

CHAPTER IV RESULTS

1. RT-PCR

The total RNAs were extracted from treated cell line at 14 days of post-transfection with siRNA. The mRNA samples were successfully transcribed without any contamination of residue genomic DNA by application of DNase-I treatment and contained with *DSCR1* gene. A house keeping gene, *GAPDH*, was also expressed in both case and control samples as expected (Fig 9).

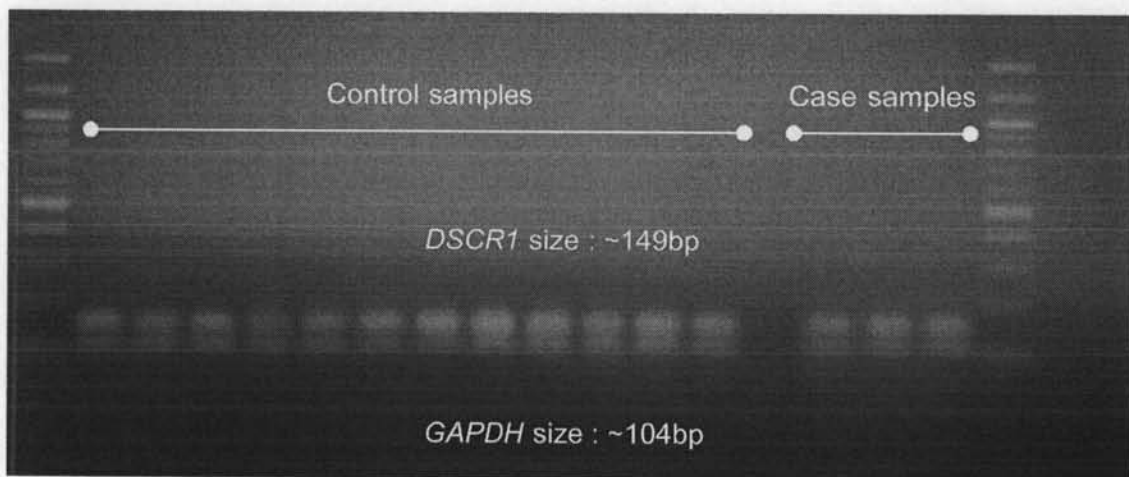


Figure 9 Conventional and duplex RT-PCR from normal and case cDNA samples.

2. Real-time PCR

Duplex RT-PCR were successfully done in all cDNA samples before proceed to real-time PCR. The result from real-time PCR in controls showed no signs of probes degradation or genomic DNA contamination. The 10 normal control samples average Ct of *GAPDH* and *DSCR1* were shown in Table 19.

2.1 No significant differences among 10 normal control samples

Relative quantification of 10 control samples showed no significant differences among ten normal control samples (Table 19 and Fig 10).

Table 19 : The average C_t of *GAPDH* and *DSCR1*.

Sample	Note	FAM (C_t)	CY5 (C_t)	ΔC_t	Gene expression	Cal1	Cal2	Cal3	Cal4	Cal5	cal6	cal7	cal8	cal9	cal10
1137	Control	23.39	19.45	3.94	0.869197	1	1.3566043	0.5946036	0.7022224	0.6925547	1.5800826	0.4796321	1.0069556	1.6021398	0.9201877
1138	Control	24.78	20.4	4.38	0.6407152	0.7371346	1	0.4383029	0.5176325	0.5105061	1.1647336	0.3535534	0.7422618	1.1809927	0.6783022
1139	Control	22.63	19.44	3.19	1.4618093	1.6817928	2.2815274	1	1.1809927	1.1647336	2.6573716	0.8066418	1.6934906	2.6944672	1.547565
1141	Control	24.22	20.79	3.43	1.2377802	1.4240502	1.9318727	0.8467453	1	0.9862327	2.250117	0.6830201	1.4339552	2.2815274	1.3103934
1143	Control	25.3	21.89	3.41	1.2550589	1.4439292	1.9588406	0.8585654	1.0139595	1	2.2815274	0.6925547	1.4539725	2.3133764	1.3286858
1170	Control	23.91	19.31	4.6	0.5500959	0.6328783	0.8585654	0.3763117	0.4444213	0.4383029	1	0.3035487	0.6372803	1.0139595	0.5823668
1171	Control	23.83	20.95	2.88	1.8122162	2.0849315	2.8284271	1.2397077	1.4640857	1.4439292	3.2943641	1	2.0994334	3.3403517	1.9185282
1172	Control	23.62	19.67	3.95	0.863193	0.9930925	1.3472336	0.5904963	0.6973718	0.6877709	1.5691682	0.476319	1	1.591073	0.9138315
1173	Control	22.24	17.62	4.62	0.5425226	0.6241653	0.8467453	0.3711309	0.4383029	0.4322686	0.9862327	0.2993697	0.6285067	1	0.5743492
1174	Control	23.34	19.52	3.82	0.9445867	1.0867349	1.4742692	0.6461764	0.7631296	0.7526234	1.7171309	0.5212329	1.0942937	1.7411011	1

Note: The gene expression is the average value of normalized target gene expression level in sample using all 10 control samples as calibrators.

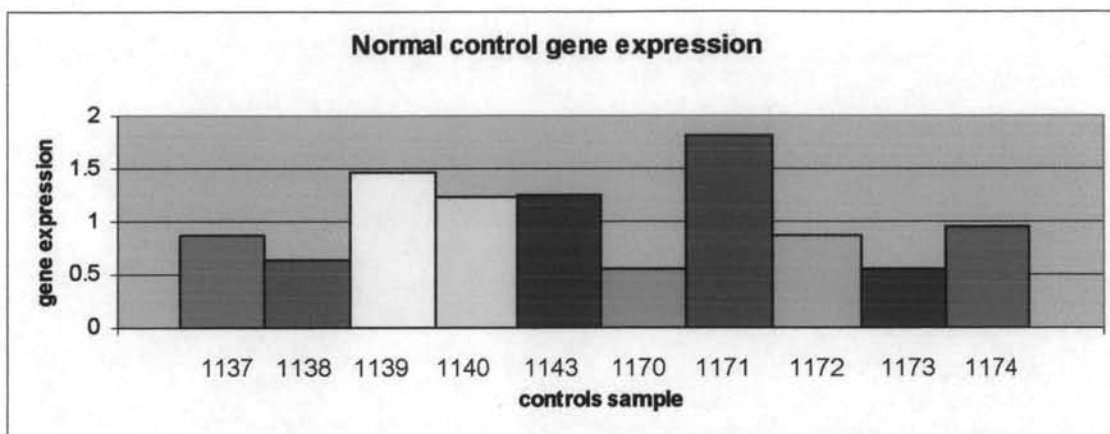


Figure 10 Normal controls *DSCR1* gene expression

Table 20 : Statistic calculation of standard derivative of 10 control samples.

Variable	N	Mean	Std Dev
Gene expression	10	1.0177	0.4178

The standard deviation was less than 2 indicate that there were no significant differences among 10 normal control samples at 95% confidence (Table 20).

2.2 Gene expression using five control samples as calibrators

In experiment, 5 control samples were selected from 10 control samples to compare with treated siRNA sample. The average C_t of *GAPDH* and *DSCR1* were shown as follow;

Table 21 : The average C_t of *GAPDH* and *DSCR1*

Sample	Note	FAM(C_t)	CY5(C_t)	ΔC_t	OVERALL MEAN	Cal1	Cal2	Cal3	Cal4	Cal5
1138	Control	23.04	17.28	5.76	0.787564717	1	0.198884121	0.77916458	1.222640278	0.737134609
1141	Control	24.22	20.79	3.43	3.959917532	5.028053498	1	3.91768119	6.147500725	3.706352248
1171	Control	21.76	16.36	5.4	1.010780954	1.283425898	0.255253031	1	1.569168196	0.946057647
1172	Control	20.8	14.75	6.05	0.644150804	0.817902059	0.162667732	0.637280314	1	0.602903914
1173	Control	23.55	18.23	5.32	1.068413704	1.356604327	0.269807059	1.057018041	1.658639092	1
1431	Case	28.06	21.33	6.73	0.402056563	0.510506063	0.10153155	0.397768242	0.624165274	0.376311687
1438	Case	21.47	14.53	6.94	0.347592868	0.441351498	0.087777805	0.343885455	0.539614118	0.325335464
1439	Case	28.42	22.34	6.08	0.630894372	0.801069878	0.159320078	0.624165274	0.979420298	0.590496331
1461	Case	27.95	19.39	8.56	0.113084287	0.143587294	0.028557233	0.111878134	0.175555609	0.105843164
1462	Case	26.15	19.02	7.13	0.304701897	0.386891248	0.076946526	0.301451957	0.473028823	0.285190929
1492	Case	26.28	16.04	10.24	0.035291642	0.044811102	0.008912217	0.034915223	0.054787858	0.033031814
1497	Case	26.34	15.93	10.41	0.031368718	0.03983002	0.007921558	0.03103414	0.048697786	0.029360086
1498	Case	26.86	17.21	9.65	0.05312263	0.067451765	0.013415085	0.052556026	0.082469244	0.04972103
1499	Case	26.4	16.7	9.7	0.051313078	0.06515411	0.012958118	0.050765775	0.079660039	0.048027349
1500	Case	25.58	16.7	8.88	0.090588416	0.115023456	0.022876339	0.089622203	0.140632311	0.08478777
1138	Control									
0.5x	0.5x	20.11	13.76	6.35	0.523213034	0.664342907	0.132127255	0.517632462	0.812252396	0.489710149
1141	Control									
0.5x	0.5x	19.55	16.03	3.52	3.720432607	4.723970646	0.939522749	3.680750602	5.775716782	3.482202253

Table 21 : The average C_t of *GAPDH* and *DSCR1* (continue)

Sample	Note	FAM(C_t)	CY5(C_t)	ΔC_t	OVERALL MEAN	Cal1	Cal2	Cal3	Cal4	Cal5
1171	Control									
0.5x	0.5x	20.07	15.69	4.38	2.049781861	2.602683711	0.517632462	2.02791896	3.182145935	1.918528239
1172	Control									
0.5x	0.5x	19.55	14.42	5.13	1.218807587	1.547564994	0.307786103	1.205807828	1.892115293	1.140763716
1173	Control									
0.5x	0.5x	20.09	14.21	5.88	0.724707327	0.920187651	0.183010712	0.716977624	1.125058485	0.678302164
1138	Control									
1x	1x	22.02	15.85	6.17	0.592739615	0.752623374	0.149684838	0.586417475	0.920187651	0.554784736
1141	Control									
1x	1x	18.38	13.72	4.66	1.688181928	2.143546925	0.426317446	1.670175839	2.620786808	1.580082624
1171	Control									
1x	1x	20.57	15.77	4.8	1.532057438	1.945309895	0.386891248	1.515716567	2.37841423	1.433955248
1172	Control									
1x	1x	19.87	17.59	2.28	8.787607211	11.15794933	2.219138944	8.6938789	13.64215827	8.224910613
1173	Control									
1x	1x	23.76	17.55	6.21	0.576531119	0.732042848	0.145591698	0.570381858	0.895025071	0.539614118
1138	Control									
1.5x	1.5x	22.88	19.6	3.28	4.393803606	5.578974665	1.109569472	4.34693945	6.821079134	4.112455307

Table 21 : The average C_t of *GAPDH* and *DSCR1* (continue)

Sample	Note	FAM(C_t)	CY5(C_t)	ΔC_t	OVERALL MEAN	Cal1	Cal2	Cal3	Cal4	Cal5
1171	Control									
1.5x	1.5x	21	17.01	3.99	2.68602063	3.410539567	0.678302164	2.657371628	4.169863043	2.514026749
1141	Control									
1.5x	1.5x	19.01	14.22	4.79	1.54271374	1.958840595	0.38958229	1.526259209	2.394957409	1.443929196
1172	Control									
1.5x	1.5x	20.95	16.94	4.01	2.649041391	3.363585661	0.668963777	2.620786808	4.112455307	2.4794154
1173	Control									
1.5x	1.5x	21.59	14.95	6.64	0.427937017	0.543367431	0.108067154	0.423372656	0.664342907	0.400534939
1431	Case									
0.5x	0.5x	19.72	16.9	2.82	6.0438606	7.674112955	1.526259209	5.979396995	9.382679594	5.656854249
1438	Case									
0.5x	0.5x	19.05	15.21	3.84	2.980326493	3.784230587	0.752623374	2.948538435	4.626752736	2.789487333
1439	Case									
0.5x	0.5x	18.81	14.15	4.66	1.688181928	2.143546925	0.426317446	1.670175839	2.620786808	1.580082624
1461	Case									
0.5x	0.5x	27.37	27.15	0.22	36.64311855	46.52712055	9.253505471	36.25228433	56.88593159	34.2967508
1462	Case									
0.5x	0.5x	20.78	18.85	1.93	11.20033816	14.2214829	2.828427125	11.08087574	17.3877578	10.48314723

Table 21 : The average C_t of *GAPDH* and *DSCR1* (continue)

Sample	Note	FAM(C_t)	CY5(C_t)	ΔC_t	OVERALL MEAN	Cal1	Cal2	Cal3	Cal4	Cal5
1492	Case									
0.5x	0.5x	18.77	17.32	1.45	15.62160071	19.8353232	3.944930818	15.45498126	24.25146506	14.6213032
1497	Case									
0.5x	0.5x	23.87	23.02	0.85	23.67791899	30.06472797	5.979396995	23.42537114	36.75834736	22.16175149
1498	Case									
0.5x	0.5x	18.89	14.01	4.88	1.449414654	1.840375301	0.366021424	1.433955248	2.250116969	1.356604327
1499	Case									
0.5x	0.5x	18.54	15.25	3.29	4.363453387	5.540437872	1.101905116	4.316912946	6.773962499	4.084048503
1500	Case									
0.5x	0.5x	25.57	25.88	-0.31	52.91006869	67.18186775	13.36140671	52.34573175	82.13925744	49.52207979
1431										
1x	Case 1x	21.82	17.2	4.62	1.735643182	2.203810232	0.438302861	1.717130873	2.694467154	1.624504793
1438										
1x	Case 1x	19.17	14.27	4.9	1.429460134	1.815038311	0.360982299	1.414213562	2.219138944	1.337927555
1439										
1x	Case 1x	21.51	18.17	3.34	4.214818146	5.351710219	1.064370182	4.169863043	6.543216468	3.944930818

Table 21 : The average C_t of *GAPDH* and *DSCR1* (continue)

Sample	Note	FAM(C_t)	CY5(C_t)	ΔC_t	OVERALL MEAN	Cal1	Cal2	Cal3	Cal4	Cal5
1461										
1x	Case 1x	23.32	18.71	4.61	1.747715535	2.219138944	0.441351498	1.729074463	2.713208655	1.635804117
1462										
1x	Case 1x	17.92	13.61	4.31	2.151690218	2.732080514	0.543367431	2.128740365	3.340351678	2.0139111
1492										
1x	Case 1x	18.12	13.36	4.76	1.575129435	2	0.397768242	1.558329159	2.445280555	1.474269217
1497										
1x	Case 1x	21.16	18.92	2.24	9.034660477	11.47164198	2.281527432	8.938297105	14.02569154	8.456144324
1498										
1x	Case 1x	18.43	13.57	4.86	1.469647729	1.866065983	0.371130893	1.453972517	2.281527432	1.375541818
1499										
1x	Case 1x	19.15	16.6	2.55	7.287734421	9.253505471	1.840375301	7.210003701	11.3137085	6.821079134
1500										
1x	Case 1x	19.18	15.43	3.75	3.172170653	4.0278222	0.801069878	3.138336392	4.924577653	2.969047141
1431	Case									
1.5x	1.5x	21.71	16.44	5.27	1.106091232	1.404444876	0.279321785	1.094293701	1.717130873	1.035264924

Table 21 : The average C_t of *GAPDH* and *DSCR1* (continue)

Sample	Note	FAM(C_t)	CY5(C_t)	ΔC_t	OVERALL MEAN	Cal1	Cal2	Cal3	Cal4	Cal5
1438	Case									
1.5x	1.5x	19	15.15	3.85	2.959739874	3.758090997	0.747424624	2.928171392	4.59479342	2.770218936
1439	Case									
1.5x	1.5x	19.31	15.09	4.22	2.290194909	2.907945035	0.578344092	2.265767771	3.555370725	2.143546925
1461	Case									
1.5x	1.5x	21.09	16.59	4.5	1.886183955	2.394957409	0.476318999	1.866065983	2.928171392	1.765405993
1462	Case									
1.5x	1.5x	17.7	14.16	3.54	3.669212311	4.658934346	0.926588062	3.630076621	5.696200782	3.434261746
1492	Case									
1.5x	1.5x	17.57	13.55	4.02	2.630743125	3.340351678	0.664342907	2.602683711	4.084048503	2.462288827
1497	Case									
1.5x	1.5x	18.66	14.32	4.34	2.107409073	2.67585511	0.532185091	2.084931522	3.271608234	1.972465409
1498	Case									
1.5x	1.5x	19.01	14.22	4.79	1.54271374	1.958840595	0.38958229	1.526259209	2.394957409	1.443929196
1499	Case									
1.5x	1.5x	20.66	19.69	0.97	21.78812865	27.6651914	5.502167273	21.55573723	33.8245773	20.39297004
1500	Case									
1.5x	1.5x	20.73	17.3	3.43	3.959917532	5.028053498	1	3.91768119	6.147500725	3.706352248

Note: The overall mean is the average value of normalized target gene expression level in sample using 5 control samples as calibrators.

Table 22 : The normalized target gene expression level in normal control and case samples.

Samples	Controls	Cases	Control 0.5X	Control 1X	Control 1.5X	Case 0.5X	Case 1X	Case 1.5X
1138 / 1431	0.787565	0.402057	0.52321303	0.59274	4.39380361	6.0438606	1.7356432	1.10609123
1141 / 1438	3.959918	0.347593	3.72043261	1.688182	2.68602063	2.9803265	1.4294601	2.95973987
1171 / 1439	1.010781	0.630894	2.04978186	1.532057	1.54271374	1.6881819	4.2148181	2.29019491
1172 / 1461	0.644151	0.113084	1.21880759	8.787607	2.64904139	36.643119	1.7477155	1.88618396
1173 / 1462	1.068414	0.304702	0.72470733	0.576531	0.42793702	11.200338	2.1516902	3.66921231
1492		0.035292				15.621601	1.5751294	2.63074313
1497		0.031369				23.677919	9.0346605	2.10740907
1498		0.053123				1.4494147	1.4696477	1.54271374
1499		0.051313				4.3634534	7.2877344	21.7881286
1500		0.090588				52.910069	3.1721707	3.95991753
Mean	1.494166	0.206001	1.64738848	2.635423	2.33990328	15.657828	3.381867	4.39403344

Note: Normal control samples were 1138, 1141, 1171, 1172, and 1173, respectively. Case samples were 1431, 1438, 1439, 1461, 1462, 1492, 1497, 1498, 1499, and 1500, respectively.

2.3 *DSCR1* gene expression in case samples are less than control samples

In untreated condition, *DSCR1* gene expression of case samples are less than control samples at 95% confidence (Table 22) even though the copy number of *DSCR1* in case samples are higher.

Table 23 : ANOVA calculation with control and case samples group.

Source	DF	Anova SS	Mean Square	F Value	Pr > F
treatment	1	5.52640362	5.52640362	8.87	0.0107

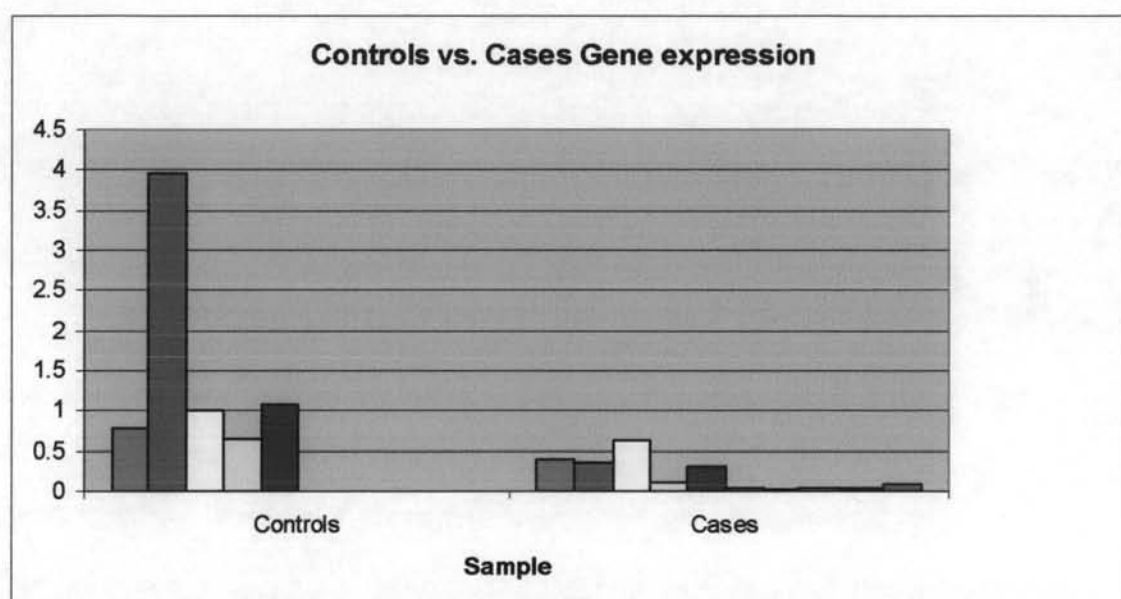


Figure 11 Normal controls gene expression compare with cases gene expression

2.4 siRNAs did not affect on control samples

In treated and untreated control groups, there were no significant differences between both groups (Table 24-26).

Table 24 : T-tests between untreated control samples group and treated with siRNA 0.5 fold in control samples group.

T-Tests			
Difference	DF	t Value	Pr > t
Control 0.5X- Control	4	-0.55	0.6100

Table 25 : T-tests between untreated controls samples group and treated with siRNA 1 fold in control samples group.

T-Tests			
Difference	DF	t Value	Pr > t
Control 1X - Control	4	0.63	0.5626

Table 26 : T-tests between untreated control samples group and treated with siRNA 1.5 fold in control samples group.

T-Tests			
Difference	DF	t Value	Pr > t
Control 1.5X - Control	4	0.95	0.3947

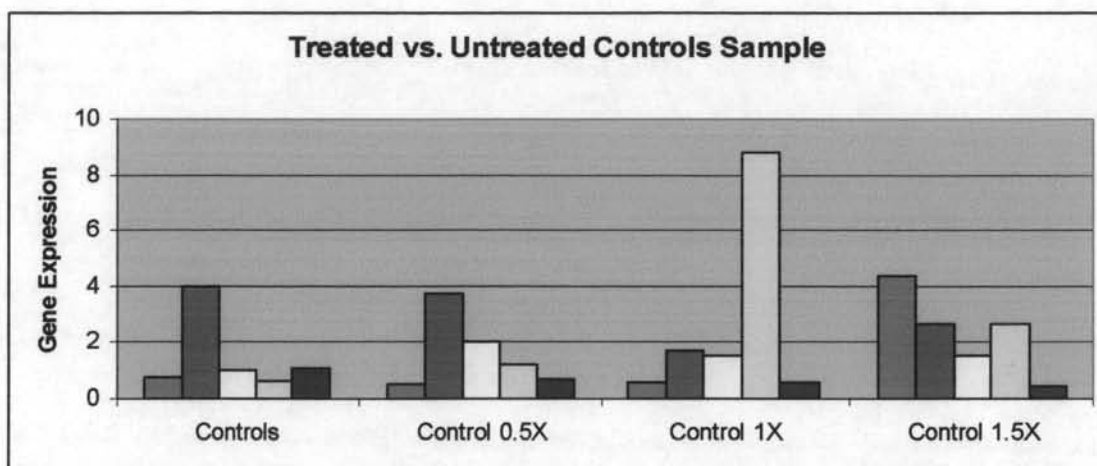


Figure 12 Untreated controls gene expression compare with treated controls gene expression

2.5 siRNAs affected on case samples at 0.5 and 1 fold but did not affect at 1.5 fold

Interestingly, in treated and untreated case groups, there were over-expression of *DSCR1* gene in treated 0.5 and 1 fold of siRNA in case samples compare to untreated samples at 95% confidence (Table 27,28) but no significant difference between 1.5 fold of siRNA in case samples compare to untreated samples (Table 29).

Table 27 : T-tests between untreated case samples group and treated with siRNA 0.5 fold in case samples group.

T-Tests			
Difference	DF	t Value	Pr > t
Case 0.5X - Case	9	2.81	0.0203

Table 28 : T-tests between untreated case samples group and treated with siRNA 1 fold in case samples group

T-Tests			
Difference	DF	t Value	Pr > t
Case 1X - Case	9	3.64	0.0054

Table 29 : T-tests between untreated case samples group and treated with siRNA 1.5 fold in case samples group

T-Tests			
Difference	DF	t Value	Pr > t
Case 1.5X - Case	9	2.12	0.0627

2.6 Effect of siRNA with DSCR1 gene expression at 0.5 fold is higher than 1 fold in case samples

Table 30 shown that siRNA at 0.5 fold is affect to *DSCR1* gene expression differences from 1 fold in case samples at 94% confidence.

Table 30 : T-tests between treated with 0.5 folds in cases sample group and treated with siRNA 1 folds in cases sample group

T-Tests			
Difference	DF	t Value	Pr > t
Case 0.5X - Case 1X	9	-2.24	0.0522

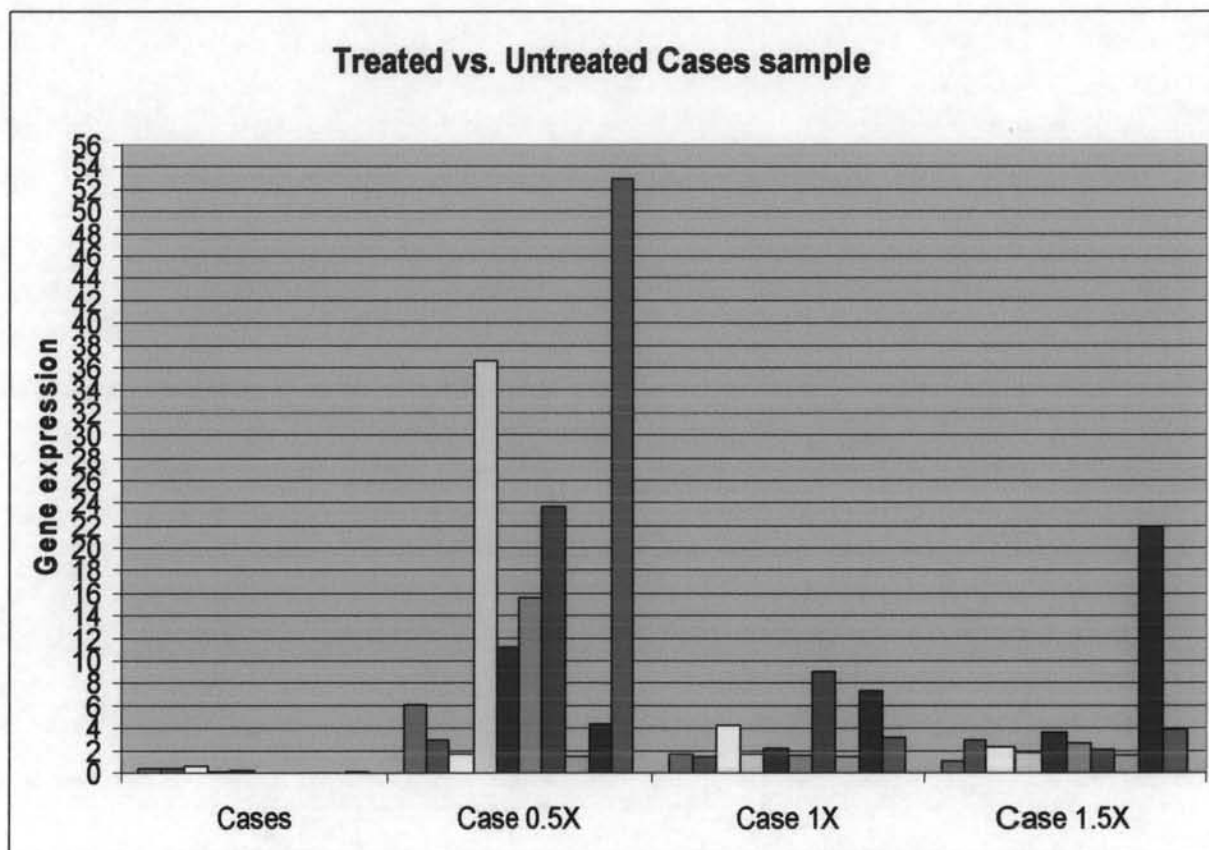


Figure 13 Untreated cases gene expression compare with treated cases gene expression

3. siRNA designing and cloning

The down stream primer of siRNA was designed by Promega software. The 300 bp of siRNA and U6 promoter was amplified, and successfully cloned into plasmid vector with confirmed by sequencing (Fig 14). siRNA sequence was blasted by NCBI database with no sequence matching to unwanted target. siRNA concentration was varied from 0.5, 1, 1.5 folds of concentration that recommended from manufactory and transfected into controls and cases fibroblast cells for determining the appropriate concentration of si-RNA to silence *DSCR1* gene in fibroblast cells. The size of inserted RFP siRNA plasmid is as alike as siRNA plasmid alone due to cutting of the ~900bp of neomycin resistant gene and inserting the ~700bp of RFP gene. Consequently, RFP is transcribed by SV40 promoter. The closely size of two plasmids can refer that uptake

rate of two plasmids are the same. However, in experiment of siRNA transfection, RFP was excluded from plasmid to ensure that the changing of *DSCR1* expression was caused by siRNA only.

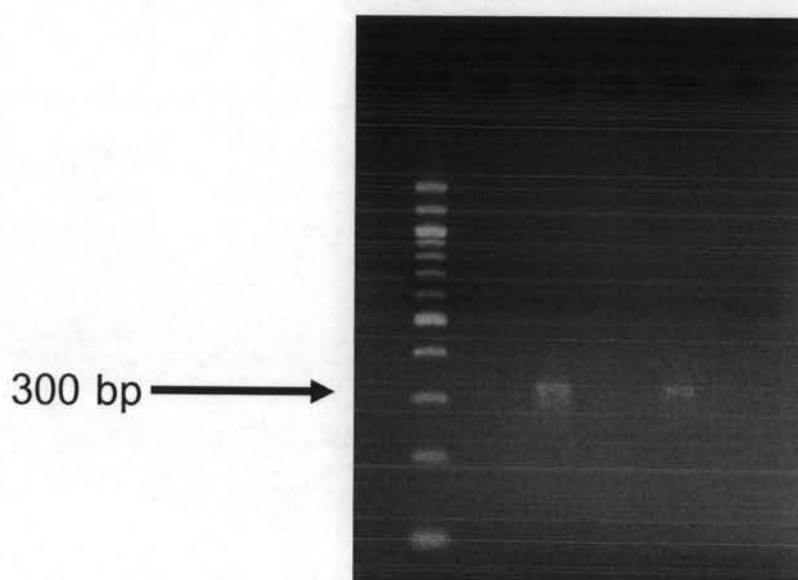


Figure 14 The 300 bp PCR product of siRNA and U6 promoter

4. Selection of transfected cell lines

G-418 at concentrations of 100, 200, 400, 600, 800 and 1,000 $\mu\text{g/ml}$ in DMEM+ 10% (v/v) FBS were used for selection of transfected cell lines within 14 days but there were no existent cells.

5. Uptake rate of siRNA into cell lines

The 700 bp of RFP gene was successfully amplified, cloned into siRNA plasmid, and confirmed by sequencing (Fig 15).

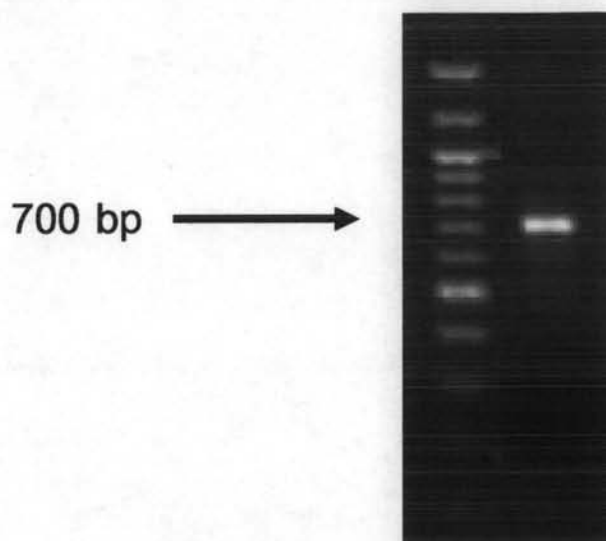


Figure 15 The 700 bp PCR product of RFP gene

RFP protein was detected at excitation maximum wavelength=558 nm and emission maximum wavelength = 583nm using florescent microscopy. Fibroblast cell lines were effectively obtain plasmid vector about 60% of treated cells with siRNA 0.5x fold (Figure 16), and about 80% of treated cells with siRNA 1x and 1.5x fold (Figure 17,18). The signal of RFP was detected within 5 days of post-transfection.

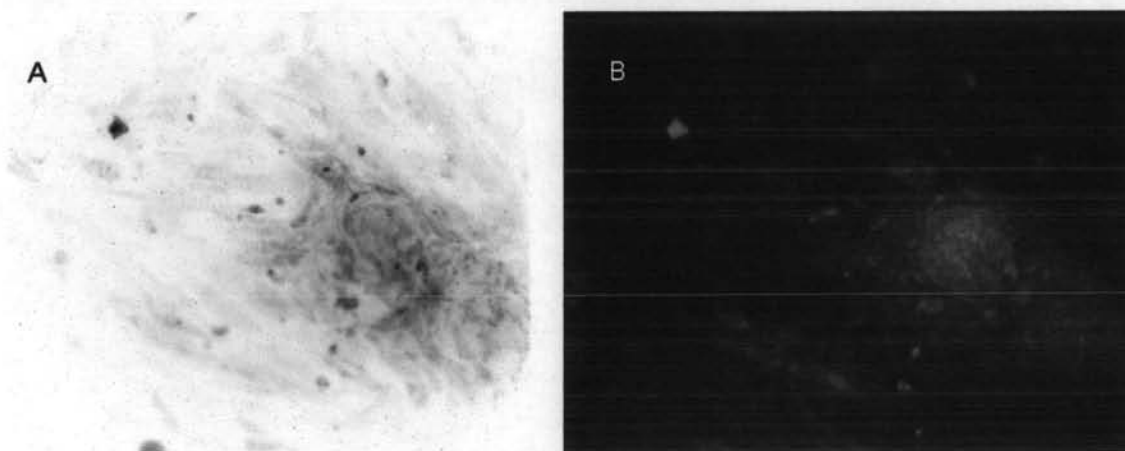


Figure 16: Uptake rate of treated cells with siRNA 0.5x fold. A: Bright field B: Spectrum red.

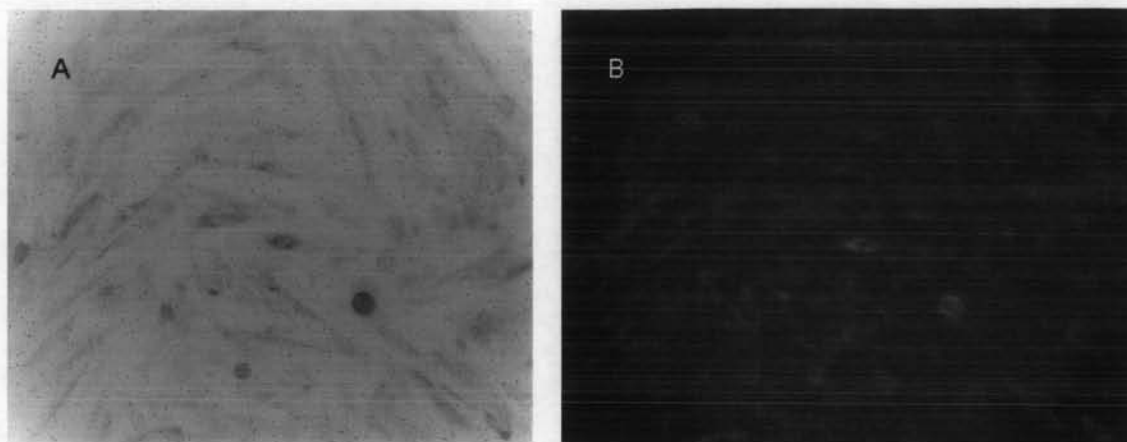


Figure 17: Uptake rate of treated cells with siRNA 1x fold. A: Bright field B: Spectrum red.

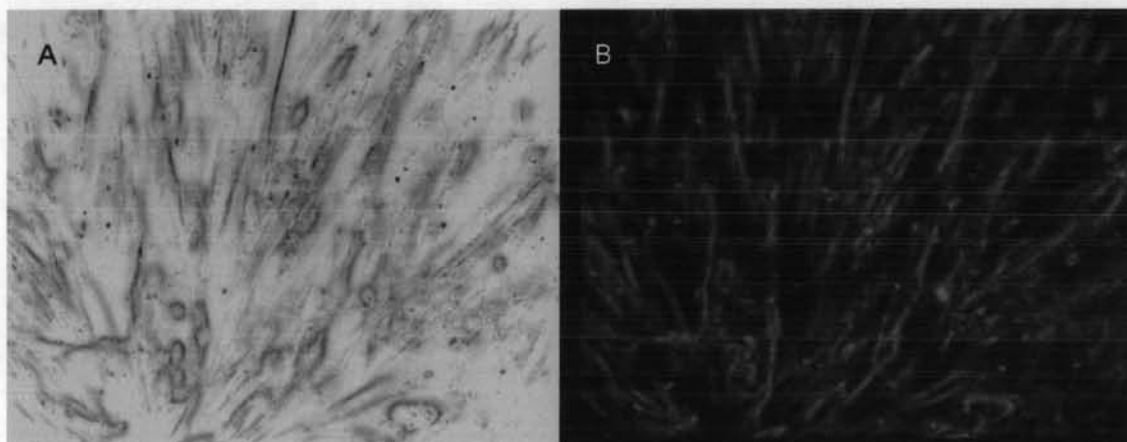


Figure 18: Uptake rate of treated cells with siRNA 1.5x fold. A: Bright field B: Spectrum red.

6. Coomassie blue gel staining

Nine protein samples were selected from control and case samples, and 5 μg of proteins were loaded and stained with coomassie blue. The coomassie gel staining was shown that all the samples were similarly quantity loaded (Fig 19).

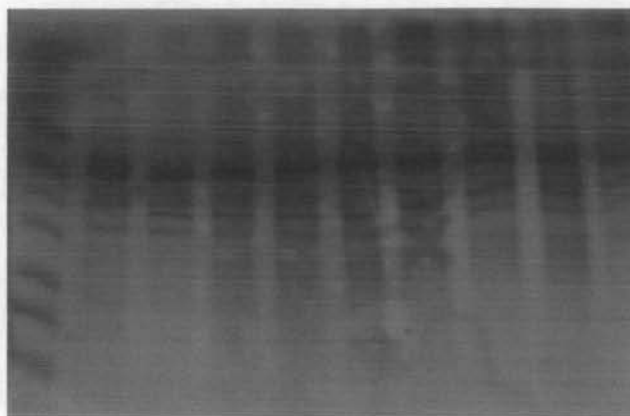


Figure 19 The coomassie gel staining

7. Western Blot

All treated case samples of No. 1431 and 1439 were not able to detect with western blot assay because cell lines slowly grown. All treated and untreated of 5 controls and 8 cases cell line were extracted protein at 28 days of post-transfection with siRNA and successfully done in western blot analysis. The ~22 KDa protein size of *DSCR1* product was detected by *DSCR1* antibody and shown in Figure 18-21. By semi-quantitative analysis, all treated proteins are seemed to be as same as untreated protein.



Figure 20 The ~22 KDa of *DSCR1* protein was detected in untreated 5 control samples.

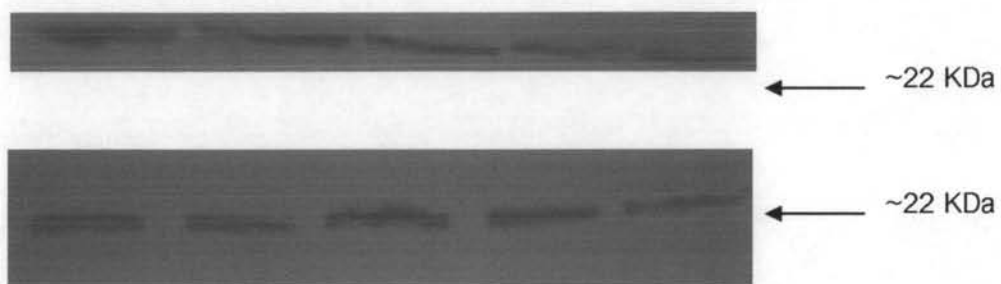


Figure 21 The ~22 KDa of *DSCR1* protein was detected in untreated 10 case samples.



Figure 22 The ~22 KDa of *DSCR1* protein was detected in treated 5 control samples.

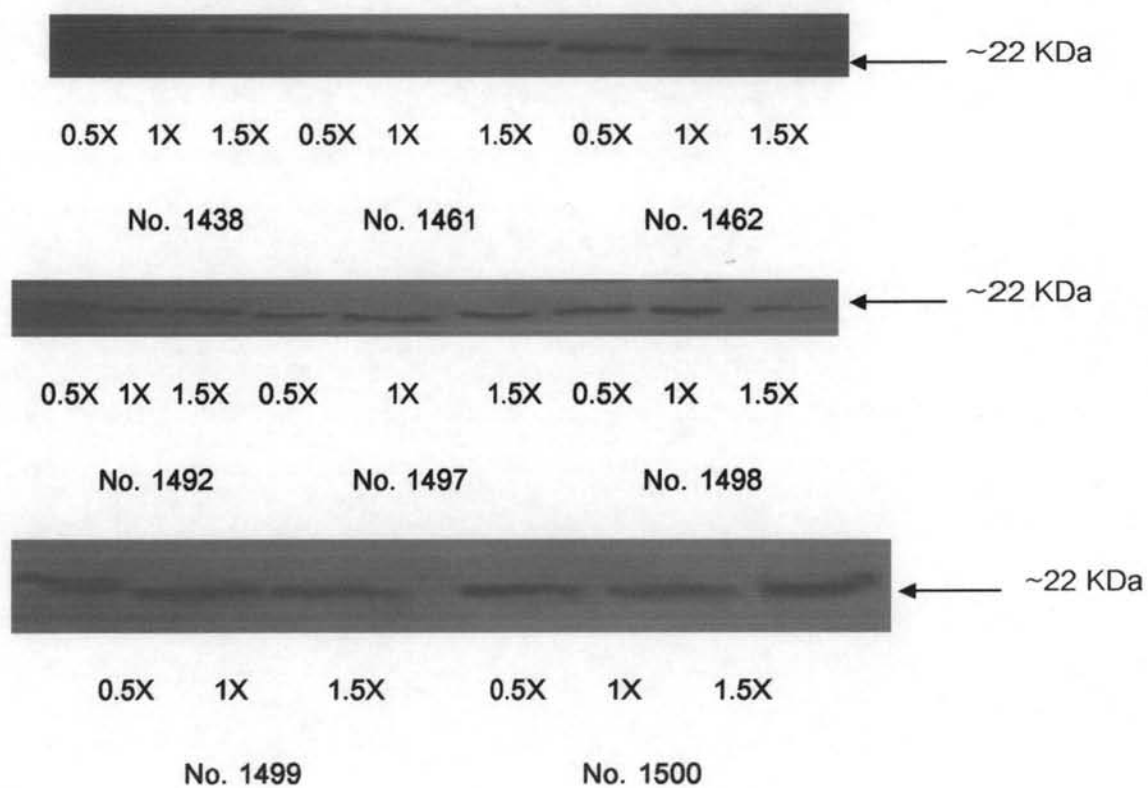


Figure 23 The ~22 KDa of *DSCR1* protein was detected in treated 8 case samples.

The result of western blot analysis could not refer to the result of real time due to the asymmetrical point of cell lines collection.