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The role of p14ARF methylation in neuroblastoma minimal residual disease

[Nöroblastom'da p14ARF metilasyonunun minimal rezidüel hastalıktaki rolü]

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ABSTRACT

Objectives: The aim of the present study is to investigate the clinical significance of epigenetic changes in neuroblastoma, we evaluated the relationship between therapeutic variables and the pattern of gene methylation in neuroblastoma cell lines.

Methods: In this minimal residual disease model, cytotoxic effects of blocked $p14^{ARF}$ by a demethylating agent on KELLY and SHSY-5Y human neuroblastoma cell lines were assessed following addition of the drugs used in the Turkish Pediatric Oncology Group (TPOG) chemotherapy protocol. Drug induced effects on cell viability, cell damage and apoptotic cell death ratio were assessed with trypan blue dying. Cytotoxic effects differed among the used neuroblastoma cell lines. For the investigation of mechanisms of this effect, $p14^{ARF}$ gen methylation and expression levels, and MYCN expression levels were targeted. Expressions of mRNA and protein were determined with real-time PCR and ELISA, respectively.

Results: The $p14^{ARF}$ gene expression levels in KELLY 5-aza-CdR, Vincristin, Vincristin+5-aza-CdR, Dacarbazine, Ifosfamide+5-aza-CdR, Doxorubicine+5-aza-CdR, Etoposide+5-aza-CdR groups were found to be significantly higher than those measured in corresponding groups of SHSY-5Y ($p<0.05$). The percentages of $p14^{ARF}$ gene promoter unmethylation were higher in MYCN (-) groups than MYCN (+) groups ($p=0.034$).

Conclusion: In this pioneering study we suggest not only a new therapeutic approach for early or late relapses following the standard treatment protocols, but also demonstrate the effect of chemoterapeutic agents on $p14^{ARF}$ gene. The present study suggests that clinically aggressive neuroblastoma cell lines have aberrant methylation of $p14^{ARF}$ genes and provides a rationale for exploring treatment strategies that include demethylating agents.

Key Words: Neuroblastoma, $p14^{ARF}$ gene, DNA methylation, minimal residual disease

Conflict of interest: The authors do not have a conflict of interest.

ÖZET

Amaç: Bu çalışmanın amacı; iyi ve kötü prognozlu iki farklı nöroblastom hücre dizisinde, farklı tedavi uygulamaları ile p14ARF gen metilasyon paterni arasındaki ilişkinin karşılaştırılmasıyla, nöroblastom minimal rezidüel hastalıktaki epigenetik değişikliklerin klinik önemini ortaya koymaktır.
Yöntem: Kelly (Nöroblastik, MYCN +) ve SH-SY5Y (Nöroblastik, MYCN -) insan nöroblastom hücre dizilerinde oluşturulan in vitro "minimal rezidüel hastalık modeli" nde, p14ARF geni 5-aza-CdR ile demetile edilerek Türk Pediatrik Onkoloji Grubu (TPOG) kemoterapi protokolünde kullanılan ilaçların tümör hücreleri üzerindeki sitotoksik etkileri değerlendirildi. Ayrıca, p14ARF geninin MYCN amplifikasyonu olan ve olmayan hücre dizilerindeki etkisi değerlendirilerek tümörün prognozunu ne yönde etkilediği ve sitotoksik etki değişikliği ile prognoz arasında bir ilişki bulunup bulunmadığı araştırıldı. İlaçların hücre canlılığı ve hücre hasarına etkisi tripan mavisi boyaması ile belirlendi. p14ARF ve MYCN mRNA ekspresyon düzeyleri real-time PCR ile, protein ekspresyonu ELISA yöntemi ile ölçüldü.

Bulgular: KELLY'deki 5-aza-CdR, Vinkristin, Vinkristin+5-aza-CdR, Dakarbazin, İfosfamid+5-aza-CdR, Doksorubisin+5-aza-CdR, Etoposid+5-aza-CdR gruplarında p14ARF gen ekspresyon düzeyleri, SHSY-5Y'deki aynı gruplara göre anlamlı yüksek bulundu ($p<0.05$). p14ARF gen promoter demetilasyon oranları MYCN (-) gruplarda, MYCN (+) gruplara göre anlamlı yüksek bulundu ($p=0.034$).

Sonuç: Bu öncü çalışma ile; sadece yeni bir tedavi yaklaşımı değil, özellikle standart tedavi protokollerini izleyen erken veya geç relapslar için kullanılan kemoterapötiklerin p14ARF geni üzerindeki etkileri ortaya konuldu. Klinik olarak agresif olan nöroblastom hücre dizilerinde anormal p14ARF metilasyonlarının bulunduğu ve nöroblastom tedavi protokollerine toksik olmayan demetile edici ajanların eklenmesi gerektiği sonucuna varıldı.

Anahtar kelimeler: Nöroblastom, p14ARF geni, DNA metilasyonu, minimal rezidüel hastalık

Çıkar çatışması: Yazarların çıkar çatışması bulunmamaktadır.

Introduction

Neuroblastoma, a solid pediatric tumor arising from neural crest cells exhibits diverse clinical behaviors ranging from spontaneous remission to rapid tumor progression and death [1]. Various genetic abnormalities, such as MYCN oncogene amplification, allelic losses of choromosomes 1p and 11q, and gain of chromosome 17q, are predictive of outcome [2]. Recently, epigenetic aberrations have been reported to contribute to neuroblastoma pathogenesis [3,4]. Modulating methylation patterns of tumor suppressor genes may cause blockage of tumorigenesis. To date, some abnormally methylated genes had been described including the angiogenesis, apoptosis, cell cycle, differentiation, invasion and metastasis related genes. A methylator phenotype, characterized by the methylation of multiple CpG islands, is widely accepted as a hallmark of neuroblastoma with poor prognosis [5]. Further studies are needed on epigenetic studies in order to better understand the neuroblastoma pathogenesis and to explore new prognostic methylation markers for improving the therapeutic strategies [6,7,8].

The INK4a/ARF locus encodes the ARF tumor suppressor (p14ARF in human and p19ARF in mouse) as well as the cyclin inhibitor p16INK4a and is situated on choromosome 9p21 in the human genome [9]. p14ARF binds to MDM2 and inhibits the ubiquitination of p53, thereby stabilizing p53. Deletion or methylation inactivates p14ARF with the resultant increase of MDM2 and inactivation of p53 [10]. p53/MDM2/p14ARF pathway abnormalities are reported to have significance in the mechanisms of minimal residual disease and chemotherapy resistance [11,12,13].

The aim of this study was to investigate possible epigenetic treatment approaches in an in vitro model of neuroblastoma. For this purpose we designed an in vitro model of minimal residual disease (MRD) in MYCN amplification positive (KELLY) and negative (SHSY-5Y) human neuroblastoma cell lines and applied a demethylating agent (5'-Aza-2-deoxycytidine (5-aza-CdR)) together with classic chemotherapeutic agents (Vincristine, Dacarbazine, Ifosfamide, Doksorubicin, Cyclofosfamid, Etoposide, Cisplatinee) to investigate their effect on MYCN expression, p14ARF expression and methylation.

Materials and Methods

This study was approved by Clinical and Laboratory Research Ethics Committee of Faculty of Medicine in Dokuz Eylül University (25.04.2008/151).

Cells and culture conditions

KELLY (ACC 355) and SHSY-5Y (ACC 209) neuroblastoma cell lines were purchased from DSMZ, Germany. KELLY cell lines were cultured in RPMI-1640 (Gibco) medium suplemented with 10%FBS (Sigma-Aldrich), 1%penicillin-streptomycin (Sigma-Aldrich) and 1%L-glutamine (Sigma-Aldrich). SHSY-5Y cell lines were cultured in DMEM (Gibco) medium suplemented with 10%FBS (Sigma-Aldrich), 1%penicillin-streptomycin (Sigma-Aldrich). Both cell lines were maintained at $37C^0$ in humidified air with $5\%CO_2$. After growing with 90%confluence, cells were split and recovered in fresh media [14].

The formation of in vitro minimal residual disease model

For developing neuroblastoma minimal residual disease model, chemotherapeutic treatments were used as follows: exponentially growing cells were seeded at $1x10^8$ cells per 75 cm^2 culture flask in 16 MYCN (+) and 16 MYCN (-) to be a total of 32 groups. Chemotherapeutic drugs, in which doses were suatible for 90%cell death, were added to flasks. After chemotherapeutic treatment, the number of viable cells was calculated as $1x10^6$. Doses of chemotherapeutic drugs for groups of cells, TPOG-NB-2009 neuroblastoma treatment protocol, and over 1 year old high-risk group for patients who are organized in blocks of A9 and A11 according to the drug dose, were calculated for a 75 cm^2-flask [15]. Approximately 90%of the cell density reached culture flask, 90%cell death will provide doses Vincristine (1 mg/mL, Orna:0,01125 mg), Dacarbazine (10 mg/mL, Aventis:1,5 mg), Ifosfamide (40 mg/mL, Eczacıbaşı:11,25 mg), Doxorubicin (2 mg/mL, Farmar:0,225 mg), Cyclophosphamide (100 mg/mL, Eczacıbaşı:2,25 mg), Etoposide (20 mg/mL, Atafarm:0,6 mg) ve Cisplatinee (0,5 mg/mL, Koçak Farma:0,225 mg) was added and incubated for 12 hours in a 37 ^oC, $5\%CO_2$ humudified incubator. After 12 h, trypan blue cell viability was performed and the percentage of viability was 10%. The rest of cells after treating with chemotherapeutic drugs, were incubated for proliferation in a 37 ^oC, $5\%CO_2$ humudified incubator. When cell proliferation reached 90%confluency, 5-aza-CdR (5 µmol/L; Sigma, St. Louis, MO:0,18 mg) was added in to all groups but except control groups [16]. After 72 h incubation, cells were removed and total RNA was isolated from the untreated and treated cells.

Cell viability test with trypan blue

The viability of cells was evaluated by 0,4%trypan blue solution [17]. Cells, in which at least 10%percentage of cell viability was counted, were chosen for tests.

Total RNA isolation, cDNA synthesis, amplification and Q-PCR

RNA isolation was performed from both neuroblastoma cell lines with High pure RNA isolation kit (Roche Diagnostic GmBH), according to manufacturer's instructions. RNA quantification was measured by spectrophotometer (PG Instruments T 80) and complementary DNA synthesis was done by SABiosciences's RT2 First Strand Kit. MYCN and $p14^{ARF}$ gene expression analysis were investigated by Real time PCR on Roche Light Cycler 480. Reaction was performed using

SABiosciences's RT2 qPCR Master Mix, primary for MYCN (NM-005378), p14^{ARF} (NM-000077), followed by the manufacturer's protocol. And as housekeeping gene GADPH (NM-002046) was used. The differences of mean Cp of each gene and each status from control GADPH gene and their R (ratio) were calculated [7].

Genomic DNA isolation and methylation analysis with Q-PCR

Total genomic DNA was extracted from neuroblastoma cell lines using the High pure PCR template preparation kit (Roche Diagnostic GmBH), according to manufacturer's instructions. DNA quantification was measured by spectrophotometer (PG Instruments T 80). DNA digestion was done using DNA methylation sensitive and DNA methylation-dependent enzymes (SABiosciences's DNA methylation enzyme kit). After digestion, p14^{ARF} gene promoter site methylation status was assessed with Methyl-Profiler DNA Methylation qPCR Assays, according to manufacturer's instruction by using p14^{ARF} promoter site primary (NM-058195) and SYBR Green qPCR master mix (#PA-010) on Roche Light Cycler 480 [18]. The analysis of results was done at www.sabiosciences.com/ dna_methylation_data_analysis.php.

Nuclear extraction and ELISA for measurement protein levels

Nuclear extraction was performed from MYCN (+) and MYCN (-) cell lines by nuclear extraction kit (Active Motif cat no 40010) according to manufacturer's instruction. To quantify p53 activation ELISA test was done with Active Motif p53 Transcription Factor Assay Kit (Active Motif TransAM # 41196) according to manufacturer's instruction. For preparation of standard curve recombinant p53 protein (active Motif TransAM Cat no 31103) was used. The colorimetric readout of samples was quantified by spectrophotometer at 450 nm with a reference wavelength of 655 nm.

Statistical analysis

All determinations were performed in triplicate, and results are expressed as the mean ±standard deviation (SD). We determined statistical significance by Mann-Whitney U test; a p-value of 0,05 or less was considered significant.

Results

MYCN and p14^{ARF} gene (CDKN2A) expression levels and methylation percentages of p14^{ARF} were measured in MYCN (+) and MYCN (-) cell lines (Table 1 and 2). In amplification curves of real-time PCR, it was observed that MYCN gene expression started beginning from the 15th cycle and p14^{ARF} expression beginning from the 20th cycle in KELLY cell line, continuing until the 30th cycle in SHSY-5Y cell line. The 30th cycle was the chosen cut off for expression in this study (Figure 1, 2). As to be a unique common protein in the signaling pathway of MYCN and p14^{ARF}, p53 protein levels were detected in this study. The p53 protein levels were correlated

Table 1. The expression levels of MYCN and p14^{ARF} and methylation percentages of p14^{ARF} in MYCN (+) cell line at different drugs of therapy.

KELLY	MYCN gene expression levels	p14^{ARF} gene expression levels	Methylation percentages of p14^{ARF} (%)		
			Hypermethylated	Unmethylated	Intermediately methylated
Control	0,810	1,060	72,08	27,92	0
5-aza-CdR	0,847	1,053	0,07	99,93	0
Vincristine	0,807	1,070	0,14	99,86	0
Vincr.+5-aza-CdR	0,907	1,063	0,02	99,98	0
Dacarbazine	0,827	1,090	0,15	99,85	0
Dacar.+5-aza-CdR	0,850	1,027	0,07	99,93	0
Ifosfamide	1,00	1,093	0,08	8,6	91,31
Ifosfa.+5-aza-CdR	0,923	1,017	0,2	99,8	0
Doksorubicine	0,753	1,007	0,22	30,63	69,14
Dokso.+5-aza-CdR	0,817	1,030	0,05	99,95	0
Cyclophosfamide	0,740	1,030	0,09	33,25	66,66
Cyclo.+5-aza-CdR	0,853	1,033	0,14	99,86	0
Etoposide	0,847	1,143	0,1	3,54	96,36
Etopo.+5-aza-CdR	0,787	1,063	0,05	54,37	45,58
Cisplatine	0,833	1,097	0,08	99,92	0
Cisp.+5-aza-CdR	0,903	1,063	0,05	99,95	0

Table 2. The expression levels of MYCN and p14^{ARF} and methylation percentages of p14^{ARF} in MYCN (+) cell line at different drugs of therapy.

SHSY-5Y	MYCN gene expression levels	p14^{ARF} gene expression levels	Methylation percentages of p14^{ARF} (%)		
			Hypermethylated	Unmethylated	Intermediately methylated
Control	1,073	1,190	2,05	62,38	35,57
5-aza-CdR	1,113	1,170	0,42	99,58	0
Vincristine	1,163	1,227	1,78	98,22	0
Vincr.+5-aza-CdR	1,130	1,153	0,46	99,54	0
Dacarbazine	1,137	1,210	0,49	99,51	0
Dacar.+5-aza-CdR	1,083	1,093	0,87	99,13	0
Ifosfamide	1,130	1,177	0,7	99,3	0
Ifosfa.+5-aza-CdR	1,077	1,173	0,6	99,4	0
Doksorubicine	1,033	1,100	0,15	99,85	0
Dokso.+5-aza-CdR	1,100	1,133	0,27	99,73	0
Cyclophosfamide	1,037	1,140	1,26	98,74	0
Cyclo.+5-aza-CdR	1,107	1,123	0,44	99,56	0
Etoposide	1,040	1,193	0,07	48,67	51,26
Etopo.+5-aza-CdR	1,097	1,187	1,15	98,85	0
Cisplatine	1,050	1,127	0,18	99,82	0
Cisp.+5-aza-CdR	1,037	1,070	0,33	99,67	0

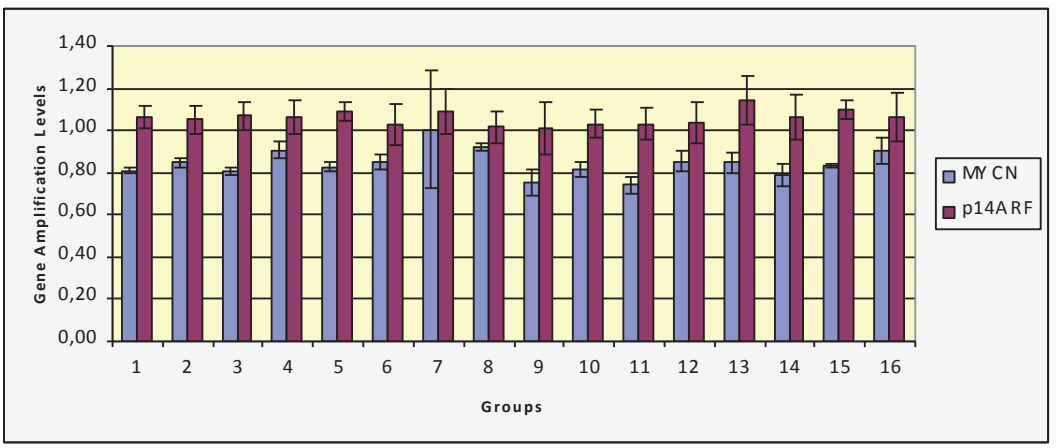

Figure 1. Comparison of gene expression levels in MYCN (+) cell groups.

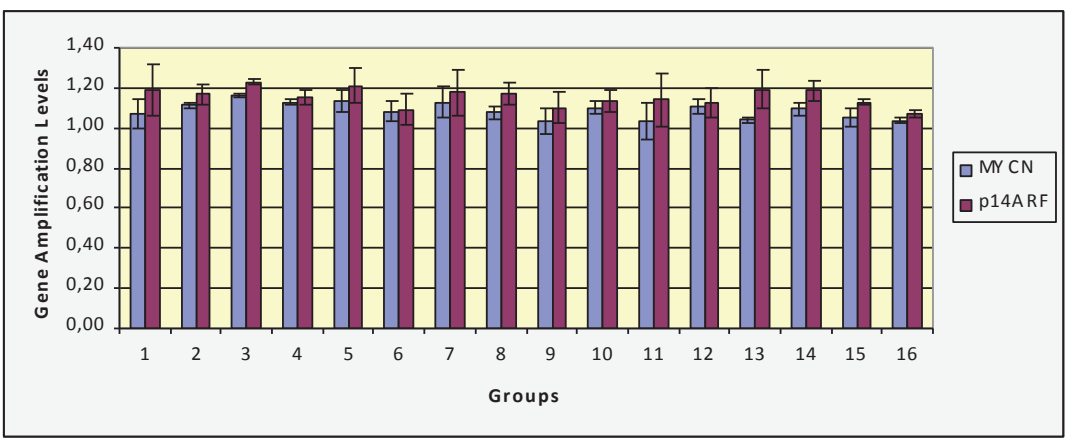

Figure 2. Comparison of gene expression levels in MYCN (-) cell groups.

with MYCN and p14^{ARF} gene expression levels. Comparison of p53 protein levels in neuroblastoma cell lines are shown at table 3. p53 protein levels were higher in KELLY MYCN (+) cell drug groups than SHSY-5Y MYCN (-) ones (p = 0.001) (Figure 3).

The percentages of p14^{ARF} gene promoter unmethylation were higher in MYCN (-) groups than MYCN (+) groups (p = 0.034) (Table 1, 2 and Figure 4). Hypermethylated and unmethylated percent of p14^{ARF} gene promoter region were found 72,08%and 27,92%, respectively in MYCN (+) cell groups. Unmethylated percent of p14^{ARF} gene promoter region were increased to 99,93%when 5-aza-CdR added. Hypermethylated and unmethylated percent of p14^{ARF} gene promoter region were found 2,05%and 62,38%, respectively in MYCN (-) cell groups. Unmethylated percent of p14^{ARF} gene promoter region was increased to 99,58%when 5-aza-CdR added.

Comparison of MYCN gene expression levels: The MYCN gene expression levels in KELLY control, 5-aza-CdR, Vincristin, Vincristin+5-aza-CdR, Dacarbazine, Dacarbazine+5-aza-CdR, Ifosfamide+5-aza-CdR, Doxorubicine, Doxorubicine+5-aza-CdR, Cyclophosfamide, Cyclophosfamide+5-aza-CdR, Etoposide, Etoposide+5-aza-CdR, Cisplatinee, Cisplatinee+5-aza-CdR groups were found to be significantly higher than those measured in corresponding groups of SHSY-5Y (Table 4).

Comparison of p14^{ARF} gene expression levels: The p14^{ARF} gene expression levels in KELLY 5-aza-CdR, Vincristin, Vincristin+5-aza-CdR, Dacarbazine, Ifosfamide+5-aza-CdR, Doxorubicine+5-aza-CdR, Etoposide+5-aza-CdR groups were found to be significantly higher than those measured in corresponding groups of SHSY-5Y (Table 5).

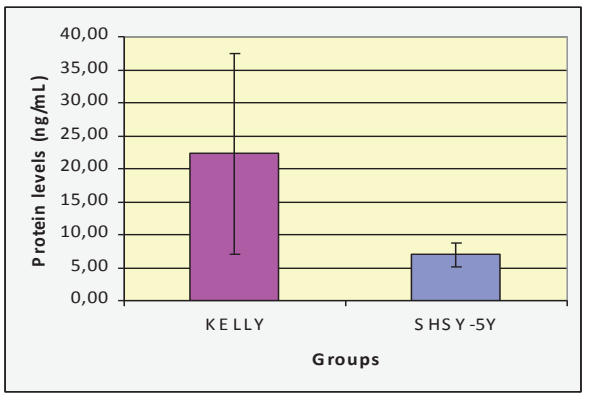

Figure 3. Comparison of protein levels in MYCN (+) and MYCN (-) cell groups.

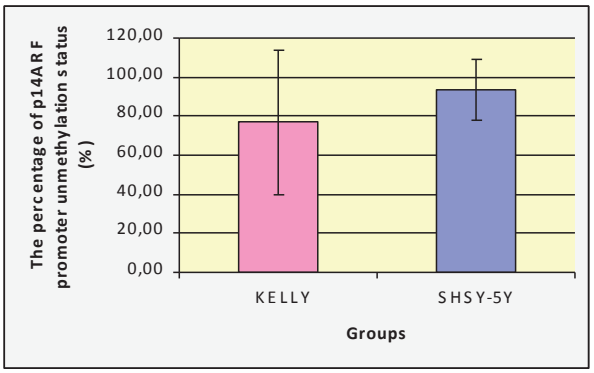

Figure 4. Comparison of the percentage of p14ARF gene promoter unmethylation status in MYCN (+) and MYCN (-) cell groups.

Table 3. The p53 protein levels in MYCN (+) and (-) cell lines.

GROUPS	p53 protein levels in KELLY (ng/mL)	p53 protein levels in SHSY-5Y (ng/mL)
Control	2,612	0,383
5-aza-CdR	0,532	0,360
Vincristine	1,502	0,254
Vincr.+5-aza-CdR	0,450	0,324
Dacarbazine	0,788	0,322
Dacar.+5-aza-CdR	0,728	0,232
Ifosfamide	1,512	0,251
Ifosfa.+5-aza-CdR	0,511	0,299
Doksorubicine	2,555	0,387
Dokso.+5-aza-CdR	1,016	0,301
Cyclophosfamide	2,138	0,362
Cyclo.+5-aza-CdR	0,426	0,298
Etoposide	1,389	0,458
Etopo.+5-aza-CdR	0,450	0,368
Cisplatine	0,768	0,486
Cisp.+5-aza-CdR	0,453	0,556

Table 4. The p values of MYCN expression levels in MYCN (+) and (-) cell groups.

KELLY MYCN	SHSY-5Y MYCN	p values
Control	Control	0,003
5-aza-CdR	5-aza-CdR	0,004
Vincristin	Vincristin	0,003
Vincr.+5-aza-CdR	Vincr.+5-aza-CdR	0,003
Dacarbazine	Dacarbazine	0,004
Dakar.+5-aza-CdR	Dakar.+5-aza-CdR	0,004
Ifosfamide	Ifosfamide	0,325
Ifosfa.+5-aza-CdR	Ifosfa.+5-aza-CdR	0,004
Doxorubicine	Doxorubicine	0,004
Dokso.+5-aza-CdR	Dokso.+5-aza-CdR	0,004
Cyclophosfamide	Cyclophosfamide	0,004
Cyclophos.+5-aza-CdR	Cyclophos.+5-aza-CdR	0,003
Etoposide	Etoposide	0,003
Etopo.+5-aza-CdR	Etopo.+5-aza-CdR	0,004
Cisplatine	Cisplatine	0,003
Cispl.+5-aza-CdR	Cispl.+5-aza-CdR	0,004

Table 5. The p values of p14ARF expression levels in MYCN (+) and (-) cell groups.

KELLY p14ARF	SHSY-5Y p14ARF	p Values
Control	Control	0,106
5-aza-CdR	5-aza-CdR	0,009
Vincristin	Vincristin	0,004
Vincr.+5-aza-CdR	Vincr.+5-aza-CdR	0,023
Dacarbazine	Dacarbazine	0,023
Dakar.+5-aza-CdR	Dakar.+5-aza-CdR	0,332
Ifosfamide	Ifosfamide	0,106
Ifosfa.+5-aza-CdR	Ifosfa.+5-aza-CdR	0,004
Doxorubicine	Doxorubicine	0,106
Dokso.+5-aza-CdR	Dokso.+5-aza-CdR	0,023
Cyclophosfamide	Cyclophosfamide	0,101
Cyclophos.+5-aza-CdR	Cyclophos.+5-aza-CdR	0,106
Etoposide	Etoposide	0,743
Etopo.+5-aza-CdR	Etopo.+5-aza-CdR	0,009
Cisplatine	Cisplatine	0,189
Cispl.+5-aza-CdR	Cispl.+5-aza-CdR	0,317

Marked changes in the cellular morphology were observed when 5-aza-CdR was added in control groups of MYCN (+) and MYCN (-) cells (Figure 5-8).

Discussion

MRD is one of the factors responsible for early or especially late relapses in high risk group of patients in whom remission has been achieved with either high dose chemotherapy or peripheral stem cell transplantation [19]. Today in the control of MRD, cytostatic or cytotoxic drugs, apoptotic agents, immunotherapeutic approaches, antiangiogenic drugs, and different rating agents are used [20]. However, against all of these therapeutic approaches, two years event free survival is still 30-40%[21]. Thus less toxic and more effective treatment strategies are needed.

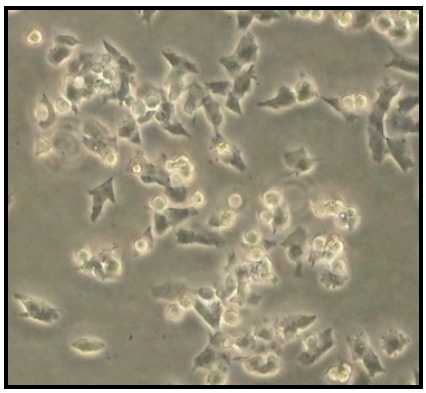

Figure 5. MYCN (+) control groups

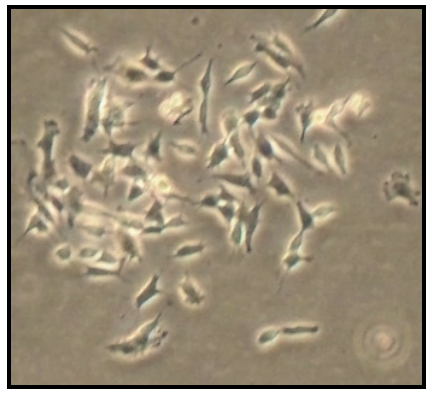

Figure 6. MYCN (-) control groups

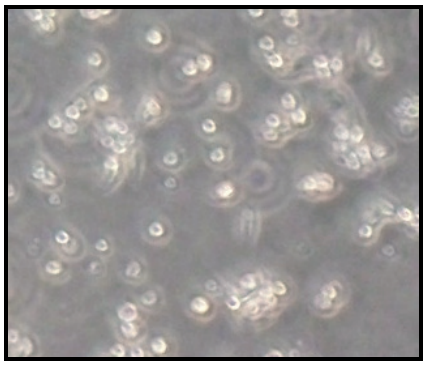

Figure 7. MYCN (+) 5-aza-CdR groups

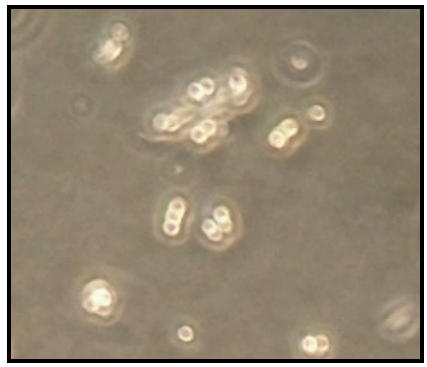

Figure 8. MYCN (-) 5-aza-CdR groups

MYCN and p14ARF gene expressions were found to be high in MYCN (+) Kelly cells when compared with MYCN (-) SHSY-5Y cells. The fact that p14ARF gene expression has been found to be significantly high in MYCN (+) cells, p14ARF is expected to be a possible predictor of the prognosis in neuroblastoma. p14ARF gene shows its tumor supressive effect via inhibiting degredation of p53 gene in cytoplasma which results from interrupting the cell cycle. Amente et al. [22] found a close relation between MYCN and p14ARF and reported an important role for p14ARF gene expression levels in the regulation of the transcriptional effect of MYCN. Therefore, p14ARF gene may show tumor supressive effect even irrelevant to tumor supressive effect of p53. Datta et al. [23] demonstrated a relation between p14ARF and c-Myc like transcription factors that had led to inhibiting transcriptional effect of MYCN gene. In the present study, p14ARF gene expression levels were found to be high parallel with those of oncogenic MYCN gene which has been considered as the main marker of poor prognosis. This finding proposed an important role for p14ARF gene in defending the cell when exposed to mitogenic signal.

In both MYCN (+) and MYCN (-) cell groups, addition of demethylating agent decreased MYCN gene expression while increasing p14ARF gene expression. Addition of 5-aza-CdR resulted with decrease in MYCN gene expressions in both MYCN (+) and MYCN (-) cell groups. The fact that the decrease was significantly high in MYCN (+) cell group when compared to that in MYCN (-) cell group suggested that demethylating agent was more effective in cells with poor prognosis. This finding is also in accordance with high p14ARF gene levels in MYCN (+) cells. Demethylation might have increased the transcriptive effect of p14ARF with the resultant inhibition of MYCN gene transcription. Yang et al. [6] showed high demethylation rates for various genes in primary tumor samples of agressive neuroblastomas and emphisized the necessity of using demethylating agent in the management protocols.

Addition of 5-aza-CdR resulted in increase in p14ARF gene expression in both MYCN (+) and MYCN (-) cell groups. Since the increase was significantly high in MYCN (+) cell group when compared to that in MYCN (-) cell groups, demethylating agent should have increased the expression levels of p14ARF gene which has tumor suppressive effect. This finding suggested a promising role for demethylating agents in improving the prognosis. Badal et al. demonstrated a time dependent increase in p14ARF gene transcription with addition of 5-aza-CdR and claimed that this had led to programmed death in tumor cells. Khan et al. [24] reported that p53 mediated apoptotic pathway was activated by p14ARF gene expressions in tumor cells and stressed the neces-

sity of targeting p14ARF gene in new therapeutic protocols.

In MYCN (+) controls, the promoter region for p14ARF gene was found to be hypermethylated in 72,08%of the cells. Addition of 5-aza-CdR increased the demethylation ratio to 99,93%. Vincristin, dacarbazine and Cisplatine established demethylating effect on the promoter region of p14ARF gene in both MYCN (+) and MYCN (-) cell groups. Addition of a demethylating agent enhanced this effect. In Vincristine treated MYCN (+) cells, demethylation was found to be in 99,86%of the cells. This effect of vincristine suggested its interaction with the molecules of the antitumoral action pathway related with tumor suppressive p14ARF gene. Dacarbazine and Cisplatine showed similar effects with those of vincristine and increased demethylation at the promoter region of p14ARF gene as a demethylating agent. Addition of dacarbazine resulted with a demethylation rate of 99,85%. Temozolomide which is also an imidazotetrazine derivative has a demethylating effect and exerts this effect by competing DNMT (DNA methyl transferase) [25,26]. Although temozolomide changes into its active form at the physiological pH, dacarbazine is subjected to metabolic activation in liver and not only loses its methylating effect but also gains demethylating effect.

Cisplatinee led to demethylation in 99,92%of the cells. Shang et al. [27] reported a synergistic suppression on tumor growth when Cisplatine and 5-aza-CdR were administered together and they attributed this effect to the interruption of G2/M phase of cell cycle irrelevant to p53 tumor suppressor gene. Ifosfamide, doxorubicin and cyclophosphamide produced demethylation on the promoter region of p14ARF gene in MYCN (-) cell groups while methylation in MYCN (+) cell groups and this methylating effect disappeared when a demethylating agent added. Etoposide induced methylation on the promoter region of p14ARF gene in both MYCN (+) and MYCN (-) cell groups and this effects was found to decrease when combined with a demethylating agent. Addition of ifosfamide, doxorubicin, cyclophosphamide and etoposide resulted in the methylation of the promoter region of p14ARF gene. Hitherto, no data accumulated to explain the mechanism of this effect. Only in a study investigating p53 mutations, resistances against melphalan, carboplatin or etoposide were attributed to p14ARF gene in relapsed neuroblastoma cell lines [10].

In the present study, demethylating effects of vincristin, dacarbazine and Cisplatinee in MYCN (+) cell groups increased with the addition of a demethylating agent. Methylating effects of ifosfamide, cyclophosphamide and etoposide on the promoter region of p14ARF gene decreased with the addition of a demethylating agent. Therefore, the combination of a demethylating agent with these drugs used in recent therapeutic protocol seems to be much more effective.

In MYCN (-) control cells, promoter region of p14ARF gene was found to be demethylated in 62,38%of the cells. When 5-aza-CdR was added, demethylation rate increased to 99,58%. In cells treated with vincristin, dacarbazine, ifosfamide, doxorubicine, cyclophosphamide and Cisplatine, demethylation increased when compared with control cells. In conttrast, demethylation enhanced when they were combined with 5-aza-CdR. As a result, these drugs were found to exert their effect as a demethylating agent.

Doxorubicin which is an antimitotic agent seemed to increase its apoptotic effect via increasing caspase-8 mRNA expression when combined with a demethylating agent. Liu et al. [16] reported that antitumoral effect of doxorubicin increased when combined with 5-aza-CdR in MYCN (-) SHSY-5Y neuroblastoma cell line and this is likely due to the increase in Caspase-8 mRNA expression. Caspase-8 expression is inactivated by methylation in a variety of solid tumors such as medulloblastoma, small cell lung carsinoma and primarily in neuroblastoma. Retinoic acid which is also used as a differentiating agent in the control of neuroblastoma MRD, has been shown to entrance apoptosis by increasing caspase-8 levels [28]. On the other hand, Etoposide induced methylation on the promoter region of the p14ARF gene in MYCN (-) cell group like a methylating agent. This effect was reversed when combined with 5-aza-CdR. In addition, demethylation increased.

In the present study, etoposide demonstrated methylating effect on the promoter region of p14ARF gene in both MYCN (+) and MYCN (-) cell groups and this effect was reducible with addition of a demethylating agent. This finding was unique for etoposide. Further studies are needed to performed in various tumor cell lines as well as on subjects to explore its mechanism. To date, no report was available about the effect of demethylating agents on p14ARF gene in MYCN (+) and MYCN (-) neuroblastoma or MRD model. However, in studies performed in pediatric or adult tumors other than neuroblastoma showed that addition of demethylating agents to chemotherapy protocols had enhanced antitumor activity and raised the necessity of developing new demethylating agents with low toxic profile for epigenetic management approaches. Majid et al. [29] compared the effect of two different demethylating agents on BTG3 tumor suppressor gene in renal cell carcinoma cell lines and they reported that genistein which is naturally found in the structure of isoflavones exerted a comparable effect with 5-aza-CdR which is highly toxic and unstable agent. In another study regarding the effects of demethylating agents on myeloid malignancies, demethylating agents were found to be effective especially when administered with low doses as well as combined with other demethylating agents [30].

We have two main conclusions;

Firstly, this study strongly suggests that p14ARF gene

methylation plays a very important role in MRD of neuroblastoma. Further in vitro and in vivo studies are needed to explore tumor suppressor p14ARF gene-related action and resistance mechanisms in order to increase the effects of drugs against MRD and to find new and more effective treatment protocols.

Secondly, in the control of MRD, metronomic treatments can be combined with the demethylating agents which are reported to be more effective against MYCN (+) neuroblastoma with poor prognosis. Thus, less toxic and more effective new alternative therapeutic approaches should be developed.

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