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p53 Mutations and Loss of p53 Function Confer Multidrug Resistance in Neuroblastoma

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Background. Neuroblastomas often acquire sustained drug resistance during therapy. Sensitivities to carboplatin, etoposide, or melphalan were determined for 18 neuroblastoma cell lines; eight were sensitive and ten were resistant. As p53 mutations are rare in neuroblastomas studied at diagnosis, we determined if acquired p53 mutations and loss of function conferred multidrug resistance. ***Results.*** Loss of p53 function (p53-LOF), defined as a failure to induce p21 and/or MDM2 in response to melphalan, was seen in 1/8 drug-sensitive and 6/10 drug-resistant cell lines. In four cell lines p53-LOF was associated with mutations in the DNA binding region of p53, while three cell lines with LOF and four cell lines with functional p53 had no evidence of p53 muta-tions. Nonfunctional and mutated p53 was detected in one resistant cell line, while a sensitive cell line derived from the same patient prior to treatment had functional and wild type (wt) p53. We transfected HPV 16 E6 (which mediates degradation of p53, causing LOF) into two drug-sensitive neuroblastoma cell lines with functional p53. LC_{90} values of HPV 16 E6 transfected cell lines were 3–7-fold (melphalan), 8–109-fold (carboplatin), and 2–158-fold (etoposide) greater than that of LXSN-transfected controls. ***Conclusions.*** These data suggest that some neuroblastomas acquire p53 mutations during therapy, which is associated with a loss of p53 function, and can confer high-level multidrug resistance. Med. Pediatr. Oncol. 35:563–568, 2000.

Key words: neuroblastoma; cancer chemotherapy; drug resistance; mutation

INTRODUCTION

Neuroblastoma is an aggressive childhood neoplasm of the sympathetic nervous system. Intensive chemoradiotherapy supported with autologous bone marrow transplantation (ABMT) has improved survival for high-risk neuroblastoma, especially if followed by 13-cis-retinoic acid [1]. However, most high-risk neuroblastoma patients develop recurrent disease that is refractory to further therapy. We have shown that during therapy, neuroblastomas can acquire a sustained drug resistance that correlates with the drugs and the intensity of the therapy received [2].

A transcriptional regulatory protein, p53 has target genes that regulate cell cycle, apoptosis, and DNA repair [3,4]; p53 mutations and/or deletions have been linked to drug resistance in acute lymphoblastic leukemia [5], melanoma [6], osteosarcoma [7], breast [8], ovarian [9], and testicular [10] cancers. Mutations of p53 are found in over 50% of most human cancers, but p53 mutations are rare in neuroblastoma and occasionally have been associated with tumor progression [11–17]. As an alternative to mutations, cytoplasmic sequestration and defective translocation of p53 has been suggested as a mechanism of nonmutational inactivation [18], yet neuroblastoma cell lines show functional p53 when subjected to genotoxic stress [19,20,21].

We have examined p53 function by measuring alkylating agent-mediated induction of p21 and MDM2 in a panel of neuroblastoma cell lines with a spectrum of resistance to melphalan (L-PAM), carboplatin (CBDCA), and etoposide (ETOP), all drugs commonly used in neuroblastoma. In addition, we examined exons 5 through 8 for p53 mutations by DNA sequencing. To determine if a loss of p53 function confers drug-resistance in neuroblastomas, we abrogated p53 function by transfecting drug-sensitive neuroblastoma cell lines with HPV16 E6 [23] using the LXSN retrovirus. We then assayed the response of HPV 16 E6-transfected clones to L-PAM, CBDCA, and ETOP. Here we show that sustained high-

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TABLE I. Neuroblastoma Cell Line Panel*

Cell lines	Phase of therapy	Drug resistance profile[a]	p53 Function[e]	p53 Sequencing
Drug-sensitive cell lines:				
SK-N-BE(1)	DX[h]		Yes	ND[f]
SMS-SAN	DX		Yes	ND
CHLA-122	DX		Yes	ND
SMS-KCNR	PD[i]		Yes	ND
SMS-LHN	PD		Yes	ND
CHLA-225	PD		No	Wt
CHLA-8	PD-BMT[j]		Yes	ND
CHLA-51	PD-BMT		Yes	ND
Drug-resistant cell lines:				
SK-N-BE(2)	PD	L-PAM[b]	No	Mutated
SK-N-RA	PD	L-PAM, CBDCA,[c] ETOP[d]	Yes	Wt[g]
LA-N-6	PD	L-PAM, CBDCA, ETOP	Yes	Wt
CHLA-119	PD	L-PAM, CBDCA, ETOP	No	Mutated
CHLA-171	PD	L-PAM, CBDCA, ETOP	No	Wt
CHLA-79	PD-BMT	CBDCA, ETOP	Yes	Wt
CHLA-90	PD-BMT	L-PAM, CBDCA, ETOP	No	Mutated
CHLA-134	PD-BMT	L-PAM, CBDCA, ETOP	No	Wt
CHLA-136	PD-BMT	L-PAM, ETOP	Yes	Wt
CHLA-172	PD-BMT	L-PAM, CBDCA, ETOP	No	Mutated

*Drug sensitivity profiles for most of the cell lines previously reported [2,25,27].
[a]Drugs with LC_{90} values higher than clinically achievable levels are indicated.
[b]L-PAM = melphalan (reference drug level 10 μg/ml).
[c]CBDCA = carboplatin (reference drug level 3 μg/ml).
[d]ETOP = etoposide (reference drug level 5 μg/ml).
[e]Yes = functional p53; no = nonfunctional p53. p53 Function was defined as 1.6-fold (median value) or greater induction of p21 and/or MDM2 relative to basal protein expression.
[f]Not determined.
[g]Wild type.
[h]DX = cell lines established from the patients prior to the treatment.
[i]PD = cell lines established from patients at the time of disease progression after induction chemotherapy.
[j]PD-BMT = cell lines established from patients at the time of disease progression after myeloablative chemoradiotherapy followed by bone marrow transplantation.

level drug resistance in neuroblastoma cell lines is associated with p53 mutations and loss of p53 function.

MATERIALS AND METHODS

Cell Lines

The cell lines used in this study, and their drug sensitivity profile to L-PAM, CBDCA, and ETOP are shown in Table I. We studied 18 neuroblastoma cell lines [2,27]. SMS-SAN, SMS-LHN, and SK-N-RA were cultured in complete medium made from RPMI-1640 (Irvine Scientific, Santa Ana, CA) supplemented with 10% heat inactivated fetal bovine serum (FBS) (Gemini Bio-Products, Inc., Calabasas, CA). SMS-KCNR, SK-N-BE(1), SK-N-BE(2), LA-N-6, CHLA-122, CHLA-171, CHLA-225, CHLA-119, CHLA-51, CHLA-8, CHLA-79, CHLA-90, CHLA-134, CHLA-136, and CHLA-172 were cultured in complete medium made from Iscove's modified Dulbecco's medium (IMDM) (Bio Whittaker, Walkersville, MD) supplemented with ~3 m*M* L-glutamine (Gemini Bioproducts, Inc., Calabasas, CA), insulin, and transferrin, 5 μg/ml each, and 5 ng/ml of selenous acid (ITS® Culture Supplement; Collaborative Biomedical Products, Bedford, MA), and 20% heat inactivated FBS. All cell lines used in this study were under passage 30. Cell lines were not selected for resistance to any drug in vitro.

Retroviral Infection

PA317 packaging cells transfected with the control retrovirus vector, pLXSN, or with the vector pLXSN16E6, containing the human papilloma virus (HPV) type 16 E6 gene [23], were obtained from American Type Culture Collection (ATCC). Both pLXSN and pLXSN16E6 were transfected into SMS-SAN and SMS-LHN cells. Transfected cells were maintained in selection medium containing 250 μg/ml G418.

Protein Expression

Antibodies to p53 (DO-1) mouse monoclonal, p21 (C-19) rabbit polyclonal, MDM2 (SMP14) mouse monoclonal, and HRP labeled secondary antimouse and antirabbit antibodies were purchased from Santa Cruz Biotechnology, Inc., Santa Cruz, CA. Western blots were performed using ECL visualization as previously described [21]. Films were scanned and protein expressions were quantitated using the software SigmaGel (Jandel Scientific). Induction of proteins were calculated relative to steady

state protein expression and the median protein inductions were determined (p53 = 2.5, p21 = 1.6, and MDM2 = 1.6) and used as cut off points for inductions of these proteins.

Automated p53 Sequencing

Exons 5–8 of 11 neuroblastoma cell lines were sequenced by the fluorescent dideoxy terminator method of cycle sequencing on an ABI 377XL (96 well format) automated DNA sequencer at Laragen, Inc. (Los Angeles, CA). The primers were synthesized at Only DNA (Midland, TX). The following primers were used: 5′-CTTGTGCCCTGAACTTTCAACTCTGTCTC-3′ and 5′ -TGGGCAACCAGCCCTGTCGTCTCTCCA-3′ (exon5); 5′-CCAGGCCTCTGATTCCTCACTGATTGCTC-3′, and 5′ -GCCACTGACAACCACCCTTAACCCCTC-3′ (exon 6); 5′ -GCCTCATCTTGGGCCTGTGTTATCTCC-3′ and 5′ -GGCCAGTGTGCAGGGTGGCAAGTGGCTC-3′ (exon 7); 5′ -GTAGGACCTGATTTCCTTACTGCCTCTTGC-3′ and 5′ -ATAACTGCACCCTTGGTCTCCTCCACCGC-3′ (exon 8). The sequences were identified by using the Sequencher software from Gene Codes (Ann Arbor, MI). Electropherograms were also read to identify mutations.

Cytotoxicity Assay

The cytotoxicity of L-PAM, ETOP, and CBDCA for neuroblastoma cell lines over a range of concentrations was determined using the DIMSCAN assay system [2]. The drug concentration ranges used were: L-PAM = 0 to 10 μg/ml, CBDCA = 0 to 5 μg/ml, and ETOP = 0 to 5 μg/ml. LC_{90} values (i.e., the drug concentration that was cytotoxic for 90% of the cell population) were calculated using the software "Dose-Effect Analysis with Microcomputers." Cell lines with an LC_{90} value higher than the achievable clinical drug concentration were considered resistant to that drug. Reference clinical concentrations were: 10 μg/ml for L-PAM, 3 μg/ml for CBDCA, and 5 μg/ml for ETOP [2].

RESULTS

Drug Resistance in Cell Lines

Cell lines with an LC_{90} value greater than the clinically achievable drug concentration were considered resistant to that drug. The drug resistance profile for each cell line is shown in Table I. There were eight sensitive and ten resistant cell lines.

Expression of p53, p21, and MDM2 in Neuroblastoma Cell Lines

Using Western blot analysis we examined basal expression and induction of p53 after a 16-hr challenge by L-PAM, and as indices of p53 function, p21 and MDM2 (Fig. 1).

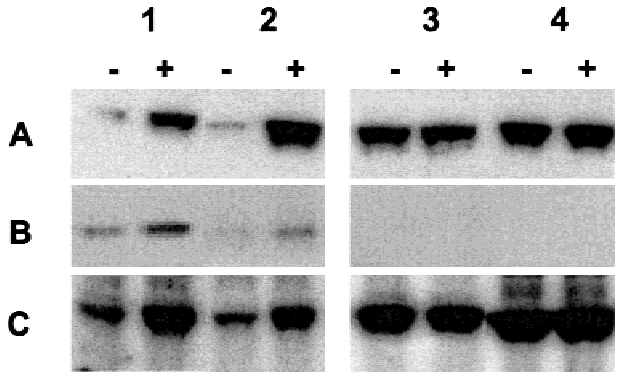

Fig. 1. Western blot analysis of **A:** p53, **B:** p21, and **C:** MDM2. Basal (−) and induced (+) levels after 16 hr 6 μg/ml L-PAM exposure are shown for representative sensitive (lanes 1 and 2) and resistant (lanes 3 and 4) cell lines. Lanes: 1. SMS-LHN, 2. SMS-KCNR, 3. CHLA-90, 4. SK-N-BE(2).

Normally, wt p53 expression is either undetectable or very low, but it increases briefly upon exposure to DNA damaging agents. In contrast, overexpression of p53 in the absence of genotoxic stress is a hallmark of mutation and can be detected by immunoblotting. We found that p53 steady-state expression was low and inducible (> 2.5) in 7/8 drug-sensitive cell lines: SK-N-BE(1), SMS-SAN, CHLA-122, SMS-LHN, SMS-KCNR, CHLA-8, and CHLA-51, and in 3/10 drug-resistant cell lines: LA-N-6, CHLA-79, and CHLA-136. A high basal level expression and a failure to induce p53 (less than 2.5-fold induction) was observed in 7/10 drug-resistant cell lines: SK-N-BE(2), SK-N-RA, CHLA-119, CHLA-171, CHLA-90, CHLA-134, and CHLA-172, and in 1/8 drug-sensitive cell lines (CHLA-225). Induction of p53 after exposure to L-PAM was demonstrated for 7/8 sensitive cell lines, and 4/10 resistant cell lines.

Cell lines that failed to induce p21 and/or MDM2 > 1.6-fold (median p21 and MDM2 inductions) were considered as p53 nonfunctional. Seven of the eight sensitive cell lines [SK-N-BE(1), SMS-SAN, CHLA-122, SMS-LHN, SMS-KCNR, CHLA-8, and CHLA-51] and 4/10 (LA-N-6, SK-N-RA, CHLA-79, and CHLA-136) drug-resistant cell lines showed p21 and/or MDM2 induction after L-PAM challenge. One of the eight drug-sensitive cell lines (CHLA-225) and 6/10 drug-resistant cell lines [SK-N-BE(2), CHLA-119, CHLA-171, CHLA-90, CHLA-134, and CHLA-172] lacked functional p53.

Analysis of p53 Mutations by Automated Sequencing

As shown in Table I, exons 5–8 of one sensitive cell line (CHLA-225 with nonfunctional p53) and all ten resistant cell lines (four with functional p53 and six with nonfunctional p53) were analyzed by automated dideoxynucleotide sequence analysis with fluorescence labeling. Mutations were observed in four cell lines [SK-N-BE(2), CHLA-119, CHLA172, and CHLA-90], all of

TABLE II. LC_{90} Values of SMS-SAN and SMS-LHN HPV 16 E6 Transfected Clones*

Transfected clones	L-PAM	CBDCA	ETOP
SAN/LXSN[a]	1.8	1.3	0.7
SAN/E6 D4[b]	8.3	19.6	29.1
SAN/E6 B3[b]	10.8	31.7	110.4
LHN/LXSN[a]	4.7	6.4	3.7
LHN/E6 2-6[b]	13.1	52.5	7.6
LHN/E6 5-B5[b]	32.1	693.5	166.2

*Drug concentration that was cytotoxic for 90% of treated cells.
[a]Empty vector controls of SMS-SAN and SMS-LHN cell lines.
[b]HPV 16 E6 clones of SMS-SAN and SMS-LHN cell lines.

which had nonfunctional p53. Wild type (wt) p53 was found in four cell lines with functional p53 (LA-N-6, SK-N-RA, CHLA-79, and CHLA-136), and in three cell lines with nonfunctional p53 (CHLA-171, CHLA-225, and CHLA-134).

Paired Cell Lines

Within the panel studied, there was a pair of cell lines derived from the same patient at diagnosis prior to treatment, SK-N-BE(1), and then after disease progression during induction therapy, SK-N-BE(2). LC_{90} values of SK-N-BE(2) were 30 times greater for L-PAM, 13 times greater for CBDCA, and seven times greater for ETOP relative to SK-N-BE(1) (Table I) [2]. SK-N-BE(1) contains functional, and SK-N-BE(2) has nonfunctional and mutated p53.

Drug Sensitivity Profile of Neuroblastoma Cell Lines with Abrogated p53 Function

We used HPV 16 E6 to abrogate p53 activity in drug-sensitive neuroblastoma cell lines SMS-SAN (*MYCN* gene amplified) and SMS-LHN (*MYCN* gene nonamplified), and then compared the sensitivity to L-PAM, CBDCA, and ETOP of HPV 16 E6 transfected clones to the parental cell lines and LXSN (empty vector) transfected controls. Reduced p53 activity was confirmed by a lack of p53, p21, and MDM2 induction in L-PAM challenged samples using Western blotting. Transfection of HPV 16 E6 conferred high-level drug-resistance to both SMS-SAN and SMS-LHN. LC_{90} values of SAN/E6 clones were four and a half to six times higher for L-PAM, 15.4 to 25 times higher for CBDCA, and 41.6 to 157.7 times higher than ETOP relative to the SAN/LXSN empty vector control cells. Similarly, LC_{90} values of LHN/E6 clones were three to seven times higher for L-PAM, 8 to 109 times higher for CBDCA, and 2 to 45.3 times higher for ETOP relative to LHN/LXSN empty vector control cells (Table 2).

DISCUSSION

Neuroblastomas acquire sustained drug resistance during therapy, which appears to be a result of selection for tumor cells resistant to those drugs employed in therapy. This correlates with the intensity of chemotherapy the tumors had been exposed to in vivo during the therapy [2]. Identification of the molecular mechanisms for drug-resistance in neuroblastoma may allow the development of therapies to overcome the particular form of drug-resistance manifested by this tumor.

As p53 mutations are infrequent in primary neuroblastomas [11–17], it has been suggested that functionally inactive p53 is due to cytoplasmic localization of p53 [18]. However, Goldman et al. reported that although p53 was mainly cytoplasmic in tested neuroblastoma cell lines, p53 levels increased mainly in the nucleus after irradiation, and transcriptional activity of p53 was intact [19]. Other studies have confirmed the presence of functional p53 in neuroblastoma cell lines in response to UV [20] or cisplatin [21].

Many investigators have demonstrated that p53 mediates response to cytotoxic agents [22] and that mutant p53 can confer resistance to DNA damaging agents [8]. Using Western blot analysis for p53 target genes, we found that p53 was functional in 7/8 drug-sensitive cell lines as determined by induction of p21 and/or MDM2 greater than 1.6 (median p21 and MDM2 induction for all cell lines tested) after 16 hr of 6 μg/ml L-PAM. Moreover, the low steady-state expression and inducible p53 observed in these cell lines also suggested the presence of functional p53.

We found that p53 was nonfunctional in 6/10 drug-resistant cell lines [SK-N-BE(2), CHLA-119, CHLA-171, CHLA-90, CHLA-134, and CHLA-172], but in only 1/8 drug-sensitive cell lines (CHLA-225) as demonstrated by a failure to induce downstream genes, p21 and MDM2, by L-PAM. High steady-state p53 expression, a hallmark of p53 mutation, was found in 5/6 cell lines with nonfunctional p53 [SK-N-BE(2), CHLA-171, CHLA-90, CHLA-134, and CHLA-225]. Although not highly drug-resistant, the CHLA-225 cell line was derived from a patient treated with an intensive chemotherapeutic regimen and showed a moderate level of drug resistance in vitro.

Sequence analysis of exons 5 through 8 (the sequence-specific DNA-binding domain of the protein) in all seven cell lines with nonfunctional p53 showed mutations in SK-N-BE(2), CHLA-119, CHLA-90, and CHLA-172. We failed to demonstrate p53 mutations in the DNA-binding region in CHLA-134, CHLA-171, and CHLA-225. As 4/10 drug-resistant cell lines (LA-N-6, SK-N-RA, CHLA-79, and CHLA-136) had functional and wt p53, mechanisms other than loss of p53 function can also mediate sustained high-level drug resistance in neuroblastoma.

To demonstrate that a loss of p53 function mediates multidrug resistance in neuroblastoma, we transfected two sensitive neuroblastoma cell lines carrying func-

tional p53 with the HPV 16 E6 gene, which targets cellular p53 for ubiquitination, thus disrupting the p53-mediated response [23]. HPV 16 E6 transformed cells have been used to examine the influence of p53 loss on genomic stability, apoptosis, and sensitivity to chemotherapeutic agents or ionizing radiation. As *MYCN* amplification has been linked to tumor progression and poor outcome in neuroblastomas, we used both a *MYCN* gene amplified (SMS-SAN) and a *MYCN* nonamplified (SMS-LHN) cell line for these experiments. The LC_{90} values of two separate HPV 16 E6 clones of both cell lines were increased relative to the LXSN empty vector controls: three- to sevenfold relative to melphalan, 8–109-fold relative to carboplatin, and 2–158-fold relative to etoposide.

Our data showed that p53 is functional in all tested neuroblastoma cell lines established prior to chemotherapy [SK-N-BE(1), SMS-SAN, and CHLA-122], and that the loss of p53 function appears to be one of the major mechanisms responsible for high-level multidrug resistance in neuroblastoma. In one pair of cell lines, derived from the same patient prior to the treatment and then after disease progression on induction chemotherapy, we demonstrated acquisition of a drug resistant phenotype [2], which was associated with a p53 mutation and loss of p53 function. This observation, and the high frequency of p53 mutations and loss of function in postchemotherapy cell lines relative to those established at diagnosis, suggests that selection for neuroblastoma cells with p53 mutations and/or loss of p53 function occurs during therapy. As most drug-sensitive neuroblastoma cell lines had functional p53, it is unlikely that loss of p53 function was acquired as a result of growth in vitro.

These data suggest that the relationship of p53 mutations and/or functionality to drug-resistance should be investigated in tumor samples from patients with recurrent neuroblastomas. One possible approach to studying clinical samples is the Affymetrix GeneChip p53 Assay, which provides sensitive, rapid, and accurate identification of p53 mutations [24]. Our preliminary data with the GeneChip p53 Assay has shown mutations in all tested cell lines that had mutations by automated DNA sequencing. It is unclear why p53 was nonfunctional in CHLA-225, CHLA-171, and CHLA-134, and yet mutations were not detected by the GeneChip or by sequencing. The latter observation suggests that methods for detecting p53 functionality in clinical specimens will be required to complement detection of mutations. Finally, demonstrating that high-level drug-resistance is associated with p53-LOF suggests that p53-independent therapies (e.g., ceramide modulators [21], BSO/L-PAM [25]) or immunotherapy [26] should be employed in recurrent neuroblastomas.

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