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Full Length Research Paper

Genes involved in the anti-cancer effect of a potent new compound boehmeriasin A on breast cancer cell

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Boehmeriasin A is a new compound showing good anticancer effect on breast cancer cell: MDA-MB-231 in vitro. The anti-cancer effect of it was revealed by gene chip and suppression subtractive hybridization (SSH). Totally, there are 328 genes of differential expression obtained from gene chip analysis. Two subtractive libraries were constructed and 163 EST cDNA clones were screened out. RT-PCR, virtual Northern, Northern and Western blot were applied to confirm the result. In conclusion, Boehmeriasin A lowered the expression of genes involved in apoptosis, while promoted expression of some differentiation related genes. This study supplies further evidence that boehmeriasin A inhibit the growth of breast cancer cells through inducing differentiation. In addition, the compound may lead to cell growth arrested via restraining the expression of genes related to cell proliferation and cell cycle regulation. Expression of genes involved in TGF- 1 signal transduction were varied, suggesting the effect of boehmeriasin A is closely associated with TGF- 1 pathway.

Key words: Boehmeriasin A, anti-cancer, SSH, gene chip.

INTRODUCTION

Boehmeriasin A is a new phenanthroquinolizidine alkaloid recently isolated from Boehmeria siamensis Craib (Urticaceae). It's structure was elucidated as 3,6,7-trimethoxy-11,12,13,14,14a,15-hexahydro-9H-phenanthro[9,10-b] quinolizidine on the basis of spectral data including 1D and 2D nuclear magnetic resonance, ultraviolet, infrared, and mass spectrometry data.

In our initial in vitro study, it was demonstrated that this novel compound had strong antitumor activity on breast cancer cell: MDA-MB-231 (Yan et al., 2006). Proliferation assay and fluorescence activated cell sorter (FACS) showed that cell growth inhibition and G1 phase arrest of cell cycle were caused by boehmeriasin A. Exposed in 7 × 10-3 g/ml boehmeriasin A for 12 h, cells in G1 phase increased from 44.8 to 66.3%. Few apoptotic cells were detected, and most cells underwent differentiation, which was characterized by specific changes in cell morphology, significant lipid droplet accumulation. The result demonstrated that boehmeriasin A potently inhibited the proliferation of breast cancer cell (MDA-MB-231) via the G1 phase cell cycle arrest and differentiation induction.

In order to shed more light on the effect of Boehmeriasin A, gene chip and suppression subtractive hybridization (SSH) were used to pinpoint the genes expressed differentially after boehmeriasin A treatment. Identification and characterization of gene expression patterns in boehmeriasin A treated breast cancer cells will contribute to a better understanding of its anticancer mechanisms, and ultimately permit development of a promising drug for breast cancer.

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Abbreviations: SSH, suppression subtractive hybridization; DMEM, Dulbecco's modified eagle media; FBS, fetal bovine serum; TGF- , Transforming growth factor- .

MATERIALS AND METHODS

Experimental cell model, RNA isolation

The breast cell line MDA-MB-231 (obtained from ATCC) was grown in Dulbecco's Modified Eagle Media (DMEM) supplemented with

10% fetal bovine serum (FBS), 100 U/ml penicillin/streptomycin. When cell confluence reached about 80%, 7×10-3 g/ml Boehmeriasin A was added. Our previous study indicated that the number of living cells almost did not change exposed in 0.007 g/mL boehmeriasin A for more than 96 h (Yan et al., 2006). So we decided the time points included 1d, 3d, and 7d after compound added. The treated and control cells were collected and named as following.

D0: control cells
D1: cells collected 1 day after addition of boehmeriasin A
D3: cells collected 3 day after addition of boehmeriasin A
D7: cells collected 7 day after addition of boehmeriasin A

The cellular RNA were isolated with Trizol Reagent (BRL) according to the manual and stored at -80°C.

Suppression subtractive hybridization

Briefly, the SMART PCR cDNA synthesis kit (BD Biosciences Clontech; Ontario, Canada) was used to generate double-stranded cDNA for D0, D1 samples according to the manufacture's instruction. To produce the first-strand cDNA,1 g RNA from each group was reverse transcribed in a total volume of 10 l with the addition of 3' SMART CDS Primer A (10 mol/l) and PowerScript reverse transcriptase. Second-strand cDNAs were generated with SMART A Oligonucleotide, followed by a PCR amplification of 17 cycles for D0,D1 sample with the 5' PCR Primer A. Purified cDNAs were digested with RsaI to generate blunt-ended cDNA fragments (0.2 - 2 kb) suitable for optimal subtractive hybridization.

In subtractive hybridization, with D1 cDNA as the tester, D0 cDNA as the driver, the D1- D0 subtractive cDNA pool representing the cDNA of increased expression after treatment was got, Inversely, with D0 cDNA as the tester, D1 cDNA as the driver, the D0-D1 subtractive cDNA pool represents the cDNA of decreased expression after treatment.

Subtraction efficiency was assessed via PCR amplification using G3PDH specific primer by comparing the abundance of cDNAs before and after subtraction. Aliquots (5 l) were removed after 15, 20, 25 and 30 cycles for analysis on agarose gel. Subtraction efficiency was estimated by noting the different number of cycles needed to generate approximately equal amounts of the corresponding PCR product in subtracted and unsubtracted samples.

Cloning of subtracted cDNAs

The subtractive cDNAs were cloned into the pGEM-T Easy vector (Promega) to construct the subtracted libraries.

Differential hybridization screening

The subtractive libraries were screened for the false positive using Select Differential Screening Kit (BD Biosciences Clontech) . Briefly the insert of each cDNA clone was amplified by PCR (25 cycles) with the Nested PCR Primer 2R (NP2R) and Nested PCR Primer 2 (NP1) supplied by the manufacturer. Each amplification product was denatured at 95°C for 2 min, then put on ice for 5 – 10 min immediately, and then transferred onto nylon membrane to make cDNA microarray with Multi- blotreplicator (V&P Scientific, INC, San Diego, USA). PPAR- cDNA (which expression has been identified by RT- PCR before) as positive control, G3PDH cDNA as internal control and 1R, 2R cDNAs as negative control were also transferred onto each membrane. Four identical microarray membrane containing 756 cDNA from clone PCR and 12 control cDNA were produced for each library. Then, the subtractive D1 - D0, D0 - D1 cDNA and unsubtractive D0, D1 cDNA were used as hybridization probes for differential screening of microarrays of the cDNA library. The probe was labeled by DIG-High Prime Kit (Roche). Equal quantities of the four heat-denatured probes were used to hybridize separately with four similar membranes. After hybridization and washing, the hybridization signal was detected with NBT/BCIP and scanned by Bio-Rad GS-700.

DNA sequencing and sequence analysis

Ultimately, 87 clones were sequenced and their nucleic acid sequences were analyzed by BLAST2.0 against GenBank database. To be considered homologous to a GenBank sequence, a cDNA sequence was required to have at least 200bp matched. The differentially expressed cDNA clones were then categorized into three groups: 1) old genes with known function and sequence, 2) old genes with known sequence and unknown function, 3) new genes.

Gene chip test

Total RNA was isolated from untreated cells (D0) and cells (D1) treated with boehmeriasin A for 1 day. The differentially expressed genes were analyzed by Shanghai United Gene Holdings Limited with gene chip technology.

Gene expression analysis

Semiquantitative RT-PCR and Virtual Northern, Northern and Western Blot were applied to confirm the differential expression pattern of selected cDNA clones identified by SSH. GAPDH was control gene.

Total RNA extracted from D0, D1, D3, D7 sample were subjected to Northern blot analysis as previously described using Northern Max-Gly kit (Ambion Inc.;Austin,TX). Probes were labeled with digoxin by random priming. Hybridization signals were detected by chemiluminescence reagent CDP-Star (NEB). Virtual Northern is similar to conventional Northern blots except that mRNA is inverse-transcribed to cDNA in large amounts with SMART PCR cDNA synthesis kit. These cDNAs could be separated by electrophoresis to obtain virtual Northern blots, which could imitate conventional Northern blots.

Total protein extracted from D0, D1, D3, D7 sample were subjected to Western blot analysis. Membranes were incubated with rabbit monoclonal antibody to cyclinE2 (ab32103, Abcam) or polyclonal antibody to ADRP/ADFP (ab52355, Abcam), and then with anti-rabbit IgG conjugated with horseradish peroxidase. Immunoreactive bands were detected using ECL Plus System (GE Biosciences).

RESULTS

Identification of differentially expressed genes using SSH.

Two cDNA libraries containing transcripts upregulated and downregulated 1 day after boehmeriasin A treatment were constructed. PCR amplification analysis was applied to verify efficiency of the subtraction procedure by comparing the expression of G3PDH before and after

subtraction. As expected, G3PDH product were detected after 15 cycles in the unsubtracted samples, whereas in the subtracted samples such as D1 - D0, D0 - D1, 25 cycles, 20 cycles were necessary for detection on agarose gels. These results confirm the effectiveness of subtraction steps.

Subtracted cDNA were cloned into T- vector to generate the two cDNA libraries (D0 - D1, D1 - D0). To eliminate false-positive clones, hybridization screening was performed on 756 colonies. cDNA from these colonies were spotted onto four identical microarrays. The subtracted (D0 - D1, D1 - D0) and unsubtracted (D0, D1) cDNA preparations were used as probes to hybridize the microarrays. For D1 - D0 library, positive clones are defined by the following criteria: clones hybridized more strongly with D1 - D0 subtracted probes than D1 unsubtracted probes, and weakly with D0 - D1 subtracted probes and D0 unsubtracted probes. For D0 - D1 library, the positive clones bind more strongly with D0 - D1 subtracted probes than D0 unsubtracted probes, and weakly with D1 - D0 subtracted probes and D1 unsub-tracted probes. 113 and 50 positive clones were screened out from D1 - D0 and D0 - D1 subtractive libraries separately.

DNA sequencing and sequence analysis

Ultimately 87 clones from subtractive libraries generated satisfactory sequencing results. By matching the cDNA sequences against GenBank database, all the subtractive cDNA were finally divided into three kinds. As showed in Table 1, 53 genes are old genes, of which 44 were nonredundant (Table 1A), and 15 genes are old genes without known function (Table 1B), 25 genes are new genes (Table 1C).

Identification of differentially expressed genes using microarray

The experimental group treated with boehmeriasin A (7×10-3 g/ml) for 1 day and the control group treated with nothing. RNA of the two groups were analyzed by the microarray with 2000 cDNA fragments. The results demonstrated there are 328 genes (Ratio value is above 2 or below 0.5) affected by boehmeriasin A. Among them, the expression of 37 genes (ratio value is above 2.5) and 54 genes (ratio value is below 0.3) change apparently, which are listed in the Table 2A and 2B respectively.

Gene expression analysis

To confirm the differentially expressed genes identified by SSH, RT-PCR, Virtual Northern and Northern blot assays were used to detect expression of these genes in Boehmeriasin A treated cells. 48 genes including total 23 old genes were checked, and 35 genes including 17 old genes showed the same change trend as the SSH result result. Some important proteins such as cyclinE2 and ADRP/ADFP were investigated by Western blot. CyclinE2, one of cell cycle related genes, was inhibited with boehmeriasin A treatment. However, the expression of ADRP/ADFP increased remarkably.

RT-PCR also was applied to detected expression of 7 genes affected by the boehmeriasin A as showed by gene chip test. It demonstrated similar result to cDNA microarray results.

DISCUSSION

Boehmeriasin A is a new compound isolated from Boehmeria siamensis Craib, Urticaceae. It exhibited pronounced anti-cancer activity in vitro as shown previously. The differentially expressed genes in cancer cell before and after treatment with drug were identified by SSH and cDNA microarray test. Three hundred and twenty-eight genes of differential expression were obtained from gene chip analysis. Among them, expression of 91 genes changed apparently. Two subtractive libraries were constructed with SSH. Sequence similarity comparison of 87 clones from the subtractive libraries revealed that 52 clones sharing high similarity to genes with known function, 15 clones to genes with unknown function, and 20 clones to new genes.

On the basis of the differentially expressed genes data, we propose boehmeriasin A inhibits the breast cancer cell by the following pathways.

1) Cell cycle was interferred after boehmeriasin A treatment. We found the expression of many cell cycle related genes including cyclinE2, HMG2, cyclinB, cdc25, Op18, MKLPI were inhibited with boehmeriasin A treatment. Cyclin E2 (Payton et al., 2002) and HMG2 (Lee et al., 1987) play important roles in regulating G1/S transition. CyclinB is one of the component of MPF (maturation-promoting factor) complex (Kishimoto et al., 1997), which is the dominant factor responsible for release from G2 and entry into M-phase. Cdc25 is an M-phase inducer and triggers entry into M phase (Nilsson et al., 2000). MKLP-1(Lee et al., 1995; Deavours et al., 1999) and Op18 (Marklund et al., 1996) are indispensable to mitosis. As outlined above, down-regulation of these genes explained why the boehmeriasin A treated cell no longer divided and stayed at G1 or G2 phase.

2) Boehmeriasin A changed the expression of important genes involved in apoptosis signal, which made cell stop growth but refrained from direct apoptosis. On the other hand, it promoted expression of some differentiation related genes. It was demonstrated that boehmeriasin A decreased the expression of some apoptosis related genes such as Daxx (death-associated protein). Daxx plays important roles in the Fas apoptosis transduction pathway (Yang et al., 1997; Chang et al., 1998; Ko et al., 2001).

Table 1. Differentially expressed genes affected by TMMHPQ resulted from SSH analysis.

Table 1A. Old genes with known functions.

Clone No	Length(bp)	Sequence identity	Differential expression				
70030	509		DM	D0	D1	D3	D7
70053	341	ARHE	RT[b]	-	0.43	0.42	0.39
70125	359	tetratricopeptide repeat domain 1	nd [c]				
70137	213	zinc finger protein 10 (KOX 1) (ZNF10)	nd				
		ADFP	VN[d]	+	+++	+++	+++
70141	245		N[e]	+	+++	+++	+++
70154	321	ribosomal protein L6(RPL6)	id [f]	+	++	++	+++
70156	442	sequestosome 1(SQSTM1)	70211				
70161	573	ADFP	nd				
70211	327	ATPase, H+ transporting (ATP6H)	id 70137				
70221	646	ribosomal protein L6(RPL6)	nd				
70225	616	pleiotropic regulator 1 (PLRG1)	VN				
70229	588	leucyl tRNA synthetase	nd				
70235	693	hydroxymethylglutaricaciduria	nd				
70243	172	oxidative-stress responsive 1(OSR1)	nd				
70248	467	sequestosome 1(SQSTM1)	nd				
70270	501	ADFP	id 70154				
70274	285	artemis protein	id 70137				
70330	233	GADD153	nd	-	+	+	+++
70341	227	ribosomal protein L15 (RPL15)	id 70521	0.22	1.02	1.17	1.04
70347	498	forkhead box O1A (FOXO1A)	RT	0.15	0.31	0.41	0.59
70363	484	C3HC4-type zinc finger protein (LZK1)	RT				
70397	497	C3HC4-type zinc finger protein (LZK1)	nd				
70416	563	C3HC4-type zinc finger protein (LZK1)	id 70347				
70436	327	SHOC2	id 70347	0.43	0.74	1.79	1.32
70512	476	ribosomal protein L27 (RPL27)	RT	0.25	0.40	0.65	0.75
70521	300	COPA	RT				
70651	408	GADD153	nd				
70660	484	zinc finger protein (ZNF139)	VN				
70675	520	DDX5	nd				
70691	281	reticulon 4 (RTN4/Nogo)	nd	-	-	-	-
70692	409	ferritin L chain	VN	+	++	+++	++++
		VDUP1	VN	+	+++	+++	+++

Table 1A. Contd.

70701	539		VN	-	0.31	0.68	0.51
70718	257	N-myristoyltransferase 1	RT	0.92			
70731	500	ribosomal protein L5 (RPL5)	nd	+	1.31	1.63	2.02
		TIEG	RT	0.07	+	+++	+++
70739	321		VN	0.42	0.17	0.41	0.34
70742	324	sequestosome 1(SQST1M1)	RT id 70154				
70757	587	DnaJ protein	RT	-	0.62	0.71	
70760	224	HSPC168	Nd	+			0.66
70375	653	TIMM9	VN	0.66	+++	-	
07005	414	nuclear receptor coactivator 4	VN	+++	+	++	+++
07023	573	NQO1	RT	++	0.67	0.62	0.69
07027	491	oncoprotein 18 /Pr22	N	++++	++	++	+
07029	173	ribosomal protein L41 (RPL41)	VN	+++	++	+	+
07032	531	heat shock 70kD protein 8	VN		++	+	++
07038	182	MHC II DR alpha	VN	0.94	+	-	-
07060	429	TOPBP1	nd				
07068	383	heat shock protein gp96 precursor	RT		1.00	0.93	0.83
07081	333	NADH dehydrogenase subunit 5	nd				
07083	608	RAB6KIFL	nd				
07114	661	ETEF1	nd				
07163	202	SSR3	nd				
07212	641	ATP synthase subunit 8 6	nd				
		ARC34	VN	+	+	+	+
		ATP synthase subunit 8 6	id 07163				

Table 1B. Old genes without known function.

Clone no	Length(bp)	Sequence identity	Differential expression				
			DM	D0	D1	D3	D7
70039	393	cDNA DKFZp566E233	VN	-	-	-	-
70063	374	clone MGC:4677 IMAGE:3532809	nd				
70142	620	cDNA FLJ90820 fis, clone Y79AA1001272	nd				
70272	307	similar to KIAA0157 gene product is novel	nd	-	++	++	++
70283	384	cDNA FLJ14893 fis, clone PLACE1004302, weakly similar to SOF1 PROTEIN	nd				
70451	564	Similar to zfp 136 (clone pHZ-20)	VN				
70472	601	cDNA FLJ10273 fis, clone HEMBB1001137, highly similar to putative phospholipase mRNA	nd	-	-	-	-
		cDNA FLJ10286 fis, highly similar to H COP9					
70522	531	complex subunit 4 mRNA	nd				
70649	616	clone IMAGE:3856788	nd				

Table 1B. Contd.

70653	680	hypothetical protein DKFZp434F0272	nd				
		GW128 protein (GW128)	RT				
70664	507	hypothetical protein FLJ11301 (FLJ11301)	nd				
70665	662	cDNA: FLJ22967 fis, clone KAT10573, highly similar to AF151892 CGI-134 protein mRNA	nd				
70689	316	hypothetical protein ASH1 (ASH1)	nd				
70726	600	Similar to hypothetical protein	nd				
70767	679	DKFZp434K1421	nd				

Table 1C. New genes.

Clone No	cDNA length (bp)	mRNA length (kb)						Gene Bank
			DM	D0	D1	D3	D7	Accession No
70041	380		VN	+	+	-	+++	BQ605322
70067	696		VN	-	-	-	-	BQ605324
70124	1100	2.0	RT	0.24	0.19	0.89	1.26	BQ605325
			N	-	-	+++	+	
70133*	854	5.5	RT	0.06	0.26	0.33	0.35	BQ605338
			N	-	+	+	+++	
70204	369	5.5	VN	-	-	+	++	BQ605333
			N	-	-	-	++	
70233	261		nd					BQ605327
70258	703		VN	-	-	-	-	BQ605328
70267	444		nd					
70326	479		nd					BQ605329
70433	228		nd					BQ605336
70437	396		nd					
70496	445		VN	-	-	-	-	
				-	-	-	-	
70556	378		VN	-	-	-	-	
70567	168		VN	-	-	-	-	BQ605332
70765	668		id 70133					
70769	668		id 70133					
70770	277		nd					
07040	596		RT	--	-	-	-	
07078	182		VN	++++	+++	++	+	
07211	327		VN	-	-	-	-	BQ605343
07219	1917		RT	0.27	0.54	0.55	0.52	BQ605339
07224	357		VN	+++	+	+	+	
07294	439		nd					BQ605341
07337	442		VN	-	-	-	-	
07338	799		VN	-	-	-	-	BQ605342

[a]DM: detect method. [b]RT: RT-PCR. [c]nd: not detect. [d]VN: virtual northern. [e]N: northern. [f]id: identical to. **ARHE**: ras homolog gene family, member E; **ADFP**: adipose differentiation-related protein; **hydroxymethylglutaricaciduria**: 3-hydroxymethyl-3-methylglutaryl-Coenzyme A l yase; **DDX5**: DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 5; **COPA**: coatomer protein complex, subunit alpha; **TIEG**: TGFB inducible early growth response ; **TIMM9**: translocase of inner mitochondrial membrane 9 (yeast) homolog; **NQO1**: NAD(P)H dehydrogenase, quinone 1; **MHC II DR alpha**: major histocompatibility complex, class II, DR alpha; **TOPBP1**: topoisomerase (DNA) II binding protein; **RAB6KIFL: RAB6 interacting, kinesin-like (rabkinesin6); ETEF1** :eukaryotic translation elongation factor 1 alpha 1; **SSR3**: translocon-ssociated protein gamma; **ARC34**: Arp2/3 protein complex subunit p34-Ar

Table 2. The differentially expressed genes in breast cancer cell line MDA-MB-231 related with TMMHPQ resulted from gene chips test.

Table 2A. The genes of lower expression.

Ratio	cy5	cy3	cy3*	Class	Definition
0.096	632	6155	6580.1	15	Human mRNA for KIAA0175 gene, complete cds
0.097	361	3484	3724.6	15	Homo sapiens clone 24655 mRNA sequence
0.097	5705	54999	58797.9	10	Human beta-tubulin gene (5-beta) with ten Alu family members
0.100	5587	52086	55683.7	14,5	Homo sapiens ankyrin 2, neuronal (ANK2) mRNA
0.103	3904	35486	37937.1	3	Human cyclin B mRNA, 3' end
0.106	560	4934	5274.8	3	Homo sapiens growth-arrest-specific protein (gas) mRNA, complete cds
0.112	240	2008	2146.7	15	Human novel protein with short consensus repeats of six cysteines mRNA,
0.113	7856	65171	69672.5	13	Human gene for heterogeneous nuclear ribonucleoprotein (hnRNP) core protein A1
0.126	3041	22491	24044.5	10	Human tumor antigen (L6) mRNA, complete cds
0.135	4710	32592	34843.2	7	Homo sapiens fibroblast growth factor (acidic) intracellular binding protein (FIBP)mRNA
0.136	1449	9969	10657.6	15	Homo sapiens ZW10 interactor (ZWINT), mRNA
0.136	368	2524	2698.3	15	Homo sapiens mRNA for KIAA0834 protein, complete cds
0.163	200	1147	1226.2	10,15	Human MHC classII HLA-DR-beta (DR2-DQw1/DR4 DQw3) mRNA,clone ROF-beta-2b
0.167	1377	7729	8262.9	13	H.sapiens QRSHs mRNA for glutaminyl-tRNA synthetase
0.169	388	2151	2299.6	11	H.sapiens syndecan-1 gene (exons 2-5)
0.169	610	3375	3608.1	7	Homo sapiens Rad51-interacting protein mRNA, complete cds
0.172	6501	35449	37897.5	3	H.sapiens HMG-2 mRNA
0.175	200	1070	1143.9	15	Human mRNA for KIAA0146 gene, partial cds
0.181	2232	11554	12352.1	13,11	Human alternative splicing factor mRNA, complete cds
0.182	385	1981	2117.8	12,15	Human glutathione transferase Zeta 1 (GSTZ1) mRNA, complete cds
0.182	583	2991	3197.6	5,5,10	Human gene for hepatitis C-associated microtubular aggregate protein p44
0.183	1323	6755	7221.6	6,15	Homo sapiens mRNA for Daxx, complete cds
0.193	4184	20280	21680.8	12	Human fatty acid binding protein homologue (PA-FABP) mRNA, complete cds
0.195	652	3133	3349.4	13	Homo sapiens SMARCA1 mRNA
0.199	5052	23745	25385.1	10,1	Homo sapiens CD9 antigen (p24) (CD9) mRNA
0.205	6098	27872	29797.2	15	Human mRNA for KIAA0325 gene, partial cds
0.207	788	3565	3811.2	7	Homo sapiens mRNA for mitotic kinesin-like protein-1 (MKLP-1 gene)
0.207	3217	14515	15517.6	11,12	Homo sapiens protein tyrosine phosphatase,receptor type,O (PTPR+F53O)mRNA
0.220	482	2045	2186.3	12	Human cytochrome b5 mRNA, 3' end
0.221	1298	5485	5863.9	11	Human lipoprotein-associated coagulation inhibitor (LACI) gene
0.226	673	2780	2972.0	12	Human pLK mRNA, complete cds
0.228	686	2813	3007.3	11	Homo sapiens emopamil-binding protein (EBP) mRNA
0.232	537	2164	2313.5	2	Human (clone CTG-A4) mRNA sequence
0.234	419	1672	1787.5	13,11	Human mRNA for carboxypeptidase E (EC 3.4.17.10)
0.235	638	2543	2718.6	12	Homo sapiens biotinidase precursor (BTD) mRNA
0.239	591	2313	2472.8	3	Human cdc25 Hs mRNA, complete cds
0.246	562	2140	2287.8	15	Homo sapiens clone 24860 Ena-VASP like protein mRNAsequence,partial cds
0.250	1113	4161	4448.4	12	H.sapiens mRNA for 17-beta-hydroxysteroid dehydrogenase

Table 2A. Contd.

0.252	3321	12321	13172.0	12	H.sapiens mRNA for cathepsin C
0.253	1719	6368	6807.8	8,5,8	Homo sapiens cysteine and glycine-rich protein 2 (CSRP2), mRNA
0.257	2007	7300	7804.2	10	Human DNA sequence from clone 53C18 on chromosome 11p12-13.
0.259	434	1568	1676.3	5	Homo sapiens kinesin superfamily motor KIF4 mRNA, complete cds
0.262	1674	5969	6381.3		Human laminin B2 chain (LAMB2) gene
0.263	4457	15849	16943.7	15	zd84f08.r1 Homo sapiens cDNA, 5' end
0.275	2783	9460	10113.4	11	Human mRNA for thymidylate synthase (EC 2.1.1.45)
0.277	1311	4424	4729.6	10	H.sapiens mRNA for bleomycin hydrolase
0.278	9632	32436	34676.4	8,13	Human AU-rich element RNA-binding protein AUF1 mRNA, complete cds
0.280	1716	5735	6131.1	11,1	Homo sapiens p53 tumor suppressor-binding protein 1 mRNA, complete cds

Table 2B. The genes of higher expression

Ratio	cy5	cy3	cy3*	Class	Definition
2.537	19332	7128	7620.3	7	Homo sapiens translin-associated factor X (TSNAX) mRNA
2.559	4733	1730	1849.5	13,8,2	Homo sapiens mRNA for tip associating protein (TAP)
2.561	6362	2324	2484.5	10	Human TCF-1 mRNA for T cell factor 1 (splice form C)
2.565	987	360	384.9	8	Homo sapiens mRNA for zinc finger protein, 3115 BP
2.590	13045	4711	5036.4	13	Homo sapiens cDNA FLJ11246 fis, highly similar to Homo sapiens pleiotropic regulator 1
2.597	21723	7825	8365.5	9,5	Human mRNA for KIAA0034 gene, complete cds
2.602	27778	9984	10673.6	13	Homo sapiens archain 1 (ARCN1) mRNA
2.620	14019	5005	5350.7	12	Human mRNA for GC box bindig protein, complete cds
2.635	2580	916	979.3	11	Human DNA binding protein (HPF2) mRNA, complete cds
2.666	1907	669	715.2	2,8	Homo sapiens cellular co-factor (RAB) gene, complete cds
2.671	911	319	341.0	12	H.sapiens mRNA for vacuolar proton ATPase, subunit D
2.818	7957	2641	2823.4	12	Homo sapiens gene for Proline synthetase associated, complete cds
2.838	11315	3729	3986.6	11	Homo sapiens SKI-INTERACTING PROTEIN (SNW1), mRNA
2.843	4693	1544	1650.6	9,14	Human myleoid differentiation primary response protein MyD88 mRNA, complete cds
2.863	6388	2087	2231.2	15	Homo sapiens anti zuai-1 mRNA, complete cds
2.866	15713	5128	5482.2	7,15,8	Homo sapiens mRNA for KIAA1019 protein, partial cds
2.931	1294	413	441.5	11,12	Human mRNA for tyrosine phosphatase, complete cds
3.031	3574	1103	1179.2	8	Human complement component C3 mRNA, alpha and beta subunits, complete cds
3.200	4505	1317	1408.0	12,8,3	zq40b12.s1 Homo sapiens cDNA, 3' end
3.251	17583	5059	5408.4	12,13,15	Homo sapiens mRNA for Asparaginyl tRNA Synthetase, complete cds
3.256	717	206	220.2	11	Homo sapiens Era GTPase A protein (HERA-A) mRNA, partial cds
3.308	7829	2214	2366.9	12	Human mRNA for alanyl-tRNA synthetase, complete cds
3.331	7023	1972	2108.2	12	Human glucose transporter-like protein-III (GLUT3), complete cds
3.352	28269	7889	8433.9	13	Human methionine aminopeptidase mRNA, complete cds
3.363	4846	1348	1441.1	15	Homo sapiens mRNA for KIAA0521 protein, partial cds
3.369	23420	6503	6952.2	12	H.sapiens mRNA for seryl-tRNA synthetase

Table 2B. Contd.

3.568	15752	4129	4414.2	15	ov70b12.s1 Homo sapiens cDNA, 3' end
3.637	972	250	267.3	15,11	Human cerebellar degeneration-associated protein mRNA, complete cds
3.720	15511	3900	4169.4	11,3,14	EGR alpha=early growth response gene alpha [human, prostate, mRNA, 3228 nt]
3.781	5983	1480	1582.2	15	Homo sapiens ataxin 2 related protein (A2LP), mRNA
3.803	20881	5136	5490.8	13	Human transcriptional activation factor TAFII32 mRNA, complete cds
4.072	3378	776	829.6	12	Homo sapiens protein phosphatase 1D magnesium-dependent,delta isoform (PPM1D) mRNA,
4.177	3974	890	951.5	14	Homo sapiens BRCA1-associated protein 2 (BRAP2) mRNA, complete cds
4.830	28497	5519	5900.2	15	Homo sapiens mRNA for KIAA0907 protein, complete cds
4.888	7076	1354	1447.5	13	Human NF-kappa-B transcription factor p65 subunit mRNA, complete cds
6.177	9106	1379	1474.2	10	Homo sapiens alkylation repair; alkB homolog (ABH), mRNA
9.014	13510	1402	1498.8	10,2	Homo sapiens mRNA for 4F2 heavy chain, complete cds
9.139	17294	1770	1892.3	11	*H. sapiens* ALK-1 mRNA

Note: Class is Classification of genes by its function with gene cluster software. A number represented a class of genes. The corresponding class is following: 10 Protooncogenes and antioncognes, 2 Proteins of ion channel and transpotation, 3 Cell cycle regulators, 4 Stress-responsing proteins, 5 Cytoskeleton and cell movement related proteins, 6 Apoptosis -related protein, 7 DNA synthsis/repair and recombination proteins, 8 DNA binding proteins and transcription factors, 9 cell receptors, 10 Immunity-related proteins, 11 Singal transduction modulators and effectors, 12 Metabolism, 13 Translation and synthsis of protein, 14 Development-related proteins, 15 Unclassified.

Expression of some cell differentiation related genes were changed with boehmeriasin A treatment. The expression of MyD88 which can improve cell differentiation was increased. The expression of ADRP/ADFP increased remarkably and finally led to intracellular lipid droplet accumulation. It is an important differentiation marker of the breast cancer cell. Boehmeriasin A may induce the breast cancer cells to start differentiation and obtain the special feature of mature mammary cells.

3) Expression of genes involved in TGF- 1 signal transduction were varied, suggesting the effect of Boehmeriasin A was closely associated with TGF- 1 pathway.

Transforming growth factor- (TGF-) signaling path-way is important for regulation of epithelial cell prolife-ration, differentiation, and apoptosis. TIEG is an impor-tant factor for mediating TGF- signaling on epithelial cell growth (Subramaniam et al., 1995). TGF binds to its cell surface receptor to induce the expression of TIEG1 and TIEG2, which inhibit epithelial cell proliferation. The expression level of TIEG is highly related with development of breast cancer. In the normal breast tissue, TIEG remains high, while in the breast cancer tissue, expression of TIEG is low. The more malignant the cancer cell, the lower level expression of TIEG. TIEG was induced remarkably 24 h after boehmeriasin A treatment. On the third day, it reached peak. It remained at high level until the seventh day.

There are two kinds of receptor in the TGF- pathway. ALK1 (Lux et al., 1999) belongs to the type I receptor, and TGF- IIR belongs to type II. Due to lacking of ALK1, MDA-MB-231 cells are insensitive to TGF- 1 signal. After boehmeriasin A treatment, expression of both receptors improved. At seventh day, expression of ALK1 reached nine times of the untreated cell. While transcription level of TGF- IIR increased gradually. At second day, it was twice of the untreated. Further experiments are needed to make sure boehmeriasin A treatment can make breast cancer cell sensitive to TGF- 1 signal.

Moreover, the cell shape was found to change from the epithelia to the fibroblast with boehmeriasin A treatment. The change on transcriptional level of cytoskeletal protein genes such as RhoE (Nobes et al., 1998) probably explained the apparent cell shape change after Boehmeriasin A treatment.

In conclusion, we have succeeded, through the use of SSH and gene chip test, in sketching a partial blueprint of the gene expression profile of the breast cell line after the treatment of boehmeriasin A. It is apparent from this study that many genes are active in response to the boehmeriasin A. Firstly, the drug restrained the expression of a serial of genes related to cell proliferation and cell cycle regulation. Secondly, boehmeriasin A lowered the expression of important proteins involved in apoptosis signal, which make cell refrained from direct apoptosis. On the other hand, it promoted expression of some differentiation related genes. Next, we will make further research on the anti-cancer mechanisms of Boehmeriasin A in protein level.

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