



Novel Insight of Cytokeratin 14 as a Biomarker in Diagnosing Bladder Cancer

Taufiq Nur Budaya^{1*}, Happy Kurnia Permatasari², Widodo Widodo³, Sumarno Reto Prawiro⁴

¹Department of Urology, Faculty of Medicine, Universitas Brawijaya, Dr Saiful Anwar General Hospital, Malang, Indonesia; ²Department of Biomedical Science, Faculty of Medicine, Universitas Brawijaya, Malang, Indonesia; ³Department of Biology, Faculty of Mathematics and Natural Science, Universitas Brawijaya, Malang, Indonesia; ⁴Department Clinical Microbiology, Faculty of Medicine, Universitas Brawijaya, Dr Saiful Anwar General Hospital, Malang, Indonesia

Abstract

Bladder cancer is the ninth most common type of cancer occurring in men. Screening for bladder cancer is often difficult due to limited proper modality and symptoms that is mild and unspecific. Leaving it untreated leads to greater morbidity and mortality. Therefore, finding a simple biomarker for screening purpose is necessary. Cytokeratin (CK) has been studied extensively as cancer biomarker. Detectable soluble CK was found in body fluid, including urine, and histological specimen using immunoassays. The level of its expression is mainly affected by cell proliferation, apoptosis, and cellular stress. CK 14, which is type I CK, was detected in the basal layer of epithelial cells and specific for squamous type of epithelial cell. CK 14 analysis in bladder cancer has revealed numerous functions such as predicting the development of bladder cancer, differentiating cancer origins, stratifying bladder cancer patients based on disease severity, and predicting bladder cancer recurrence. Its potential to become excellent biomarker makes it subject of interest in research development.

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***Correspondence:** Taufiq Nur Budaya, Doctoral Program, Department of Urology, Faculty of Medicine, Universitas Brawijaya, Dr Saiful Anwar General Hospital, Malang, Indonesia.

E-mail: taufiq_uro.fk@ub.ac.id

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Introduction

Novel diagnostic modalities to detect bladder malignancies are developing these past years. Up to this moment, the gold standard to diagnose bladder cancer is by cystoscopy assessment. However, this procedure tends to be avoided because of the invasive nature of the cancer. Therefore, patients often feel inconvenient undergoing the procedure [1]. Diagnosing bladder cancer is even more difficult as patients may not exhibit severe symptoms. Typically, it only manifests as painless hematuria which is not specific bladder malignancy [2], [3], [4]. Urinary biomarkers offer promising method to screen for bladder cancer. These biomarkers constitute various substances detected in the course of urine flow. Cytokeratin (CK) has been studied extensively in cases of malignancies. These proteins make up a class of intermediate filaments which contribute in the formation of epithelial cells cytoskeletons. Previously, CKs are believed to represent the occurrence of cell proliferation, apoptosis, or necrosis [5]. Among the 20 types of CKs, CK 14 is a

marker of squamous epithelial cells differentiation [6], [7]. Many studies have analyzed CK 14 as diagnostic or prognostic marker in various cancers. CK expression can be detected by immunoassay method. CK is potential urine biomarker to detect bladder cancers. This review aims to discuss the potential role of CK 14 in the diagnosis of bladder cancers.

Burdens of Bladder Cancer

Bladder cancer is the ninth most common type of all cancers. Male gender is more susceptible to this cancer, as it is ranked the seventh most common type of cancer in males [8]. In 2012, over 430,000 new patients are diagnosed with bladder cancers and 165,000 bladder cancer-related mortalities were reported. The incidence is higher in industrialized countries such as the United States (US) and European countries [9]. From American Cancer Society, 17,240 patients died from bladder cancer. In Indonesia, the exact number of cases is not

known. GLOBOCAN statistics estimated that there were 5.8 cases in every 100,000 Indonesians [10]. However, the unreported case was not taken into account in this statistics. In general, prevalence and incidence of bladder cancer in each country depend on risk factors. The risk factors include genetic predisposition, age, smoking habit, occupational factors (e.g., exposure to benzidine), diet (e.g., coffee and chocolate), pollution, geographic location (e.g., endemicity of *Schistosoma haematobium*), and gender (male predominance) [11]. Bladder cancer is divided into two major subtypes based on the type of cell involved: Urothelial bladder cancer (UBC) and non-UBC (NUBC). UBC are most prevalent, occurs in over 90% of all bladder cancer cases, with the remaining cases are NUBC. NUBC types are squamous cell carcinoma (SCC), adenocarcinoma, and small cell carcinoma (SmCC), also known as neuroendocrine tumor [12]. NUBC poses substantially lower value of disease-specific survival rate compared to UBC [13].

Aside from the risk of mortality, bladder cancer also impairs activities of daily living, both physically and mentally, leading to increased morbidity. This phenomenon is more dominant in elderly patients. Most common psychological issues observed after the diagnosis of bladder cancer are anxiety and depression. Moreover, these patients also tend to be not active in social life. In some cases, patients may become suicidal, although it is considerably rare. Limitations in daily activities include taking shower, going to toilet, moving a chair, dressing, eating, and walking. Obviously, these problems are more prevalent in patients with cancers of higher staging classification. Females are more prone to suffer from these problems. In addition, female patients are reported to have poorer outcome in the first 2 years following the diagnosis [14]. These detrimental conditions may potentially hinder patients from actively controlling and managing the cancer [15]. Thus, it will lead to worsened morbidity and increased mortality.

Diagnostic Problems of Bladder Cancer

Ideally, bladder cancer is diagnosed using cytology assessment and histopathological analysis of bladder biopsy as the gold standard [16]. However, it can be troublesome especially in remote areas due to limited facilities. Therefore, this condition may significantly delay bladder cancer diagnosis and negligence of disease severity. This phenomenon is still observed in Indonesia. Many health centers far away from metropolitan cities lack of health facilities, from diagnostic to treatment modalities. This results in the increasing number of severe cases of bladder cancer. A great proportion of patients are presented with locally advanced or advanced bladder cancer at the time of diagnosis [17]. These patients may have suffered from

bladder cancer for up to 5 years. Plus, each patient's level of knowledge also plays an important role in the diagnosis. Simple diagnostic kit could become an effective strategy for immediate and appropriate diagnosis even in remote areas, such as urinary biomarker. As a marker of epithelial differentiation, diagnostic kits based on CKs could be developed.

CK 14

CKs are a class of intermediate filaments which form the cytoskeletal networks of the cells. Cytoskeleton itself helps maintain the structural integrity of the cell upon the presence of mechanical stress and aids in cell movement and division [18], [19]. There are two types of CKs, acidic CKs (40–56 kDa), well known as the type I intermediate filament, consisting of CK9-CK20, and basic CKs (53–68 kDa), well known as the type II intermediate filament, which consist of CK1-8. This classification is based on the molecular weight and isoelective pH of each CK. Type I CK is of low molecular weight, whereas type II CK has higher molecular weight. CKs with lower molecular weight are observed in non-stratified epithelial cells, and vice versa [20]. Apart from CKs, intermediate filaments are also classified into type III (desmin, glial fibrillary protein/GFAP, peripherin, and vimentin), type IV (neurofilament proteins and internexin), and type V (lamins, exclusive to the cell nuclei) intermediate filaments. In addition, filensin and phakinin are currently included in the type VI intermediate filaments [21]. All CKs are encoded by 2 large families of genes consisting of nearly 50 distinct genes [5]. Similar to all intermediate filaments, the characteristic structures of the CKs lie on the three domains namely the non-helical N-terminal segment, the non-helical C-terminal segment, and the helical central rod [21].

During the apoptosis of epithelial cells, reorganization of the cell cytoskeletons occurs. This process therefore also reorganizes the CKs into granular structures. The enzyme caspases are needed to aid the cleavage of the CKs. The cleavage of the CK14 occurs at the sequence VEMD/A which is also identified in the CK12 to CK17. In contrast to this site, CK18 and CK19 are cleaved by the caspase at the sequence VEVD/A of the non-helical sequences. In addition, modifications, including glycosylations, transglutaminations, and phosphorylations, of the N-terminal and C-terminal segments may also affect the nature of the CKs. These processes result in the increased solubility of the CKs as they will be present as degraded protein fragments of lower molecular weight [5], [21].

CKs are expressed on epithelial cells which are dispersed throughout the body. Each CK is expressed in normal epithelial tissues as well as cancers derived from

the tissues [22]. Its expression varies depending on the type of the tissue and the development of each tissue itself. Among all CKs, the CK18 and CK19 are the most abundant CKs in the body and malignancies 5 CK 14 is a member of type I CK identified in the basal layer of the epithelial cells and the myoepithelial components of body tissues. Expression of CK14 is tightly associated with the presence of squamous epithelial cells. Previous literatures described CK14 to be expressed in squamous cell differentiation [6], [23], [24].

Mutation of CKs is associated with certain diseases. Along with CK5, CK14 mutation in the basal layer of the epidermis is involved in the epidermolysis bullosa [25]. Whereas abnormalities of CK8 and CK18 are related to hepatic and bowel diseases [26].

Regulation of CK 14

A few mechanisms in regulating CK14 expression have been proposed although exact pathway remains unclear. Therefore, up to this moment, regulation of cytoskeletal dynamic equilibrium is a field of active research. In general, CK expression is affected by cell proliferation and cellular stress in which each CK has different response. CK 14 is known to be expressed more during cell cycle and on cellular stress such as hypoxia [7]. In the context of cancer, reorganization of CK into stable soluble form of intermediate filament may help cancer cell migration which leads to cancer invasiveness. This process is mediated by actin/CK/14-3-3 σ complex and regulated by PKC ζ [27]. Formation of soluble CK through this process may also yield detectable CK in the circulation. Another study suggested that CK14 expression is correlated with forkhead box A1 (FOXA1) regulation. In FOXA1-knockout bladder cancer induced mice, CK14 expression increased. Whereas retention of FOXA1 expression was associated with lower CK14 expression. In this study, as CK14 expression increased, urothelial carcinoma became more aggressive and invasive [28]. Ho *et al.* (2012) linked CK14 expression with Stat3 regulation in bladder cancer model. Stat3-driven bladder cancer was proven to be invasive hence a worse outcome. The cancer was predominantly composed of stem cells characterized by CK14 staining [29]. CK 14 expression was also observed on stem cells differentiating into epidermal component following administration of BMP-4 [30].

Detection of CKs

CKs might be detected in body fluid such as cyst fluid, cerebrospinal fluid, edema fluid, and

urine [21]. Most common methods for analyzing soluble CK fragments (CYFRA) in the serum are tissue polypeptide antigen, tissue polypeptide specific antigen, and CYFRA 21-1. These assays detect the fragmented CKs released by the carcinoma cells as diagnostic or prognostic markers for various types of cancers [31]. Several studies have analyzed CK role in detecting circulating tumor cells using antibody against CK [32], [33], [34]. Antibodies against CKs are most effective in detecting metastatic process of a cancer. Nevertheless, it can also be used to identify cancers containing epithelial components [35]. Whereas in histopathological specimens, their expressions are commonly detected using immunoassays such as immunohistochemistry and immunofluorescence assay. In the context of bladder malignancies, detection of CKs in the urine is the most appropriate screening method. Numerous literatures have reported CK detection as urinary biomarkers for detecting bladder cancers [33], [36], [37], [38]. CK detection with adjunctive modalities such as nuclear matrix protein-22 or bladder tumor antigen may increase the accuracy in diagnosing bladder cancer.

The Role of CK 14 for the Diagnosis of Bladder Cancer

CKs have been widely used as biomarkers for detecting and classifying malignancies. At present, a number of processes are believed to enable the release of CKs fragments to the circulation system, thus it can be detected in the blood. Those processes include abnormal mitosis, proteolytic degradation, and spillover from the cells which undergo proliferation, apoptosis, or neoplasm. However, the exact mechanism of this phenomenon is yet to be explained. CK 14 is usually expressed in the tumor peripheral parts [39]. In cases of malignancies, CK14 is less analyzed compared to CK8, CK18, CK19, and CK20 [18]. However, its use offers a potential benefit in clinical practices.

CK 14 Role in Bladder Cancer Screening

As discussed before, the patterns of CK14 expression can be regarded as specific marker for epithelial differentiation. A study that analyzed CK14 expression in patients with spinal cord injuries, found that 7 of 54 patients had CK14 expression of bladder biopsies in either individual or cluster of cells accordant to squamous cell differentiation [6]. Similar finding was reported as CK14 was expressed

in SCC of the bladder or UBC with squamous cell component [24]. Another study revealed robust CK14 expression in bladder cancer model mice induced by N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN). In healthy mice, CK14 is expressed sporadically, while in BBN-treated mice, its expression is significantly increased [40]. These studies showed that CK14 expression can help predicting and detecting bladder epithelial metaplasia which results in bladder carcinomas, especially SCC of the bladder. Hence, it is potentially beneficial to screen for bladder malignancies.

CK 14 Role in Differentiating Cancer Origins

Due to its preponderance on particular tissues, CKs may be used to differentiate the origin of a cancer. A group of CKs, namely CK1, CK5, CK10, and CK14 (34 β E12), was proven to exclude the diagnosis of large-cell neuroendocrine carcinomas and detect the basaloid carcinoma (BC) of the lungs. Morphologically, both carcinomas may express similar features, especially in specimens of low quality [41]. Linskey *et al.* (2013) reported that CK14, along with CK17, was prominently expressed in basal cell carcinoma with squamous metaplasia (BCCm) [23]. This feature differentiated BCCm with basal SCC. A study analyzing CK expression in the breast cancer showed that CK5/14 is almost exclusively expressed in BRCA1-associated tumors. In contrast, only a few BRCA2-associated cancer displayed CK4/15 expression [42]. In oral carcinogenesis, CK14 expression was observed in hyperkeratosis with epithelial dysplasia, carcinoma *in situ* (CIS), and SCC [43]. However, it is not expressed in hyperkeratosis without epithelial dysplasia. Another study suggested that CK14 is expressed in cancers with squamous cell component [44]. Thus, CK14 expression may be a good indicator of SCC. In the setting of bladder cancer, CK14 expression in the urine may raise the suspicion of SCC which typically have poorer prognosis. Gaisa *et al.* (2011) reported consistent result as CK14 expression was most prominent in SCC of the bladder and decreased as squamous cell component decreased [45]. CK 14 can also be expressed in neuroendocrine tumor specimens along with CK1, CK5, and CK10 [46]. Although considerably rare, CK14 expression may give a cue to the presence of SmCC of the bladder as it is commonly mistaken for another type of bladder cancer. These findings may suggest that CK14 expression may be used to differ several types of cancers. Therefore, clinicians could predict survival rate and recommend appropriate treatment of choice for certain cancer patients.

CK 14 Role in Determining Bladder Cancer Grading

Rapid progressivity to undifferentiated histopathological structure and invasiveness are some traits of malignant tumor. It is essential to identify cancer aggressiveness to plan an appropriate individual treatment. As many as 10–20% of bladder cancer cases develop to invasive tumor. Muscle invasive bladder cancer owns a high propensity of distant metastasis and is therefore associated with higher mortality [47]. A number of studies analyzed CK14 expression in a certain grade of malignancies. Southern *et al.* (2001) reported that CK14 expression is prominent in low-grade squamous intraepithelial lesions of the cervix caused by human papilloma virus (HPV) infection, especially in basal and parabasal regions. Whereas, the expression reduced and became absent in intermediate- or high-grade lesions [48]. Epidemiological study revealed that approximately, 35% of bladder tumor cases were related to HPV infection [49]. In this case, safe sexual practice is a key to avoid bladder malignancy. The result is consistent with studies regarding oral and lung carcinogenesis in which CK14 expressions were decreased as the degree of dysplasia increased [50], [51], [52]. Moreover, Gruver *et al.* (2012) also reported that invasive urothelial carcinoma demonstrated low expression of CK14 [53].

Contrast to these findings, Nakayama *et al.* (2011) reported increased expression of CK14 as squamous intraepithelial neoplasia progressed with the greatest expression observed in CIS [54]. Similar results were identified in other studies about invasive salivary adenoid cystic carcinoma and breast cancer [55], [56]. Recent study also reported that urothelial carcinoma with higher CK14 expression was associated with very poor outcomes related to its invasive properties (Volkmer *et al.*, 2012; Reddy *et al.*, 2015; Jangir *et al.*, 2019) [28], [57], [58]. A number of literatures have mentioned CK14 expression in leading cells in the case of collective invasion (Cheung *et al.*, 2013; Cheung and Ewald, 2016; Wu *et al.*, 2019; Nagai *et al.*, 2021) [2], [55], [59], [60]. In general, increased CK14 expression is associated with higher degree of malignancy [39]. This phenomenon may be explained by possible function of CK14. Alam *et al.* (2011) reported that in CK14 knockdown cells, cell proliferation and cell cycle progression were suppressed. This was possible as CK14 was removed, there were a reduction in phosphatidylinositol 3-kinase/Akt (PI3K/Akt) signaling involved in cell proliferation and increased Notch-1 signaling involved in cell differentiation [61]. CK 14 expression is potentially able to stratify bladder cancer patients based on its grade of metaplasia and detect invasiveness of bladder cancer. However, further study is still needed.

CK 14 Role in Predicting Bladder Cancer Recurrence

Recurrence is a common notion in any type of malignancy. This phenomenon may occur especially when cancer cells are not fully eradicated during treatment. Bladder cancer itself has been known to recur in 50% to 70% of cases in 5 years of time. As discussed before, higher CK14 expression may suggest higher grading of bladder cancer. Greater CK14 expression also indicated greater recurrence rate of bladder cancer [57]. In addition, high CK20 and low CK5 expression were also proven to a marker of unfavorable recurrence free survival [62].

Conclusion

CK 14 is a member of acidic CK and is specific to squamous epithelial cell. Its ubiquitous expression in malignancy makes it a potential biomarker in the management of bladder cancer. CK 14 expression is able to predict bladder cancer, differentiate cancer origin, identify bladder cancer severity, and predict bladder cancer recurrence. However, further research is yet to be conducted to understand the full potential of CK14 as a biomarker of bladder cancer.

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