Expression Analysis of RbBP6 in human cancers : a Prospective biomarker

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Abstract

Retinoblastoma binding protein 6 (RBBP6) is a cancer-related protein that has been implicated in the regulation of cell cycle and apoptosis. RBBP6 isoform 1 has been demonstrated to interact with two tumour suppressors, p53 and pRB. Isoform 1 been shown to regulate p53 through its ubiquitin ligase activity, thus implicating in cell cycle regulation and apoptosis. Isoforms 1 and 2 are multidomain proteins containing a domain with no name (DWNN) domain, a Zinc Finger, a RING Finger, an Rb-binding domain and a p53-binding domain. The RBBP6 isoform 3 comprises the DWNN domain only. Isoform 4 lacks the Rb-binding domain but its role is less understood. RBBP6 isoform 3 has been reported as a cell cycle regulator with anticancer potential. There have been several studies that have clearly demonstrated that RBBP6 may be an important biomarker for cancer diagnosis and a potential drug target for cancer treatment. This work focused on differential expression of RBBP6 transcripts in different cancers, providing detailed analysis of their potential as diagnostic biomarkers for different cancers. These cancers include breast, liver, cervical and colon carcinomas. The expression of RBBP6 transcripts may further provide better understanding of the role of the RBBP6 in carcinogenesis and cell homeostasis.

Introduction

Cancer is a major public health problem across the globe. According to GLOBOCAN (Global Burden of Cancer Study), it was estimated that about 14.1 million new cancer cases and 8.2 million deaths occurred in 2012, worldwide. Over the years, the burden has moved to less developed countries, which currently accounts for about 57% of cases and 65% of cancer deaths, worldwide [1]. Lung cancer remains the leading cause of cancer death among males in both more and less developed countries [2]. Although occurrence rates for all cancers combined are approximately twice as high in more developed than in less developed countries in both males and females, mortality rates are only 8–15% higher in more developed countries [3].

Cancers arise through progressive mutations in response to a variety of environmental impacts (viruses, carcinogens, etc.). However, they progress to form disease by avoiding cancer suppression mechanisms (immunity, hypoxia, programmed cell death, etc.). They can do this sometimes by mutation of their DNA, but it is increasingly understood that this is heavily driven by altered gene expression and posttranscriptional regulation (epigenetic modification, alternative splicing, miRNA, etc.).

Bradner et al. [4] and Zanconato et al. [5] have reiterated the importance of transcriptional activation within cancer cells, further implicating the importance of the transcriptional coactivators, Yes–associated protein and the transcriptional coactivator with PDZ-binding domain. Proliferation dysregulation is also a key feature of cancer development. Both cell cycle and apoptosis are linked to proliferation rates and gene-regulated processes. One of the genes that has been heavily implicated in both processes is the RBBP6 gene. RBBP6 gene products have been shown to interact with two of the most studied tumour suppressors, p53 and pRB [6].

Retinoblastoma binding protein 6 (RBBP6) is a 250-kDa splicing nuclear protein, previously implicated in the regulation of cell cycle and apoptosis. Through alternative splicing, the human RBBP6 gene codes for three protein isoforms and isoform 3 consist of the DWNN (domain with no name domain) only while the other two isoforms, 1 and 2, comprise additional zinc, RING, retinoblastoma and p53 binding domains. The RBBP6 gene is localized on chromosome 16p12.2 [7–9]. Currently, it has been documented that there are four protein isoforms of this gene due to different processes that include alternative splicing (mRNA) and alternative use of RBBP6 promoters. One of its isoforms has been reported to interact with two tumour suppressor proteins, retinoblastoma protein (pRb) and p53 [9,10]. These interactions suggest diversity in its function. This gene produces multifunctional gene products that have been implicated in DNA replication [11], development [12], proliferation [13] and metastasis [14,15]. The RBBP6 gene encodes four protein isoforms, 1, 2, 3 and 4 [16]. These isoforms, at least isoforms 1, 2 and 3 are derived from the two major mRNA transcripts, a 1.1- and 6.1-kb transcripts. Isoform 1 is encoded by the 6.1-kb transcript, while alternative splicing of the 6.1-kb transcript results in variant 2, thus producing variants 1 and 2. Isoform 3 encoded by a 1.1-kb transcript is also known as a Domain With No Name (DWNN) [17,18]. It remains a matter of debate how the isoform 4 is derived. Additionally, both isoforms 1 and 3 are almost well understood, while isoforms 2 and 4 remain a mystery. Diversity in RBBP6 gene products may be critical in understanding how this gene influences the carcinogenesis process. Ntwasa [16] briefly reviewed the function of RBBP6, most critically, labelling this interesting gene as another monitor of p53.

Functional diversity of RBBP6 isoforms

There is sufficient evidence that RBBP6 multiple splice variants are involved in different cellular mechanisms and may have opposing functions where RBBP6 isoform 1 may be procarcinogenic while isoform 3 may be anticarcinogenic, depending on the cells they are expressed in. In gastric cancer, RBBP6 has been shown to be expressed in cancer stem cells [15]. Interestingly, we previously showed that the smallest isoform, RBBP6 isoform 3, is involved in cell growth inhibition and cell cycle arrest [17]. These opposing effects further advocate for the opposing functions, at least between RBBP6 isoforms 1 and 3. Most previous studies have shown that the RBBP6 isoform 1 is procarcinogenic while isoform 3 is strongly linked to anticancer activities. RBBP6 isoform 1 has been linked to regulation and stability of p53 and YB1, which further suggests its involvement in tumour progression [19]. It is one of the few proteins that interacts with two major tumour suppressor genes, p53/TP53 and the retinoblastoma gene product (pRb) [18]. Through its RING finger domain, RBBP6 isoform 1 has been reported to mediate ubiquitination of TP53 by Mdm2, an E3 ubiquitin ligase [20]. RBBP6 promotes the degradation of p53, thereby increasing cell proliferation. In mice, the bigger isoform is referred to as PACT, while the rat homologue is the P2P-R [6,9]. These homologues provided the initial understanding of the RBBP6 function. There has been a growing evidence of the role of RBBP6 in cellular processes, but there is a limited

distinction on which isoform performs which role. For example, recently, Xiao et al. [21] expertly showed that RBBP6 knockdown sensitized colon cancer cells to radiotherapy. This concurs with several pieces of evidence that has been documented showing that RBBP6 is involved in anticancer activities. One also needs to determine which isoform is involved in anticancer activities between isoforms 2 and 1. Care should be taken when referring to the function of this gene because it encodes a variety of isoforms that may have differing or opposing functions. There has been no study that shows that expression of these variants depends on each other. Table 1 summarizes the different known functions of RBBP6 in different cellular homeostasis processes.

Table 1 A summary of different isoforms and their cellular functions

RBBP6 isoform	Cellular processes	References
RBBP6 isoform 1	•The mouse/rat orthologues are involved in the regulation of cell cycle arrest, mitotic apoptosis	[6,9,10]
	•The yeast homologue was implicated in RNA processing	[22]
	•E3 ligase that leads to the ubiquitination of YBX1	[19]
	•Suspected scaffold protein for assembly of the p53/TP53-MDM2 complex, leading to increased p53 degradation	[16]
	•Regulates ZBTB38 protein stability by acting as an E3 ligase and influences DNA replication. ZBTB38 in turn negatively regulates the expression of MCM10 which is involved in DNA replication	[11]
RBBP6 isoform 2	No confirmed or known function	[16]
RBBP6 isoform 3	Cell cycle arrest regulation	[17]
RBBP6 isoform 4	No confirmed or known function	[16]

Regulation of RBBP*6* in carcinogenesis

Recently, Varghese et al. [22] showed that RBBP6 may be under the regulation of miR-424, especially in cervical cancer. In their study, they showed that aberrant DNA promoter methylation of the miR-424 resulted in its inactivation. Most importantly, this study showed that the miR-424 interacts with RBBP6 3′-UTR and silences its activity in SiHa cells. According to this report, it is more likely that the low expression of miR-424 in cervical cancer due to its promoter silencing may support the high expression of RBBP6 that has been reported in cervical cancer cells [23]. The mitogen-activated protein kinase signalling pathway has been implicated in promoting cellular proliferation and cell survival in cervical cells [24]. Teng et al. [23] documented that RBBP6 promoted cervical carcinoma proliferation, viability and migration through the JNK signalling pathway. The data show that: (1) RBBP6 is under the miR-424 regulation and the loss of miR-424 expression due to promoter methylation, resulting in RBBP6 upregulation; (2) the involvement of RBBP6 has been shown to be highly expressed in different cancers; and (3) recent implication of RBBP6 in the upregulation of p-JNK, resulting in the activation of JNK signalling pathway that then phosphorylates c-Jun, which increases its transactivation that is associated with cell survival and cell proliferation. Additionally, downregulation of mR-424 has been reported to contribute to angiogenesis [25], inhibit the proliferation of renal cancer cells [26]. As shown in Figure 1, downregulation of miR-424 by its promoter methylation results in the upregulation of RBBP6, which has been shown to activate the JNK pathway.

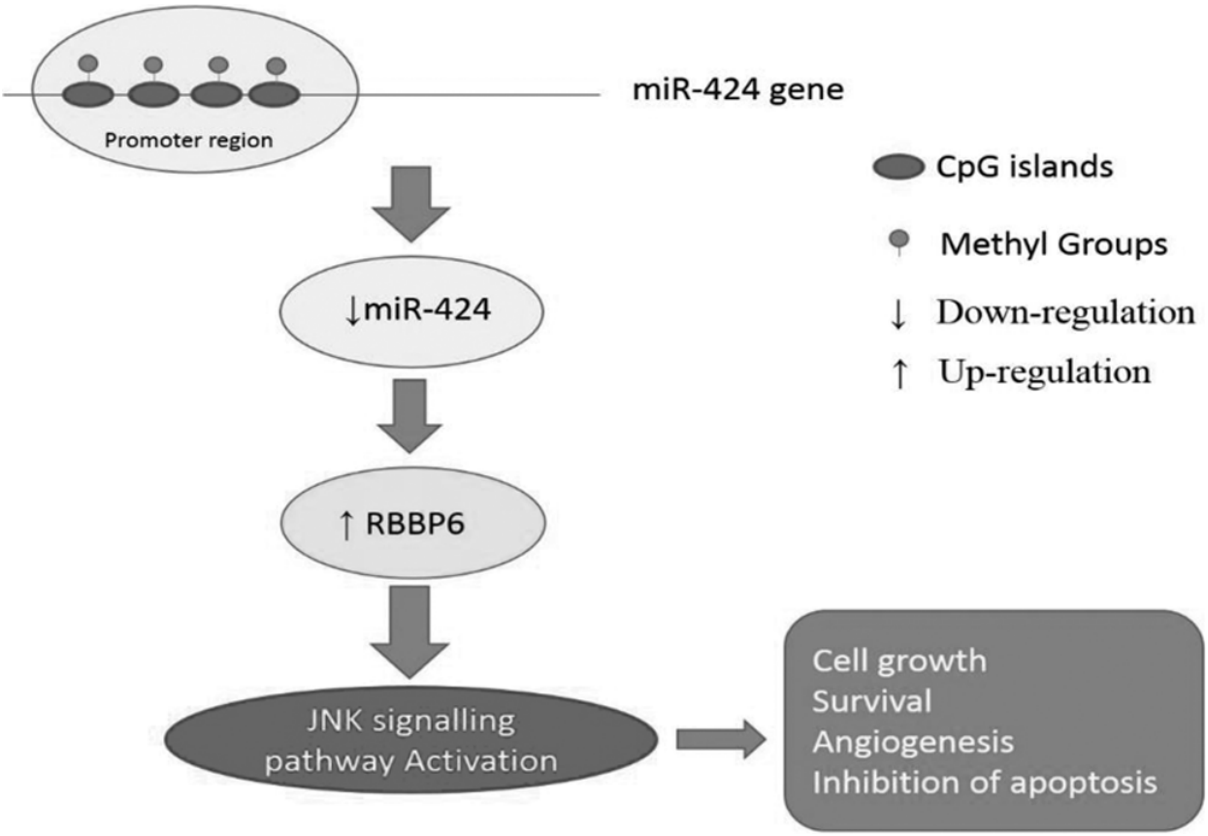

Fig. 1. Downregulation of miR-424 results in the upregulation of RBBP6, which supports the carcinogenic process through the JNK signalling pathway. This supports cell growth, survival, angiogenesis and apoptotic inhibition.

Localization of the RBBP6 transcript 3 in various human cancersThe RBBP6 has been reported to be overexpressed in most cancers. There are conflicting reports on the expression of RBBP6 in human cancers, but there is a growing evidence for upregulation of this gene. Here, we reviewed the expression and implication of RBBP6 in different cancers, at both mRNA (Fig. 2; Table 2) and protein (Fig. 3 and Table 3) levels. There seems to be a difference in the expression patterns between the RBBP6 variant 3 mRNA versus the expression pattern of the two RBBP6 transcripts, 1 and 2. Additionally, DWNN competes with RBBP6 for binding to the core machinery [17,27]. Table 2 and Figure 2 below give a summary on the expression of RBBP6 mRNAs in several human cancer tissues and human cancer cell lines.

Table 2 A summary of differential expression of retinoblastoma binding protein 6 mRNAs in different cancers

Tissue	Expression	References
Oesophageal carcinoma	•DWNN-containing mRNA expressed in small quantities in solid tumour •RBBP6 localization widespread in a number of squamous cell carcinoma •DWNN mRNA was expressed by few cells around the island of the tumour, showing nuclear staining, while the island of the tumours did not show any staining •RBBP6 mRNA was detected in some nuclei while other cells showed cytoplasmic localization of the RBBP6 mRNA	[17]
Breast carcinoma	•DWNN and DWNN-containing RBBP6 variants 1 and 2 mRNAs at low levels •Little accumulation of the RBBP6 mRNAs in the mass of tumours •Nuclear accumulation in normal fatty breast tissue •Qualitatively this suggests that in breast cancers the DWNN-associated RBBP6 mRNA products are downregulated when compared to a normal tissue	[17]
Hepatocellular carcinoma	•Liver tissues (both cancerous and noncancerous) express the RBBP6 variants and its isoforms •RBBP6 mRNAs were detected in cellular areas between islands of tumours. •RBBP6 mRNAs were not localized in the tumour islands, while areas of tissue adjacent to the tumours show few cells localizing the RBBP6 mRNAs •The negative controls clearly show no labelling	[17]
Cervical carcinoma	•Liver tissues (both cancerous and noncancerous) express the RBBP6 variants and its isoforms •No detectable localization of the RBBP6 DWNN mRNAs in the tumour islands	[17,29]
Serous papilloma adenocarcinoma and colon adenocarcinoma	•RBBP6 overexpressed in colon cancerous tissue and observed mostly in the nuclei of cancerous cells •Normal mucosa showed negative staining for RBBP •Island of tumour did not stain positive for DWNN-containing mRNA •RBBP6 mRNAs localized in the infiltrating lymphocytes situated in the lamina propia •The goblet cells and the absorptive cells within the tubular crypts/glands did not show any localization of the RBBP6 DWNN-containing transcript	[14,17]
Lung cancer	•Upregulation of RBBP6 mRNAs in well differentiated squamous cell carcinoma •Localization indicates increased abundance of RBBP6 mRNA in the cytoplasm of squamous cell carcinoma with more staining in keratinized region •Upregulation of the RBBP6 in brochoalveolar carcinoma in the cuboidal cells •Low levels of RBBP6 in small cell and large cell carcinoma	[30]

DWNN, domain with no name; RBBP6, retinoblastoma binding protein 6.

Table 3 A summary of differential expression of retinoblastoma binding protein 6 proteins in different cancers

Tissue	Expression	References
Hepatocellular carcinoma	•Moderate expression of RBBP6 isoform 1 and high expression of RBBP6 isoform 3 in HepG2 cell line •Weak expression detected in 27% of cancer patients	[17] [31,32]
Breast carcinoma	•Moderate expression of RBBP6 isoform 1 and low expression of RBBP6 isoform 3 in MCF-7 cell line •Decreased expression of RBBP6 sensitizes MCF-7 to camptothecin induced cell death •Medium expression was detected in 50% of cancer patients. Low expression levels were detected in a further 25%	[17] [33] [31,32]
Cervical carcinoma	•Three-fold increase in protein expression in SiHa and C33a cell lines •Moderate expression of RBBP6 isoform 1 and high expression of RBBP6 isoform 3 in HeLa cell line •High expression levels were detected in 16% of cancer patients. Medium expression levels were detected in 58% of cancer patients Low expression was detected in 25% of cancer patients	[24] [17] [31,32]
Serous papilloma adenocarcinoma and colon adenocarcinoma	•RBBP6 was found to be overexpressed in colon tumorous tissues. The level of expression increases with the stage of cancer and degree of metastasis •Medium expression was detected in 50% of cancer patients. Low expression levels were detected in a further	[14] [31,32]
Lung cancer	•RBBP6 and p53 proteins were overexpressed in lung adenocarcinomas and lung squamous cell carcinomas •Medium expression levels were observed in 40% of cancer patients. Low expression levels were observed in a further 20%	[30] [31,32]

RBBP6, retinoblastoma binding protein 6.

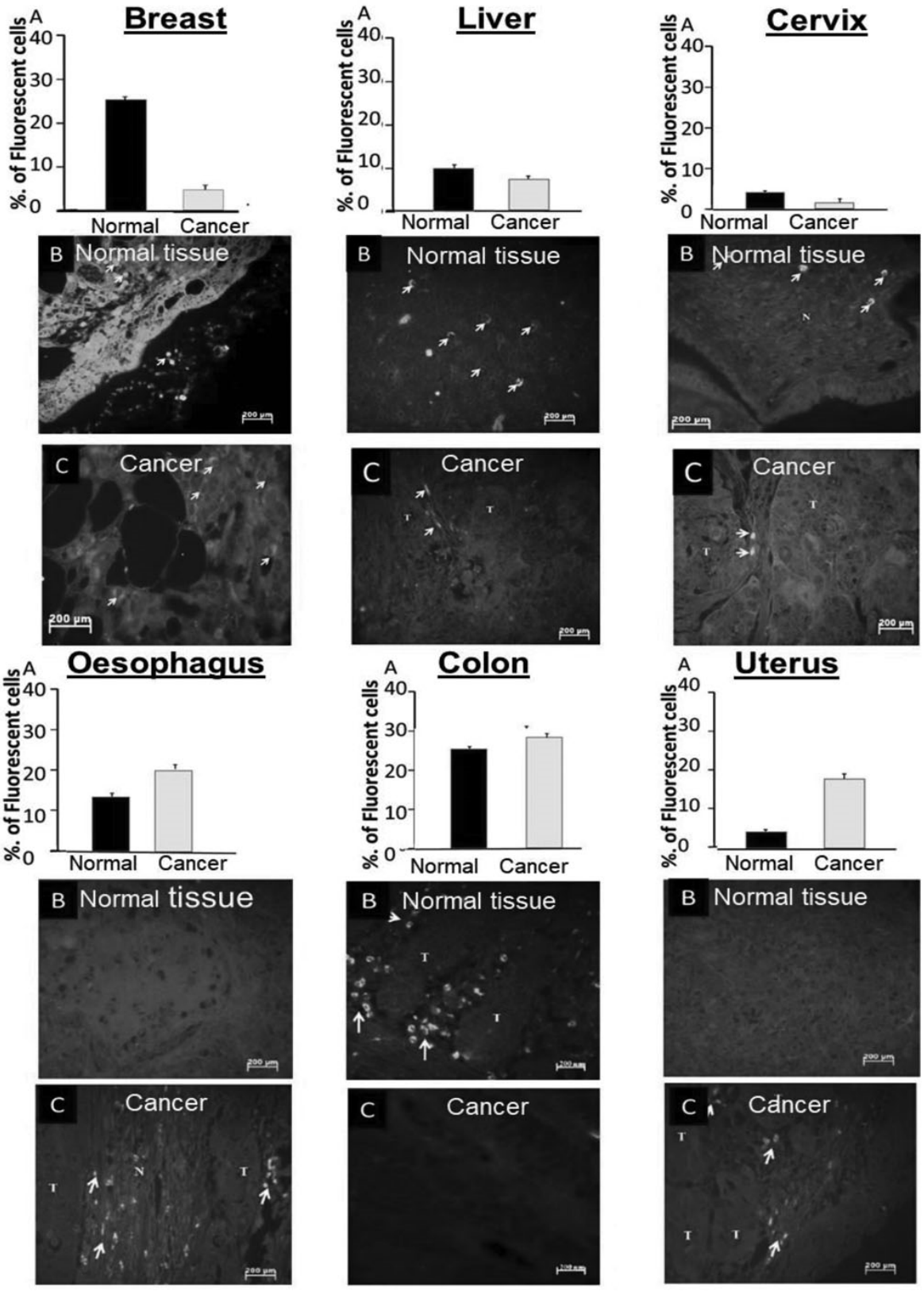

Fig. 2A comparison of the localization of RBBP6 transcript 3 mRNA between various normal and cancer tissues. The distribution of RBBP6 transcript 3 mRNA is shown in different normal healthy human tissues and the localization in their cancerous equivalent, using FISH staining. Above each set of micrographs is a quantitative analysis of the staining. The graph was constructed using three biological replicates and shows the number of positive cells (y-axis) in different tissues. Human cancer cells generally show lower transcription levels of the DWNN-containing RBBP6 mRNA than noncancerous cells.

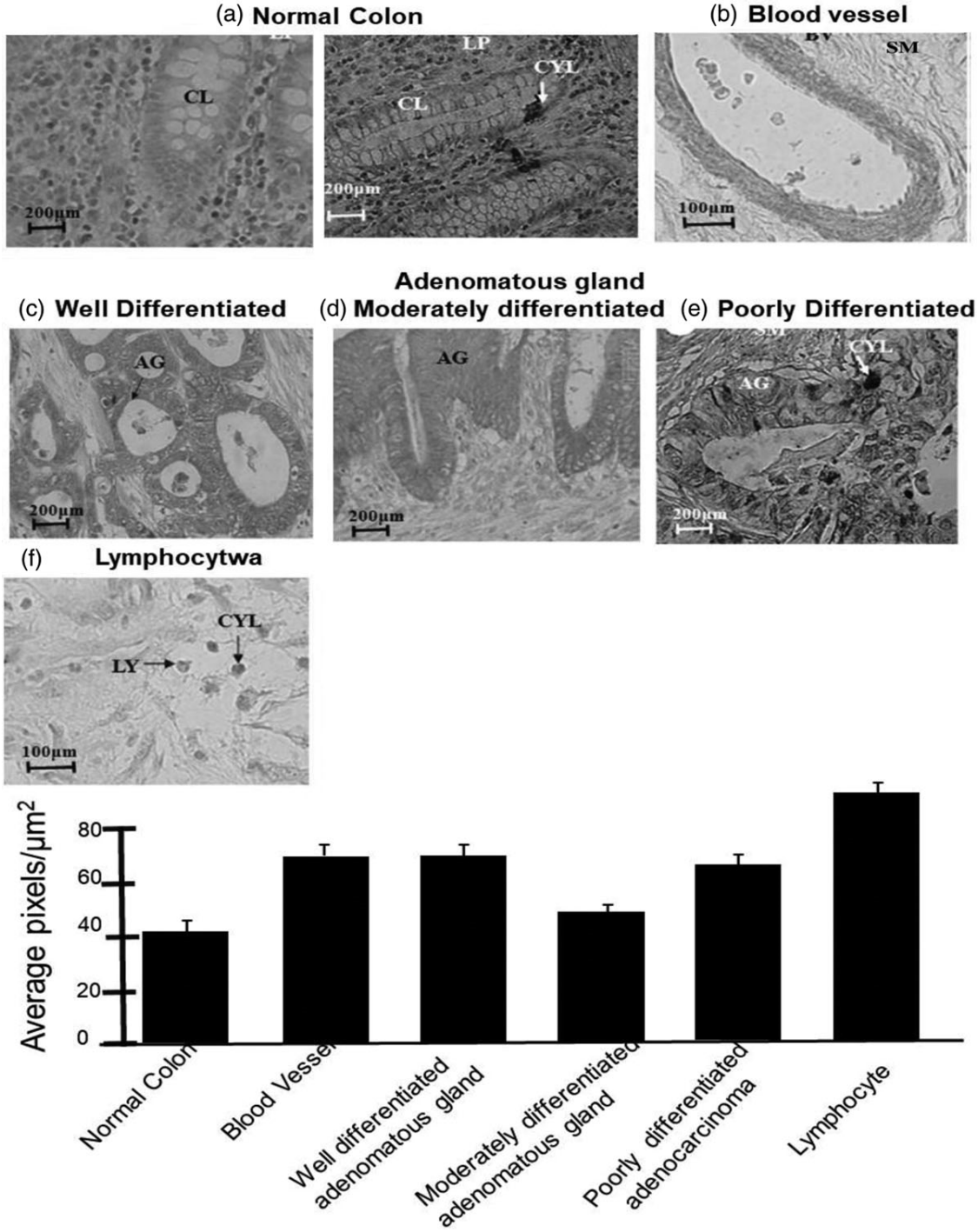

Fig. 3. Localization of the RBBP6-3 (DWNN) protein and quantification of expression levels in colon cancer. (a) Normal healthy colon shows nuclear and cytoplasmic localization of the DWNN-containing proteins in lamina propria (LP) in between the crypts of Lieberkühn (CL). (b) DWNN-containing proteins are located in the cytoplasm of cells within the blood vessels in subserosa. The localization of these proteins in adenomatous glands (AGs) changes depending on the state of differentiation. (c) In well differentiated tissue, the DWNN-containing proteins are found in the nucleus and cytoplasm of cells in submucosa (SM). (d) In moderately differentiated AG, DWNN-containing proteins are found in the cytoplasm of cells in the SM. (e) In poorly differentiated AG, DWNN-containing proteins are located in the cytoplasm in the lamina propria. (f) In lymphocytes, the DWNN proteins are found in the cytoplasm. The graph depicts the expression level of DWNN-containing proteins in colon cancer as the average immunolabeling of 3,3'-diaminobenzidine per area. The highest expression of DWNN was found in the lymphocytes. Equally high expression of DWNN was observed in the blood vessels and the poorly and well differentiated AGs. The DWNN protein level was the lowest in the moderately differentiated AGs and the normal colon tissue. DWNN, domain with no name.

Localization of the RBBP6 isoform 3 in various human cancers

Previously, the localization of DWNN-containing proteins in different human cancers and their normal tissue controls has been established using a purified antihuman DWNN domain antibody [17]. Mostly, the DWNN-containing RBBP6 proteins were found specifically within the cytoplasm of cancer cells within different carcinomas. This is summarized in Table 3 and Figure 3 where localization of the DWNN domain-containing proteins showed differential localization in different cancers, including hepatocellular carcinoma, cervical cancer, breast cancer and ovarian cancer.

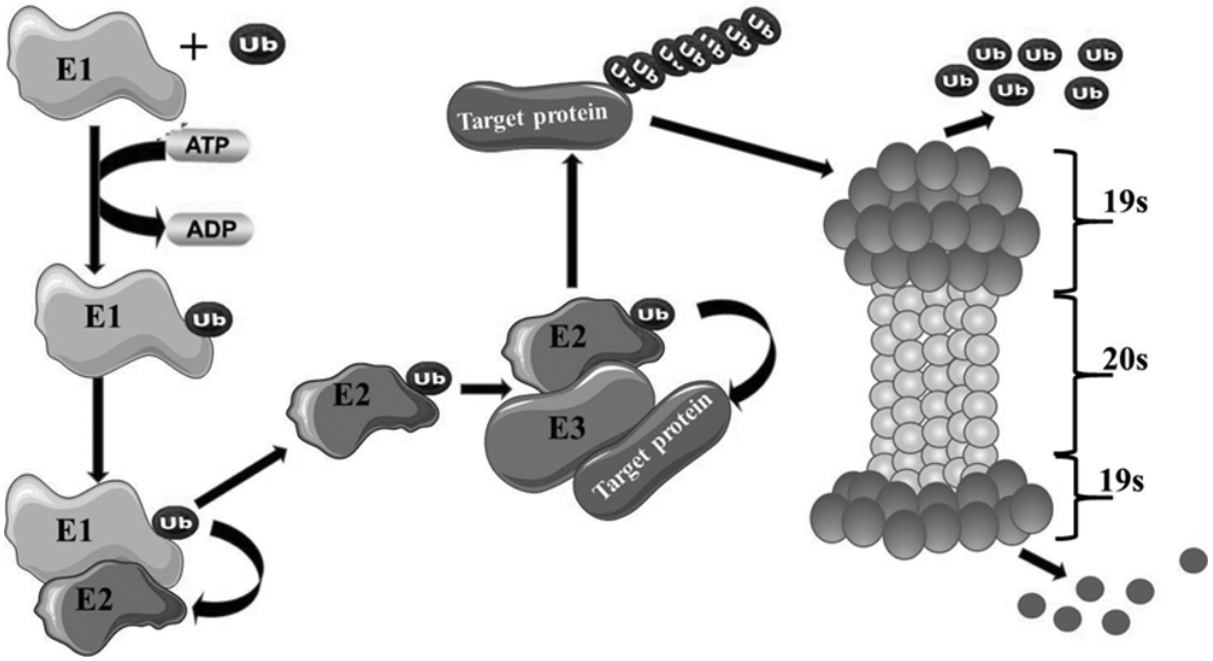

Fig. 4. The ubiquitin proteasome regulates cellular processes through protein catabolism. The ubiquitination of proteins is an ATP-dependent process involving three classes of enzymes. The E1 ligases are the ubiquitin activating enzymes and they bind ubiquitin in an energy ATP-dependent process. They then catalyze the transfer of the ubiquitin peptide to an E2 ligase. E2 ligases are ubiquitin carrier or conjugation proteins and they form a complex with an E3 ligase. The E3 ligase is responsible for binding a particular target protein and tags it by catalyzing the transfer of the ubiquitin protein to the target protein. This process is repeated until the target protein has a polyubiquitin chain. These polyubiquitnated proteins are then degraded by the 26S proteasome. The proteasome consists of 20S core that forms a pore, and is responsible for proteolysis. The 19S particle makes up the 9 subunit base and the 10 subunit lid of the proteasome. The lid recognizes the polyubiquitin chain and through an ATP-dependent process ‘opens’.

Interaction of RBBP6 and tumour suppressors, pRb and p53

During apoptosis and cell cycle arrest, the cell responds by activating a cascade of genes involved in cell growth. RBBP6, through its pRb- and p53-binding domains, interacts with both p53 and pRB. One of the mouse homologues of RBBP6, P2P-R, is a truncated form of RBBP6, lacking the DWNN that binds p53 and pRB [9]. The role of p53 during cell proliferation is well documented and any increase or decrease in the expression of this protein will have a positive or negative effect on cell survival [28].

There are many known tumour suppressor genes encoding proteins that inhibit the growth of potential cancerous cells by effecting cell cycle regulation and apoptosis. Of these genes, Rb and p53 are the most studied tumour suppressor genes implicated in cancer progression

[29,30]. Based on its structure, it was predicted that RBBP6 is a ubiquitin ligase [18], and it was only later that the RBBP6-mediated ubiquitination (Fig. 3) of two cell homeostasis-related proteins, YB-1 [19] and p53 [20], was experimentally established that demonstrated, respectively.

Conclusion

In conclusion, it is clear that RBBP6 is involved in cancer development and is correlated with poor prognosis in various cancers. The frequent upregulation of RBBP6 highlights its potential as a novel therapeutic target for most cancers. RBBP6 may be a target of deregulation in cancer progression, as its expression pattern changes in human cancers. Moreover, alterations of its expression in different cell states also advocate well for its involvement in carcinogenesis and apoptosis. Isoform 1 is upregulated in cancers and promotes cell proliferation and may be considered as antiapoptotic, while isoform 3 is antiproliferative and is downregulated in cancers. The potential clinical value of RPPB6 alone or in combination with p53 and Rb as novel biomarkers in cancers should be investigated extensively.

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