Original Article

Medical Science

P53, Bax and *Cathepsin D* Dysregulation in Neurons Subjected to Cyanide Toxicity and Oxygen Deprivation

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ABSTRACT [ENGLISH/ANGLAIS]

Cyanide is a potent neurotoxin capable of potentiation NMDA R1 (N-methyl-D-aspartate receptor 1) a form of glutamate receptor that is calcium gated, thus causing excitotoxicity. It is also well established that the glutamate-glucose exchange is dependent on the activity of the Na^+/K^+ ATPase pump, thus we examine the role of the Na^+/K^+ pump in the metabolism of the neuron during cyanide toxicity. Six separate perfusion set up of the rat brain cortical tissues were made with ACSF (ACSF, ACSF+KCN, ACSF+KCN + pump blocker, ACSF + pump blocker). The tissues were perfused for duration of 180 minutes. The tissues were processed immunohistochemically using antibodies against p53, Bax and Cathepsin D (CD) to demonstrate disregulation of cell cycle proteins associated with the induced DNA breakage as a result of cyanide toxicity. The pump blockers (methyldigoxin and promethazine) induced excitotoxicity when used in culture, and amplified cyanide toxicity when combined with KCN. Cell death induced by toxicity of cyanide and the blockade of the Na/K ATPase pump has been seen to be complimentary in driving the toxicity effects that drives the cell into apoptosis. The tumor suppressor/apoptosis inducing factors p53 and Bax were over expressed while cathepsin was suppressed to show that the cells are apoptotic as against an increased cathepsin D level that would have implied senescence.

Keywords: Neuron, hydrogen cyanide, oxygen, P53, Bax and Cathepsin D

RÉSUMÉ [FRANÇAIS/FRENCH]

Le cyanure est une puissante neurotoxine capable de potentialisation NMDA R1 (N-méthyl-D-aspartate 1) une forme de récepteur du glutamate qui est calcique dépendant, provoquant ainsi l'excitotoxicité. Il est également bien établi que l'échange glutamate-glucose dépend de l'activité de la pompe Na + / K + ATPase, ainsi, nous examinons le rôle de la Na + / K + pompe dans le métabolisme du neurone pendant la toxicité du cyanure. Six perfusion séparée mise en place des tissus cérébraux corticaux de rat ont été faites avec l'ACSF (ACSF, ACSF + KCN, ACSF + bloqueur de KCN pompe +, bloquant ACSF pompe +). Les tissus ont été perfusés pendant la durée de 180 minutes. Les tissus ont été traitées par immunohistochimie utilisant des anticorps contre p53, Bax et la cathepsine D (CD) pour démontrer dérégulation des protéines du cycle cellulaire associés à la cassure de l'ADN induite à la suite de la toxicité du cyanure. Les bloqueurs de la pompe (méthyldigoxine et de la prométhazine) induite excitotoxicité lorsqu'il est utilisé dans la culture, et amplifié la toxicité du cyanure lorsqu'il est combiné avec KCN. La mort cellulaire induite par la toxicité du cyanure et le blocus de la pompe Na / K ATPase a été vu pour être complémentaires dans la conduite des effets de toxicité qui entraîne la cellule en apoptose. Le suppresseur de tumeur / induisant l'apoptose p53 et Bax facteurs ont été plus exprimé en cathepsine a été supprimée pour montrer que les cellules sont apoptotique contre un niveau accru la cathepsine D que cela aurait impliqué la senescence.

Mots-clés: Neuron, le cyanure d'hydrogène, d'oxygène, P53, Bax et la cathepsine D

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INTRODUCTION

The term cyanide refers to free cyanide (CN) or hydrogen cyanide (HCN); depending on physical factors like temperature, pH and salinity of the medium in which it exists [1]. The most common route through which cyanide enters the human system is via oral means. The most common sources of oral cyanide are food substances obtained from cyanophoric plants [1, 2]. The major defense of the body against the toxicity of cyanide is the enzyme "rhodenese" which catalyzes the conversion of cyanide to thiocyanate (SCN) in the presence of sulphur containing amino acids [2, 3]. Since this enzyme is present

in sufficient quantities but sequestered in tissue sites that are not readily accessible, the rate limiting factor in the conversion of cyanide to thiocyanate is the relative abundance of the sulphur containing amino acid [4].

Cyanide is a potent neurotoxin capable of crossing the blood brain barrier and inducing damage and cell death in the neurons [1]. Toxicity of cyanide has been described in two main aspects; firstly, it potentiates N-Methyl-D-Aspartate Receptor 1 *(NMDA R1)*, thus excitotoxic as it causes prolonged activation of this glutamate receptor. Secondly it inhibits Cytochrome C oxidase (Heme *a3-Cuβ bi*-nuclear centre) to generate superoxide anions, otherwise reactive oxygen species (ROS) [5].

Both the excitotoxicity and ROS generation during cyanide toxicity accounts for cell death in the neurons. However, the mechanism of cell death adopted by the neuron will vary under different conditions as either apoptotic or necrotic [6]. Cell death studies on NP3 cancer cells shows that higher temperature induced necrosis and lower temperature induced apoptosis. This was found to be different for the cortical neurons since they contain more RNA than DNA and are not capable of dividing; thus, extreme doses induced apoptosis in the cortex [7]. In addition, apoptosis and necrosis are confirmed to be preceded by the same initial stimuli of DNA breakage which can be induced by NO generated from ROS [7, 8]. The extent of generation of such endogenous modulators of cellular activity in cyanogenic toxicity will most likely determine the adopted pattern of cell death. DNA breakage pattern has been characterized to be of two types using TUNEL staining techniques. [9, 8] The 3'P-5'OH cleavage pattern is described as apoptotic while the 3'OH-5'P cleavage pattern is described as necrotic.

Resultant DNA cleavage has a direct effect on the cell cycl [8]. Cyanide has been reported to predispose cells to tumorgenensis although this has not been proven. In this context we shall consider the DNA cleavages effect on cell cycle and how this leads to disregulation of cell cycle proteins p53 and cathepsin D as an indicator of the adopted mode of cell death in the neurons. Normal (wild-type) p53 suppresses outgrowth of genetically damaged, hence potentially neoplastic cells in two distinct ways: by causing a pause in the cell cycle, and by promoting exit from the cell cycle altogether (programmed cell death, or apoptosis) [10]. This dichotomy is thought to allow an appropriate biological response to the two sequlae of DNA damage: Either genome integrity is restored by DNA repair, in which case cells can be released from transient cell cycle arrest, or when damage persists, cells can be permanently eliminated from the population by apoptosis [11]. This function of p53 as "guardian of the genome" may extend to a role in initial monitoring and repair of DNA damage in addition to direct control of cell growth and death [11, 2]. Thus up regulation of p53 synthesis in neuronal cells treated with cyanide will represent a cell undergoing DNA cleavage and apoptosis. The p53 ceases cell cycle by binding to DNA tetrameters via a recognition motif such that the cycle is blocked prior to S-phase [13, 14]. When genetic damage such as DNA strand breaks occurs in a cell with normally functioning p53, levels of the active protein rise, and the cell cycle is stalled or the process of cell death is induced [9]. It is not known what cellular signals decide between these alternatives or convert cell arrest to cell death. Cells deficient in functional p53 are genetically unstable and become permissive for inopportune gene amplification or chromosome loss through DNA strand breakage and rejoining [15, 16].

Cathepsin D (CD) is a major lysosomal protease, the targeted deletion of CD in mice results in extensive neuropathology, including biochemical and morphological evidence of apoptosis and autophagic stress (aberrant autophagosome accumulation), effects that are similar to those observed in neurodegenerative diseases [17]. To determine the contribution of cyanogenic neurotoxicity in Bax-dependent apoptosis in these rats, Bax- and CD-immunohistochemistry was conducted on cyanide perfused tissue blocks using ACSF+CN. Studies have shown that lysosomal dysfunction and autophagic stress were apparent in CD-deficient brain as indicated by the accumulation of autofluorescent storage material and by increased levels of LC3-II (light chain 3-II, a selective autophagosome marker), respectively, although other studies show that cathepsin D induced neurodegenration is independent of Bax [16,15]. Thus we seek to investigate the roles of p53 and cathepsin D as regulators of apoptotic cell death in degenerating neurons under oxygen deprived cyanogenic toxic conditions in other to understand the regulation of cell death resulting from DNA cleavage and cell cycle changes under this condition.

METHODS

All reagents were procured from Sigma Aldrich, Na^+/K^+ pump blockers were procured from Standard Pharma, Nigeria).

Preparation of ACSF

The ACSF was prepared so that it contains 18 mM glucose; 119 mM NaCl, 2.5 mM KCl, 1.3 mM $MgSO_4$, 2.5

mM $CaCl_2$, 26.2mM $NaHCO_3$, 1 mM NaH_2PO_4. The solution was prepared in distilled water to make up to 100ml [10]. Oxygen was not included in the perfusion set up as we seek to study the activity of the pump blockers in cyanogenic-oxygen deprived environment.

Na^+/K^+ Pump Blockers

Digoxin and Promethazine were procured from Standard Biochem Co, Nigeria. 250µg of powdered Digoxin was dissolved in 50ml dextrose saline solution [24]. 2ml ampoule of promethazine contains 50mg promethazine in dextrose saline; the solution was diluted by making up to 50ml in a clean glass measuring cylinder such that the new concentration is 1 mg/ml [18].

Preparation of Tissue for Perfusion

Adult male wistar rat weighing 250 gm was dissected to expose the brain. Cortical tissue blocks of 0.5gms were obtained and washed in dextrose saline and was immediately transferred into a test tubes. Six tubes labeled A, B, C_1, C_2, D and E such that A contains ACSF only Control I), B (ACSF + KCN), C_1 (ACSF + KCN + Digoxin), C_2 (ACSF + KCN + Promethazine), D (ACSF + Promethazine), E (ACSF + Digoxin). KCN was added to the perfusion fluid at a concentration of 25mg/Kg of tissue, Digoxin concentration in ACSF was 0.3571µg/Kg while Promethazine concentration was 0.714mg/Kg. C_1 and C_2 were re-perfused with the blockers every 30 minutes while the overall process lasted for 180 minutes such that the total volume of blocker perfused is between 150-250µm.

Perfusion

The test tubes A, B, C_1, C_2, D and E were connected to a perfusion set up to run the respective ACSF (as described above for each tube) through the tube for 180 minutes, the set up was rocked gently at intervals to aid circulation and exchange in the tissue block. 10 µl metronidazole was added to each tube as an antibiotic to prevent bacteria action. The set up was incubated at 37^0C.

Immunohistochemistry (Antigen Retrieval Method)

The tissues obtained were prefixed in formolcalcium to arrest all protein activities, after 48 hours the tissues were then processed with embedding done at 50^0C to obtain paraffin wax sections. The sections were mounted on a slide and dewaxed to expose the tissues. The exposed tissues were placed in the humidity chamber to rehydrate the tissue. It was then placed in a microwave using the method of Serrano et al. [19].

DAB immunohistochemistry was carried out using the immunoglobin G (IgG) peroxidase anti–peroxidase method (PAP). The sections were washed in phosphate buffer saline (PBS), incubated in normal goat serum for 30 min, and then incubated for 6 hr at 37°C in a solution containing a monoclonal antibody against rat Bax, Cathepsin D and p53 proteins. Separate slides were incubated with at a dilution of 1:100 in PBS in separate reaction chambers. The sections were then treated with trypsin (0.1% in 0.1% calcium chloride) at 37°C for 15 minutes before blocking the non-specific reactions with hydrogen peroxide (0.3%). The antibodies were used at a dilution of 1:100 in PBS containing 0.1% Bovine serum albumin and 0.005% sodium azide. The peroxidase in all the reactions was developed with 0.025% di aminobenzidine tetrachloride in phosphate buffer saline, pH 7.2, containing 0.03% hydrogen peroxide.

Transformation

Methenamine silver intensification was used on the immunoperoxidase preparation after the peroxidase/H_20_2/DAB reaction has been carried out to give a brown deposit. The sections were then counterstained in hematoxylin. The counterstained sections were washed in running tap water, thoroughly rinsed in distilled water, and placed in preheated methenamine silver solution at 60°C for five minutes. Although it could be occasionally longer if the intensification had been carried out at room temperature. In this study, to further increase the clarity, hematoxylin was removed from counterstained nuclei with acid alcohol before the silver intensification was carried out.

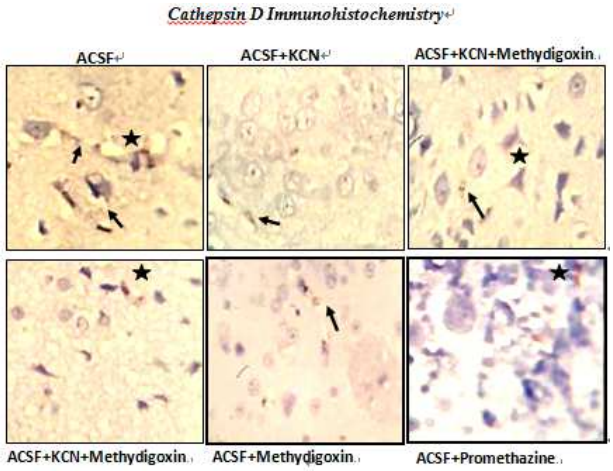
The composition of the stock solution was 0.125% silver nitrate in 1.5% hexamine. The solution was stored at 4°C. Prior to use, 2 ml of 5% tetraborate was added to 50 ml of the stock silver solution giving a pH of 8.0, which was then filtered into a coupling jar and protected from sunlight.

RESULTS

Increase in the expression of p53 in sections treated with KCN and a pump blocker indicates that the toxicity of cyanide is aided by the pump blocker thus driving the genetically predictable neurons into apoptosis. The production of Nitric oxide and reactive oxygen species that stimulates breakage of genetic materials during toxicity of cyanide thus forcing the expression of the

tumor suppression protein (p53), the extent of the DNA damage seems to correspond to the extent of p53 expression. Bax which has been suspected to be a determinant of cathepsin D induced apoptosis was up regulated in a similar pattern as p53 in the neurons (Fig 1), while Cathepsin D was greatly reduced further supporting that the neurons entirely undergo apoptosis (cortex).

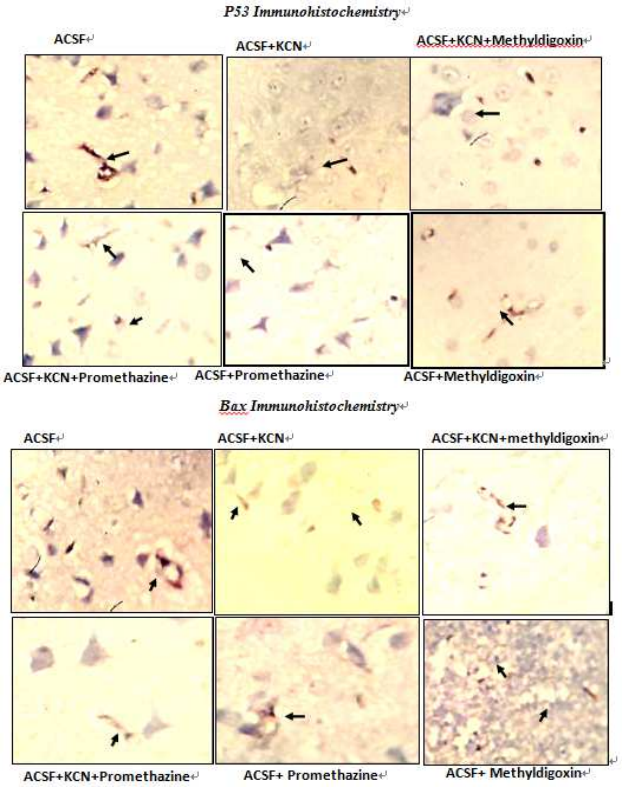
Figure 1: This figure shows anti body against rat Cathepsin D shows suppression as the tissues in ACSF only showed greater expression of the protein (★) compared to the treatment groups. The expression of the protein was least in the tissues treated with both KCN and a pump blocker. The colour reaction was developed in 3′3′-Diaminobenzidene tetrachloride (DAB). (Magnification X400)



DISCUSSION

Two major mechanisms have been described for cyanide toxicity. The first involves the production of ROS via inhibition of the Heme a3-Cuβ binuclear centre of cytochrome *C* oxidase. The enzyme being a member of the P450 super family has been implicated with the role of transferring molecular oxygen to water to drive the proton gradient required to drive electron transport [1]. Thus inhibition of this enzyme will imply diffusion of oxygen molecules across the mitochondria membrane and accumulation of the diffused oxygen since it cannot be converted to water under this circumstance. The accumulated oxygen reacts with accumulating electrons since a proton gradient was not created to drive electrochemical potential for the electron transport chain.

Figure 2: This figure shows up regulation of p53 and Bax was observed in the treatment groups, although the protein was expressed in the ACSF, the expression was higher in the treatment groups, highest activity was seen in the treatments with KCN+ pump blockers. (Magnification X400).



The resultants are superoxide anions (oxygen radicals), otherwise called reactive oxygen species (ROS) [4]. ROS reacts with nitrogen compounds to produce nitric oxide and reactive nitrogen species (RNS). NO being an endogenous modulator of cellular activity is also capable of inducing DNA cleavage and apoptosis if produced in excess amount; a situation obtainable in cyanide toxicity. DNA breakage affects the cell cycle, if it is of such gravity that can drive an aplastic neuron to proceed into cell division it could lead to tumorgenesis or drive the cell to apoptosis. The p53 protein is a tumor suppressor protein that proof reads the neurons with fragmented DNA thus driving them into apoptosis [9]. The second mechanism in which DNA breakage can occur in the neuron is via the lipid peroxidation effects of ROS which peroxidates the lipid bilayer of the nuclear membrane and lysosomal membrane thus releasing endonucleases that can cleave the genetic materials in tow basic patterns 3′P-5′OH and 3′OH-5′P [8,9]. Each of the cleavage pattern has been observed to be specific for Apoptosis and Necrosis respectively. Normal levels of P53 can be observed in neuronal cells (ACSF perfused tissues) but will up regulate to prevent the cell from driving into mutation

during the toxicity of cyanide that can cause breakage of DNA in two ways

1. Peroxidation exposing the genetic materials to endonucleases [8]
2. Formation of NO which can signal apoptosis [1]

Increased level of p53 indicates the cell was been driven into apoptosis to prevent mutation from the gene breakage, also the extent of p53 expression in tissues will indicate the severity of the damage or the number of break points in genetic material. In situations where p53 is de regulated or the expression is suppressed, the cells are prone to mutation and tumorgenensis [20]. P53 itself is a protease that is released to drive the destruction of genetic materials and specific proteins in the cell thereby preventing mutation. In this study a rapid preliminary test was conducted immunohistochemically to detect p53 expression using monoclonal antibodies against p53 to detect nuclear accumulation of the protein [21]. The approach was adopted because the mutated forms of the enzyme are resistant to digest than the wild type thus leading to an increase in the protein half-life [22, 16]. In this context we take several factors into consideration while interpreting our results; increase in the half-life of the protein does not entirely imply mutation of loss of the WT allele but might be as a result of increase in the production of the protein (in a cellular context), we selected uniform panel of diluted antibodies and the staining was conducted at the same time in all the sections and the tissues were prefixed in formolcalcium.

Cathepsin D (CD) is a ubiquitous lysosomal aspartic endoproteinase. CD is over expressed in cancer cells but suppressed in cells undergoing apoptosis. Over activation of CD enhances cell proliferation, tumorgenensis, and metastasis. Several studies suggest that CD participates in the signalling pathways leading to cell death and has been linked to Bax (often described as Bax dependent) [23]. In addition, CD levels are significantly increased during the normal aging process. Here, CD was dramatically reduced during treatment as cells shows features of apoptosis. Taken together, it suggests that regulation of CD expression would be an effective strategy for manipulating senescence and apoptosis pathways in toxicity studies involving toxicity [23]. Although research work has shown that Bax-Bax heterodimers will usually lead to apoptosis while Bcl-2-Bax dimer signals the cell for survival, in this study however expression of Bax shows up regulation in the apoptotic cells and also corresponds to the expression pattern of p53 another protein that drives a cell into apoptosis. Bax has been suggested to be a transcriptional target for p53 which induces apoptosis and cell cycle arrest under assault to the DNA [2].

In conclusion, cell death induced by toxicity of cyanide and the blockade of the Na/K ATPase pump has been seen to be complimentary in driving the toxicity effects that drives the cell into apoptosis. The tumour suppressor/apoptosis inducing factors p53 and Bax were over expressed while cathepsin was suppressed to show that the cells are apoptotic as against an increased cathepsin D level that will suggest senescence.

REFERENCES

[1] Isom GE, Gunasekar PG, Borowitz JL. Cyanide and neurodegenerative disease. In Chemicals and Neurodegenerative Disease (S. C. Bondy, Ed.), pp.101–129. Prominent Press, Scottsdale, AZ. 1999.

[2] Osuntokun BO. Cassava diet, chronic cyanide intoxicationand neuropathy in Nigerian Africans. World Rev Nutr Diet 1981;36:141-73.

[3] Okafor PN, Okoronkwo CO, Maduagwu ON. Occupational and dietary exposure of humans to cyanide from large scale cassava processing and ingestion. Food and Chemical Toxicology (2002) 40: 1001-3.

[4] Prabhakaran K, Li L, Zhang L, Borowitz JL, Isom GE. Upregulation of BNIP3 and translocation to mitochondria mediates cyanide-induced apoptosis in cortical cells. Neuroscience 2007;150:159-67.

[5] Sotoblanco RA, Aparicio MA. Relationships between dietary cassava cyanide levels and Brain performance. Nutrition Reports International 2002;37:63-75.

[6] Katherine L, Barrett Jonathan M, Willingham A, Julian Garvin, Mark C. Willingham. Advances in cytochemical apoptosis. Journal of Histochemistry and Cytochemistry 2001; 49:821-32.

[7] Ogundele OM, Enuaibe BU, Igwe J, Olu-Bolaji AA, Caxton-Martins EA. Histological studies of the Visual relay centres in cyanide toxicity: Mode of neuronal cell death in the V1, LGB and SC of adult wistar rats. Journal of Cell and Animal Biology 2011;5(11):231-46.

[8] Denison TA, Koch CF, Shapiro IM, Schwartz Z, Boyan BD. Inorganic phosphate modulates responsiveness to 24, 25(OH) 2D3 in chondrogenic ATDC5 cells. J Cell Biochem 2009;107:155-62.

[9] Shiokawa D, Ohyama H, Yamada T, Takahashi K, Tanuma S. Identification of an endonuclease

responsible for apoptosis in rat thymocytes. Eur J Biochem 1994;226:23-30.

[10] Kim SJ, Park YJ, Oh YJ. Proteomic analysis reveals a protective role for DJ-1 during 6-hydroxydopamine-induced cell death. Biochem Biophys Res Commun. 2012 Apr 21. [Epub ahead of print].

[11] Flores-Rozas H, Kelman Z, Dean FB, Pan ZQ, Harper JW, Donnell MM, Hurwitz J. Cdk-interacting protein 1 directly binds with proliferating cell nuclear antigen and inhibits DNA polymerase (Holoenzyme). Proc. Natl. Acad. Sci. USA 1994;91:8655–9.

[12] Kuerbitz SJ, Plunkett BS, Walsh WV, Kastan MB..Wild-type p53 is a cell cycle checkpoint determinant following irradiation. Proc. Natl. Acad. Sci. USA 1992;89:7491–5.

[13] Baker SJ, Markowitz S, Fearon ER, Willson JK, Vogelstein B. Suppression of human colorectal carcinoma cell growth by wild-type p53. Science 1990;249:912–5.

[14] Gu H, Jin L, Li Q, Mei Y, Wu M. p53-Facilitated miR-199a-3p Regulates Somatic Cell Reprogramming. Stem Cells 2012;30 7(): 1405-13..

[15] David Sidransky. Clinical Implications of the p53 GENE. Annu. Rev. Med.1996;47:285–301.

[16] Jones K, Elmere LW, Povine L, Holt SE., Gewirtz DA. p53-dependent accelerated senescence induced by ionizing radiation in breast tumour cells. Int J Radiat Biol 2005;81:445–58.

[17] Koch S, Molchanova SM, Wright AK, Edwards A, Cooper JD, Taira T, Gillingwater TH, Tyynelä J. (2011) Morphologic and functional correlates of synaptic pathology in the cathepsin D knockout mouse model of congenital neuronalceroid lipofuscinosis. J Neuropathol Exp Neurol. 2011; 70(12):1089-96.

[18] Petersen M, Davids LM, Kidson SH. Simultaneous Immunofluorescent Labeling Using Anti-BrdU Monoclonal Antibody and a Melanocyte-specific Marker in Formalin-fixed Paraffin-embedded Human Skin Samples. Appl Immunohistochem Mol Morphol 2012;20(6):614-7.

[19] Serrano M, Lin AW, McCurrach ME. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and 16INK4a. Cell 1997;88: 593–602.

[20] Macleod KF, Sherry N, Hannon G. p53-dependent and independent expressionnof p21 during cell growth, differentiation and DNA damage. Genes Dev. 1995;9:935–44

[21] Hollstein M, Rice K, Greenblatt MS.1994. Data base of p53 gene somatic mutations in human tumors and cell lines. Nucleic Acids Res.22:3551–5

[22] Johansson AC, Steen H, Ollinger K, Roberg K. Cathepsin D mediates cytochrome c release and caspase activation in human fibroblast apoptosis induced by staurosporine. Cell Death Differ 2003;10: 1253–9.

[23] Xue W, Zender L, Miething C. Senescence and tumour clearance is triggered by p53 restoration in murine liver carcinomas. Nature 2007; 445:656–60.

[24] Kano I, Satoh K, Nagai F, Ushiyama K, Nakao T, Hara Y, Kano K. Antigenic determinant of a monoclonal antibody: extracellular domain at the M3-M4 junction of the alpha-subunit of Na,K-ATPase. Biochem. Cell Biol. 1990;68:1262-7.

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CONFLICT OF INTEREST

No conflict of interests was declared by authors.