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Acetylcholinesterase and HHV-8 in Squamous Cell Carcinoma and Retinoblastoma

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Abstract. *Acetylcholinesterase (AChE) and human herpesvirus type 8 (HHV-8) antigens were studied in tissue sections from 56 squamous cell carcinomas (SCC) and five retinoblastomas (Rb). Approximately 62.5% of SCC and 80% of Rb showed positive staining for AChE. AChE staining in tumors was much higher than in normal control tissue. However, only 21.4% of SCC and 60% of Rb contained HHV-8 antigens. Of the 56 SCC, 17.9% were positive for both AChE and HHV-8 antigens, whereas 60% Rb were positive for both markers. The co-existence of AChE and HHV-8 antigens may play a role in the development of SCC and Rb. A possible mechanism for the development of these tumors is discussed.*

Acetylcholinesterase (AChE) plays a key role in terminating neurotransmitter activity at cholinergic synapses. A single gene mapped at 7q22 chromosal position encodes AChE in muscles and nerves (1), homopoietic cells (2), embryonic tissues (3), germ cells (4) and a variety of tumors (5). A growing body of evidence suggests that this enzyme plays a very important role in tumorogenesis. The gene for AChE has been shown to be amplified, mutated and/or aberrantly expressed in a variety of human tumors (6). The aberrant expression of this gene can be manifested at the DNA, mRNA or protein level (6-8). These alterations and elevation in AChE gene products are seen in a variety of tumors despite the fact that AChE is not present in their normal counterparts. Currently it is considered to be a useful tumor marker (5-14). High AChE activity is reported in brain, breast, lung and ovarian tumors and leukemia (6-17). Neuroblastoma cells genetically engineered to overexpress AChE, developed tumors *in vivo* at a notably greater rate compared to transfection controls. Similar observations have been reported for glioma cell lines (18, 19). The AChE peptide contains S/T-P-X-Z motif which is one of the many substrates for cdc-2 kinases. Phosphorylation of retinoblastoma (Rb) protein by cdc-2 kinases may be the molecular mechanism linking AChE with tumor cell proliferation (6-16).

Human herpesvirus type 8 (HHV-8) is a lymphotropic and oncogenic virus (20-24). HHV-8 DNA sequences have been found in 50% small cell carcinoma (SCC) cases (22-26). HHV-8 expresses unique viral proteins such as kaposin and latent nuclear antigen (LNA-1) in malignant cells (21, 27). LNA-1 seems to act as a transcription cofactor which may target Rb protein to disrupt the E2F transcriptional regulatory pathway. On the other hand, kaposin may inactivate the P53 regulatory pathway (23, 27).

SCC and retinoblastoma are relatively common in Saudi Arabia and about 12% of the adult population is seropositive for HHV-8. Based on these observations, an attempt was made to determine the frequency of co-existence of AChE and HHV-8 antigens in 56 SCC and 5 retinoblastomas. A possible molecular mechanism involved in the induction of SCC and retinoblastoma in the presence of AChE and HHV-8 antigens is discussed.

Materials and Methods

Fresh SCC and retinoblastoma tissues were obtained at the time of surgery at the King Khaled Eye Specialist Hospital (KKESH), Riyadh, Saudi Arabia. Tumor tissues were collected randomly from patients with confirmed SCC or retinoblastoma. Normal retinal tissues were collected from healthy donors. Controls for HHV-8 antigens were cells from primary effusion lymphoma with HHV-8 infection (PEL) which were kindly provided by Dr. Ethel Cesarman of Cornell University, New York, USA. Tissues and cell pellets were fixed in 10% buffered formalin, embedded in paraffin and sectioned. Four micron sections were plated on polylysin-coated slides (2 sections/slide) and used for immunoperoxidase staining. One slide from each sample was stained with hematoxylin to study tissue morphology.

Immunoperoxidase staining. Deparaffinized tissue sections with appropriate positive and negative controls were incubated with 0.3% hydrogen peroxide and rinsed twice with phosphate-buffered saline (PBS). Slides were then treated with pepsin (13 mg/48 ml

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in 0.2 N HCl) for ten minutes at room temperature and washed twice in PBS. The sections were incubated with 10% goat serum (Chemicon Temecula; CA, USA) at 37˚C for 30 minutes. Monoclonal antibodies to AChE (Chemicon) and HHV- LNA-1 (ABI, Columbia MD, USA) were diluted (1:100) and applied to tissue sections and incubated overnight at 37˚C in a moist chamber. The sections were rinsed with PBS containing 0.2% Triton® X-100 and incubated for three hours at 37˚C with a biotin-labeled secondary antibody (goat antimouse immunoglobulin). After rinsing with PBS with 0.2% Triton® X-100, the slides were then incubated for three hours at 37˚C with the avidin-biotin-horseradish peroxidase complex and sites of peroxide activity were visualized after incubation with 3-amino-9-ethylcarbazole in 0.01% hydrogen peroxide (ABC Kits, Foster City, CA, USA). The slides were counterstained for two minutes in Mayer's hematoxylin, rinsed in water and then six drops of crystal mount (Fisher Scientific, Springfield, NJ, USA) were applied to each section and slides were then baked at 60˚C for 30 minutes. The sections were mounted in Permount (Fisher Scientific) and examined (28).

Results

Fifty-six SCC tissues and five Retinoblastoma tissues were analyzed by using immunohistochemical staining for the presence or absence of AChE and HHV-8 antigens. Positive and negative controls were normal retinal sections incubated with and without AChE antibody respectively (Figure 1 a, b).

Approximately 62.5% of SCC gave clear and strong positive staining for AChE; the rest of the samples showed weak diffuse staining (Table I). Normal retina was used as positive control for the detection of AChE. Two representative photographs of positive staining in SCC and Rb are given in Figure 1 c and d along with the positive and negative controls.

Approximately 21.4% of the 56 samples were positive for HHV-8 antigen LNA-1 (Table I). Positive controls for HHV-8 were cells from primary effusion lymphoma with HHV-8 infection (PEL). When incubated with HHV-8 antibody, these cells showed bright nuclear staining. Representative photographs are shown in Figure 2 a and b. Only 17.9% of the 56 samples contained both AChE and HHV-8 antigen (Table I).

Five retinoblastomas were also analyzed for the presence or absence of AChE and HHV-8 antigen. Approximately 80% of Rb were positive for AChE and 60% stained positively for HHV-8 antigen (Table I); however, 60% of retinoblastomas were positive for both AChE and HHV-8 antigen (Table I).

Table I. *Distribution of AChE and HHV-8 antigens in squamous cell carcinomas (SCC) and retinoblastomas (Rb).*

Type/Number of tumors	Percentage		
	AChE	HHV-8	AChE+HHV-8
SCC/56	62.5	21.4	17.9
Rb/5	80.0	60.0	60.0

Discussion

All SCC studied in this study were positive for AChE; however, only 62.5% cases showed very strong AChE staining. Similar results were reported previously in ovarian, brain, lung and breast tumors and leukemia (6-17).

Approximately 80% of retinoblastomas analyzed also showed positive staining for AChE. HHV-8 antigen LNA-1 was present in 21.4% of SCC and 60% of retinoblastomas. Only 17.9% of SCC and 60% retinoblastoma analyzed contained both AChE and HHV-8 antigen.

To our knowledge, no reports are available showing HHV-8 and/or AChE presence in retinoblastomas; however, 50% of SCC studied previously by other investigators contained HHV-8 antigen (21, 22, 27).

Our observations suggest that HHV-8 may possibly be involved in the development of these tumors through inactivation of tumor suppressor genes such as *P53* and Rb protein and activation of AChE.

The regulatory pathways existing in normal and tumor cells are outlined in Figure 3.

Mutational deregulation of the cell cycle is a hallmark of cancer. The protein product of the retinoblastoma gene (*Rb*) is the prototype tumor suppressor gene by virtue of its central role in regulating the cell cycle. *P53* is a key regulator of cell cycle and apoptosis control, and is also mutated in more than half of human cancer cases.

These two pathways form an interconnected tumor suppressor network. In most types of cancer, the Rb pathway is disrupted such that Rb is inactivated, either by mutation of the *Rb* gene or by functional inactivation of the *Rb* gene by hyperphosphorylation of the protein. Cellular P53 levels are very low in normal cells, due to an inhibitory interaction with HDM2 that targets P53 for degradation. In cancer cells, P53 undergoes post-translational modifications which abolish its ability to bind to HDM2 and be inhibited by it (29-31).

In normal cells, P53 and Rb proteins (Rb bound to E2F) are active and reduce the cell cycle rate which suppresses tumor development. In tumors, the *P53* and *Rb* genes are inactive either because of mutations or because P53 is hydrolyzed and the Rb-E2F complex is broken due to hyperphosphorylation or interaction with certain proteins from SV40 large T antigen, adenovirus E1A, HPV, E6 and E7 proteins, HHV-8 kaposin and LNA-1

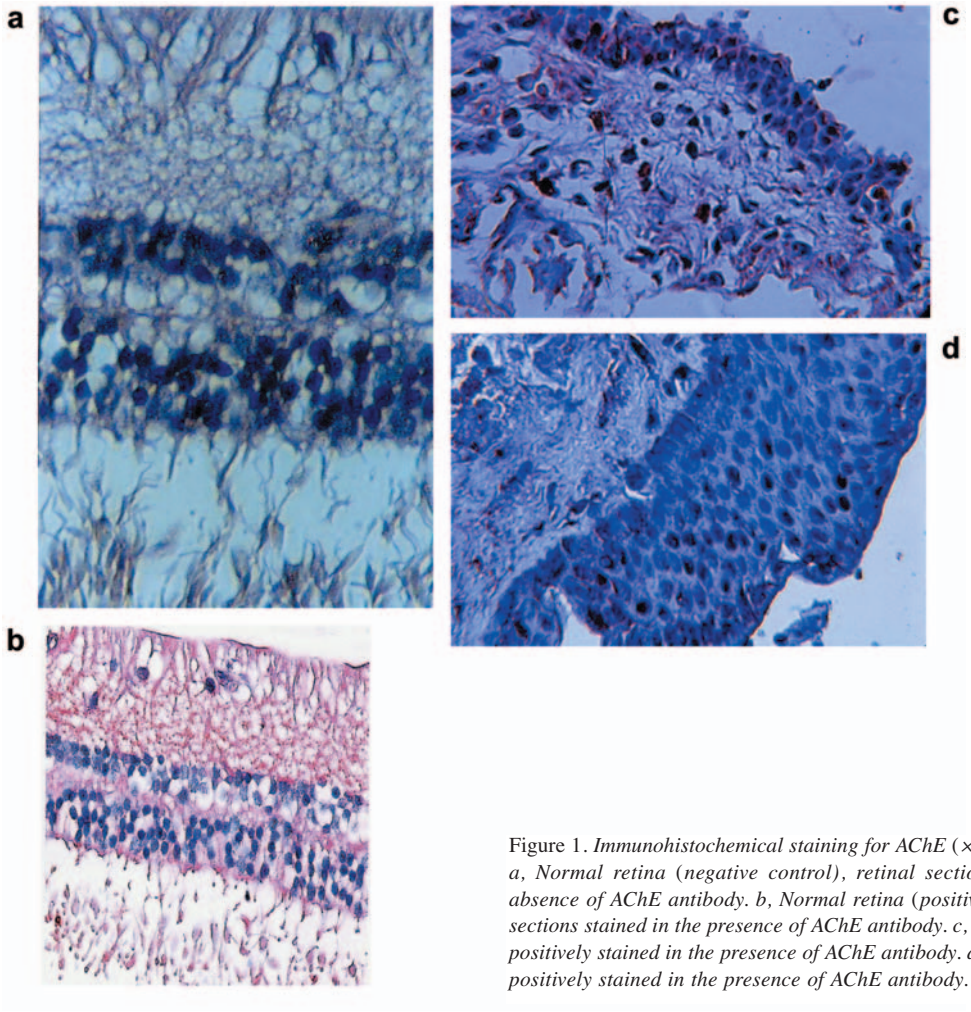

Figure 1. *Immunohistochemical staining for AChE (×40 magnification). a, Normal retina (negative control), retinal sections stained in the absence of AChE antibody. b, Normal retina (positive control), retinal sections stained in the presence of AChE antibody. c, SCC tissue section positively stained in the presence of AChE antibody. d, Rb tissue section positively stained in the presence of AChE antibody.*

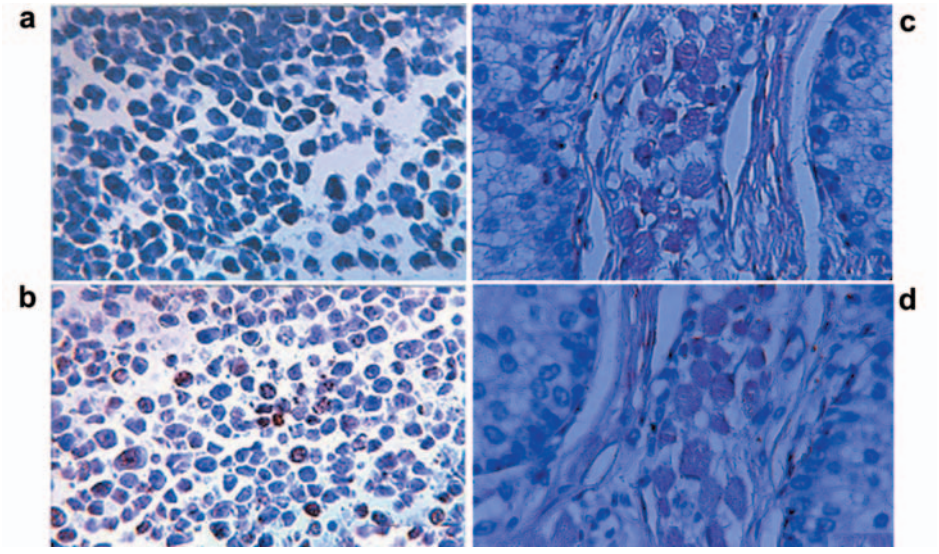

Figure 2. *Immunohistochemical staining for HHV-8 antigen (×40 magnification). a, PEL (negative control) stained in the absence of HHV-8 antibody. b, PEL (positive control) stained in the presence of HHV-8 antibody. c, SCC tissue section stained in the presence of HHV-8 antibody. d. Rb tissue section stained in the presence of HHV-8 antibody.*

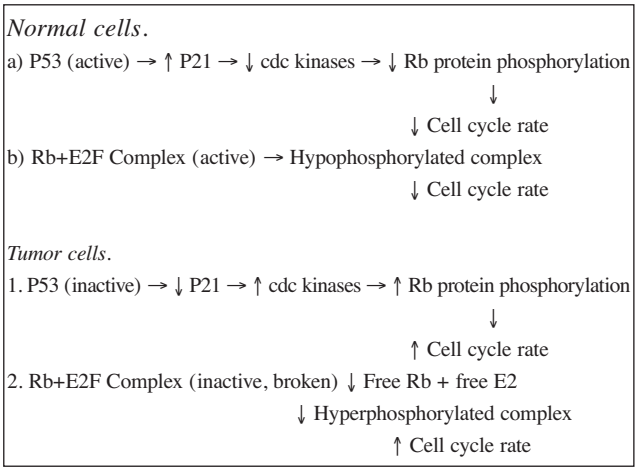

Figure 3. *Regulatory pathways in normal and tumor cells*.

proteins and P33 of Bacculovirus (32-36). Certain cellular proteins such as AChE may also affect the cdc kinase activities and reverse the regulatory pathway which exists in normal cells (6, 16).

As has been reported earlier (21-27), along with AChE, the HHV-8 kaposin protein may inactivate P53 and/or LNA-1 may break up the Rb-E2F complex, allowing the development of tumors through the regulatory pathways shown in Figure 3.

In our study, although the results are preliminary, they do indicate that the increased levels of AChE in SCC and retinoblastoma may activate cdc kinases, which in turn may increase the phosphorylation of Rb protein and hence activate the cell cycle rate *via* interruption of P53 regulatory pathway (tumor cells, Figure 3 a). In some tumors, the presence of HHV-8 LNA-1, however, suggests that HHV-8 may interrupt the Rb-E2F regulatory pathway in normal cells allowing them to become cancerous cells (tumor cells, Figure 3 b). Similar mechanisms have been proposed and reported in the development of other tumors (6-16, 21-23, 27).

Since AChE appears to play developmentally important roles in multiple cell types, the amplification and overexpression of the AChE gene may also affect the progression of various tumors. If the mechanism is confirmed, AChE may be an excellent target for tumor treatment using specific inhibitors of AChE and cdc kinases, as well as antisense mRNA treatment strategy (37-44).

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References

1 Taylor P: The cholinesterase. J Biol Chem *266*: 4025-4028, 1991.

2 Roberts WL, Doctor BP, Foster JD and Rosenberry TL: Bovine brain acetylcholinesterase primary sequences involved in intersubunit disulfide linkage. J Biol Chem *266*: 7481-7487, 1991.

3 Zakut H, Ehrlich G, Ayalon A, Prody CA, Malinger G, Seidman S, Ginzberg D, Kehlenbach R and Soreq H: Acetylcholinesterase and butylcholinesterase gene co-amplify in primary ovarian carcinoma. J Clin Invest *86*: 900-908, 1990.

4 Lapidot-Lifson Y, Prody CA, Ginzberg D, Meytes D, Zakut H and Soreq H: Co-amplification of human acetylcholinesterase and butylcholinesterase genes in blood cells; correlation with various leukemias and abnormal megakaryocytopoiesis. Proc Natl Acad Sci USA *86*: 4715-4717, 1989.

5 Malinger G, Zakut H and Soreq H: Cholinoceptive properties of human primordial, preantial and antral ocytes *in situ* hybridization and biochemical evidence for expression of cholinesterase genes. J Mol Neuro Sci *1*: 77-84, 1991.

6 Soreq H, Lapidot Lifson Y and Zakut H: A role for cholinesterase in tumorogenesis. Cancer Cells *3*: 511-516, 1991.

7 Seidman S, Sternfeld M, Ben Aziz-Aloya R, Timberg R, Kaufer-Nachun D and Soreq H: Synaptic and epidermal accumulations of human acetylcholinesterase are encoded by alternative 3-terminal axons. Mol Cell Biol *15*: 2993-3002, 1995.

8 Karpel R, Aloya R, Sternfeld M, Ehrlich G, Ginzberg D, Torroni P, Clemente F, Zakut H and Soreq H: Expression of three alternative acetylcholinesterase messenger RNAs in human tumor cell lines of different tissue origins. Exp Cell Res *210*: 268-277, 1994.

9 Onganer P, Mustafa BA, Djamgoz M, Whyte K and Greenfield S: An acetylcholinesterase-derived peptide inhibits endocytic membrane activity in a human metastatic breast cancer cell line. Biochim Biophys Acta *1760*: 415-420, 2006.

10 Battisti V, Schetinger MR, Maders LD, Santos KF, Bagatini MD, Correa MC, Spanevello RM, do Carmo Araújo M and Morsch VM: Changes in acetylcholinesterase (AChE) activity in lymphocytes and whole blood in acute lymphoblastic leukemia patients. Clin Chim Acta *402 (1-2)*: 114-118, 2009.

11 Martínez-López de Castro A, Nieto-Cerón S, Aurelio PC, Galbis-Martínez L, Latour-Pérez J, Torres-Lanzas J, Tovar-Zapata I, Martínez-Hernández P, Rodríguez-López JN and Cabezas-Herrera J: Cancer-associated differences in acetlycholinesterase activity in bronchial aspirates from patients with lung cancer. Clin Sci (Lond) *115(8)*: 245-253, 2008.

12 Barbosa M, Rios O, Velasquez M, Villalobos J and Ehrmanns J: Acetylcholinesterase and butylcholinesterase histochemical activities and tumor cell growth in several brain tumors. Surgical Neurology *55*: 106-112, 2001.

13 Perry C, Sklan EH, Birikh K, Shapira M, Trejo L, Eldor A and Soreq H: Complex regulation of acetylcholinesterase gene expression in human brain tumors. Oncogene *21*: 8421-8441, 2002.

14 Ransom JT, Cherwinski HM, Delmendo RE, Sharif NA and Eglen R: Characterization of the M4 muscarinic receptor Ca^{2+} response in a subclone of PC-12 cells by single cell flow cytomentry. Inhibition of response by bradykinin. J Biol Chem *266*: 11738-11745, 1991.

15 Zhang XJ, Yang L, Zhao O, Coen JP, He HY, Jin QH, Guo LH, Almany M, Zhang LY and Shi YF: Induction of acetylcholinesterase expression during apoptosis in various cell types. Cell Death Diff *9*: 790-800, 2002.
16 Santos SC, Vala I, Miguel C, Barata JI, Garcao P, Aqostinho P, Mendes M, Coelho AV, Calado A, Oliveira CR, Silva JM and Saldanha C: Expression and subcellular localization of a novel nuclear acetylcholinesterase protein. J Biol Chem *282(35)*: 25597-25603, 2007.
17 Musthafa CP, Moosa A, Chandrashekharan AP, Nando Kumar R, Narayanan AV and Balakrishnan V: Intestinal pseudo-obstruction as initial presentation of thymoma. Indian J Gastroenterol *25(5)*: 264-265, 2006.
18 Kaur H and Bigbee IW: Neuronal and glial cell biology. J Neurochem *81*: 16-21, 2002.
19 Johnson G and Moore SW: Association of the HNK-1 epitope with the detergent soluble G4 isoform of acetylcholinesterase from human neuroblastoma cells. Int J Dev Neur Sci *19*: 439-445, 2001.
20 Brasnu E, Wechsler B, Bron A, Charlotte F, Bliefeld P, Lehoang P, Marcelin AG and Bodaghi B: Efficacy of interferon-alpha for the treatment of Kaposi's sarcoma herpesvirus-associated uveitis. Am J Ophthalmol *140(4)*: 746-748, 2005.
21 Ablashi DV, Chatlynne LG, Whitman JE and Cesarman E: Spectrum of Kaposi's sarcoma-associated herpesvirus or human herpesvirus 8 diseases. Clin Microbiol Rev *15*: 439-464, 2002.
22 Qavi HB: The Human Herpesvirus 8 and Kaposis Sarcoma: Duance's Foundation of Clinical Ophthalmology. Tasman W and Jaeger E (eds.). J. B. Lippincott Company, USA *93A*: 1-10, 2003.
23 Güvenç MG, Midilli K, Ozdoğan A, Inci E, Tahamiler R, Enver O, Sirin G, Ergin S, Kuşkucu M, Divanoğlu EO, Yilmaz G and Altas K: Detection of HHV-8 and HPV in laryngeal carcinoma. Auris Nasus Larynx *35(3)*: 357-362, 2008.
24 Verma V, Shen D, Sieving PC and Chan CC: The role of infectious agents in the etiology of ocular adnexal neoplasia. Surv Ophthalmol *53(4)*: 312-331, 2008.
25 Inaqi R, Kosuqe H, Nishimoto S, Yashikawa K and Yamamishi K: Kaposi's sarcoma-associated herpesvirus (KSHV) sequences in premalignant and malignant skin tumors. Arch Virol *141*: 2217-2223, 1996.
26 Rady PL, Yen A, Rollefson JL, Orango I, Bruce S, Hughes TK and Tyring SK: Herpesvirus-like DNA sequences in non-kaposi's sarcoma skin lesions of transplant patients. Lancet *345*: 1339-1340, 1995.
27 Verma SC, Lan K and Robertson E: Structure and function of latency-associated nuclear antigen. Curr Top Microbiol Immunol *312*: 101-136, 2007.
28 Qavi HB, Green MT, Lewis DE, Hollinger FB, Pearson G and Ablashi DV: HIV-1 and HHV-6 antigens and transcripts in retinal cells from AIDS patients in the absence of CMV infection. Inves Ophthal Vis Sci *36*: 2040-2047, 1995.
29 Bortek J, Bartkova J and Lukas A: The retinoblastoma protein pathway in cell cycle control and cancer. Exp Cell Res *237*: 1-6, 1997.
30 Levine AJ: P53, the cellular gatekeeper for growth and division. Cell *88*: 323-331, 1997.
31 Harbour JW: Eye cancer: Unique insight into oncogenesis, the Cogan Lecture. IOVS *47*: 1737-1745, 2006.
32 Beniston RG, Morgan IM and O'Brian V: E7 and P53 in papilloma virus oncogenesis cell transformation. Carcinogenesis *22*: 1069-1076, 2001.
33 Dyson N, Howley PM, Munger K and Harlow E: The human papilloma virus-16, E7 oncoprotein is able to bind to the retinoblastoma gene product. Science *243*: 934-947, 1989.
34 Molho-Pessach V and Lotem M: Viral carcinogenesis in skin cancer. Curr Probl Dermatol *35*: 39-51, 2007.
35 Underbrink MP, Hoskins SL, Pou AM and Albrecht T: Viral interaction: a possible contributing factor in head and neck cancer progression. Acta Otolaryngol *128(12)*: 1361-1369, 2008.
36 Grigoli G, Prikhod K and Yan W: Baculovirus P33 binds human P53 and enhances P53-mediated apoptosis. J Viral *73(2)*: 1227-1234, 1999.
37 Perry CA, Sklan EH and Soreq H: CREB regulates AChE-R-induced proliferation of human glioblastoma cells. Neoplasia *6*: 279-286, 2004.
38 Garcia-Allyson MS, Saez-Valero J, Munoz-Delgado E and Vidal CJ: Identification of hybrid cholinesterase forms consisting of acetyl- and butylcholinesterase subunits in human glioma. Neuroscience *107*: 100-208, 2001.
39 Johnson G and Moore SW: Localization of a novel adhesion-promoting site on acetylcholinesterase using catalytic antiacetylcholinesterase antibodies displaying cholinersterase-like activity. Appl Biochem Biotechnol *83*: 131-144, 2000.
40 Mack A and Robitzki A: The key role of butyrylcholinesterase during neurogenesis and neural disorders: an antisense-5 butyrylcholinesterase-DNA study. Prog Neurobiol *60*: 607-628, 2000.
41 Wasserman LM, Newsham I, Huang HJ and Cavenee WK: cAMP effect on myogenic gene expression in rhabdomyosarcoma cells. Exp Cells Res *25*: 55-62, 1996.
42 Kamal MA, Nasim FH and al-Jafari AA: Investigation of the effect of anti-neoplastic drugs, cyclophosphamide, cisplatin and methotrexate on the turnover kinetics of human erythrocyte acetylcholinesterase. Biochem Mol Biol Int *39*: 293-302, 1996.
43 McQueen MJ: Clinical and analytical considerations in the utilization of cholinesterase measurements. Clin Chem Acta *7*: 91-105, 1995.
44 Soreq H and Zakut H: Amplification of butyrylcholinesterase and acetylcholinesterase genes in normal and tumor tissues: putative relationship to organophosphorous poisoning. Pharm Res *7*: 1-7, 1990.

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