced DAB staining buffer (Pierce, IL) was used to visualize signals. Staining intensity was evaluated blindly by at least a researcher and a pathologist, and scored from '0' to '3', representing negative and low, intermediate, and high staining, respectively.

Plasmid construction

Pokemon expressing plasmid was generated by inserting the encoding region into pcDNA3.1 expression vector (Invitrogen, CA) at the site of Hind III. Pokemon primer pairs were: 5'-CTT AAG CTT GCC ACC ATG GCC GGC GGC GTG G-3' and 5'-GTC AAG CTT TTA GGC GAG TCC GGC TGT GAA GTT AC-3'. Survivin promoter was subcloned into pGL4.10-basic plasmid (Promega, WI) at BamH I and EcoR V sites to drive luciferase expression. The amplification primer pairs were: 5'-GTC AGA TCT AGT GAA AAG GAG TTG TTC CTT TCC TCC CTC-3' and 5'-GTC AAG CTT GCC GCC GCC GCC ACC TC-3' for 2082 bp fragment, 5'-GTC AAG CTT GCC GCC GCC GCC GCC ACC TC-3' and 5'-GTC AGA TCT AAA GAC AGT GGA GGC ACC AGG C-3' for 1054 bp fragment, and 5'-GTC AAG CTT GCC GCC GCC GCC ACC TC-3' and 5'-GTC AGA TCT TTG GGA TTA CAG GCA TGC ACC AC-3' for 441 bp fragment.

Site–directed mutagenesis

Mutants (pLuc-95m and pLuc-231m) of survivin promoter were generated using in vitro site–directed mutagenesis system (Promega, CA). GGGTG sequence in the binding site of Pokemon was replaced by AAAAA and confirmed by sequencing.

cDNA microarray analysis

Human cDNA microarrays covering 22k cDNA spots (CapitalBio, China) were used. In brief,

total RNA was extracted from MCF-7 cells with ectopic Pokemon expression and vector control using the TRIzol reagent (Invitrogen, CA) and fluorescence-labeled cDNA probes were made for hybridization using 30 μg of total RNA with a oligo-(dT)18 primer and SuperScript II reverse transcriptase (Gibco BRL, CA). Hybridized slides were scanned using laser confocal scanner LuxScan-10KA and signal intensities for each spot were calculated by subtracting local background using LuxScan 3.0 software (CapitalBio, China). Three independent replicates were conducted, and the spot with ≥ 2.0-fold increase or decrease was considered a significant change. Gene expression signaling pathways were analyzed with MAS2.0 software (CapitalBio, China) (GEO accession number: [GSE27442]).

Pokemon silencing and Western blot

Pokemon expression in breast cancer cells MDA-MD-231 was knocked down using chemically synthesized siRNA#1 (targeting 426-444 bp, sense: 5'-GCU GGA CCU UGU AGA UCA Att-3' and antisense: 5'-UUG AUC UAC AAG GUC CAG Ctt-3'); siRNA#2 (targeting 476-494 bp, sense: 5'-AGU ACC UCG AGU UCU UCC Att-3 and antisense: 5'-UGG AAG AAC UCG AGG UAC Utt-3') [13]; and siRNA#3 (targeting 624-642 bp, sense: 5'-GGA GUA CCU CGA GUU CUU Ctt-3' and antisense: 5'-GAA GAA CUC GAG GUA CUC Ctt-3'). The siRNAs were introduced as described previously [33], and protein knockdown was examined using western blot [34]. Soluble proteins (30 μg) were probed with anti-Pokemon (1:500), anti-survivin, anti-p14ARF and anti-Bcl-2 antibodies (1:500) (Abcam, MA). Loading variations were normalized against β-actin which was identified by anti-β-actin monoclonal antibody (Sigma,

MO).

Transient transfection and luciferase activity assay

Transient gene delivery was carried out to assess the effect of Pokemon on survivin promoter activity in MDA-MB-231 and MCF-7 cells, as described previously [35, 36]. Briefly, 1×10^5 cells were mixed with survivin promoter constructs and Pokemon expression vector or Pokemon siRNA#1. At 48 hours after transfection, cell extracts were prepared with 1 × lysis buffer, and a 10 μl aliquot of the supernatant was mixed with 50 μl of luciferase assay reagent (Promega, CA) and analyzed with Microplate Luminometer (Beckman, CA). Luciferase activity was normalized by a Renilla luciferase internal control.

Chromatin immunoprecipitation (ChIP) assay

MCF-7 and MDA-MB-231 cells were fixed by the addition of 1% formaldehyde to the medium for 10 min. Formaldehyde was quenched by addition of 1 × glycine for 5 min at room temperature. ChIP assay was performed as described previously [37], with three microliters of immunoprecipitated DNA and primers (forward: 5'-GTC AAG CTT GCC GCC GCC GCC ACC TC-3' and reverse: 5'-GTC AGA TCT TTG GGA TTA CAG GCA TGC ACC AC-3') located at -441 to -418 and -17 to +1 of survivin promoter.

Statistical analysis

Spearman rank correlation coefficients were used to assess the relationship between Pokemon and survivin expression, Wilcoxon rank-sum tests or Kruskal-Wallis tests were utilized with categorical variables, and Kaplan-Meier survival curves were produced to examine the relationship between Pokemon expression and mortality. Results were considered statistically significant for $p<0.05$.

Results

Overexpression of Pokemon in breast cancer is positively correlated with disease progression.

Tissue microarray YTMA-23, provided by Yale Cancer Center Critical Technology Group, was evaluated for Pokemon expression. This array consisted of 246 breast cancer cases and normal controls, among which 182 malignant tissues were histologically interpretable. The results showed that Pokemon was undetectable in normal breast lobules, but overexpressed in 158 of 182 (86.8%) cancerous tissues. The level of Pokemon expression was assessed according to the intensity of immunostaining and assigned the score of 0 to 3 (negative to most intensive) (Figure 1A). This microarray YTMA-23 came with complete clinical records and follow-ups and therefore, the correlation between Pokemon expression and clinical pathological parameters was analyzed. As summarized in Table 1, Pokemon expression correlated positively with tumor size ($p=0.0148$) and lymph node metastasis ($p=0.0014$), but not with patients' age, tumor type, and nuclear grades. Pokemon was also found to be overexpressed in 28 of 35 (80%) interpretable recurrent breast tumors in another microarray YTMA-89.

Tumor size and lymph node metastasis are well known prognostic factors for breast cancer and therefore, the effect of Pokemon expression on patient survival was further analyzed using Kaplan-Meier plots. As shown in Figure 1B, Pokemon expression was negatively correlated with overall (n=175, p=0.01) and in particular, disease-related (n=79, p=0.0134) survival of breast cancer patients, indicating that Pokemon is a negative prognostic indicator.

ER, PR and HER-2 are well-established biomarkers and therapeutic targets for breast cancer. The correlation of Pokemon expression with these three molecular markers was assessed using Kruskal-Wallis tests, and the results showed that Pokemon expression did not correlate with ER, PR or HER-2 alone or with any combination of the three genes.

Survivin is a downstream target of Pokemon

The positive correlation of Pokemon expression with disease progression suggests that Pokemon may promote cancer development. To determine the underlying mechanism, we attempted to identify genes regulated by Pokemon in breast cancer cells MCF-7. A total of 6 MCF-7 cell clones with stable Pokemon overexpression were isolated (Additional file 2) and three of them were subjected to cDNA microarray analyses. Figure 2 shows a representative data of microarray analysis, in which a total of 121 genes were found have more than 2 fold increased or decreased expression in Pokemon-overexpressing cells, compared to the vector control. Based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) databases, these genes are classified into 7 signaling and/or metabolic pathways (Figure 2C). Notably, these Pokemon target genes are mainly involved in apoptosis, cell cycle, differentiation, and biosynthesis,

suggesting the importance of Pokemon in cell growth, proliferation and carcinogenesis.

Interestingly, BIRC5 (survivin), a negative prognostic factor for breast cancer [38], was found to be one of the downstream targets of Pokemon. Therefore, transfection experiments were performed to determine the role of Pokemon in inducing the expression of survivin. MCF-7 cells were transfected with Pokemon-expression vector to increase the level of Pokemon, while MDA-MD-231 cells were transfected with Pokemon siRNAs to knock down its expression. As showed in Figure 2D-I, three different siRNAs targeting 426-444, 476-494 and 624-642 bp of Pokemon mRNA successfully knocked down its expression and in turn led to decrease of survivin, proving the cDNA microarray data. A time-course study showed that significant silencing of Pokemon by siRNA was observed at 48 hour. Interestingly, Pokemon silencing in MDA-MD-231 led to upregulation of p14ARF, but had not effect on Bcl-2 expression. In addition, a transient expression of Pokemon in MCF-7 cells induced survivin up-regulation in a manner paralleled with Pokemon levels (Figure 2D-II), supporting the cDNA microarray data from stable clones. Taken together, these results suggest that Pokemon indeed induces survivin expression.

Survivin and Pokemon expression is highly correlated in human breast cancer tissues

To confirm that the induction of survivin by Pokemon indeed occurs in breast cancer, adjacent tissue microarrays containing 50 breast cancers and matching adjacent normal breast tissues were subjected to immunohistological analyses of Pokemon and survivin expression. As shown in Figure 3, the levels of survivin and Pokemon expression were found to be highly correlated

(r=0.6799, p<0.0001). In normal breast tissues, both the expression level of Pokemon and survivin was low (data not shown).

Pokemon regulates survivin expression via DNA sequence-specific binding to its promoter

We further explored the mechanism by which Pokemon regulates the expression of survivin. The promoter region of survivin, containing 2080 bp upstream of the transcription start site was cloned and used to drive the expression of luciferase reporter in MCF-7 and MDA-MB-231 breast cancer cells. Progressive deletions of 5' end of the survivin promoter revealed that the -441 bp fragment possesses full promoter activity (Figure 4A). Promoter motif analyses recognized two GT-boxes (GGGTG), located at - 231 to -227 bp and -95 to -91bp, which are potential binding sites of Pokemon. Therefore, this promoter fragment was used to investigate the regulatory role of Pokemon on its activity. As shown in Figure 4B, co-transfection of PCDNA3.1 (+)/Pokemon greatly increased the activity of -pluc-441 in both MCF-7 and MDA-MB-231 cells while the delivery of Pokemon siRNA significantly reduced its basal activity, suggesting Pokemon as a positive regulator of survivin promoter. Furthermore, replacing GGGTG with AAAAA in these two GT boxes of -pluc-441 abrogated the promoter's responsiveness to Pokemon in both MCF-7 and MDA-MB-231 cells (Figure 4B), and interaction between Pokemon and survivin promoter was further confirmed by ChIP assay. As shown in Figure 4C, survivin promoter fragment was amplified by PCR from chromatin precipitated by an anti-Pokemon antibody, but not by non-specific IgG. These data suggest that Pokemon up-regulates survivin expression through direct binding to the GT-boxes in its promoter.

Discussion

Pokemon is an oncogenic transcription factor. Embryonic fibroblasts (MEF) from Pokemon null mice are resistant to cellular or viral oncogene-induced carcinogenic transformation. On the other hand, ectopic expression of Pokemon makes the MEF cells susceptible to oncogenic transformation. These findings suggest an important role of Pokemon in carcinogenesis [10]. In this study, we showed that Pokemon was overexpressed in primary and recurrent breast cancer tissues and up-regulated the expression of anti-apoptotic survivin, leading to disease progression and poor survival.

Using tissue microarray technology, we showed that Pokemon was overexpressed in 86.8% of breast cancer, but not in the normal beast tissues, indicating its tumor-specific expression. More importantly, the Pokemon expression correlated positively with the tumor size and lymph node metastasis, both are indicators of unfavorable clinical outcome. Indeed, the level of Pokemon expression was negatively correlated with a survival rate. These findings indicate that Pokemon may be a novel prognostic marker for breast cancer and a potential therapeutic target. We therefore attempted to elucidate the underlying mechanism of its tumor promoting function.

Previous studies have shown that Pokemon functions as an onco-protein by inhibiting ARF/p53 pathway [10]. Using cDNA microarray analyses, we showed that in breast cancer cells Pokemon regulates the expression of at least 121 genes, some of which are involved in important cellular signaling/metabolic pathways. Survivin, an anti-apoptotic protein and a negative

prognostic indicator of breast cancer [38, 39], is one of those genes whose expression was induced by Pokemon. A study on breast cancer tissues from 50 patients showed that the expression levels of survivin and Pokemon were highly correlated ($p<0.0001$, $r=0.6799$), suggesting that Pokemon serves as an upstream inducer of survivin in breast cancers.

Survivin is widely implicated in cell carcinogenesis, tumor progression, and resistance to radiation and chemotherapies [30, 40]. Previous studies showed that p53 is a suppressor of survivin expression, and loss of p53 function would lead to the induction of survivin, resulting in cancer growth and resistance to chemotherapeutic agents [28, 41]. Pokemon has been shown to repress the expression of p53 [10, 42]. Therefore, Pokemon may induce the expression of survivin indirectly via suppressing p53. However, in this study, we demonstrated that Pokemon can directly induce the expression of survivin by binding to the GC boxes in its promoter. This finding reveals a new signaling pathway of Pokemon-mediated oncogenesis and advances understanding of survivin regulatory mechanisms (Figure 5).

In summary, we found that Pokemon is overexpressed specifically in breast cancers, but not in normal breast tissues. Its oncogenic function may be partly due to its ability to directly induce the expression of survivin, an important cancer-promoting gene. The correlation of Pokemon expression with tumor size, lymph node metastasis, and poor patient survival suggests its potential role as a prognostic marker and therapeutic target for the treatment of this disease.

Conclusions

Pokemon was overexpressed in primary and recurrent breast cancer and correlated with

tumor size, lymph node metastasis, and poor patient survival, being a potential target for the treatment of this malignancy. Pokemon might prompt breast cancer progression through upregulating the expression of survivin, an important cancer-promoting gene.

Abbreviations

ChIP, Chromatin immunoprecipitation Assay; EGFR, epidermal growth factor receptor; FBI, factor that binds to inducer of shot transcripts-1; HER2, human epidermal growth factor receptor 2; IAP, inhibitor of apoptosis protein; KLF5, kruppel-like factor 5; LRF, leukemia/lymphoma related factor; PMSF, Phenylmethanesulfonyl fluoride.

Authors' contributions

ZX and JYY designed the studies and drafted the paper and ZX, MJ, LH, LF, TC, YL, WJ and XZ performed the studies. CD procured the tissue microarrays and revised the paper. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Acknowledgments

This work was supported by grant from National 863 Project of the Ministry of Science and Technology (China, 2007AA02Z160). The authors would like to thank the financial supports from the Ministry of Science and Technology of China (2009ZX09501-004 and 2007AA02Z160) and the Chinese National Natural Science Foundation (20872077 and 90813013).

References

1. Maeda T, Hobbs RM, Merghoub T, Guernah I, Zelent A, Cordon-Cardo C, Teruya-Feldstein J, Pandolfi PP: **Role of the proto-oncogene Pokemon in cellular transformation and ARF repression**. *Nature* 2005, **433**:278-285.
2. Wei CL, Wu Q, Vega VB, Chiu KP, Ng P, Zhang T, Shahab A, Yong HC, Fu Y, Weng Z, Liu J, Zhao XD, Chew JL, Lee YL, Kuznetsov VA, Sung WK, Miller LD, Lim B, Liu ET, Yu Q, Ng HH, Ruan Y: **A global map of p53 transcription-factor binding sites in the human genome**. *Cell* 2006, **124**:207-219.
3. Apostolopoulou K, Pateras IS, Evangelou K, Tsantoulis PK, Liontos M, Kittas C, Tiniakos DG, Kotsinas A, Cordon-Cardo C, Gorgoulis VG: **Gene amplification is a relatively frequent event leading to ZBTB7A (Pokemon) overexpression in non-small cell lung cancer**. *J Pathol* 2007, **213**:294-302.
4. Aggarwal A, Hur MW ,Aggarwal H, Silva ED, Davey MS, Murphy RF, Agrawal DK.: **Expression of leukemia/lymphoma-related factor (LRF/POKEMON) in human breast carcinoma and other cancers.** *Exp Mol Pathol* 2010, **89**:140-148.
5. Laudes M, Bilkovski R, Oberhauser F, Droste A, Gomolka M, Leeser U, Udelhoven M, Krone W: **Transcription factor FBI-1 acts as a dual regulator in adipogenesis by coordinated regulation of cyclin-A and E2F-4**. *J Mol Med* 2008, **86**:597-608.
6. Pendergrast PS, Wang C, Hernandez N, Huang S: **FBI-1 can stimulate HIV-1 Tat activity and is targeted to a novel subnuclear domain that includes the Tat-P-TEFb-containing nuclear speckles**. *Mol Biol Cell* 2002, **13**:915-929.
7. Kukita A, Kukita T, Ouchida M, Maeda H, Yatsuki H, Kohashi O: **Osteoclast-derived zinc finger (OCZF) protein with POZ domain, a possible transcriptional repressor, is involved in osteoclastogenesis**. *Blood* 1999, **94**:1987-1997.
8. Choi WI, Jeon BN, Park H, Yoo JY, Kim YS, Koh DI, Kim MH, Kim YR, Lee CE, Kim KS, Osborne TF, Hur MW: **Proto-oncogene FBI-1 (Pokemon) and SREBP-1 synergistically activate transcription of fatty-acid synthase gene (FASN)**. *J Biol Chem* 2008, **283**:29341-29354.
9. Maeda T, Merghoub T, Hobbs RM, Dong L, Maeda M, Zakrzewski J, van den Brink MR, Zelent A, Shigematsu H, Akashi K, Teruya-Feldstein J, Cattoretti G, Pandolfi PP: **Regulation of B versus T lymphoid lineage fate decision by the proto-oncogene LRF**. *Science* 2007, **316**:860-866.
10. Maeda T, Hobbs RM, Pandolfi PP: **The transcription factor Pokemon: a new key player in cancer pathogenesis**. *Cancer Res* 2005, **65**:8575-8578.
11. Lee DK, Suh D, Edenberg HJ, Hur MW: **POZ domain transcription factor, FBI-1, represses transcription of ADH5/FDH by interacting with the zinc finger and interfering with DNA binding activity of Sp1**. *J Biol Chem* 2002, **277**:26761-26768.
12. Lee DK, Kang JE, Park HJ, Kim MH, Yim TH, Kim JM, Heo MK, Kim KY, Kwon HJ, Hur MW: **FBI-1 enhances transcription of the nuclear factor-kappaB (NF-kappaB)-responsive E-selectin gene by nuclear localization of the p65 subunit**

of NF-kappaB. *J Biol Chem* 2005, **280**:27783-27791.

13. Jeon BN, Yoo JY, Choi WI, Lee CE, Yoon HG, Hur MW: **Proto-oncogene FBI-1 (Pokemon/ZBTB7A) represses transcription of the tumor suppressor Rb gene via binding competition with Sp1 and recruitment of co-repressors**. *J Biol Chem* 2008, **283**:33199-33210.
14. Widom RL, Lee JY, Joseph C, Gordon-Froome I, Korn JH: **The hcKrox gene family regulates multiple extracellular matrix genes**. *Matrix Biol* 2001, **20**:451-462.
15. Pessler F, Hernandez N: **Flexible DNA binding of the BTB/POZ-domain protein FBI-1**. *J Biol Chem* 2003, **278**:29327-29335.
16. Ambrosini G, Adida C, Altieri DC: **A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma**. *Nat Med* 1997, **3**:917-921.
17. Rodel F, Hoffmann J, Distel L, Herrmann M, Noisternig T, Papadopoulos T, Sauer R, Rodel C: **Survivin as a radioresistance factor, and prognostic and therapeutic target for radiotherapy in rectal cancer**. *Cancer Res* 2005, **65**:4881-4887.
18. Li F: **Survivin study: what is the next wave?** *J Cell Physiol* 2003, **197**:8-29.
19. Grabowski P, Kuhnel T, Muhr-Wilkenshoff F, Heine B, Stein H, Hopfner M, Germer CT, Scherubl H: **Prognostic value of nuclear survivin expression in oesophageal squamous cell carcinoma**. *Br J Cancer* 2003, **88**:115-119.
20. Adida C, Haioun C, Gaulard P, Lepage E, Morel P, Briere J, Dombret H, Reyes F, Diebold J, Gisselbrecht C, Salles G, Altieri DC, Molina TJ: **Prognostic significance of survivin expression in diffuse large B-cell lymphomas**. *Blood* 2000, **96**:1921-1925.
21. Kennedy SM, O'Driscoll L, Purcell R, Fitz-Simons N, McDermott EW, Hill AD, O'Higgins NJ, Parkinson M, Linehan R, Clynes M: **Prognostic importance of survivin in breast cancer**. *Br J Cancer* 2003, **88**:1077-1083.
22. Monzo M, Rosell R, Felip E, Astudillo J, Sanchez JJ, Maestre J, Martin C, Font A, Barnadas A, Abad A: **A novel anti-apoptosis gene: Re-expression of survivin messenger RNA as a prognosis marker in non-small-cell lung cancers**. *J Clin Oncol* 1999, **17**:2100-2104.
23. Kawasaki H, Altieri DC, Lu CD, Toyoda M, Tenjo T, Tanigawa N: **Inhibition of apoptosis by survivin predicts shorter survival rates in colorectal cancer**. *Cancer Res* 1998, **58**:5071-5074.
24. Islam A, Kageyama H, Takada N, Kawamoto T, Takayasu H, Isogai E, Ohira M, Hashizume K, Kobayashi H, Kaneko Y, Nakagawara A: **High expression of Survivin, mapped to 17q25, is significantly associated with poor prognostic factors and promotes cell survival in human neuroblastoma**. *Oncogene* 2000, **19**:617-623.
25. Kajiwara Y, Yamasaki F, Hama S, Yahara K, Yoshioka H, Sugiyama K, Arita K, Kurisu K: **Expression of survivin in astrocytic tumors: correlation with malignant grade and prognosis**. *Cancer* 2003, **97**:1077-1083.
26. Asanuma H, Torigoe T, Kamiguchi K, Hirohashi Y, Ohmura T, Hirata K, Sato M, Sato N: **Survivin expression is regulated by coexpression of human epidermal growth factor receptor 2 and epidermal growth factor receptor via phosphatidylinositol**

3-kinase/AKT signaling pathway in breast cancer cells. *Cancer Res* 2005, **65**:11018-11025.

27. Hoffman WH, Biade S, Zilfou JT, Chen J, Murphy M: **Transcriptional repression of the anti-apoptotic survivin gene by wild type p53**. *J Biol Chem* 2002, **277**:3247-3257.
28. Mirza A, McGuirk M, Hockenberry TN, Wu Q, Ashar H, Black S, Wen SF, Wang L, Kirschmeier P, Bishop WR, Nielsen LL, Pickett CB, Liu S: **Human survivin is negatively regulated by wild-type p53 and participates in p53-dependent apoptotic pathway**. *Oncogene* 2002, **21**:2613-2622.
29. Zhou M, Gu L, Li F, Zhu Y, Woods WG, Findley HW: **DNA damage induces a novel p53-survivin signaling pathway regulating cell cycle and apoptosis in acute lymphoblastic leukemia cells**. *J Pharmacol Exp Ther* 2002, **303**:124-131.
30. Li F, Altieri DC: **Transcriptional analysis of human survivin gene expression**. *Biochem J* 1999, **344 Pt 2**:305-311.
31. Siddiqui SF, Pawelek J, Handerson T, Lin CY, Dickson RB, Rimm DL, Camp RL: **Coexpression of beta1,6-N-acetylglucosaminyltransferase V glycoprotein substrates defines aggressive breast cancers with poor outcome**. *Cancer Epidemiol Biomarkers Prev* 2005, **14**:2517-2523.
32. Giltnane JM, Moeder CB, Camp RL, Rimm DL: **Quantitative multiplexed analysis of ErbB family coexpression for primary breast cancer prognosis in a large retrospective cohort**. *Cancer* 2009, **115**:2400-2409.
33. Yan R, Zu X, Ma J, Liu Z, Adeyanju M, Cao D: **Aldo-keto reductase family 1 B10 gene silencing results in growth inhibition of colorectal cancer cells: Implication for cancer intervention**. *Int J Cancer* 2007, **121**:2301-2306.
34. Zu X, Yan R, Robbins S, Krishack PA, Liao DF, Cao D: **Reduced 293T cell susceptibility to acrolein due to aldose reductase-like-1 protein expression**. *Toxicol Sci* 2007, **97**:562-568.
35. Zu X, Yu L, Sun Q, Liu F, Wang J, Xie Z, Wang Y, Xu W, Jiang Y: **SP1 enhances Zbtb7A gene expression via direct binding to GC box in HePG2 cells**. *BMC Res Notes* 2009, **2**:175.
36. Liu Z, Zhong L, Krishack PA, Robbins S, Cao JX, Zhao Y, Chung S, Cao D: **Structure and promoter characterization of aldo-keto reductase family 1 B10 gene**. *Gene* 2009, **437**:39-44.
37. Chung SW, Chen YH, Yet SF, Layne MD, Perrella MA: **Endotoxin-induced down-regulation of Elk-3 facilitates heme oxygenase-1 induction in macrophages**. *J Immunol* 2006, **176**:2414-2420.
38. Paik S, Shak S, Tang G, Kim C, Baker J, Cronin M, Baehner FL, Walker MG, Watson D, Park T, Hiller W, Fisher ER, Wickerham DL, Bryant J, Wolmark N: **A multigene assay to predict recurrence of tamoxifen-treated, node-negative breast cancer**. *N Engl J Med* 2004, **351**:2817-2826.
39. Lu J, Tan M, Huang WC, Li P, Guo H, Tseng LM, Su XH, Yang WT, Treekitkarnmongkol W, Andreeff M, Symmans F, Yu D: **Mitotic deregulation by survivin in**

ErbB2-overexpressing breast cancer cells contributes to Taxol resistance. *Clin Cancer Res* 2009, **15**:1326-1334.

40. Spaulding B, Pan D, Ghadersohi A, Nielsen G, Jensen S, Gellert F, Ling X, Zhang M, Black A, Li F: **Characterization of the 12C4 survivin monoclonal antibody and insight into the expression of survivin in human adult tissues**. *Histopathology* 2006, **49**:622-633.
41. Zhu N, Gu L, Findley HW, Chen C, Dong JT, Yang L, Zhou M: **KLF5 Interacts with p53 in regulating survivin expression in acute lymphoblastic leukemia**. *J Biol Chem* 2006, **281**:14711-14718.
42. Agrawal A, Yang J, Murphy RF, Agrawal DK: **Regulation of the p14ARF-Mdm2-p53 pathway: an overview in breast cancer**. *Exp Mol Pathol* 2006, **81**:115-122.

Figure legends

Figure 1. Pokemon expression in breast cancer tissues and its relationship with patient survival. (A) Immunohistochemistry, indicating Pokemon expression in normal and malignant breast tissues. Results were scored by a researcher blinded to the samples and a pathologist. Images show Pokemon expression in normal breast lobules (a) and in breast cancers, scored at '1' (b), '2' (c), and '3' (d), respectively. **(B)** Kaplan-Meier plots of overall (i) (n=175, p=0.01) and disease-related (ii) (n=79, p=0.0134) survival. Data were from microarray YTMA-23 containing 175 breast cancer cases with clinical data and more than 30-year's follow-ups. In disease-related survival plots, disease-free survivals or patients who died from breast cancer were included.

Figure 2. Target genes of Pokemon in MCF-7 cells. cDNA microarray analyses were performed as described in Materials and Methods. Data from three independent replicates were combined, and any spot with at least 2.0-fold increase or decrease, compared to vector control, was considered a significant change. Signaling pathways were classified with MAS2.0 software.

(A) Ectopic expression of Pokemon in MCF-7 breast cancer cells, detected by Western blot. **(B)** Scatter plots, showing differential expression profile of Pokemon target genes in MCF-7 cells. **(C)** Pathway classification of Pokemon target genes in MCF-7 cells. **(D)** Survivin expression regulated by Pokemon. MDA-MB-231 cells treated with siRNAs for 48h were harvested for western blot with the indicated antibodies (I); cells transiently transfected with either Pokemon expression vector (MCF-7 cells) or siRNA (MDA-MB-231 cells) were used for time-dependent expression by Western blot as described in Materials and Methods(II).

Figure 3. Correlation of survivin and Pokemon expression in breast cancer tissues. Pokemon and survivin expression in breast tissues was examined by immunohistochemistry as described in the Materials and Methods. **(A)** Images show Pokemon and survivin expression in two adjacent sections, which are histologically similar but not the same. **(B)** Summary of Pokemon and survivin expression data. Spearman rank correlation coefficients were used to test the relationship between Pokemon and survivin expression.

Figure 4. Pokemon stimulates survivin expression by direct binding to its promoter. (A) Schematic representation of survivin promoter–luciferase reporter plasmids, including wild type survivin promoter and 5'-progressive deletions, as well as site-directed mutants at Pokemon binding sites, pLuc-95m (at -95 bp) and pLuc-231m (at -231 bp). Refer to the Materials and Methods for the plasmid construction. Survivin promoter activity was analyzed in MCF-7 cells by luciferase assay. **(B)** Regulation of survivin promoter activity by Pokemon in MCF-7 and MDA-MB-231 breast cancer cells. Wild type and mutant promoter (pLuc-441) was co-transfected with Pokemon gene or Pokemon siRNA#1 and luciferase activity was measured

with β-galactosidase as an internal control. Data represent the mean of three independent experiments ± SD. Statistical significance was tested using Student *t* tests with *p<0.05 and **p<0.01. **(C)** ChIP assay, indicating that Pokemon bound to survivin promoter in MCF-7 and MDA-MB-231 breast cancer cells. Rabbit IgG was used as a negative control. Refer to the Materials and Methods for details.

Figure 5. Hypothetic pathways that Pokemon regulates survivin expression. p53 suppresses survivin expression and induces cell apoptosis or senescence [28, 29]. Pokemon activates survivin signaling by suppressing the ARF/p53 pathway [1]. This current study shows that Pokemon also enhances survivin expression via direct binding to its promoter, promoting breast oncogenesis.

Table1. Correlation of Pokemon expression with clinicopathological parameters

Variables	Pokemon (*n* = 182)				*P*-value
	3	2	1	0	
Subtotal (%)	72 (39.6)	69 (37.9)	17 (9.3)	24 (13.2)	
Age (Years)					0.2451
>50 (%)	58 (40.8)	55 (38.7)	13 (9.2)	16 (11.3)	
≤50 (%)	13 (33.3)	14 (35.9)	4 (10.3)	8 (20.5)	
Tumor Types					0.8733
Collid (%)	3 (37.5)	3 (37.5)	1 (12.5)	1 (12.5)	
Ductal (%)	35 (47.9)	26 (35.6)	5 (6.8)	7 (9.6)	
Lobular (%)	14 (53.8)	8 (30.8)	3 (11.5)	1 (3.8)	
Tumor Size (cm^3)					0.0148
> 2 (%)	47 (42.7)	46 (41.8)	9 (8.2)	8 (7.3)	
≤ 2 (%)	23 (33.8)	22 (32.3)	8 (11.8)	15 (22.1)	
Node Metastasis					0.0014
Positive (%)	49 (44.1)	38 (34.2)	8 (7.2)	16 (14.4)	
Negative (%)	22 (31.9)	30 (43.5)	9 (13.0)	8 (11.6)	
Nuclear Grade					0.057
1 (%)	5 (23.8)	9 (42.6)	2 (9.5)	5 (23.8)	
2 (%)	40 (40.4)	38 (38.4)	12 (12.1)	9 (9.1)	
3 (%)	25 (45.5)	20 (38.4)	3 (12.1)	7 (9.1)	
ER					0.429
Positive (%)	38 (37.6)	39 (38.6)	11 (10.9)	13 (12.9)	
Negative (%)	34 (42.0)	30 (37.0)	6 (7.4)	11 (13.6)	
PR					0.3444
Positive (%)	41 (40.2)	34 (33.3)	14 (13.7)	13 (12.8)	
Negative (%)	31 (38.8)	35 (43.8)	3 (3.7)	11 (14.7)	
HER-2					0.2309
Positive (%)	31 (38.7)	28 (35.0)	7 (8.8)	14 (17.5)	
Negative (%)	41 (38.3)	41 (38.3)	11 (10.3)	14 (13.1)	

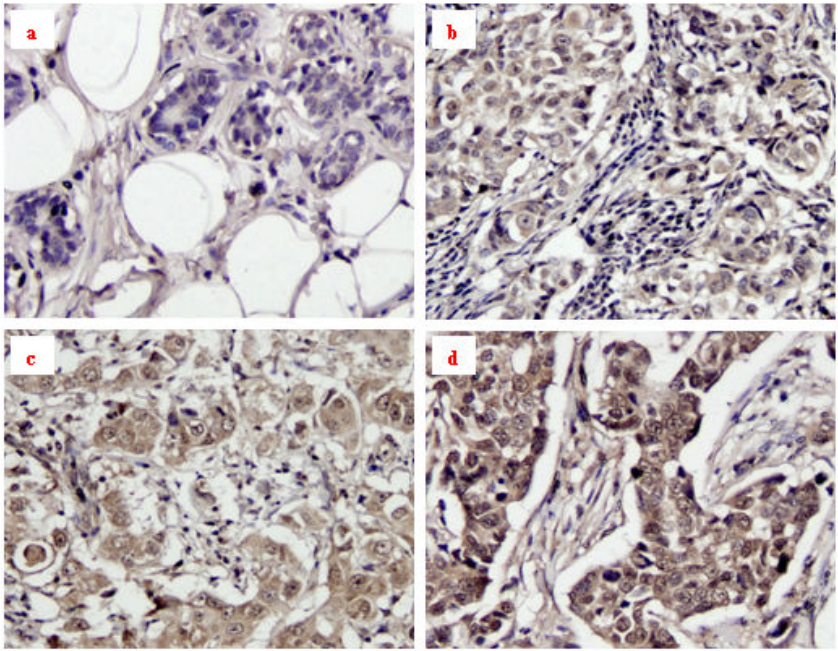
Data were from the tissue array YTMA-23 in which 182 breast cancer cases were histologically interpretable and analyzed for the correlation of Pokemon expression with clinicopathological parameters. ER, estrogen receptor; PR, progesterone receptor; and HER-2, human epidermal growth factor receptor 2.

Additional files

Additional files 1. Descriptive statistics of tissue microarray. Tissue microarray, YTMA-23, contained 246 breast cancer cases with clinical records. Summarized below are the descriptive statistical data.

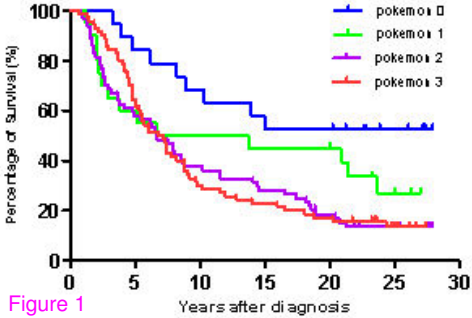
Additional files 2. MCF-7 cell clones with stable over expression of Pokemon. MCF-7 cells were transfected with pcDNA3.1/Pokemon or the empty vector, and the transfected cells maintained with neomycin were subjected to western blot analysis for the detection of Pokemon expression.

A) Immunohistochemistry



B) Patient survival

i) Overall survival



ii) Disease-related survival

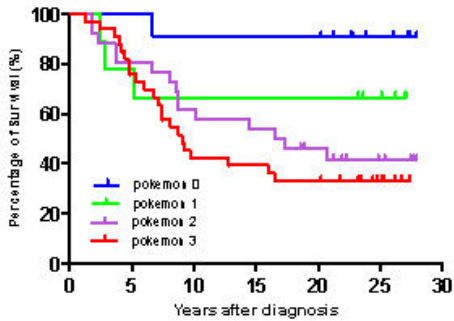
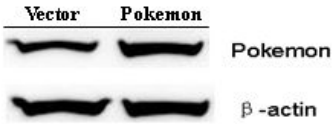
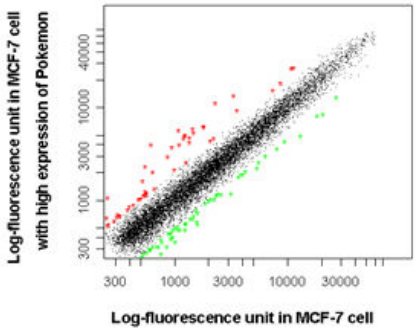


Figure 1

B) Scatter plots of Pokemon target genes

A) Pokemon expression

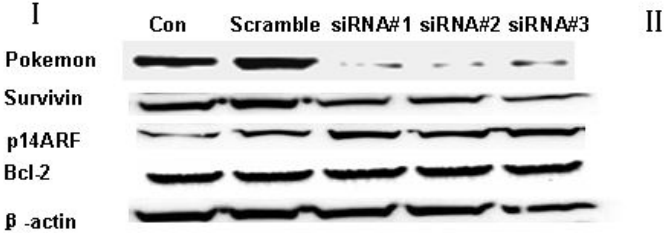




C) Functional classification of Pokemon target genes

Apoptosis	Cell cycle	Cell growth Diffrentiation	Transcription regulation	Signal Transduction	Biosynthesis Metabolism	Transport and ion channel
SULF1	DUSP1	SLC2A1	CARS	STC2	MGC22265	KCNJ4
BIRC5	JUN	DDIT3	RREB1	WNT6	RBKS	PKD2
TRIB3	CKS2	NGFR	DUSP1	PLD2	FUT1	COL4A6
TNFRSF12A	CDC14B	TRIB3	WARS	SULF1	RREB1	VMD2
NGFR		C5	CARS	EFNA3	RHOB	
RHOB		BCAT1	DDIT3	CXXC5	MAWBP	
BCLAF1		PCK2	TRIB3	CRLF1	PCK2	
P8		PAX1	SR140	STC2	FUT1	
BCLAF1		DDIT3			SAT	
MYO1A		ZMPSTE24			CNNM2	
PLCXD1		TRIB3			DUSP1	
					PLD2	

D) Survivin expression affected by Pokemon



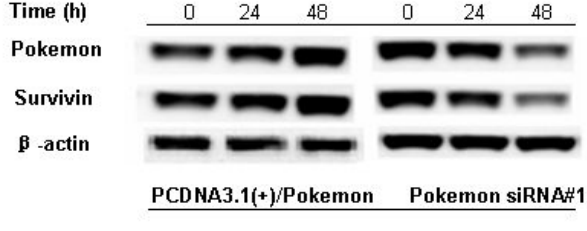
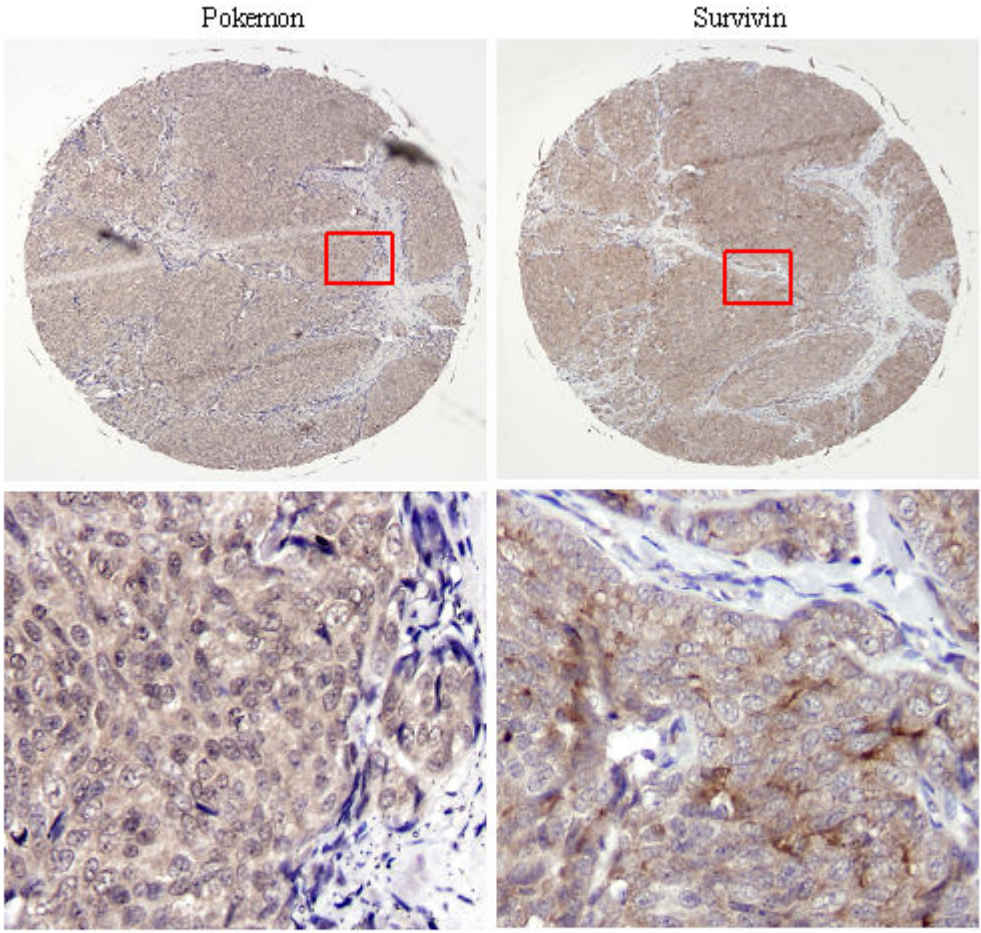


Figure 2

A) Immunohistochemistry

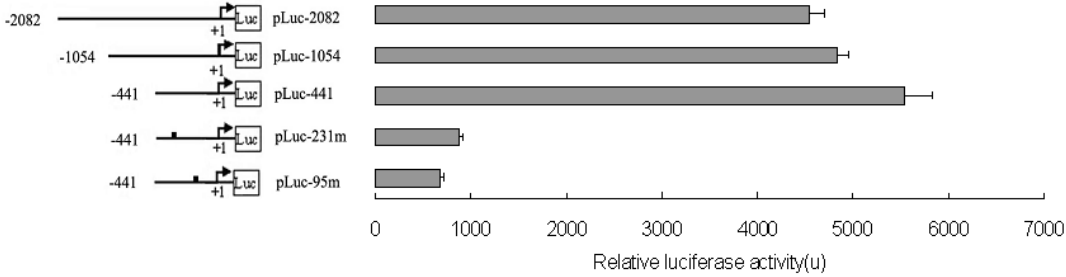


B) Correlation of Pokemon and survivin expression

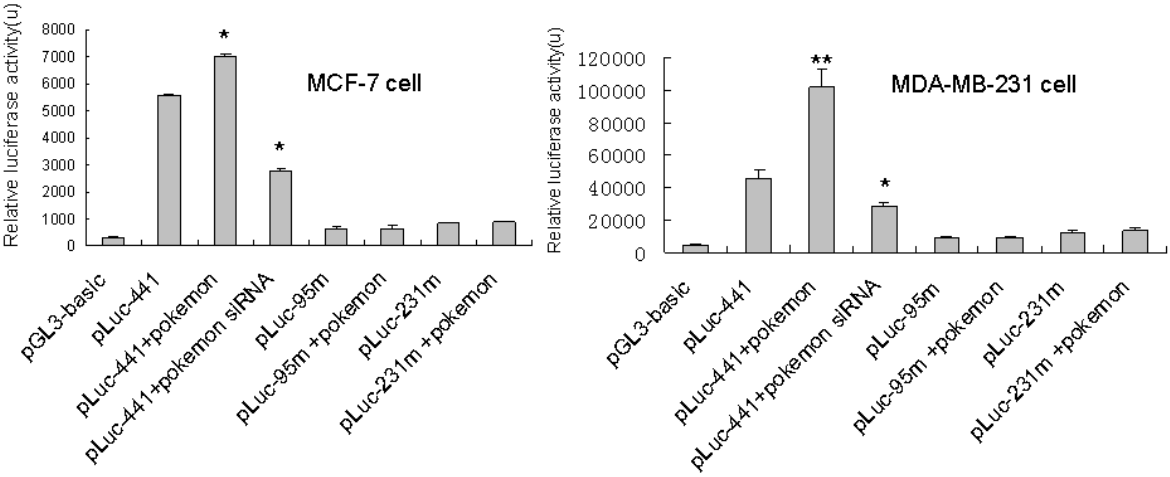
	Case number	Staining score (%)				p value	Spearman r
		0	1	2	3		
Pokemon	49	9 (18.4)	6 (12.2)	16 (32.7)	18 (36.7)	<0.0001	0.6799
Survivin	49	6 (12.2)	12 (24.5)	14 (28.6)	17 (34.7)		

Figure 3

A) Schemes of wild type and mutant survivin promoters



B) Effect of pokemon on survivin promoter activity



C) ChIP assay

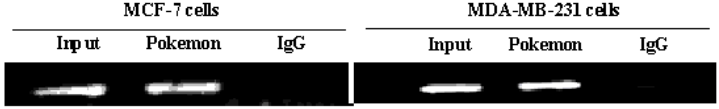


Figure 4

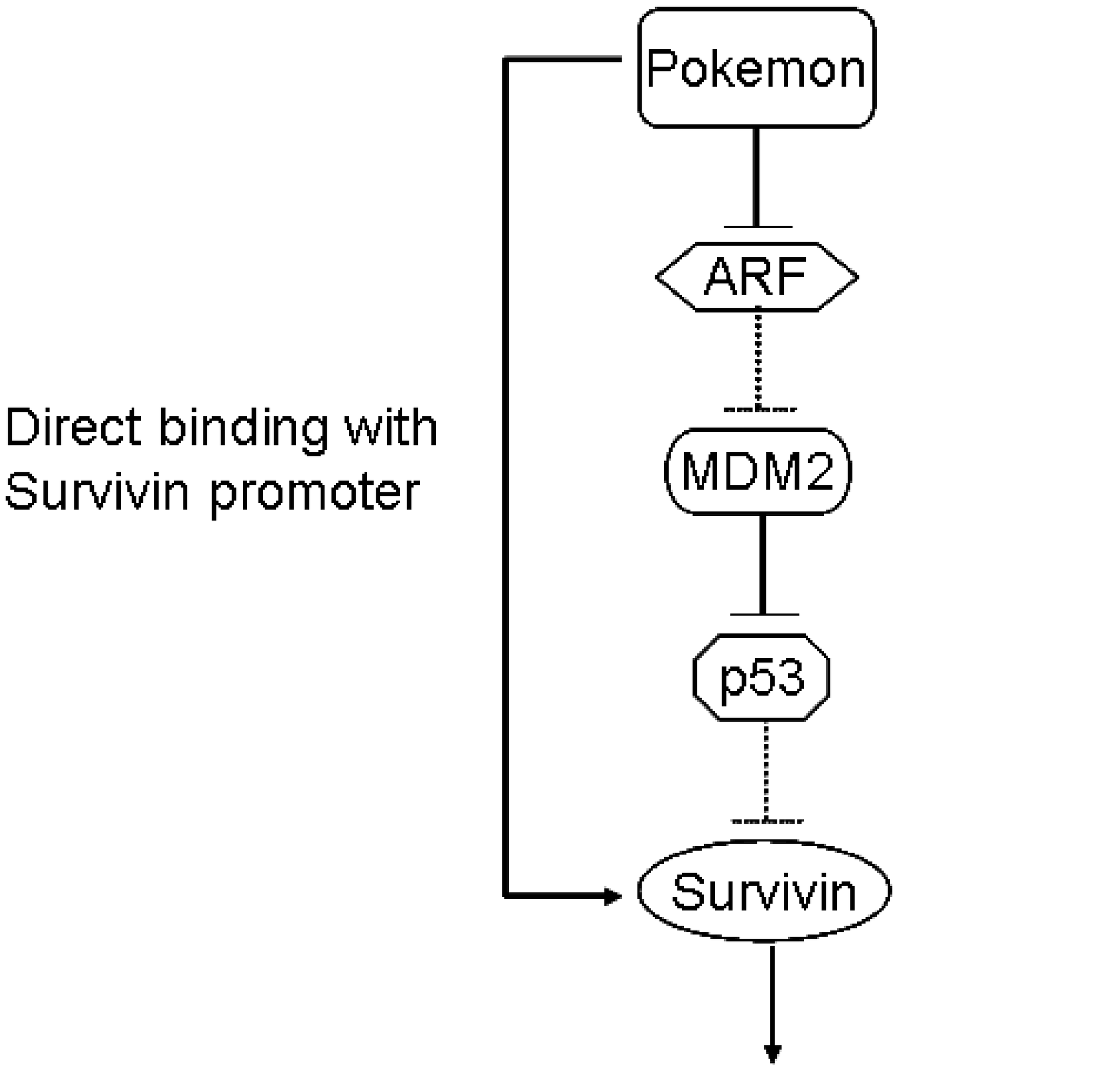

Pokemon
ARF
Direct binding with
Survivin promoter
MDM2
p53
Survivin
Anti-apoptosis and cell survival

Figure 5

Additional files provided with this submission:

Additional file 1: Supplementary Table 1.doc, 52K
http://breast-cancer-research.com/imedia/1786277505527096/supp1.doc
Additional file 2: Figure S1.doc, 45K
http://breast-cancer-research.com/imedia/1360721243527096/supp2.doc