

Original Article

Gene profiling analysis for patients with oral verrucous carcinoma and oral squamous cell carcinoma

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Received February 23, 2014; Accepted April 9, 2014; Epub July 15, 2014; Published July 30, 2014

Abstract: Oral verrucous carcinoma (OVC) is one malignant tumor which was carved out from the oral squamous cell carcinoma (OSCC). However, the clinical and pathological features as well as the treatment strategies of OVC are different from OSCC. Here, global transcript abundance of tumor tissues from five patients with primary OVC and six patients with primary OSCC including their matched adjacently normal oral mucosa were profiled using the Affymetrix HGU133 Plus 2.0. Ingenuity Systems IPA software was used to analyse the gene function and biological pathways. There were 109 differentially expressed genes (more than 2-fold) between OVC and the adjacently normal tissue, among them 66 were up-regulated and 43 were down-regulated; 1172 differentially expressed genes (more than 2-fold) between OSCC and the adjacently normal tissue, among them 608 were up-regulated and 564 were down-regulated. There were 39 common differentially expressed genes in OVC and OSCC compared with their matched normal oral mucosa, among them 22 up-regulated and 17 down-regulated, and 8 of them different between OVC and OSCC. In addition, the gene expression profile was further validated by quantitative real-time PCR (Q-RT-PCR) analysis for four of those 39 selected genes.

Keywords: Oral verrucous carcinoma, oral squamous cell carcinoma, biological pathways, expression profile, quantitative real-time PCR

Introduction

Verrucous carcinoma is a special form of squamous cell carcinoma with its own clinical and histological features. It was first described as a different entity of tumor from squamous cell carcinoma by Lauren V. Ackermann in 1948, so verrucous carcinoma is also known as "Verrucous carcinoma of Ackermann" or "Ackermann's tumor" [1]. Verrucous carcinoma frequently affects multiple organs and tissues including the oral mucosa, esophagus, leg, temporal bone, eye, penis, buttocks, foot, toe, skin, hand etc. [2-9]. Oral cavity is the most common site for verrucous carcinoma, which is thus called Oral Verrucous Carcinoma (OVC). OVC is slow growing, locally invasive and is not supposed to metastasize, but it can grow very large and can destroy adjacent tissue such as

bone and cartilage [10], and some cases have local lymphatic metastases and recurrence [11]. The diagnosis of OVC is established by close communication between surgeons and pathologists. Like oral squamous cell carcinoma (OSCC), surgery is considered as the treatment of choice, but it is not thought to be necessary to perform a neck dissection [12, 13]. Nonetheless, the molecular mechanisms of OVC remain unclear. Recent research has shown that there were some genes differentially expressed between OVC and OSCC, such as α B-crystallin [14], matrix metalloproteinase-2 [15], matrix metalloproteinase-9 [16] and vascular endothelial growth factor [16], but there is no overall study on OVC and OSCC. The aim of the present study was to identify differential gene expression profiles between OVC and OSCC and find new possible molecular biomark-

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Table 1. Clinical and pathological features of patients

Case	Diagnosis	Primary Site	Age	Sex
1	OVC	lower lip	48	Male
2	OVC	gingiva	45	Male
3	OVC	gingiva	53	Male
4	OVC	gingiva	63	Male
5	OVC	tongue	46	Male
6	OSCC	tongue	63	Male
7	OSCC	lower lip	53	Male
8	OSCC	tongue	68	Male
9	OSCC	gingiva	60	Female
10	OSCC	buccal	45	Male
11	OSCC	gingiva	46	Male

Table 2. Primers used for RT-qPCR to verify array-identified genes

Gene	Primer	Sequence (5'→3')	Amplification Size
MMP1	F	GTGCTACACGGATACCCCAAG	205
	R	GGCCAATCCAGGAAAGTCAT	
SERINE1	F	GCGCTGTCAAGAAGACCCA	242
	R	AACACCCTCACCCGAAGT	
MAL	F	GTCACCTGGACGCAGCCTA	249
	R	AACACCATCTGGGTTTTTCAGC	
DNASE1L3	F	GAGCCCTTTGTGGTCTGGTT	159
	R	AATGAAATTCTCCGCCTTCC	
GAPDH	F	TGTTGCCATCAATGACCCCTT	202
	R	CTCCACGACGTACTCAGCG	

ers genes in OVC, which was useful for the clinical diagnosis and therapies.

Materials and methods

Patients and samples

We collected the primary cancer and the matched normal oral mucosa tissues obtained from 5 patients with OVC and 6 patients with oral squamous cell carcinomas (OSCC). All the patients underwent surgery from January 2007 to December 2009 in Xiangya Hospital, Central South University. Consent was taken from all the patients after getting the institutional ethical approval. The selected patients did not receive any preoperative chemotherapy or radiotherapy or experience any other cancer. Each sample was confirmed by pathologic analysis and anonymized prior to the study. Two samples of 1 cm size were immediately cut (within 15 minutes) from the tumor resected by

a standard surgical procedure, snap frozen in liquid nitrogen, and stored at -80°C until use. The clinicopathological features of patients are shown in **Table 1**. Written informed consent was acquired from all participants in this study, according to the declaration of Helsinki. The study was reviewed and approved by the Medical Ethics Committee of the Changsha Xiangya Hospital.

Total RNA isolation

Total RNA was isolated from the frozen tissues using the TRIzol Reagent (Invitrogen Life Technologies, Carlsbad, CA; P/N 15596-018) according to the manufacturer's protocol. The aqueous phase containing the RNA separated from the TRIzol reagent was further purified using the RNeasyMinElute Cleanup Kit (Qiagen, Valencia, CA; 74204). The quality of total RNA was then assessed by agarose gel electrophoresis of A260/280 ratio and by analysis on the Bioanalyser 2100 (Agilent).

Target sample preparation

One microgram of total RNA was first reverse transcribed using a T7-Oligo (dT) Promoter Primer in the first-strand cDNA synthesis reaction. Following RNase H-mediated second-strand cDNA synthesis, the double-stranded cDNA is purified and serves as a template in the subsequent in vitro transcription (IVT) reaction. The IVT reaction is carried out in the presence of T7 RNA Polymerase and a biotinylated nucleotide analog/ribonucleotide mix for complementary RNA (cRNA) amplification and biotin labeling. The biotinylated cRNA targets are then cleaned up, fragmented, and hybridized to GeneChip expression arrays.

Microarray hybridization and processing

Gene expression profiling was performed using the HGU133 Plus 2.0 Affymetrix GeneChip platform, this microarrays contain approximately 38,500 genes profiled (47,000 distinct transcripts assayed). Hybridization with the biotin-labeled RNA, staining and scanning of the HGU133 Plus 2.0 chips followed the prescribed procedure outlined in the Affymetrix technical

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Table 3. The first 5 Associated Network Functions altered genes in OVC vs matched normal oral mucosa

Net-work	Genes in Ingenuity networks ¹	Function	Score ²	Focus molecules
1	CCR7, CD1C, CDSN, collagen, Collagen type IV, CTSC, CYP2E1, CYTIP, Cytokeratin, DEFB1, FABP4, Gm-csf, IFI6, IFN Beta, IgG, IL24, IL12 (complex), INPP5D, Interferon alpha, KLK7, KRT4, KRT13, KRT17, LDL, LGALS1, LIPG, MMP1, MMP11, NFkB (complex), PI3, PLAC8, Tgf beta, TGFBI, TNFAIP6, TXNIP	Dermatological Diseases and Conditions, Genetic Disorder, Inflammatory Response	53	24
2	Akt, Ap1, BLNK, CAV1, CCL20, COL4A1, COL4A2, Collagen Alpha1, CST6, CTSL2, CXCR4, ERK1/2, Focal adhesion kinase, FSH, Hcg, IL1, INHBA, Laminin, Lh, P38 MAPK, Pdgf (complex), PDGF BB, PFN2, PP2A, PPP2R2C, PROCR, SEC14L2, SELL, SERPINE1, SLC1A1, Sos, STC1, TCL1A, TEAD4, Vegf	Cellular Movement, Hematological System Development and Function, Immune Cell Trafficking	39	19
3	AKR1C3, ALDH3A1, AVPI1, BTLA, CCL23, CTSL2, CXCR7, DLG4, DNASE1L3, EHF, EIF4E, GPX4, GPX4, Hmgb1, HNF1A, HTT, IL13, KLF11, MMP10, MTMR2, NEFH, NEFL, NEFM, PI3, POU3F1, ROBO2, SEMA3C, SPRR2A (includes others), STRA6, TCR, TIMP4 TNF, WISP1, WNT1, WNT10B	Neurological Disease, Cell-To-Cell Signaling and Interaction, Cellular Assembly and Organization	26	14
4	ADRA1D, AKR1B10, ATRIP, BATF3, CCNG2, CDK2, CITED2, COL1A2, COL7A1, CTBP1, EPB49, ESRRG, GNB2L1, HLF, HLTf, JUN, KIF1B, KRT17, LARP6, LGALS1, mevalonic acid, MMD, NODAL, NOV, NR2F1, NRIP1, PKN1, SAA1, SLC27A6, SP1, SPAG4, TGFBI, Thyroid hormone receptor, TMED4, WFDC5	Cell Cycle, Cellular Development, Embryonic Development	22	12
5	26s Proteasome, AKT3, AQP2, ARRD4, BSG, Caspase, DDIT4, DUSP1, DUSP4, ERK, FOXE1, HERPUD1, HLA-DQA1, HPGD, Hsp90, IL17R, Insulin, Jnk, JUN/JUNB/JUND, Mapk, MATK, mevalonic acid, Mmp, PI3K (complex), Pka, Pkc(s), PODXL, Ras, RGS13, RNA polymerase II, SNCG, sphingomyelinase, Ubiquitin, UTS2, WISP1	Cell Cycle, Cellular Movement, Immunological Disease	11	7

¹Genes in bold were identified by microarray analysis; other genes were either not on the expression array or did not change significantly. ²A score >3 was considered significant.

manual. Hybridization was performed at 45°C for 16 hour using the Hybridization Oven 640 (Affymetrix). Washing and staining was done using Fluidics Station 450 (Affymetrix). Images were acquired using the Affymetrix Gene Array scanner. Images were acquired using the GeneArray scanner 3000 5 (Affymetrix).

Microarray data analysis

Scanned output files were visually inspected for hybridization artifacts, the statistical analysis of microarrays method was used to do the data preprocessing, include masking and background subtraction. Then used the pre-processed data did the signal value (expression values) calculated, and get the *p*-value and fold change. Genes exhibiting marked differences (fold change >2 or <0.05) of expression between two analyzed groups were selected by SAM application supplied by Multi Experiment Viewer. The IPA software was used for network analyses.

Confirmatory Q-RT-PCR analysis

We quantified the expression of 4 genes by Q-RT-PCR analysis: MMP1, SERINE1, MAL and DNASE1L3. Total RNA was performed as described above. Reverse transcription (RT) was carried out using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA)

after pretreatment with RNase-free DNase I (Roche, Indianapolis, IN, USA). The primer sequences for each gene are shown in **Table 2**, all primers were designed using the IDT SciTools software (<http://www.idtdna.com/SciTools/SciTools.aspx>) and are synthesized by Invitrogen. Actin acted as endogenous control and all the samples were assayed in duplicate. The SPSS/PC software package version 17.0 was used for collection, processing, and statistical data analysis. Statistical analysis was performed using the non-parametrical Wilcoxon test for comparison of paired samples. *p*<0.05 values were considered statistically significant.

Results

Differentially expressed genes in OVC vs matched normal oral mucosa

Gene expression analysis revealed a total of 109 altered genes (genes that were over or under-expressed more than 2-fold) with 66 up-regulated and 43 down-regulated genes in OVC compared with its matched normal oral mucosa (OVCN). Functionally analyzed build on the existing pathway using the IPA knowledge base, the network with the highest score (network 1, score = 53) was generated with 24 focus genes. The first 5 Associated Network Functions altered genes are shown in **Table 3**.

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Table 4. The first 5 Associated Network Functions altered genes in OSCC vs matched normal oral mucosa

Net-work	Genes in Ingenuity networks ¹	Function	Score ²	Focus molecules
1	AIM1, AMPK, ANGPT2, ANGPTL2, ANPEP, AURKA, CALU, CCDC64B, ECM1, EGR1, EMP1, EMP2, ENPEP, EPHX2, ERBB2, ERBB3, ETS1, GDPD3, HES1, HMMR, ID4, MELK, MXD1, NPNT, PRKCDP, PTGES, RAB10, SF3A3, Sphk, SRGN, TMEM158, TNS3, VAV2, Vegf, VEGFC	Cardiovascular Disease, Cellular Growth and Proliferation, Cancer	40	32
2	AASS, BGN, C2, C1q, C1QA, C1QB, C1QC, CAND2, CD207, COL11A1, COL16A1, COL4A6, COL5A1, COL5A2, COL5A3, COL6A2, COL6A3, Complement component 1, DFNA5, EXT1, FNDC3B, FXYP5, GATM, Igm, KDELR3, LOXL2, MPHOSPH9, OVOL1, PCOLCE2 (includes EG:26577), PDLIM5, PLXNC1, PMM1, SLC16A3, ST3GAL5, TGFB1	Connective Tissue Disorders, Genetic Disorder, Dermatological Diseases and Conditions	39	32
3	Akt, ANGPTL1, ARSI, Aryl Sulfatase, CADM1, CBP-ICSBP-IRF-1-PU.1, CDH13, CYBA, CYBB, EPB41L3, FABP7, FOXC1, GNS, HOXA1, HOXB7, HOXD10, LPXN, MAG11, MYH11, N-acetylglucosamine-6-sulfatase, NCF2, NMB, PAX9, PBX1, Phox, Rac/Cdc42, SORBS2, SRPK2, ST8SIA4, SULF1, SULF2, THBS2, TLE2, WDR26	Free Radical Scavenging, Genetic Disorder, Immunological Disease	35	29
4	CCNB2, Cdc25, CDK1, CKS2, COL10A1, COL18A1, COL1A1, COL1A2, COL3A1, Collagen type I, Collagen(s), CRABP1, CTSS, CTSL1, Cyclin B, DLGAP5, ESPL1, Gelatinase, HMGA2, ITGAV, MXI1, NID2, P4HA1, PRELP, PTHLH, Rbp, RBP1, RBP7, SAMSN1, SCNN1B, SPARC, TGFBI, TM4SF1, TNC, USP6NL	Connective Tissue Disorders, Genetic Disorder, Cellular Assembly and Organization	33	29
5	ACP5, ACP6, AQP3, BASP1, Calpain, CDKN2A, DPP3, ERMP1, FNDC1, FSH, G protein beta gamma, Integrin, ITGB4, KLF5, LDL, LEPRE1, MMP2, MMP9, MMP14, MYCN, NUCB1, OGN, P4HA2, PHGDH, PLAGL1, PLAU, PMEPA1, RAD51AP1, RILPRNA polymerase II, SC4MOL, SERPINE1, SLFN12, SORCS2, Tgf beta	Cardiovascular System Development and Function, Tumor Morphology, Organismal Development	32	28

¹Genes in bold were identified by microarray analysis; other genes were either not on the expression array or did not change significantly. ²A score >3 was considered significant.

Table 5. Twenty-four focus genes in network 1

Gene ID	Gene symbol	Gene name	Fold change
206193_s_at	CDSN	corneodesmosin	14.67
211964_at	Collagen type IV (COL4A2)	collagen, type IV, alpha 2	2.38
225647_s_at	CTSC	cathepsin C	2.23
210397_at	DEFB1	defensin, beta 1	2.21
203980_at	FABP4	fatty acid binding protein 4, adipocyte	4.15
204415_at	IFI6	interferon, alpha-inducible protein	2.75
206569_at	IL24	interleukin 24	2.10
205778_at	KLK7	kallikrein-related peptidase 7	3.50
205157_s_at	KRT17	keratin 17	2.82
201105_at	LGALS1	lectin, galactoside-binding, soluble, 1	2.01
219181_at	LIPG	lipase, endothelial	2.87
204475_at	MMP1	matrix metalloproteinase 1 (interstitial collagenase)	7.47
203878_s_at	MMP11	matrix metalloproteinase 11 (stromelysin 3)	2.17
203691_at	PI3	peptidase inhibitor 3, skin-derived	2.29
201506_at	TGFBI	transforming growth factor, beta-induced, 68kDa	2.10
206026_s_at	TNFAIP6	tumor necrosis factor, alpha-induced protein 6	2.50
206337_at	CCR7	chemokine (C-C motif) receptor 7	0.30
205987_at	CD1C	CD1c molecule	0.47
209975_at	CYP2E1	cytochrome P450, family 2, subfamily E, polypeptide 1	0.42
209606_at	CYTIP	cytohesin 1 interacting protein	0.39
203332_s_at	INPP5D	inositol polyphosphate-5-phosphatase, 145kDa	0.40
207935_s_at	KRT13	keratin 13	0.42
213240_s_at	KRT4	keratin 4	0.37
219014_at	PLAC8	placenta-specific 8	0.32
201008_s_at	TXNIP	thioredoxin interacting protein	0.43

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Differentially expressed genes in OSCC vs matched normal oral mucosa

Gene expression analysis revealed a total of 1172 altered genes (Genes that were over or under-expressed more than 2-fold) with 608 up-regulated and 564 down-regulated genes in OVC compared with its matched normal oral mucosa (OSCCN). The network with the highest score (network 1, score = 40) was generated with 32 focus genes. The first 5 Associated Network Functions altered genes are shown in **Table 4**.

The common genes in OSCC, OVC compared with its matched normal oral mucosa

Gene expression analysis revealed a total of 167 altered genes (Genes that were over or under-expressed more than 2-fold) with 108 up-regulated and 59 down-regulated genes in OSCC compared with OVC. We focused on a total of 39 common genes (Genes that were over or under-expressed more than 2-fold) with 22 up-regulated and 17 down-regulated genes in OSCC and OVC compared with its matched normal oral mucosa. There were 8 of the 39 genes were differently expressed between OVC and OSCC, which were ADAMTS12, COL4A1, COL4A2, INHBA, MMP1, SERPINE1, TGFBI, HLF, and all were up-regulated except HLF. All the 39 common genes are shown in **Table 5**. Among of these 39 genes, 11 genes focus on the associated network functions: dermatological diseases and conditions, genetic disorder, inflammatory response and 8 genes focus on the associated network functions: cellular movement, hematological system development and function, immune cell trafficking. All these results suggest that OSCC and OVC not only have common genetic and molecular basis, but probably have independently regulatory mechanisms in vivo.

Common genes in OSCC and OVC displayed different gene expression level

To validate the microarray results, we performed Q-RT-PCR analysis for 4 genes (MMP1, SERPINE1, MAL and DNASE1L3) from 5 samples of OVC, OSCC and their matched paracancerous and normal oral mucosa tissue, independently. The Q-RT-PCR expression of the selected genes was in accordance with corresponding microarray data. SERPINE1 mRNA level is reduced gradually in OVC, paracancerous tis-

sue of Oral verrucous carcinoma (OVC-P) and normal mucosa tissue of Oral verrucous carcinoma (OVC-N), just similar to expression level of OSCC and their matched paracancerous and normal oral mucosa tissue. Specifically, expression of MMP1 was increased over 400-fold in OSCC related to paracancerous tissue of Oral squamous cell carcinoma (OSCC-P) and normal mucosa tissue of Oral squamous cell carcinoma (OSCC-N). MMP1 mRNA level is also high in OVC related to those in OVC-P and OVC-N. The results indicated that MMP1 maybe play a significant role in developing oral cancer [17]. The expression level of MAL was increased gradually in OVC, OVC-P and OVC-N, and the similar expression pattern occurred among OSCC, OSCC-P and OSCC-N. Most specially, transcription level of DNASE1L3 is reduced gradually in OVC, OVC-P and OVC-N, but is increased gradually in OSCC, OSCC-P and OSCC-N. In all, these differently expressed genes provide clues about the carcinogenesis of OVC and OSCC.

Discussion

Oral verrucous carcinoma is a rare variant of Oral squamous cell carcinoma (OSCC), but little is known about the molecular mechanisms for its malignant development. The genetic and epigenetic alterations are related to most of cancer progression which may also be closely linked with oral verrucous carcinoma. It is, therefore, essential for understanding OVC to make comprehensive gene expression profiling. We compared with squamous cell carcinoma in order to better differentiate it from squamous cell carcinoma. In undertaking this analysis, we have identified several genes that are differentially expressed in OVC and OSCC compared with its matched normal oral mucosa. In addition, we have identified 39 common genes are differentially in OSCC and OVC compared with its matched normal oral mucosa. 8 of these 39 common genes were different between OVC and OSCC. These findings can be a good description of that OVC is a rare variant of OSCC but it's different of OSCC from the molecular point of view. All the 8 genes were ADAMTS12, COL4A1, COL4A2, INHBA, MMP1, SERPINE1, TGFBI, HLF, which we thought were one of the major research directions.

MMPs are a family of zinc-dependent proteases that can collectively degrade all components of the extracellular matrix [18]. MMP activity is

tightly regulated at the level of transcription and activation by proteolytic cleavage. Proteins of the matrix metalloproteinase (MMP) family are involved in the breakdown of extracellular matrix in normal physiological processes, such as embryonic development, reproduction, and tissue remodeling, as well as in disease processes, such as arthritis and metastasis. Matrix metalloproteinase (MMP1) is one of the most abundant proteases in the matrix metalloproteinase family. It is capable of degrading type I, II and III collagens, and plays a pivotal role in extracellular matrix (ECM) remodelling in both normal development and pathology [19]. It plays a clinically important role in inflammatory disease, and has been implicated in numerous pathological processes including wound healing, arthritis and tumour metastasis [20-22]. Some studies have shown that MMP-1 is a hallmark of human metastatic cancer, and its over expression represents a high risk factor that adversely correlates with overall survival of patients with invasive breast carcinoma [23-25]. Some research showed that the complex roles of MMP in tumor progression of sarcomas, not only does metastasis seem to be affected by MMP1 silencing, but also local tumor growth and angiogenesis are affected inversely [26]. In our research, MMP-1 was up-regulated in OVC and OSCC control with their normal oral mucosa, and highly up-regulated in OSCC. This finding implies OVC is a low-grade variant of OSCC, with slow growth, no metastatic potential and lowest invasive potential.

SERPINE1 (serpin peptidase inhibitor, clade E, member 1) plays an important role in tumorigenesis and invasion as a primary inhibitor of plasminogen activators [27]. High expression of SERPINE1 is predictive of a poor prognosis for survival of patients with cancer [28, 29]. Previous studies have shown that that SERPINE1 is mainly expressed in cancer cells, such as ovarian cancer, colorectal cancer and OSCC, but not in normal oral mucosa. But the mechanisms responsible for the up-regulation of SERPINE1 in OVC remain unclear. In this paper, we found that the SERPINE1 is predominantly expressed in OVC and OSCC compared with their normal oral mucosa. This conclusion is in agreement with the microarray data.

INHBA (inhibin, beta A) is a subunit of both activin and inhibin, two closely related glycoproteins with opposing biological effects. The

INHBA subunit joins the alpha subunit to form a pituitary FSH secretion inhibitor. Inhibin has been shown to regulate gonadal stromal cell proliferation negatively and to have tumor-suppressor activity. In addition, serum levels of inhibin have been shown to reflect the size of granulosa-cell tumors and can therefore be used as a marker for primary as well as recurrent disease [27]. INHBA is also a ligand in the transforming growth factor-beta (TGF- β) superfamily [28], INHBA also stimulates inflammatory corneal angiogenesis by increasing vascular endothelial growth factor (VEGF) levels [29]. VEGF expression may have prognostic significance for patients with HNSCC [30].

In summary, the study of gene expression in OVC and OSCC on a genome-wide scale was achieved successfully. There are obvious differences in gene expression between OVC and OSCC. 167 known genes were differentially expressed between OSCC and OVC, among of them 108 were up-regulated and 59 were down-regulated. The common differential expressed genes between OVC and OSCC compared with their matched normal mucosa were 39, among of them 22 were up-regulated, 17 were down-regulated, and 8 of them were differentially expressed between OVC and OSCC. These 8 genes may determine the identity differences of the two cancers. It remains to be determined whether their 8 genes can discriminate between OVC and OSCC in a much larger study.

Acknowledgements

This project was supported by The National Natural Science Foundation of China (3087-2895), The key Program of Department of Science and Technology of Hunan (2008FJ-2011) and the Natural Science Foundation for Distinguished Young Scholars of Hunan Province (S2013J504B).

Disclosure of conflict of interest

None.

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