Differential Expressions of p53, p53R2, hRRM2 and PBR in Chronic Lymphocytic Leukemia: A Correlation with Intracellular Cholesterol

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Abstract Regulation of intracellular cholesterol homeostasis exists under balance between intracellular biosynthesis and uptake from extracellular origin by cell surface transport proteins. Expected role of cholesterol on either tumor suppressor gene and/or DNA synthesis has been aimed in the present study to explore intracellular cholesterol homeostasis in CLL subjects. Higher expressions of p53R2 (p53 dependent subunit of ribonucleotide reductase) and p53 were found in lymphocytes of chronic human lymphocytic leukemia as comparison to their normal counterparts. Inverse relation was found with p53 independent R2 subunit (in human hRRM2) of ribonucleotide reductase, which was found to be decreased from its control group. More expression of peripheral type benzodiazepine receptor, a cholesterol transporter, was noticed in isolated nuclear fraction with simultaneous increase of cholesterol concentration in cytoplasmic and nuclear compartments. A parallel increase of cholesterol in cell nucleus with increased p53R2 expression shows priority of

the involvement of cholesterol in the process of cell replication.

Keywords p53 · p53R2 · hRRM2 · Chronic lymphocytic leukemia

Introduction

Chronic lymphocytic leukemia (CLL) is mainly a B cell lymphoma characterized by the assemblage of CD5+ B-lymphocytes in the peripheral blood, bone marrow and secondary lymphoid organs (lymph nodes and spleen) [1, 2]. Lymphocyte count in the peripheral blood is found to be $>5 \times 10^9 \text{ L}^{-1}$. CLL is the most common type of leukemia in adults in the western countries representing about 25–30 % of all types of known leukemia [3].

Cholesterol homeostasis is one of the major regulatory events of cell's lipid metabolism. The key regulatory steps of intracellular cholesterol homeostasis exist through its intracellular biosynthesis, uptake of LDL (source of exogenous cholesterol) by LDL receptors (LDLR), storage of cholesterol esters and efflux of VLDL or HDL [4, 5]. The recent studies show that LDLR is highly expressed in the cancer cells [6]. The increased level of cellular cholesterol, due to more LDL uptake, supports rapid membrane formation by the developing cancer cells [7]. Peripheral-type benzodiazepine receptor (PBR) was identified in peripheral tissues because of its ability to bind the benzodiazepine diazepam (ValiumTM) [8]. The PBR appears to be a heteromeric complex of at least three different subunits, viz., an isoquinoline binding subunit, a VDAC—voltage-dependent anion channel, and ANT—adenine nucleotide translocase [9–11]. Recent studies demonstrated that PBR is a high affinity cholesterol binding protein [12, 13]. There

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are studies showing abundance of PBR in tumor cells and also specifically on nuclear membrane of tumor cells [14–18]. The subunits of PBR act as cholesterol receptor as well as cholesterol channel. PBR has been found to be aggressively expressed in human breast cancer cells [19]. Cholesterol is even found in the nucleus in association with chromatin lipids [20]. But till date no detailed study has been done in effect to find relation of cell proliferative proteins with the turnover of this nuclear cholesterol in leukemic cells of CLL subjects.

Ribonucleotide reductase (RR) is a regulated rate-limiting enzyme in the conversion of ribonucleoside diphosphate to 2'-deoxyribonucleoside diphosphate, which is essential for DNA synthesis [21]. In humans the enzyme is a dimer having one large subunit (hRRM1, Mr 170,000 dimer) and one of the two small subunits (hRRM2 and p53R2) identified separately [22, 23]. Inhibition of RR activity has been tested as a potential treatment modality in anticancer settings [24]. p53R2 plays a key role in the resolution of cellular DNA damage by providing deoxyribonucleotide triphosphates (dNTPs) to allow DNA repair in cells by making cell cycle arrest in response to p53 activation [21, 24]. Overexpression of p53R2 is associated with clinical response in myelodysplastic syndrome/acute myelogenous leukemia [25]. It has been suggested that the opposite regulation of hRRM2 and p53R2 in the invasion potential may play a critical role in determining the invasion and metastasis phenotype in cancer cells [26].

The impact, if any, of the turnover of intracellular cholesterol with the expression of tumor suppressor agent e.g. p53 protein and its simultaneous effects on the profile of ribonucleotide reductase, a protein responsible for DNA synthesis, are the initial targets of this study to understand the mechanism of regulation of genes by cholesterol in the pathogenesis of chronic lymphocytic leukemia.

Materials and Methods

Selection Criteria

For Patients

Inclusion criteria: Blood samples of CLL patients collected from Cancer clinic, AIIMS, New Delhi with informed consent. Samples were obtained after diagnosis of CLL.

Exclusion criteria: Patients were excluded from the study if suffering from any acute or chronic infections, patients receiving any medication known to affect lipid metabolism at the time of initial blood sampling, patients not willing to give consent.

For Controls

Inclusion criteria: Blood sample were collected from healthy subjects and not suffering from any illness.

Exclusion criteria: Subjects were excluded if suffering from any acute or chronic disease or not willing to give consent.

Collection of Blood Sample

Blood samples were collected from the male human subjects (25 normal control and 25 patients) with anti-coagulant (EDTA). It was used for lymphocyte isolation, extraction of nucleus and cytoplasm, cell lysate preparation and western blotting.

Isolation of Peripheral Blood Lymphocytes

The lymphocytes were isolated using modified method of Boyum [27]. LymphoprepTM density gradient medium from Axis-Shield, Oslo, Norway was used for lymphocyte isolation.

10 ml blood with anticoagulant (EDTA) was diluted with equal volume of 0.15 M NaCl. 20 ml of this mixture was layered on 10 ml Lymphoprep in 50 ml centrifuge tube. The tube was centrifuged at 1400 rpm for 40 min. The interphase materials containing mononuclear cells was collected using sterile pipette and mixed with 30 ml Hank's balanced salt solution. This was again centrifuged at 1400 rpm for 10 min. The resulting cell pellet was resuspended in 20 ml culture media (RPMI 1640 media containing Penicillin and Streptomycin). The suspended cells were centrifuged at 1000 rpm for 10 min. The cell pellet (lymphocytes) was used either for lysate preparation or for isolation of nucleus and cytoplasm.

Cell Lysate Preparation

The isolated lymphocytes were washed twice in ice cold PBS. The cells were pelleted by centrifugation at $2000 \times g$ for 5 min at 4 °C. The supernatant was discarded and the pellet was resuspended in lysis buffer (5 times of the pellet size) containing 50 mM Tris-Cl (pH 7.4), 300 mM NaCl, 0.5 % (v/v) Triton X-100, 5 mM EDTA with 2 mM PMSF, 10 mg/ml leupeptin and 10 U/ml aprotinin and then lysed by strong vortexing. The lysed suspension was kept on ice for 30 min and then centrifuged at $12,000 \times g$ for 15 min at 4 °C. The supernatant was taken and stored at 4 °C. All lysates were used within 2 days of preparation.

Protein Estimation

Protein estimation was performed by the Bradford method (Bradford MM, 1976) [28]. Estimation was performed in

BMG LABTECH multidetection microplate reader FLUOstar Omega.

Cholesterol Estimation

Cholesterol was estimated by following the protocol of Zlatkis et al. [29].

Western Blotting

The lymphocytes were lysed and the lysate was used on SDS-PAGE run at 10–12 mA in appropriate volume of 5x protein loading buffer containing 1 % SDS, 5 % β -mercaptoethanol, 50 mM Tris-HCl (pH 6.8), 10 % glycerol and 0.02 % Bromophenol and boiled for 5 min.

Transfer was performed at 35 V for 16 h at 4 °C in a transfer buffer containing 48 mM Tris-HCl, 39 mM glycine and 20 % methanol, pre-chilled at 4 °C. The nitrocellulose membrane was washed with blocking reagent (5 % Blot-quick blocking powder in 0.01 M PBS containing 0.05 % Tween 20) for 2–3 h at a room temperature on a rocker. The mouse monoclonal primary antibody with dilutions of 1:1000 for p53, p53R2, and R2, 1:3000 for GAPDH and goat polyclonal antibody diluted to 1:1000 for PBR in blocking reagent were used for incubation of the membranes at room temperature for 2 h. The membrane was then washed thrice in washing buffer containing 0.01 M PBS and 0.1 % Tween 20 for 10 min. Anti-mouse HRP conjugated secondary antibody was added at 1:2000 dilutions for p53, 1:5000 dilutions for p53R2 and R2, 1:12,000 for GAPDH and anti-goat HRP conjugated antibody was added at 1:10,000 dilution for PBR and incubated for 2 h at room temperature. Blots were then developed by Enhanced Chemiluminescence in the dark and the bands were captured on an X-ray film. Nitrocellulose membrane and Blot-Quick blocking reagent was a product of Genotech Incorporation, CA, USA. Secondary antibodies (anti goat and anti mouse HRP conjugates) were from Bangalore, Genei, Bangalore, India. ECL (enhanced chemiluminescence)/luminol was obtained from Santacruz Biotechnologies, CA, USA. Kodak XK-5 X-ray films were from Kodak Corporation, USA.

Isolation of Nucleus and Cytoplasm

Approximately 10 million cells from isolated lymphocytes were washed twice in 0.01 M PBS at $300\times g$. The pellet was re-suspended in nuclear isolation buffer (10 mM Tris-HCl pH 7.4, 1 mM EDTA, 15 mM NaCl, 0.15 mM Spermine and 0.5 mM Spermidine, 0.1 % NP-40, 5 % Sucrose). The nuclei were obtained after centrifugation at 3000 rpm for 5 min. The isolated pellet (nuclei) was suspended in lysis buffer and considered as nuclear fraction.

The remaining supernatant was centrifuged at $100,000\times g$ for 60 min for isolation of cytoplasmic fraction. Respective markers (DNA for nucleus and lactate dehydrogenase for cytoplasm) were assessed spectrophotometrically to see the purity of nuclear and cytoplasmic fractions. DNA was estimated by diphenylamine reaction [30] and lactate dehydrogenase was assayed as reported earlier by Wilkinson et al. [31].

Statistical Analysis

The mean expression density of western blotting results was compared using student's t-test. To compare the cholesterol level between control and patients, *p* values were calculated by student's t-test.

Results

p53, p53R2 (Fig. 1A, C) and PBR (Fig. 2) were found significantly higher in CLL patients as compared to control subjects. Among them p53R2 expression was remarkably high. On the contrary, R2 expression (Fig. 1B) got significantly decreased in CLL subjects as compared to their control counterparts. The appearance of two bands for R2/hRRM2 might be the result of mass difference of the degraded and resynthesized products in cell cycle process. Both cytoplasmic and nuclear cholesterol concentrations were increased in CLL patients as compared to control subjects (Fig. 3). The purity of nuclear and cytoplasmic isolates prepared from lymphocytes by ultracentrifugation was checked by assaying optimal response of the organelle specific markers in respective fractions. Nuclear fraction was confirmed with optimal concentration of DNA whereas cytoplasmic fraction was ascertained from maximal activity of LDH (data not shown). A parallel increase of intracellular cholesterol along with p53R2 and PBR (nuclear membrane bound cholesterol channel) only gives an indication of a kind of latent link between cholesterol homeostasis and regulation of oncogenic machinery in cell nucleus.

Discussion

p53, a 53 kDa tumor suppressor protein, is known to be elevated in stress situation like cancer. p53 is also known to remove damaged DNA through apoptosis mediated cell death. Phosphorylated p53 activates p21 gene to stop cell cycle activity. In normal cells, p53 level remains low because of its rapid degradation. But in cancer cells, its expression increases for the elimination of damaged DNA and to promote cell death, which could be a part of natural

Fig. 1 a Expression of p53 in cell lysate of control subjects and CLL patients by western blotting. p53 expression was found higher in CLL patients as compared to control subjects. p value calculated by Student's t -test $**p < 0.01$. **b** The expression of Ribonucleotide reductase 2 (R2/hRRM2) Subunit in control subjects and CLL

patients. The R2 expression decreases in CLL subjects as compared to the control counterparts. p value calculated by Student's t -test $**p < 0.01$. **c** The expression of p53R2 in control subjects and CLL patients. The expression was remarkably higher in CLL patients. p value calculated by Student's t -test $**p < 0.01$

Fig. 2 Expression of PBR in nuclear fraction of lymphocytes from control subjects and CLL patients. The expression of PBR was found to be higher in CLL patients. p value was calculated by Unpaired Student's t -test $**p < 0.01$

protection mechanism. Our western blot analysis (Fig. 1a) has shown a high expression of p53 in chronic lymphocytic leukemia (CLL) subjects.

The ribonucleotide reductase synthesizes deoxyribonucleotides (dNTPs) that are utilized in DNA synthesis. Ribonucleotide reductase has two p53 independent subunits, R1 (in human hRRM1) and R2 (in human hRRM2). The level of R1 remains same in cell cycle but R2 periodically degrades and reforms throughout the cell cycle. In our result R2 was decreased in CLL patients (Fig. 1b). This is expected because p53 expression remains high in CLL patients and higher expression of p53 arrests cell cycle activity at G1 by inducing p21 transcription. p53R2, another form of R2 induced by p53, gets expressed under

stress situations and like R2 can form complex with R1 to carry out dNTPs synthesis particularly at the time when normal DNA synthesis gets inhibited by the signal from highly expressed p53 in response to DNA damage e.g. in a pathological situation like cancer. We have compared the inverse relationship between the expressions of the two subunits, R2 and p53R2 respectively. The expression of R2 has been decreased, whereas the expression of p53R2 increases in CLL subjects respectively. One form of the R2-subunits in ribonucleotide reductase i.e. p53R2 is thus playing a compensatory role for the reduced expression of the other form of R2. The p53R2, which rises in response to highly expressed p53 in CLL subjects, probably supports the formation of dNTPs in maintaining its pool within the

Fig. 3 Cholesterol content in cell nucleus and cytoplasm from control subjects and CLL patients. Both cytoplasmic and nuclear cholesterol concentrations were increased in CLL patients as compared to control subjects. Unpaired Student's t-test * $p < 0.05$; ** $p < 0.01$

cells for repair and synthesis of DNA. The development of cancer tumor is probably due to an unregulated activity of highly expressed p53R2 subunit in the process of DNA synthesis and cell proliferation.

Earlier reports have shown the existence of cholesterol in cell nucleus of regenerating liver cells [32, 33] and a presence of highly expressed cholesterol transporter, PBR, in breast cancer cells [19, 34]. Our study has also shown an increased expression of PBR in nuclear fraction of lymphocytes from CLL patients as compared to control subjects (Fig. 2). This shows the probability of more cholesterol transfer from cell cytoplasm to cell nucleus in leukemic cells. Therefore, our next aim was to measure the cholesterol level in nucleus and cytoplasm of both normal and CLL subjects. In fact, our result shows a significant increase of cholesterol in both cell cytoplasm and nucleus in CLL subjects as compared to normal ones (Fig. 3). Hence it attracts a close concern on the hidden role of cholesterol in cell duplication and especially in case of a disease for uncontrolled proliferation of cells like cancer. It can be presumed that the high concentration of cholesterol in nucleus and cytoplasm of CLL subjects is utilized by rapidly developing cancer cells for the synthesis of their cell membrane and/or some other purposes still remained unsolved.

Thus, our overall observation shows a demand for cholesterol in cells with highly expressed p53R2; which may be used for membrane formation in those progeny cells created by the newly replicated DNA from the dNTPs produced by the activity of ribonucleotide reductase. Highly expressed PBR in CLL cell-nucleus may support the demand by passing cholesterol from its cytoplasmic pool to nuclear compartment.

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Compliance with Ethical Standards

Conflict of interest Ankit Verma declares that he has no conflict of interest. N. C. Chandra declares that he has no conflict of interest.

Human and Animal Rights The study was performed using human blood samples. The participants included were chronic lymphocytic leukemia patients and age-matched healthy controls.

Informed Consent Informed consent was obtained from all individual participants included in the study.

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Ethics approval reference number: IESC/T-66/03.02.2012.

References

- Chiorazzi N, Rai KR, Ferrarini M. Chronic lymphocytic leukemia. *N Engl J Med*. 2005;352:804–15.
- Rozman C, Montserrat E. Chronic lymphocytic leukemia. *N Engl J Med*. 1995;333:1052–7.
- Levin TT, Li Y, Riskind J, Rai K. Depression, anxiety and quality of life in a chronic lymphocytic leukemia cohort. *Gen Hosp Psychiatry*. 2007;29(3):251–6.
- Brown MS, Goldstein JL. Receptor-mediated endocytosis: insights from the lipoprotein receptor system. *Proc Natl Acad Sci USA*. 1979;76:3330–7.
- Oppenheimer MJ, Oram JF, Bierman EL. Downregulation of high density lipoprotein receptor activity of cultured fibroblasts by platelet-derived growth factor. *Arteriosclerosis*. 1987;7(4):325–32.
- Chen Y, Hughes-fullford M. Human prostate cancer cells lack feedback regulation of low-density lipoprotein receptor and its regulator SREBP2. *USA Int J Cancer*. 2000;91:41–5.
- Gal D, Simpson ER, Porter JC, Snyder JM. Defective internalization of low density lipoprotein in epidermoid cervical cancer cell. *J Cell Biol*. 1982;92:597–603.
- Papadopoulos V. Peripheral-type benzodiazepine = diazepam binding inhibitor receptor: biological role in steroidogenic cell function. *Endocr Rev*. 1993;14:222–40.
- Snyder SH, McEnery MW, Verma A. Molecular mechanisms of peripheral benzodiazepine receptors. *Neurochem Res*. 1990;15:119–23.
- McEnery MW, Snowman AM, Trifiletti RR, Snyder SH. Isolation of the mitochondrial benzodiazepine receptor: association with the voltage dependent anion channel and the adenine nucleotide carrier. *Proc Natl Acad Sci*. 1992;89:3170–4.
- Garnier M, Dimchev AB, Boujrad N, Price JM, Musto NA, Papadopoulos V. In vitro reconstitution of a functional peripheral-type benzodiazepine receptor from mouse Leydig tumor cells. *Mol Pharmacol*. 1994;45:201–11.
- Li H, Yao Z, Degenhardt B, Teper G, Papadopoulos V. Cholesterol binding at the cholesterol recognition amino acid consensus (CRAC) of the peripheral type benzodiazepine receptor and inhibition of steroidogenesis by an HIV TAT-CRAC peptide. *Proc Natl Acad Sci*. 2001;98:1267–72.
- Lacapère JJ, Delavoie F, Li H, Péranczy G, Maccario J, Papadopoulos V, et al. Structural and functional study of reconstituted peripheral benzodiazepine receptor (PBR). *Biochem Biophys Res Commun*. 2001;284:536–641.

14. Wang JK, Morgan JJ, Spector S. Benzodiazepines that bind at peripheral sites inhibit cell proliferation. *Proc Natl Acad Sci.* 1984;81(3):753–6.
15. Ikezaki K, Black KL. Stimulation of cell growth and DNA synthesis by peripheral benzodiazepine. *Cancer Lett.* 1990;49(2):115–20.
16. Bruce JH, Ramirez AM, Lin L, Oracion A, Agarwal RP, Norenberg MD. Peripheral-type benzodiazepines inhibit proliferation of astrocytes in culture. *Brain Res.* 1991;564:167–70.
17. Neary JT, Jorgensen SL, Oracion AM, Bruce JH, Norenberg MD. Inhibition of growth factor-induced DNA synthesis in astrocytes by ligands of peripheral-type benzodiazepine receptors. *Brain Res.* 1995;675:27–30.
18. Mukhopadhyay S, Mukherji S, Das SK. Increased expression of peripheral benzodiazepine receptor (PBR) in dimethyl-benz[a]anthracene-induced mammary tumors in rats. *Glycoconjugate.* 2006;23:199–207.
19. Hardwick M, Fertikh D, Culty M, Li H, Vidic B, Papadopoulos V. Peripheral-type benzodiazepine receptor (PBR) in human breast cancer: correlation of breast cancer cell aggressive phenotype with PBR expression, nuclear localization, and PBR-mediated cell proliferation and nuclear transport of cholesterol. *Cancer Res.* 1999;59(4):831–42.
20. Albi E, Viola Magni MP. The role of intranuclear lipids. *Biol Cell.* 2004;96:657–67.
21. Tanaka H, Arakawa H, Yamaguchi T, Shiraishi K, Fukuda S, Matsui K, et al. A ribonucleotide reductase gene involved in a p53-dependent cell-cycle checkpoint for DNA damage. *Nature.* 2000;404:42–9.
22. Cerqueira N, Pereira S, Fernandes PA, Ramos MJ. Overview of ribonucleotide reductase inhibitors: an appealing target in anti-tumour therapy. *Curr Med Chem.* 2005;12:1283–94.
23. Reichard P. From RNA to DNA. Why so many ribonucleotide reductases? *Science.* 1993;260:1773–7.
24. Nakano K, Balint E, Ashcroft M, Vousden KH. A ribonucleotide reductase gene is a transcriptional target of p53 and p73. *Oncogene.* 2000;19:4283–9.
25. Link PA, Baer MR, James SR. p53-inducible ribonucleotide reductase (p53R2/RRM2B) is a DNA hypomethylation-independent decitabine gene target that correlates with clinical response in myelodysplastic syndrome/acute myelogenous leukemia. *Cancer Res.* 2008;68:9358–66.
26. Liu X, Zhou B, Xue L. Metastasis-suppressing potential of ribonucleotide reductase small subunit p53R2 in human cancer cells. *Clin Cancer Res.* 2006;12:6337–44.
27. Bøyum A, Scand J. Isolation of lymphocytes, granulocytes and macrophages. *Immunol.* 1976;5(Suppl. 5):9–15.
28. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.* 1976;72:248–54.
29. Zlatkis A, Zak B, Boyle AU. A new method for direct determination of serum cholesterol. *J Lab Clin Med.* 1953;41:486.
30. Burton K. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochem J.* 1956;62(2):315–23.
31. Wilkinson JH. Enzyme kinetics and its relevance to enzyme assay. *J Clin Path.* 1971;24:14–21.
32. Albi E, Cataldi S, Rossi G, Viola Magni MP. A possible role of cholesterol-sphingomyelin/phosphatidylcholine in nuclear matrix during rat liver regeneration. *J Hepatol.* 2003;38:623–8.
33. Albi E, Pieroni S, Viola-Magni MP, Sartori C. Chromatin sphingomyelin changes in cell proliferation and/or apoptosis induced by ciprofibrate. *J Cell Physiol.* 2003;196:354–61.
34. Hardwick M, Cavalli LR, Barlow KD, Haddad BR, Papadopoulos V. Peripheral-type benzodiazepine receptor (PBR) gene amplification in MDA-MB-231 aggressive breast cancer cells. *Cancer Genet Cytogenet.* 2002;139(1):48–51.