Redox-evolution of Eca-109 cells by 8-Br-cAMP and quercetin and correlation with p16 ssDNA binding nuclear matrix protein

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Abstract

Background. The DNA binding nuclear matrix protein (NMP) has been reported to be associated with regulation of gene expression. *Objective.* This subject was designed to investigate the effect of 8-Br-cAMP (Br) or quercetin (Q) on human esophageal cancer cells and to estimate the altered correlation between p16 sequence-specific (-869bp) DNA (ssDNA) binding NMP and Eca-109 cancer cell evolution. *Methods.* Both intranuclear and extranuclaer proteins were employed in Southwestern blot with the labeled ssDNA probe. The signals of *in situ* p16 mRNA and *in situ* ssDNA NMP were examined with labeled p16cDNA and ssDNA probes respectively. The Eca109 cancer cell evolution was evaluated with survival, apoptotic and differentiated cell rates, cell cycle progression, and expressions of Rb and PCNA. *Results.* A major enhanced 66 kDa signal of intra-nuclear matrix protein binding with the ssDNA in Southwestern blot positively correlated with the up-regulated expression signals of p16 mRNA, *in situ* ssDBP, Rb, and percentages of G2/M phase, differentiated and apoptotic cells after induction with 8-Br-cAMP or quercetin for 48 hours. However, all of these signals negatively correlated with down-regulated expression signal of PCNA simultaneously. *Conclusion.* Eca-109 cancer cells could be induced by Br or Q towards redox-evolution and suggest that the 66 kDa nuclear matrix protein may be a potential biomarker for induced Eca-109 cancer cells towards differentiation-apoptosis. [Life Science Journal. 2007; 4(3): 1 – 7] (ISSN: 1097 – 8135).

Keywords: nuclear matrix protein; Southwestern blot; *in situ* DNA binding protein; differentiation; apoptosis; Eca-109 cell line

1 Introduction

It was firstly reported that the 8-Br-cAMP, a signal transduction analogue, could selectively bind with PKAIIα to inhibit the cell growth[1]. The quercetin, one kind of plant flavonoids, could inhibit the PKC and TPK transduction pathways[2].

The nuclear matrix protein (NMP) is made up of the DNA-free proteins to form a scaffold in the cell nucleus. The solenoid chromatin appears as continuous loops to attach to the NMP and the attached chromatin region is designated as the scaffold/matrix attached region, S/MAR or MAR[3]. The MAR, as a novel cis-acting element, in association with NMP involves-various molecule events, such as DNA organization and other molecular events in the nucleus. The macromolecular complex of MAR and NMP also could up-regulate or down-regulate specific gene transcription[4, 5].

Wang reported that the alteration of NMP in neuroblastomas, an increased expression of Hsc, might be a sensitive indicator for detection of early apoptosis[6]. The BLCA-4 NMP, a bladder cancer-specific marker, could be used in diagnosis[7] and the NMPs-tumor specific were proposed to be diagnostic markers for human colon cancer, breast cancer and other cancers[8, 9]. Chen reported that the human esophageal cancer cell lines, Ec1 and Ec18, possessed specific NMP about 60 kDa[10]. Whether there may be biomarker in the induced 8-Br-cAMP (Br) or quercetin (Q) group, compared to the control group of

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Eca-109 cells was one of interesting subjects in this research.

Mezencev indicated that the cell proliferation, differentiation and apoptosis were regulated through p53-p21waf1 and p16-CDK-Rb pathway[11]. It has been reported that p16 could not only inhibit the cyclin-dependent cell cycle progression, but also facilitate cell differentiation, apoptosis or senescence[12 – 14]. In our previous works it was found that the 8-Br-cAMP or quercetin could inhibit human tumor cell growth, proliferation and facilitate the tumor cells towards differentiation or apoptosis closely associated with regulating gene expressions of wild type p53, p21waf1, p16, etc[15, 16]. So far, the information about relationship to the NMP binding with p16 sequence-specicific DNA (ssDNA) and esophageal cancer cell evolution has been limited. To explore the correlation between the altered p16 ssDNA (-869bp)-NMP and the Eca-109 cancer cell evolution, including regulation of cell growth, expressions of p16mRNA, *in situ* p16 ssDBP, differentiation and apoptosis was another interesting target in this research.

2 Materials and Methods

2.1 Cell culture

The human esophageal cancer Eca-109 cell line was kindly sent by Professor Pan YQ from China Acad Sci and the cell line has been kept to avoid any contamination in our lab. The Eca-109 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL, US) supplemented with 10% fetal bovine serum (FBS, TBD, China), 100 IU/ml penicillin and 100 μg/ml streptomycin in humidified 5% CO_2 incubator at 36.5 ºC. The subcultured cells at density of 1×10^7 from each 25 cm^2 flask were divided into 3 aliquots. The first aliquot was co-cultivated with 2×10^{-5} mol/L of 8-Bromo-adenosine-cyclic monophosphate (8-Br-cAMP, Sigma, US) dissolved in 0.05% dimethyl sulfoxide (DMSO, Solarbio, China), designated as the Br group. The second aliquot was co-cultivated with 43 μmol/L of quercetin (Q, Shanghai 2nd Reagent Factory, China) dissolved in 0.05% DMSO, as the Q group. The third aliquot was treated with 0.05% DMSO alone, as the control (C) group. The incubation time with these drug doses referred to our previous work[17].

2.2 Extraction of nuclear matrix protein and extranuclear protein

The essential protocol was as follows. Under 4 ºC the harvested Eca-109 cells in suspension at density of 1×10^7 cell/ml were washed with 1 × PBS and digested with 1% Nonidet P-40 (NP40, Sigma, USA) in 0.25 mol/L sucrose and subsequent 2.5 mol/L sucrose. They were digested until only the naked nuclei could be observed under a microscope. The extranuclear protein was routinely extracted from the cytosol and the NMP was extracted from the naked nuclei solution which was treated with 0.5% Triton X-100 (Sigma, US) in cell skeleton buffer (10 mmol/L PIPES (Sigma, US) pH 6.8, 300 mmol/L sucrose, 100 mmol/L NaCl, 3 mmol/L $MgCl_2$, 1 mmol/L EGTA). After centrifugation the precipitate was treated with the extract buffer (10 mmol/L PIPES pH 6.8, 250 mmol/L ammonium sulfate, 300 mmol/L sucrose, 3 mmol/L $MgCl_2$, 1 mmol/L EGTA), followed by digesting with 200 U/ml of DNase 1 (Promega, US) and 0.5% Triton-X100 in digesting buffer (10 mmol/L PIPES pH 6.8, 300 mmol/L sucrose, 50 mmol/L NaCl, 3 mmol/L $MgCl_2$, 1 mmol/L EGTA) for 30 minutes and extracted by top centrifugation. Prior to above each step additional 1 mol/L phenylmethylsulfonyl fluoride (PMSF, Sigma, US) and 1000 U/ml RNasin (Promega, US) were supplemented and each step was performed at 4 ºC. The concentration of NMP and extranuclear protein (ENP) was quantitatively determined by ultra-violet spectrometry and Coomassie brilliant blue G-250 method. The mentioned above procedure was referred to Spector[18].

2.3 Preparation of labeled DNA probes

The DH5α *E. coli* containing p16-promoter-luc-pGL2 basic reporter plasmid, including p16 upstream -869 bp – exon 1α, was kindly sent from Dr. Yi Ding. The amplified plasmid DNA was extracted and digested with both *Eco*RI and *Hind*III enzymes (MBI, Canada). The 890 bp target DNA fragment was isolated from 2.5% agarose gel after electrophoresis. Both the 890 bp DNA fragment and the denatured p16 cDNA were biotin-labeled with random primer system (Promega, US) separately and the sensitivity of the respective labeled probes was detected by the DNA dot blot. The procedure was referred to Ausubel[19].

2.4 Southwestern blot

The protocol was described as previously[19]. After SDS-PAGE the intranuclear NMP and the extranuclear protein from the Br, Q and C groups in the gel were electro-transferred onto nitrocellulose membrane (NCM, Protran™, US). The marker lane was cut from the entire NCM and stained with amido black 10 B to demonstrate the marker bands. The residual NCM was immersed and washed with the standard binding buffer (SBB, 10 mmol/L Tris-Cl buffer pH 7.0, 1 × Denhardt, 1 mmol/L Na_2-EDTA, 0.05 mol/L NaCl, 1% Triton-X100, 0.1 mg/ml herring sperm DNA), followed by incubation with the labeled 890 bp DNA probe in SBB (500 ng/ml as final concentration) at room temperature for 1 hour. After washing

with SBB and with blocking solution (20 mmol/L Tris-Cl, pH 7.5, 500 mmol/L NaCl, 0.05% Tween 20) for 30 minutes, the NCM was incubated with 1:1000 freshly diluted streptavidin-alkaline phosphatase (Promega, US) in the blocking solution at 37 ºC for 20 minutes followed by washing with Tris-Cl buffer. The NBT (nitroblue-tetrazolium, Promega, US) and BCIP (5-bromo 4-chloro-3-indole phosphate, Promega, US) used as a substrate was plated onto the NCM to demonstrate the signals in bluish-violet color. The negative control was performed with no labeled probe.

2.5 *In situ* hybridization for p16 mRNA signals

The routine procedure was described previously[18]. The Eca-109 cells with density of 1×10^7 cell/ml were dropped onto the pretreated slides and fixed with 4% paraformaldehyde. After digestion with 5 μg/ml (final concentration) proteinase K (Promega, US) at 37 ºC for 10 minutes and postfixation with 2% paraformaldehyde for 5 minutes, the cell slides were treated with 0.25% acetyl anhydrate and 0.1 mol/L triethanoamine (pH 8.0) for 10 minutes. The cell slides placed in a closed humidified box were pre-hybridized with hybridization solution (50 % deionized formamide, 5 × Denhardt, 5 × SSPE, 0.1% SDS and 0.1 mg/ml herring sperm DNA) at 42 ºC for 3 hours. The cell slides were incubated with the hybridization solution containing the final concentration of 500 ng/ml labeled p16 cDNA probe at 42 ºC overnight. After hybridization the slides were stringently washed four times with 0.1 × SSC at 42 ºC for 15 minutes, and blocked with 1% acetylated BSA. After incubation with 1:1000 freshly diluted streptavidin-alkaline phosphatase at 37 ºC for 20 minutes, the slides were washed thoroughly with Tris-Cl buffer (pH 7.5) and subsequent Tris-Cl buffer (pH 9.5). The NBT-BCIP was used as chromogen to develop the bluish-violet color signals. The cell slides were treated with no labeled probe as the negative control.

2.6 Detection of *in situ* p16 ssDNA binding protein (ssDBP) signals

The procedure was previously described[20]. Firstly the cell slide was treated with 50 mmol/L levamizol for 30 minutes at room temperature to remove endogenous alkaline phosphatase in cells. Then, the slides were treated with 0.3% Triton X-100 at room temperature for 20 minutes, PBS washed twice, digested with final concentration of 50 μg/ml DNase 1, PBS washed, and subsequently digested with final concentration of 100 μg/ml RNase A (Promega, US). The slides were post-fixed with 2% paraformaldehyde for 5 minutes, washed with PBS again. The following procedure was almost the same as that *in situ* hybridization except that: (1) the biotin-labeled p16 promoter sequence-specific DNA in 890 bp length instead of p16 cDNA was used as the probe; (2) the hybridization incubation time was only 2 hours; and (3) the SBB solution with higher salt concentration of 2 × SSC (subsequent 1 × SSC to replace the 0.1 × SSC solution with lower salt concentration) was used as the washing solution. The negative control slide was treated with no labeled DNA probe.

2.7 Immunostaining of Rb and PCNA

The cell slides were treated with 0.3% Triton X-100 at room temperature for 20 minutes. After heat retrieval of the tissue antigen with citrate buffer (pH 6.0) at 95 ºC for 10 minutes, the Rb polyclonal and PCNA monoclonal antibodies (Zymed, US) freshly diluted to 1:100 were used as primary antibodies, and the 1:200 diluted HRP-labeled anti-rabbit and anti-mouse sera were used as secondary antibodies. The diaminobenzidin (DAB) and H_2O_2 were used as the substrate to demonstrate the brownish-colored immunoreactivity (IR) signals. The negative control cell slides were carried out with PBS instead of the specific primary antibodies.

2.8 Detection of the cell cycle phase by flowcytometry

The isolated Eca-109 cells in Br, Q and C groups were fixed with 80% ethanol. The cells were diluted to a density of 1×10^6 cells/ml, after washing with 1 × cold PBS three times. The cells were incubated with 40 μg/ml propidium iodide (PI) and 40 μg/ml RNase A according to the manufacture's instructions, and counted by flowcytometry (FACS) with a CSC alibur flowcytometer (Becton Dickinson, US).

2.9 Detection of cell percentages by TUNEL or HCl-denatured methyl green-pyronin staining

2.9.1 TUNEL for detection of apoptosis. The slides of each group were treated with the levamizol to eliminate endogenous alkaline phosphatase followed by digestion with proteinase K and post-fixation with paraformaldehyde. Then, each slide was incubated with 20 μ1 terminal deoxynucleotidyl transferase (TdT) buffer containing 1 μ1 (20 IU) TdT (Promega, US), and 1 μl biotin-16-dUTP (Roche, Switzerland) at 4°C overnight. The following procedures were performed similarly as those for *in situ* hybridization. The negative control incubated with TdT buffer alone was carried out in the substrate at the same time.

2.9.2 HCl-denatured methyl green-pyronin staining. Iseki firstly described this method[21]. The slides of each group were re-fixed with ice-cold Carnoy's fixative for 15 minutes followed by digestion of intra-nuclear DNA with

1 mol/L HCl in 80% ethanol for 5 minutes. The slides were stained with 0.5% methyl green and pyronin GS (BDH) in acetate buffer (pH 4.8) for 5 – 7 minutes, subsequently rinsed with D·H_2O and acetone. The differentiated cells were mainly stained with pyronin in reddish color, while the proliferating cells predominantly stained with methyl green in bluish color.

2.10 Data analysis

Each kind of tests was repeated for three times at least. The Southwestern blot samples were scanned with a Gene Genius Bio Imaging System (Synoptics, LTD). The expression signals of p16mRNA, *in situ* ssDBP, RB-IR and PCNA-IR on the cell slides treated with no counterstaining were semi-quantitatively determined with Bio Sens Digital Imaging System (Shan Fu, China) as the gray-scale means (GSM). The data ± standard error(s) were statistically analyzed with SPSS 11.0. Analysis of variance (ANOVA), correlation analysis and χ^2 tests were performed, and $\alpha = 0.05$ was set as significance level.

3 Results

3.1 Sensitivity of the labeled probe

The detected sensitivity of the biotin-labeled probes for both p16 cDNA and the 890 bp ssDNA fragment could approach up to 1.0 pg/ml, indicating that each of the labeled probes would give qualified effect.

3.2 Southwestern blot

In the Southwestern blot of intranuclear NMP, a distinct band of 20 kDa was found in all three lanes, including Br, Q and C lanes. There were major enhanced bands of 66 kDa, and additional 58 kDa and 40 kDa bands were found in both Br and Q lanes in comparison with those in the control lane. Besides, the major enhanced bands in the extranuclear protein were 40 kDa and 28 kDa, displaying that the 40 kDa was present not only in the intranuclear but also in the extranuclear proteins. In the Southwestern blot of intranuclear NMP the scanning value of the 66 kDa bands showed 202.2 ± 1.2 in the Br lane, 176.3 ± 1.4 in the Q lane and 27.4 ± 1.0 in the C lane. Both the blot signals of Br and Q groups were much higher than those of the C group, $P < 0.01$ (Figure 1).

3.3 *In situ* p16 ssDBP

The violet colored *in situ* ssDBP signals were predominately located in the cell nuclei and also localized in the cytoplasm of some cells, especially in the larger cells with stronger signals. In the Br or Q group some nuclei with positive signals were frequently located at the cell periphery, implying a putative feature of apoptosis. The *in situ* ssDBP signals in the Br or Q group were markedly stronger than those in the control group, $P < 0.01$ (Table 1, Figures 2A – 2C). The results showed that the p16 sequence-specific (-869bp) DNA binding protein exists mainly in the nucleus and cytoplasm as well, consistent with that visualized in the Southwestern blot.

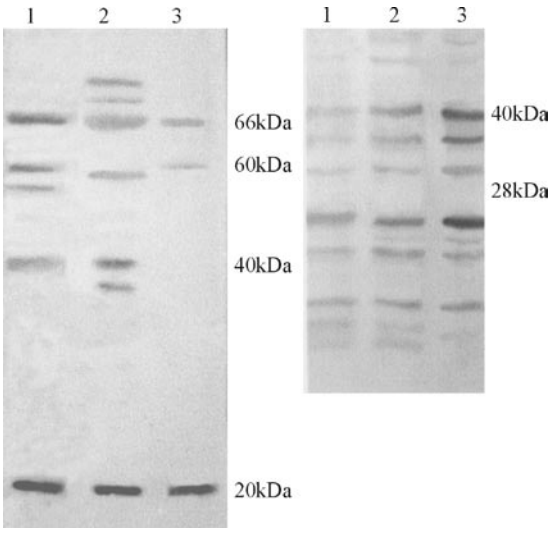

Figure 1. Southwestern blot of intranuclear NMP (left): Lane 1, Q group; Lane 2, Br group; Lane 3, C group. Southwestern blot of extratranuclear NMP (right): Lane 1, C group; Lane 2, Q group; Lane 3, Br group.

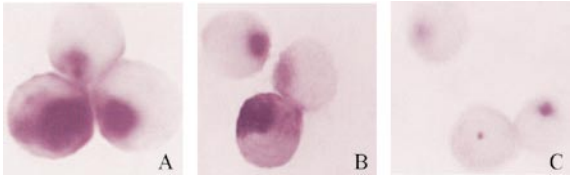

Figure 2. A) p16 ssDBP, Br group, × 1000; B) p16 ssDBP, Q group, × 1000; C) P16 ssDBP, C group, × 1000.

3.4 *In situ* hybridization of p16 mRNA expression

The *in situ* hybridization signals of p16 mRNA in violet-colored granules were localized in the cytoplasm of Eca-109 cells. The p16mRNA signals in the Br or Q group were stronger than those in the control group $P < 0.01$ (Table 1, Figures 3A – 3C). It implicated that the expression of p16 gene was activated in Eca-109 cells of the Br or Q group.

3.5 Immunostaining of Rb and PCNA

The Rb-IR signals in both Br and Q groups located mainly in the nuclei were markedly increased than those in the control group in which the Rb-IR signals were pre-

dominately located in the cytoplasm, $P < 0.01$ (Table 1, Figures 4A – 4C), suggesting that the activated Rb may be translocated from the cytoplasm into nuclei after induction by Br or Q. The expression signals of PCNA-IR were mainly localized in the nuclei of Eca-109 cells. However, the PCNA-IR signals in both Br and Q groups were decreased than those in the control group, $P < 0.01$ (Table 1, Figures 5A – 5C).

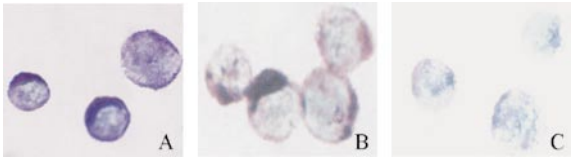

Figure 3. A) p16 mRNA, Br group, × 1000; B) p16mRNA, Q group, × 1000; C) p16mRNA, C group, × 1000.

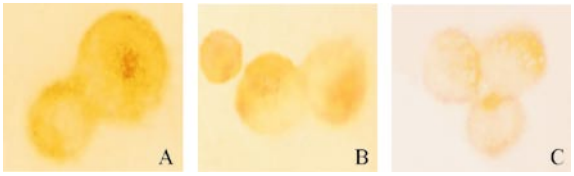

Figure 4. A) Rb-IR, Br group, × 1000; B) Rb-IR, Q group, × 1000; C) Rb-IR, C group, × 1000.

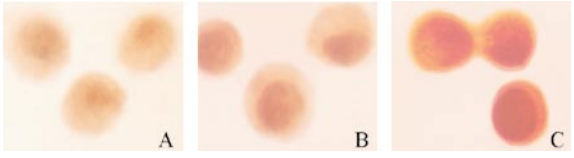

Figure 5. A) PCNA-IR, Br group, × 1000; B) PCNA-IR, Q group, × 1000; C) PCNA-IR, C group, × 1000.

Table 1. GSM $\bar{x}$ ± s of signals of p16 mRNA, p16 ssDBP, Rb-IR and PCNA-IR in each group of Eca-109 cells

Groups	p16 mRNA	p16 ssDBP	Rb-IR	PCNA-IR
Br group*	243.8 ± 5.7	103.5 ± 2.4	63.3 ± 3.0	21.2 ± 0.5
Q group**	207.8 ± 9.7	98.5 ± 3.7	57.3 ± 1.6	38.6 ± 0.7
C group***	106.3 ± 5.4	78.0 ± 2.6	40.4 ± 0.7	54.0 ± 0.9

GSM, gray scale means; DBP, DNA binding protein; IR, immunoreactivity. * or ** *vs.* ***, $P < 0.01$; * *vs.*** , $P < 0.05$.

3.6 The cell cycle phase detected by flowcytometry

In the cell cycle phase there was no significant alteration in G0 – G1 phase or S phase, while the proportion of Eca-109 cells were promoted in G2/M phase in the Br or Q group when compared with the C group, $P < 0.01$ (Table 2); displaying that the induced Eca-109 cancer cells were mainly arrested in the G2/M phase.

3.7 HCl-denatured-methyl green and pyronin staining

The nuclear DNA of proliferating cells (P) was less sensitive to HCl hydrolysis, mostly stained with methyl green in bluish color, while the differentiated cells (D) were stained in reddish color. The percentages of D in Br, Q and C groups were 71%, 69% and 17% respectively. Cell differentiation in C group was much lower than that in Br and Q groups, $P < 0.01$ (Figures 6A – 6C). In contrast the percentages of proliferating cells in Br, Q and C groups were 29%, 31% and 83% respectively. There was a reciprocal percentage for D versus P in these three groups, suggesting that Br or Q may inhibit Eca-109 cells to proliferate and facilitate the cells towards differentiation.

Table 2. The percentages in the cell cycle phase detected by flowcytometry in each group of Eca-109 cells

Group	Cell cycle phase		
	G0 – G1 phase (%)	S phase (%)	G2/M phase (%)
Br	57.38	18.72	23.90*
Q	61.34	13.06	25.60**
C	68.59	18.66	12.75***

* or ** *vs.* ***, $P < 0.01$. Besides, there was a positive correlation between the arrested cell percentages in G2/M phase and 66 kDa NMP blot signals in the variance of Br, Q and C groups, $r = 0.959$, $P < 0.01$.

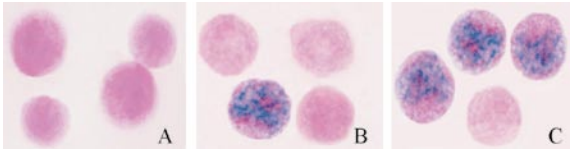

Figure 6. A) Differentiated cells in reddish staining, Br group, ×1000; B) Differentiated cells in reddish staining and one proliferating cell in bluish staining, Q group , × 1000; C) Proliferating cells in bluish staining, C group, × 1000.

3.8 TUNEL staining

The apoptosis signals in violet color were localized in the nuclei most of which were located at cell periphery. The apoptosis percentages in Br, Q and C groups were 45%, 42% and 3% respectively. The percentage in the Br or Q group was significantly higher than that of the C group, $P < 0.01$ (Figures 7A – 7C), exhibiting that Br or Q could induce Eca-109 cells towards apoptosis.

3.9 Correlations between data

There were significant positive correlations between the Southwestern 66 kDa NMP blot signals and the p16mRNA, p16 *in situ* ssDBP, Rb-IR and the percentages of G2/M phase, differentiated and apoptotic cells,$P < 0.01$; while there were significant negative correlations between the PCNA signals and Southwestern 66 kDa NMP blot, p16mRNA, p16 *in situ* ssDBP and Rb-IR in the variance of Br, Q and C groups, $P < 0.01$ (Tables 3 and 4).

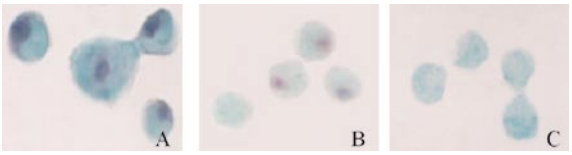

Figure 7. A) Apoptotic cells with violet colored signal, Br group, TUNEL with methyl green counterstaining, × 800; B) Apoptotic cells and one un-apoptotic cell, Q group, TUNEL with methyl green counterstaining, × 800; C) Un-apoptotic cells, C group, TUNEL with methyl green counterstaining, × 800.

Table 3. Correlations between the Southwestern blot signals and other signals of Br, Q and C groups

	Signals of Br, Q and C groups				
	p16 mRNA	p16 ssDBP	Rb-IR	differentiated cells	Apoptosis
Southwestern blot	0.991	0.981	0.975	0.994%	0.995%
$P <$	0.01	0.01	0.01	0.01	0.01

Table 4. Correlations between the the PCNA signals and other signals of Br, Q and C groups

	Signals of Br, Q and C groups			
	Southwestern blot	p16 mRNA	p16 ssDBP	Rb-IR
PCNA-IR	– 0.909	– 0.944	– 0.898	– 0.936
$P <$	0.01	0.01	0.01	0.01

4 Discussion

Cremer reported that the macromolecular complexes, including NMP and MAR, involved transcription, splicing, DNA replication and repair[22]. Kagotani indicated that the nuclear matrix played an important role in gene transcription and the FISH technique could visualize the behavior of genes at an individual cell level[23]. In the present study the alteration of p16 gene expression and p16 *in situ* ssDBP could be demonstrated at the individual cell level and the results showed that the signals of p16 gene expression in both Br and Q groups were markedly stronger than those in the control group, which met with what was found in the Southwestern blot and positively correlated with each other.

In this research the technique for *in situ* detection of p16 *in situ* ssDNA binding protein was modified by us, i.e., first treatment under condition of low salt concentration for benefit to formation of DNA binding protein at 37 ºC for 2 hours, followed by treatment under condition of higher salt concentration for stringent washing to remove the non-specific binding proteins. In most cells the p16 ssDBP signals were localized in the nuclei, and the signals were also located in the cytoplasm of some cells, even distributed within the entire cell. In our previous work it was found that the extranuclear DBP was most similar to the intranuclear DBP except that the latter had much higher and lower molecular weight, which was also found in this research. It suggests that there may be a shift of DBP between the nucleus and cytoplasm, and the cytoplasmic DBP may be potentially inactive nuclear protein[20]. The p16 *in situ* ssDBP signals in both Br and Q groups were stronger than those in the control group, which was coincident with what exhibited in the Southwestern blot and positively correlated with each other. Djeliova reported that during the cell cycle there were dynamics of replication origins with the nuclear matrix, the complex associated in the late G1 phase and dissociated after initiation of DNA replication in the S phase[24]. On the slides the stronger DBP signals were frequently found in the larger Eca-109 cells, those probably may be G1 phase cells, which remains to be studied.

Chatterjee reported that RGS12TS-S NMP, a regulator of G signaling protein, could inhibit S-phase DNA synthesis in various tumor cell lines and repress the cell cycle progression[25]. Our previous experiments showed that the 8-Br-cAMP could up-regulate expressions of wtp53, p16 INK4 and Rb; down-regulate expressions of PCNA and CDK4 in human HXO-Rb44 tumor cells through the cell cycle repress[16]. In this research the flowcytometric analysis showed that the G2/M phase cell proportion in Br or Q group was higher than that in the C group, which may be associated with up-regulated expressions of p16 and Rb. It showed that 8-Br-cAMP could inhibit the cell cycle progression through p16-Rb regulation not only found in the human Hxo-Rb44 tumor cells, but also in the human Eca-109 esophageal cancer cells.

It was reported that the expression level of PCNA was not only a biomarker for evaluation of cell proliferation level, but also for the cancer therapy efficiency[26, 27]. In both Br and Q groups the PCNA-IR signals were markedly lower than those in the control group and negatively correlated with other signals, suggesting that the 8-Br-

cAMP and quercetin might induce Eca-109 cancer cells towards redox-evolution to a certain extent.

The present results showed that about 70% differentiated cells and about 40% apoptotic cells could be found in the Br or Q group. It was supposed that the up-regulated expressions of p16 gene may be accounted for activated transcription in the nuclei through p16 -869bp DNA, acting as a cis-element and its binding 66 kDa NMP, acting as a transactivator. The complex of p16 ssDNA-NMP may be closely associated with up-regulating the specific expression of p16 gene to inhibit the cell cycle progression and proliferation, and facilitate the Eca-109 cancer cells towards differentiation and apoptosis after induction by Br or Q for 48 hours. In the Eca-109 cells the 66 kDa NMP binding with p16 ssDNA may be the underlying mechanism for the altered phenotype towards to differentiation-apoptosis evolution.

Chen reported that the human esophageal cancer cell lines, Ec1 and Ec18, possessed specific NMP about 60 kDa[10]. In the present research the 60 kDa band was not found in the Br or Q group, but only found in the control group, implicating that 60 kDa may be a potential biomarker for Eca-109 esophageal cancer cell line treated with no inducer and the 66 kDa NMP may be a potential biomarker for the induced Eca-109 cells towards redox-evolution. These biomarkers may contribute to diagnosis, prognosis and assessment of efficiency in cancer therapy.

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