RESEARCH ARTICLE

Acetylation of Retinoblastoma Like Protein2 (Rb2/p130) in Tumor Tissues

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Abstract

The activity of Rb proteins is controlled by post-translational modifications, especially through phosphorylation. Acetylation of Rb2/p130 was reported recently in NIH3T3 cells but its physiological relevance in cell cycle control and tumorigenesis is still unknown. Efforts are underway to investigate possible interplay between Rb2/p130 phosphorylation and acetylation. Here we hypothesized that Rb2/p130 acetylation, like p53 acetylation, may play a role in development of the tumor phenotype. The proposed hypothesis regarding acetylation of Rb2/p130 in tumor VS normal cells was found to be true in our case study of 36 tumor samples. Statistical analysis of results suggest strong correlation among Rb2/p130 acetylation and cancer phenotype.

Keywords: Pocket proteins - Rb2/p130 - acetylation - cell cycle - protein modification

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Introduction

Retinoblastoma is a tumor that occurs sporadically or inheritably in children. Mutations in both alleles of retinoblastoma (Rb) gene were found to occur in the neoplasm (Cobrinik, 2005; Giacinti and Giordano, 2006). The identification and subsequent cloning of Rb gene opened a new avenue in cancer research and soon the Rb gene was reported to be mutated in many other forms of human cancers (Classon and Harlow, 2002; Giacinti and Giordano, 2006).Shortly after the discovery of tumor suppressor Rb gene, two other proteins sharing the characteristic structural and functional attributes of the retinoblastoma protein (pRb) were identified (Classon and Harlow, 2002; Claudio et al., 2002; Cobrinik, 2005; Giacinti and Giordano, 2006). These proteins were named Rb1/p107 and Rb2/p130 and all three members of the family were collectively referred to as "pocket proteins". The term "pocket proteins" derives from conserved pocket domain (Paggi and Giordano, 2001; Claudio et al., 2002; Cobrinik, 2005) present in all three members of the Rb family through which they bind to viral oncoproteins (Classon and Dyson, 2001; Cobrinik, 2005; Rocha-Sanchez et al., 2007). Although the members of Rb family are described as tumor suppressor proteins, they have physiological roles in development, differentiation, apoptosis and angiogenesis (Classon and Dyson, 2001; Claudio et al., 2002; Paggi and Giordano 2002; Toppari et al., 2003; Cobrinik, 2005; Rocha-Sanchez and Beisel 2007; Longworth and Dyson, 2010). Genome wide searches, employing chromatin immunoprecipitation (ChIP) and microarray techniques for E2F targets have identified many genes that are indirectly regulated by Rb family of proteins. These also include genes involved in DNA repair, mitochondrial biogenesis and metabolism (Korenjak and Brehm, 2005; Bosco and Knudsen, 2007).

Rb2/p130 has been shown to block the cell cycle, whereas pRb over expression sometimes fails to do so. The human cervix carcinoma cell line, C33A, is inhibited by Rb2/p130 overexpression, but not by pRb (Lacy and Whyte, 1997). Similarly T98G human glioblastoma cell line is sensitive to growth responsive effects of Rb2/p130 but resistant to growth suppressive effects of pRb and Rb1/p107 (Claudio et al., 1994). Moreover in HONE-1 (nasopharyngeal carcinoma derived cell line), a drastic reduction in Rb2/p130 expression levels has been reported, while expression level of other Rb family remained constantly elevated (Claudio et al., 1994). On the other hand, Saos-2 human osteosarcoma cells were growth arrested in Go/G1 of the cell cycle by all the three members of the retinoblastoma family (Claudio et al., 1994; Claudio et al., 2002). In the light of these observations, it has been proposed that the growth suppressive properties of retinoblastoma family proteins are cell type specific. Due to the presence of E2F4-p130 complexes in abundance in quiescent cells, some authors have proposed E2F4-p130 complexes as marker of Go Phase of the cell cycle (Smith et al., 1996; Claudio et al., 2002).

Rb family members are post-translationally regulated proteins. Phosphorylation of Rb family members leads to the dissociation of Rb/E2F complexes and inactivation of Rb proteins. Rb family proteins remain in this active state until passage through mitosis, after which they are re-engaged through the action of phosphatases (Knudsen and Knudsen, 2008). Acetylation of pRb has already been reported during muscle differentiation (Nguyen et

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al., 2010). Previously, we reported that retinoblastoma like protein2 (Rb2/p130) is also acetylated in cell cycle dependent manner (Schwarze et al., 2010). Very little is known about the roles of Rb2/p130 acetylation in cell cycle control and tumor genesis. Recently, we have also shown an inter-relation between acetylation and phosphorylation of Rb2/p130 in in vitro (Saeed et al.,2012). Nothing is known about the levels of Rb2/p130 acetylation in cancer cells.

Current study was aimed to detect any measurable differences in acetylation status of Rb2/p130 proteins in tumor vs healthy tissues. Tumor biopsies as well as blood samples from related individuals were collected from hospitals after being diagnosed by oncologists. Immunoprecipitations were performed on crude protein extracts obtained from these samples and dot blot was performed using specific antibodies against Rb2/p130 and acetyl lysine. Significant differences were noticed in Rb2/p130 acetylation. This highlights acetylation as an important phenomenon in regulating activities of Rb2/p130 proteins and hence its association with tumor development.

Materials and Methods

Identification of patients and tissue sample collection

Fresh tissue samples (brain) from cancer patient after being diagnosed and verified by oncologists were obtained from National Oncology and Radiotherapy Institute and Pakistan Institute of Medical Sciences (PIMS). Samples were collected after a signed consent form from the patient. Samples were immediately treated with trichostatin A (TSA) a portent histone deacetylase inhibitor (HDAC inhibitor) and were stored at -20 or -80 oC until further analysis. Addition of a potent histone deacetylases is very important to prevent the work of deacetylases so that epigenetic signature for Rb2/p130 acetylation is preserved in a best possible way. The present study was conducted with a prior approval from ethical committees of both CIIT (COMSATS, Institute of Information Technology, Islamabad) and hospital.

Cellular fractionation and protein extraction

Tissues were homogenized using glass homogenizer and were suspended thoroughly in RIPA buffer (150 mMNaCl, 1% NP-40, 0.5% deoxycholate (DC) 0.1% SDS, 50 mMTris, 0.2 mM NaVO3, 10 mMNaF, 0.4 mM EDTA, 10% glycerol, pH=8.6, mM PMSF, 1×protease inhibitor cocktail (Pierce P-78425)). Then carefully pipette out all of the mixture in an eppendorf tube. Placed it on ice for about 45 minutes. The mixture was centrifuged at 2000 rpm for 10-15 minutes at 4°C. After centrifugation, supernatant was collected carefully in a new sterile eppendorf tube. Total protein content was estimated at 595nm using nanophotometer (Implem GmbH, Germany: No 3310).

Immunoprecipitation

Cellular extract (200 μl) were incubated with antibody solution (2.5 μg/ml) for 60 min at 4°C. Anti-Rb2/p130 (BD Transduction Laboratories, No 610262) antibodies were used for immunopecipitations. Protein G Sepharose (Invitrogen USA; 30 μl) equilibrated twice in RIPA-buffer was added and incubated for 2 h at 4°C under gentle shaking, followed by centrifugation at 800×g for 5 min. The supernatant was saved for immunoblotting. Beads were washed 3 times with 0.5 ml RIPA-buffer and finally resuspended in 50 μl of SDS-sample buffer for further immunoblot analysis.

Dot blot

For dot blot 18 ug precipitated proteins were spotted on nitrocellulose membrane. BSA were used as negative control. Spots were encircled for demarcation and were left to dry for 30 min. Blot was blocked for immuno- detection with 5% (w/v) skim dry milk in TBS-T for at least 1 h, followed by incubation with the diluted antiserum (anti-Rb2/p130 (BD Transduction Laboratories, No 610262) and anti-acetyl Lysine (Millipore No 06-933)in TBS -T containing 5% nonfat dry milk powder for 2 h at room temperature or overnight at 4°C. The membrane was then rinsed three times for 10 min with TBS-T and incubated with alkaline phosphatase conjugated secondary antibody (anti-mouse, anti-rabbit respectively; Pierce UK) diluted 1 : 10,000 in TBS-T + 5% non-fat dry milk powder for 2 h at room temperature.Immunocomplexes were detected by incubating the membrane with 1-StepTM NBT/BCIP (Thermo scientific, USA) solution.

Statistical analysis

Dot intensities were quantified using densitometer and analyzed using statistical tests such as Chi-Square analysis. The value of $p<0.05$ is thus considered as highly significant.

Results

Demographic distribution and tumor grading

The present study included 36 brain tumor samples. Majority of the patients were from the adjoining areas of Sindh and Khyber-pakhtunkhuwan. Analysis of the patient data was carried out in correlation to different parameters e.g. age, gender, location of tumor and grade.

Brain tumor was observed in both of the genders. In this study the number of males affected was more as compared to females.

All the data collected, represent tumors of different portions of the brain. Among these the rate of meningioma was observed to be highest while extra dural tumor and schwannoma were observed the least.

According to the data collected mostly tumors were of grade I, followed by grade II (Figure 1)

Rb2/p130 got hyper acetylated in brain tumor samples

In order to detect any measurable differences in Rb2/p130 acetylation in tumor Vs normal tissues, total cell extracts were incubated with antibodies against Rb2/p130. The immunopreciptates were then analyzed for the acetylation status of Rb2/p130 proteins. Acetylation signals were detected in fractions where Rb2/p130 was present (Representative blot picture of one of the experiment is shown in Figure 2). Although lower levels of Rb2/p130 proteins were noticed in tumor tissues when

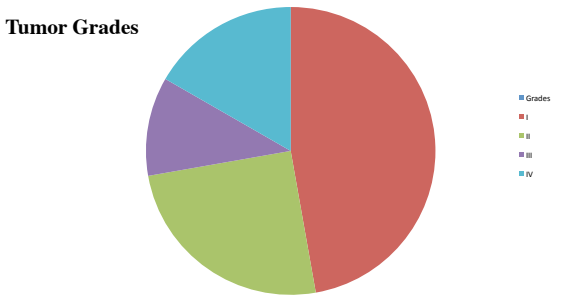

Figure 1. Patients Distribution in Different Grades of Brain Tumor. Majority of the patients were found to have grade I tumor. However, patients with grade III tumors were less observed compared to other grades

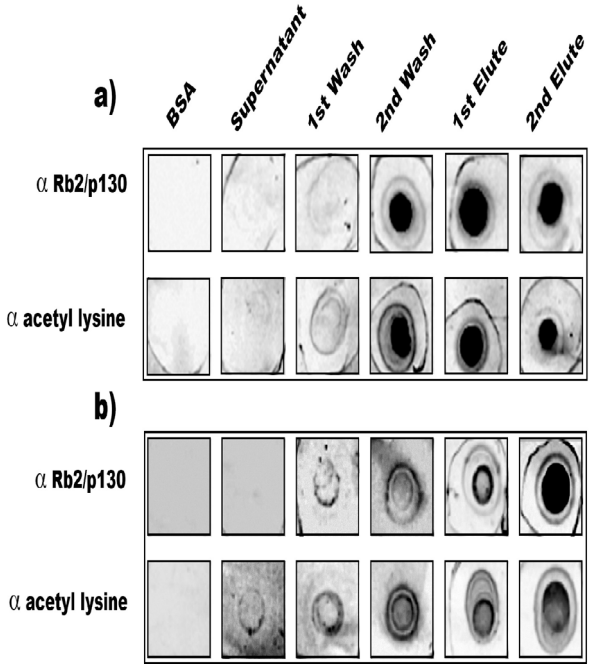

Figure 2. Rb2/p130 was Found to be Highly Acetylated in Tumor Tissues Than Diseased Free Tissues (blood samples). Total protein contents of diseased and healthy tissues were immunoprecipitated with antibodies against Rb2/p130 coupled to protein G -Sepharose beads. Different fractions were subjected to spotting on nitrocellulose membrane with subsequent immunoblotting against indicated antibodies. BSA was used as control. (a) Brain tumor (b) diseased free normal tissue

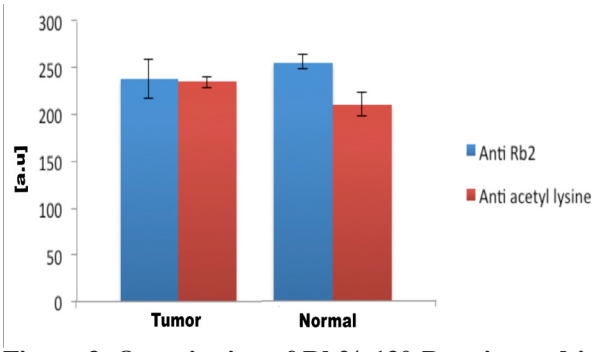

Figure 3. Quantitation of Rb2/p130 Proteins and its Acetylation was Performed using Densitometer. Error values were estimated and plotted along with indicated mean values in Excel (Microsoft). Despite lower levels of Rb2/p130 proteins, higher acetylation levels were recorded in tumor tissues

Table 1. Statiscal Analysis Performed on Cumulative Mean Values of Dot Intensities Obtained from All Tumor and Normal Tissues Under Observation Showed Significant Level of Association in Rb2/p130 Acetylation and Tumor Occurance. Moreover, Levels of Rb2/p130 Expression were also Found to be Significantly Low in Tumor Tissues than Normal, Diseased Free Tissues

Antibodies	Cumulative mean spot intensity as analyzed		p value
	Normal samples	Diseased samples	
Rb2/p130	252.26	236.96	0.010
Acetyl lysine	207.40	234.10	0.007

compared to diseased free tissues, higher levels of Rb2/p130 acetylation were detected in tumor tissues.

Statistical analysis performed showed significantly down-regulated expression of Rb2/p130 in diseased samples as compared to the normal samples, while significantly higher levels of Rb2/p130 acetylation was detected in diseased samples as compared to the normal ones (See Table 1). which thus justify our hypothesis about the downregulation of Rb2 gene and hyper-acetylation of Rb2 in tumor in patients (Figure 2)

Discussion

Activities of 'pocket protein' family are regulated via posttranslational modifications. Phosphorylation being one of the important chemical modifications on Rb proteins regulates number of cellular and developmental process in living organisms (Classon and Dyson, 2001; Paggi et al., 2002; Toppari et al., 2003; Cobrinik, 2005; Rocha-Sanchez and Beisel, 2007; Longworth and Dyson, 2010). Acetylation on Rb proteins and their role in regulating cell cycle remained elusive. Quite recently we had shown that Rb2/p130 is posttranslationally acetylated in cell cycle dependent manner (Schwarze et al., 2010). We have also presented evidence that Phosphorylation and acetylation on Rb2/p130 are not mutually exclusive and their inter-relation helps maintaining physiological environment within the cell (Saeed et al., 2012). The role of Rb2/p130 acetylation in tumor genesis remains unclear.

The results presented in this paper suggest that Rb2/p130 is highly acetylated in tumor tissues than normal, diseased free tissues (Figure 2). This finding not only suggest a significant association of Rb2/p130 acetylation with uncontrolled growth (tumor development), but also corroborates our earlier finding that acetylation is a prerequisite for phosphorylation of Rb2/p130 (Saeed et al., 2012). E2F-Rb complexes control G1/S transition in cell cycle. Phosphorylation of Rb proteins disrupts these complexes and promotes G1/S transition. Rb2/p130 acetylation being prerequisite for its phosphorylation adds an addition level in regulating their functional activities. It is also clear from the Figure 2 that Rb2/p130 is a routinely acetylated within cells to perform its various activities.

Levels of Rb2/p130 expression were also significantly lower in tumor tissues when compared to healthy tissues (Table 1 and Figure 3). This finding supports previous

studies where lower Rb2/p130 protein expression was noticed. The expression levels of Rb2/p130 have been shown to inversely correlate with cancer malignancies by immunohistochemical analysis in endometrial carcinoma, oral squamous carcinoma and in uveal melanoma (Baldi et al., 1996b). A lower level of Rb2/p130 protein expression and its hyper-acetylation seems a two-fold strategy for deregulated cell cycle and malignant growth formation. This key finding might open new avenue for developing novel therapeutic strategies for disease management and control.

Though circumstantial evidence supports association of Rb2/p130 acetylation with tumor occurrence, the key questions how Rb2/p130 acetylation effect its phosphorylation and play role in tumor genesis remained unanswered.

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