NEURAL REGENERATION RESEARCH
Volume 7, Issue 12, April 2012
www.nrronline.org

Cite this article as: Neural Regen Res. 2012;7(12):932-937.

Gene expression by simian virus 40 large T antigen-induced medulloblastomas in mice☆

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Abstract

Signaling pathways known to have components with mutations in human medulloblastoma include sonic hedgehog, Wnt/beta-catenin and insulin-like growth factor. Microarray analysis was applied to examine the gene expression changes in medulloblastomas of pTet-on/pTRE-SV40Tag transgenic mice. Altogether, 14 112 genes were detectable, including 152 genes with significantly different expression levels. These genes were associated with immunity, the cell cycle, signal transduction, cytoskeleton and metabolism. To further confirm the microarray data, real-time polymerase chain reactions were used to examine the expression changes of genes related to sonic hedgehog, Wnt/beta-catenin and insulin-like growth factor signal pathways. Immunohistochemistry detected insulin receptor substrate-1 in the nuclei of brain tumor tissue cells from pTet-on/pTRE-SV40Tag transgenic mice, suggesting that SV40 large T antigen may activate the insulin-like growth factor signal pathway to promote tumorigenesis.

Key Words: SV40 large T antigen; medulloblastoma; cDNA microarray; real-time polymerase chain reaction; insulin-like growth factor

Abbreviations: SHH, sonic hedgehog; IGF, insulin-like growth factor; WNT, Wnt/beta-catenin; SV40Tag, simian virus 40 large T antigen; IRS-1, insulin receptor substrate-1; IGFBP, insulin-like growth factor binding protein

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Received: 2012-01-06
Accepted: 2012-03-09
(NY20111019002/WJ)

Wei XL, Feng J, Hu YH. Gene expression by simian virus 40 large T antigen-induced medulloblastomas in mice. Neural Regen Res. 2012;7(12):932-937.

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doi:10.3969/j.issn.1673-5374.2012.12.009

INTRODUCTION

Recent advances in the study of the role of sonic hedgehog (SHH)[1], Wnt/beta-catenin (WNT)[2] and insulin-like growth factor (IGF)[3] signaling pathways in the development of the cerebellum have shed new light on the pathogenesis of medulloblastoma. Despite these advances, the molecular mechanisms underlying the pathogenesis of medulloblastoma remain poorly understood. The elucidation of the signaling pathways involved in the pathogenesis of medulloblastoma has substantially improved the clinical management of this neoplasm, enabled more accurate prediction of the disease risk and led to the development of new targeted treatments.

Recently, a pTet-on/pTRE-SV40Tag transgenic mouse model of medulloblastoma was generated in our laboratory[4]. Overexpression of simian virus 40 large T antigen (SV40Tag) induced the formation of medulloblastoma in mice. SV40 has been detected in medulloblastoma[5]. Furthermore, much evidence has indicated that the SV40Tag could have oncogenic potential[6]. The SV40Tag, one of the initially-transcribed protein T antigens of SV40, is a regulatory protein that binds to tumor suppressor genes, including p53[6]. Biochemical analysis showed that transformation of mouse embryonic fibroblast and several other cell lines by SV40Tag induced the phosphorylation of insulin receptor substrate-1 (IRS-1)[7]. However, the molecular mechanism underlying the induction of tumors by SV40 remains unclear. In this study, microarray analysis was applied to analyze the gene expression changes in medulloblastoma and to identify tumor-associated genes in the SHH, WNT and IGF pathways. In addition, the interaction between SV40Tag and IRS-1 was examined to confirm the roles of these proteins in tumor pathogenesis and development

RESULTS

Quantitative analysis of experimental animals

Six pTet-on/pTRE-SV40Tag transgenic mice (three males and three females) aged 6 weeks were exposed to doxycycline hydrochloride in drinking water. Six pTet-on/pTRE-SV40Tag double transgenic mice (three males and three females) without administration of doxycycline hydrochloride were used as controls. Brain

tumors and normal cerebella were dissected for the extraction of poly (A) mRNA. All mice were alive during the administration of doxycycline hydrochloride or normal water. There was no infection or death, and all 12 mice entered the final analysis.

Analysis of differentially expressed genes involved in brain tumor development

Microarrays have been used to examine the gene expression profiles of SV40Tag-induced medulloblastomas in mice. A total of 14 112 genes were examined. Our experimental results showed that 152 genes were either up-regulated 4-fold or down-regulated 0.25-fold or more in medulloblastomas. Analysis of physiological function indicated that the altered genes were associated with metabolism, cell growth, division and DNA synthesis, signal transduction, immunity and the cell cycle (Table 1). This confirmed that the cancer was caused by multiple genetic changes. Some of these genes are known to be related to cancer pathogenesis, such as *Tmpo* [8], *Pcna* [9], and *Msh5* [10].

Table 1 Partially differentially expressed genes by microarray

Gene function	Gene name	Description	Fold change
Metabolism	*Hao1*	Hydroxyacid oxidase 1	0.11
	Tmpo	Thymopoietin (gene)	5.08
Cell growth, division and DNA synthesis	*Pcna*	Proliferating cell nuclear antigen (gene)	4.87
	Hmgn2	High mobility group nucleosomal binding domain 2	4.88
	Rnf32	Ring finger protein 32	6.59
Signal transduction	*Gpr37*	G protein-coupled receptor 37	0.29
	IRS-1	Insulin receptor substrate 1	4.01
	Casp7	Caspase 7	4.30
	Rad51c	Rad51 homolog c	4.36
	IGF-IR	Insulin-like growth factor I receptor	2.21
	IGF-II	Insulin-like growth factor II	0.18
	IRS-1	Insulin receptor substrate-1	5.39
	IGFBP-1	Insulin-like growth factor binding protein 1	1.07
	IGFBP -2	Insulin-like growth factor binding protein 2	0.25
	IGFBP -3	Insulin-like growth factor binding protein 3	1.16
	IGFBP -4	Insulin-like growth factor binding protein 4	1.25
	IGFBP -5	Insulin-like growth factor binding protein 5	0.65
	IGFBP -6	Insulin-like growth factor binding protein 6	0.96
	Shh	Sonic hedgehog	2.21
	Patch1	Patched homolog 1	0.18
	Gli2	GLI-Kruppel family member GLI2	5.39
	Sufu	Suppressor of fused homolog	1.07
	β-catenin	Beta-catenin	0.25
Immunity	*H2-Ob*	Histocompatibility 2, O region beta locus	1.16
	Ttrap	Traf and Tnf receptor associated protein	4.78
	Ly6a	Lymphocyte antigen 6 complex, locus A	5.88
Cell cycle	*Cdkn1c*	Musculus cyclin-dependent kinase inhibitor 1C (P57)	4.56
	Msh5	Mut S homolog 5 (gene)	4.61
	Cdc20	Cell division cycle 20 homolog	39.0
Others	*Ddc8*	Testis-specific protein	0.14
	Jam2	Junction adhesion molecule 2	5.60
	Kifc5a	Kinesin family member C5A	7.88

Genes up-regulated 4-fold or down-regulated 0.25-fold or more were considered differentially expressed.

Real-time polymerase chain reaction was performed to further confirm the gene expression profile data derived from microarray analysis. Fourteen genes were examined and 11 of them showed similar expression changes by both microarray and real-time polymerase chain reaction. The data also revealed that the fold changes of most of the chosen genes were greater by real-time polymerase chain reaction than microarray analysis (Table 1).

A number of signaling pathways have been implicated in tumor pathogenesis, including SHH, WNT and IGF signaling pathways[11-12]. Therefore, the expression of genes associated with these signaling pathways was examined. Although the expression level of *Patch1* (P = 0.001) decreased, three important genes in the SHH signal pathway-*Sufu* (P = 0.05), *Shh* (P = 0.11) and *Gli2* (P = 0.28)-did not show significant changes (Figure 1). The expression level of *β-cantenin* (P = 0.06), which is involved in the WNT signaling pathway, was increased (Figure 2). Furthermore, the exp ression levels of *IGF-IR* and *IRS-1* were increased significantly in medulloblastoma[4]. The expression levels of *IGFBP-1* (P = 0.00004), *IGFBP-2* (P = 0.003), *IGFBP-3* (P = 0.047), *IGFBP-5* (P = 0.013), *IGFBP-6* (P = 0.046) were significantly decreased (Figure 3).

IRS-1 is translocated into the nucleus

IRS-1 is the major cytoplasmic component of the insulin and IGF-1 signaling pathways[13]. The cellular localization of IRS-1 in brain tumor tissue was examined. Interestingly, a predominantly nuclear immunostaining for IRS-1 was found in brain tumor cells (Figure 4), while it was mainly located in the cytoplasm in normal brain cells.

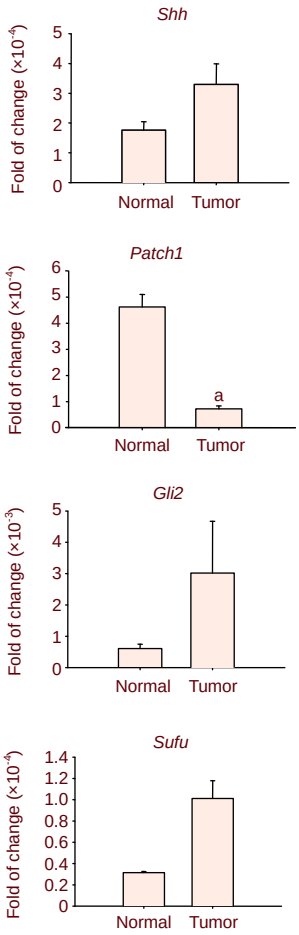

Figure 1 Relative gene expression of genes in the sonic hedgehog (*Shh*) pathway by real-time polymerase chain reaction. The $2^{-\Delta\Delta Ct}$ method was used to analyze gene expression. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as a reference gene. Compared with normal cerebella, the fold changes ($2^{-\Delta\Delta Ct}$) of genes are: *Shh* 1.83, *Patch1* 0.16, *Gli2* 4.98, *Sufu* 3.23. Data are expressed as mean ± SEM of three replicates (two-sample *t*-test). $^{a}P < 0.05$, *vs*. the normal group.

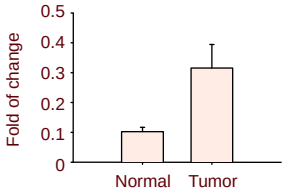

Figure 2 Relative gene expression of *β-cantenin* by real-time polymerase chain reaction. The $2^{-\Delta\Delta Ct}$ method was used to analyze gene expression. *Gapdh* was used as a reference gene. Normal indicates cerebella of transgenic mice without doxycycline hydrochloride treatment; tumor indicates those from transgenic mice with doxycycline hydrochloride treatment. Compared with normal cerebella, the fold change ($2^{-\Delta\Delta Ct}$) of *β-cantenin* is 2.45. Data are expressed as mean ± SEM of three replicates (two-sample *t*-test).

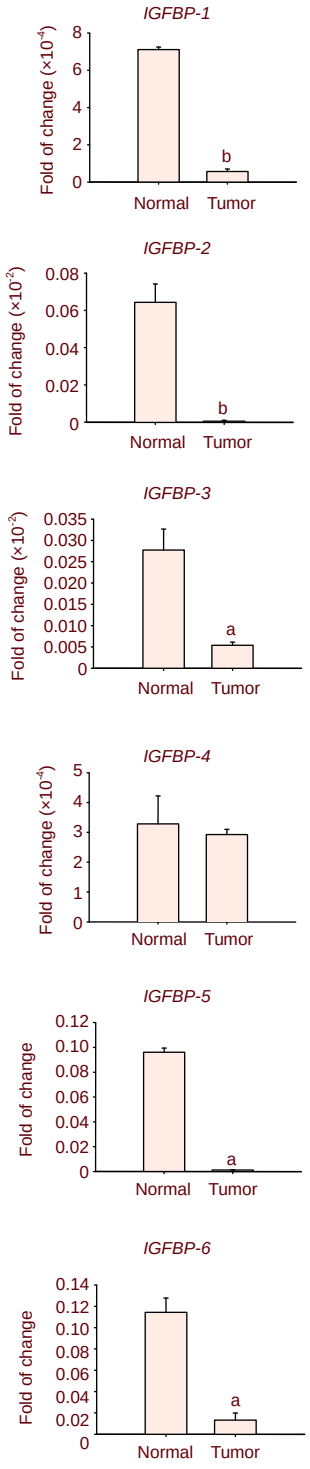

Figure 3 Relative gene expression of genes in the insulin-like growth factor (IGF) pathway by real-time polymerase chain reaction. The $2^{-\Delta\Delta Ct}$ method was used to analyze gene expression. *GAPDH* was used as a reference gene. Compared with normal cerebella, the fold changes ($2^{-\Delta\Delta Ct}$) of these genes are: *IGFBP-1,* 0.08, *IGFBP-2,* 0.01, *IGFBP-3,* 0.19, *IGFBP-4,* 0.89, *IGFBP-5,* 0.01, *IGFBP-6,* 0.12. Data are expressed as mean ± SEM of three replicates (two-sample *t*-test). $^{a}P < 0.05$, $^{b}P < 0.01$, *vs*. the normal group. IGFBP: Insulin-like growth factor binding protein.

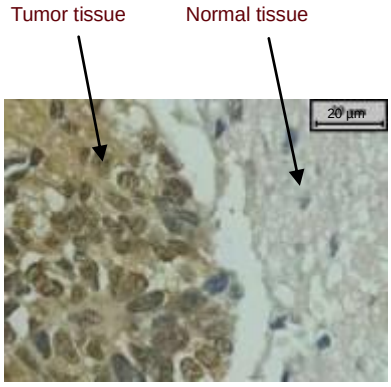

Figure 4 Insulin receptor substrate-1 (IRS-1) in a brain tumor (immunohistochemical analysis). An antibody against IRS-1 was used for this experiment. Arrows indicate the location of IRS-1 in either nuclei (tumor tissue) or cytoplasm (normal tissue).

DISCUSSION

Medulloblastoma, an embryonic tumor, occurs in the central nervous system. It is defined as a malignant tumor of the cerebellum that preferentially occurs in children, and has a strong tendency to metastasize[14]. Despite recent research advancing our understanding of the pathogenesis of medulloblastoma, the specific genes and signaling pathways involved in this brain tumor type remain to be elucidated[15].

Cancer is caused by the accumulation of genetic alterations, such as oncogenes or tumor suppressor genes. The pTet-on/pTRE-SV40Tag transgenic mouse model provides a useful model for studies on tumor development; however, the etiology of medulloblastoma has not been clearly elucidated. In this study, these mice were used to study gene expression changes in medulloblastomas using high-density microarrays. The advantages of microarrays include their high throughput and good coverage of the genome; however, the method still suffers from a lack of perfect processing methods and acceptable sensitivity. Thus, validation of the gene expression profiling data was performed using real-time polymerase chain reaction. Our results showed that most of the genes with altered expression were associated with different signaling pathways related to metabolism and the cell cycle.

Some cell signaling pathways contribute to medulloblastoma formation and malignancy. Different pathways are activated in different subsets of medulloblastomas. Confirming the deregulated pathways in individual human tumors could help with their classification and determination of patient prognosis. The SHH, WNT and IGF signaling pathways were examined in this medulloblastoma model to determine which pathways are deregulated. First, the SHH signal pathway was analyzed. Medulloblastomas originate from granule-cell progenitors located in the external granular layer[16] of the cerebellum[17]. The SHH signal pathway is an important mitogenic regulator of external granular layer progenitor cells and plays a critical role during development as well as regulation of both cell fate and proliferation[18]. The binding of secreted *Shh* to *Patch* relieves the inhibition of smoothened (*Smoh*) and results in the activation of *Patch* itself and the transcription factor *Gli*. Inappropriate activation of this pathway has been implicated in many tumors[19-20]. Mutations of the key mediators of the SHH pathway, including patched homolog (*Patch*), suppressor of fused homolog (*Sufu*) and smoothened homolog (*Smo*) have been described in about 30% of human medulloblastomas[21]. Our results show that *Patch1* was down-regulated in medulloblastomas while *Shh*, *Sufu* and *Gli2* showed no significant changes. Therefore, the SHH pathway may not participate in the pathological process in the present medulloblastoma model.

The WNT pathway is essential for proper development, cell-fate determination, proliferation and survival of neural progenitor cells[22]. It has been reported that approximately 15% of medulloblastomas show mutations affecting the WNT signal transduction pathway[23-24]. The binding of Wnt to the Frizzled receptor inactivates the complex containing adenomatous polyposis coli, glycogen synthetase kinase-3, axin and β-catenin and leads to the nuclear accumulation of β-catenin, which activates the transcription of genes such as cyclin D1[25]. Therefore, β-catenin is an important protein in this pathway. Our results show that β-catenin expression is significantly increased in medulloblastoma.

The IGF pathway is involved in cell growth, survival and tumor development[26]. IGF-binding proteins have growth-inhibitory effects by competitively binding to IGFs and preventing them from binding to IGF-1R[27]. Accumulated evidence suggests that changes in the levels of IGFs and IGF binding proteins were associated with increased risk of cancer[13, 28]. The expression levels of some genes related to the insulin and IGF pathways have been investigated[4]. Our results confirmed that the expression levels of *IGF-IR*, *IGF-II* and *IRS-1* were significantly changed in medulloblastoma[4]. Moreover, the expression levels of *IGFBP-1*, *IGFBP-2*, *IGFBP-3*, *IGFBP-5* and *IGFBP-6* were significantly decreased. Changes in the IGF pathway may contribute to the pathology of the medulloblastoma model.

IRS-1 is a major cytosolic signaling molecule for IGF-1R[29]. It is a crucial signaling molecule in both insulin and IGF-1R signal pathways. IRS-1 plays essential roles in IGF-1 mediated cell proliferation[28]. Co-transfection of IRS-1 and the SV40 Tag into R-cells could induce transformation. Furthermore, the SV40 Tag formed a complex with IRS-1, which played a critical role in transforming R-cells[30]. Therefore, there was an association between IRS-1 and the SV40Tag in brain tumor tissue, consistent with similar results in primary cultured tumor cells from the transgenic mouse model[4].

In summary, the SV40Tag may cooperate with components of IGF pathways, and play an important role in the pathological process leading to the development of medulloblastomas. Further studies using our animal model may contribute to an understanding of the molecular mechanisms of medulloblastoma and help with identification of therapeutic targets for the disease.

MATERIALS AND METHODS

Design

Microarray, real-time polymerase chain reaction, immunohistochemistry experiments.

Time and setting

This study was performed at the Shanghai Engineering Research Center of Molecular Therapeutics and New Drug Development, Key Laboratory of Brain Functional Genomics, MOE & STCSM, East China Normal University, China from December 2006 to January 2010.

Materials

A total of 12 specific pathogen-free pTet-on/pTRE-SV40Tag mice (six males and six females) aged 6 weeks were maintained in standard laboratory conditions in a 12-hour light-dark cycle. Six of them (three males and three females) were exposed to 0.05 mg/mL doxycycline hydrochloride (Sigma, St. Louis, MO, USA) in drinking water. Six mice without doxycycline hydrochloride treatment were selected as controls. Experimental procedures were performed in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by The Ministry of Science and Technology of China[31].

Methods

Microarray analysis

Total RNA was extracted and reverse transcribed into cDNA using the QIA RNA Extraction Mini Kit (Qiagen, Shanghai, China). High-density oligonucleotide microarray analysis was conducted as previously described[32]. Microarray experiments were performed using an Agilent G4121A Mouse Oligo Microarray Kit (Agilent, Shanghai, China) following the manufacturer's instruction. Data were analyzed using Imagene software (Biodiscovery, Shanghai, China) and normalized by Feature Extraction.

Real-time polymerase chain reaction

The primers for real-time polymerase chain reaction were designed using Primer 5.0 software and synthesized by Sangon (Shanghai, China). GAPDH was used as a reference gene. Polymerase chain reaction was performed in a 20-μL total reaction mixture volume, containing 1 μL of cDNA reaction products and 0.2 μL of SYBR Green I as follows: one cycle at 95°C for 10 minutes, followed by 38 amplification cycles, each cycle consisting of denaturation at 95°C for 30 seconds, primer annealing at 59°C for 30 seconds and extension at 72°C for 30 seconds. Experiments were performed in an Opticon 2 thermal cycler (MJ Research, USA). Gene expression levels were calculated and presented as $2^{-\Delta\Delta Ct}$ values as previously described[33]. The polymerase chain reaction primers used are as follows:

Gene	Sequence (5'–3')	Product size (bp)
GAPDH	Upstream: TCA ACG ACC CCT TCA TTG AC Downstream: ATG CAG GGA TGA TGT TCT GG	512
β-catenin	Upstream: TCT ACG CCA TCA CGA CAC Downstream: CAG ACA GAC AGC ACC TTC	292
Shh	Upstream: CGG CAG ATA TGA AGG GAA GA Downstream: GGT CCA GGA AGG TGA GGA AGT	554
Gli2	Upstream: CAC AGG GCG GGC ACA AGA Downstream: GGA GGG CAG TGT CAA GGA A	343
Sufu	Upstream: TCC AGG TTA CCG CTA TCG TC Downstream: TCC ACT GTT GGG CTG AAT GT	489
IGFBP-1	Upstream: GCC CGA GTT CCT AAC TGT TG Downstream: CAG CAG CCT TTG CCT CTT C	386
IGFBP-2	Upstream: CCA GAC GCT ACG CTG CTA TC Downstream: CAC TGC TAC CAC CTC CCA AC	200
IGFBP-3	Upstream: GCA GCC TAA GCA CCT ACC T Downstream: CCT CTG GGA CTC AGC ACA T	337
IGFBP-4	Upstream: GGT TGC GAG GAG TTG GTG Downstream: GTG GGT ACG GCT CTG TGA G	441
IGFBP-5	Upstream: TGA GAT GAG ACA GGA ATC CGA ACA Downstream: CGA AGG CGT GGC ACT GAA AG	257
IGFBP-6	Upstream: CCG TCG GAG GAG ACT ACA AAG Downstream: CCA TCT GGA GAC ACT GGC AAA	347

Histological and immunohistochemical analysis

Tissues were cut into 0.5 cm^3 pieces, fixed in 10% paraformaldehyde and embedded in paraffin. Immunohistochemical analysis was performed using rabbit anti-mouse IRS-1 monoclonal antibody (1:400, Upstate, Shanghai, China) at 37°C for 1 hour. Tissues were incubated with normal goat serum at room temperature for 20 minutes, and treated with antibody overnight after quenching endogenous peroxidase with 0.6% hydrogen peroxide. The tissues were then incubated with biotinylated secondary antibody (goat anti-rabbit antibody, 1:5 000; Zhongshan Golden Bridge, Beijing, China) at 37°C for 1 hour and avidin DH-biotinylated horseradish peroxidase-H complex. Immunoreactivity was visualized with diaminobenzidine

from the streptavidin biotin complex kit (Boster, Wuhan, China) according to the manufacturer's protocol. Nuclei were counterstained with hematoxylin. Slices were observed under microscopy and photographed (Olympus, Tokyo, Japan).

Statistical analysis

All experimental data are expressed as the mean ± SEM of three repeated independent experiments. Statistical analysis was performed by two-sample *t*-test for comparisons between groups. A value of $P < 0.05$ was considered to represent statistical significance.

Acknowledgments: We would like to express our sincere thanks to Dr. Qiang Sun for his theoretical support and technical guidance.

Funding: This work was supported by the National Natural Science Foundation of China, No. 31000574; the Fundamental Research Fund for the Central Universities, No. 78210042; and the Development Program of China during the 10th Five-Year Plan Period, No. 2001BA70113.

Author contributions: Xiaoluan Wei implemented the majority of experiments, wrote the manuscript, and analyzed the data. Jie Feng was responsible for providing information and technology. Yinghe Hu conceived and designed this study, and reviewed the manuscript.

Conflicts of interest: None declared.

Ethical approval: The project received full approval from the Animal Ethics Committee of East China Normal University, China.

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(Edited by Wang YT, Deng H/Qiu Y/Wang L)