Toxicology and Applied Pharmacology xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/taap

# The expression of keratin 6 is regulated by the activation of the ERK1/2 pathway in arsenite transformed human urothelial cells

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ARTICLE INFO

*Article history:*
Received 9 February 2017
Revised 13 April 2017
Accepted 9 May 2017
Available online xxxx

*Keywords:*
Keratin 6
Arsenite
MAPK
Bladder cancer
UROtsa
Biomarker

ABSTRACT

Urothelial cancers have an environmental etiological component, and previous studies from our laboratory have shown that arsenite ($As^{+3}$) can cause the malignant transformation of the immortalized urothelial cells (UROtsa), leading to the expression of keratin 6 (KRT6). The expression of KRT6 in the parent UROtsa cells can be induced by the addition of epidermal growth factor (EGF). Tumors formed by these transformed cells have focal areas of squamous differentiation that express KRT6. The goal of this study was to investigate the mechanism involved in the upregulation of KRT6 in urothelial cancers and to validate that the $As^{+3}$-transformed UROtsa cells are a model of urothelial cancer. The results obtained showed that the parent and the $As^{+3}$-transformed UROtsa cells express EGFR which is phosphorylated with the addition of epidermal growth factor (EGF) resulting in an increased expression of KRT6. Inhibition of the extracellular-signal regulated kinases (ERK1/2) pathway by the addition of the mitogen-activated protein kinase kinase 1 (MEK1) and MEK2 kinase inhibitor U0126 resulted in a decrease in the phosphorylation of ERK1/2 and a reduced expression of KRT6. Immuno-histochemical analysis of the tumors generated by the $As^{+3}$-transformed isolates expressed EGFR and tumors formed by two of the transformed isolates expressed the phosphorylated form of EGFR. These results show that the expression of KRT6 is regulated at least in part by the ERK1/2 pathway and that the $As^{+3}$-transformed human urothelial cells have the potential to serve as a valid model to study urothelial carcinomas.



## 1. Introduction

Urothelial carcinoma is the fifth most commonly diagnosed tumor, and among the genitourinary tract malignancies, it is the second most common cause of death in patients from developed countries (Siegel et al., 2013). Urothelial carcinomas are routinely classified into two categories: non-muscle-invasive tumors and muscle-invasive tumors. The 5-year survival for patients with non-muscle-invasive urothelial carcinomas approximates 90%, whereas individuals with muscle-invasive tumors have 5-year survival frequencies of approximately 60% (Luke et al., 2010). Individuals with non-muscle-invasive disease appear to have a high rate of recurrence, and some with recurrence are found to have progressed to muscle-invasive disease (Zuiverloon et al., 2012). There are few effective chemotherapeutic treatment options for patients with muscle-invasive disease, illustrating the need for new prognostic markers to identify and further study those early lesions prone to progress to a more invasive disease status. One marker that appears to have prognostic utility for urothelial carcinomas is squamous differentiation of the urothelial cancer cells, but a major drawback is that its appearance appears very late in disease progression. While urothelial carcinomas that contain a prominent squamous component are rare, there is evidence that the presence of a squamous component indicates a poor prognosis for the patient. Focal squamous differentiation has been shown to be an unfavorable prognostic feature for patients undergoing radical cystectomy (Frazier et al., 1993) or radiation therapy (Martin et al., 1989; Akdas and Turkeri, 1990) and is associated with a poor response to systemic chemotherapy (Logothetis et al., 1989). Recently, molecular profiling has shown that muscle invasive bladder cancer can be grouped into basal and luminal subtypes similar to what has been seen in breast cancers (Choi et al., 2014). Basal muscle invasive bladder cancers are enriched with squamous features with elevated expression of epithelial keratins (KRT) such as KRT5, KRT6 and KRT14 in addition to other basal genes as seen in breast cancers (Perou et al., 2000).

This laboratory has previously published data indicating that the expression of KRT6 might be developed as a marker that can detect early

*Abbreviations:* AKT, AKT serine/threonine kinase; $As^{+3}$, arsenite; $Cd^{+2}$, cadmium; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ERK, extra-cellular signal regulated kinases; JNK, c-Jun N-terminal kinase; KRT, keratin; MEK, mitogen-activated protein kinase kinase 1; RIPA, radioimmunoprecipitation assay; TBS, Tris-buffered saline; TBS-T, Tris-buffered saline with Tween-20.

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http://dx.doi.org/10.1016/j.taap.2017.05.007
0041-008X/

squamous differentiation of urothelial carcinomas before it is readily visible by diagnostic microscopy. In these studies, it was shown using the immortalized, but not tumorigenic, human urothelial cell line, that malignant transformation of the cells by both arsenite ($As^{+3}$) and cadmium ($Cd^{+2}$) resulted in tumor transplants where expression of KRT6 was co-localized to areas of overt squamous differentiation (Rossi et al., 2001; Sens et al., 2004; Somji et al., 2008, 2011; Cao et al., 2010). In these studies, 6 independently isolated $As^{+3}$-transformed cells and 7 independently isolated $Cd^{+2}$-transformed cells were used, and all produced tumor transplants where KRT6 expression was co-localized to areas of overt squamous differentiation. It was also shown using a small subset of archival specimens of human urothelial carcinomas that KRT6 staining could identify small, focal areas of squamous differentiation in some urothelial carcinomas that might be missed on routine microscopic examination (Somji et al., 2008).

The goal of the present study was two-fold. One, to further validate that the $As^{+3}$-transformed UROtsa cells are a good model that retains important characteristic features of urothelial cancer. To achieve this goal, the expression of the epidermal growth factor receptor (EGFR) and the activation of the EGFR signaling pathway was determined in the parental as well as the $As^{+3}$-transformed UROtsa cells, and their tumor transplants. The rationale for this examination is that it is well documented that the EGFR pathway is frequently overexpressed in human urothelial cancer and overexpression correlates with higher tumor grade/stage and poorer prognosis (Neal et al., 1990; Miyamoto et al., 2000; Izumi et al., 2012; Mooso et al., 2015). The demonstration of expression of the EGFR pathway in the $As^{+3}$-transformed cells would also reinforce the known role of arsenic exposure in the etiology of urothelial cancer. The second goal was to determine the mechanism leading to the increased expression of KTR6 in $As^{+3}$-transformed UROtsa cells and their derived tumor transplants. Specifically, to determine if expression of keratin 6 expression is influenced by the EGFR pathway and if this might further define its potential for development as an early biomarker of adverse prognosis.

## 2. Materials and methods

### 2.1. Animals

Mouse heterotransplants of the UROtsa transformed cell cultures were produced by subcutaneous injection at a dose of $1 \times 10^6$ cells in the dorsal thoracic midline of athymic nude (NCR-*nu*/*nu*) mice. Tumor formation and growth were assessed externally with a ruler on a weekly basis and the maximal tumor size was 2.5 cm. All mice were sacrificed by 10 weeks after injection or when clinical conditions dictated euthanasia (excessive weight loss, lethargy, self-mutilation or mutilation by cage mates). This study adhered to all recommendation dictated in the Guide for the Care and Use of Laboratory Animals of the NIH. The specific protocol was approved by the University of North Dakota Animal Care Committee (IACUC#1110-2C). All efforts were taken in order to minimize animal suffering, and mice were euthanized when clinical condition dictated. Animals were sacrificed by 10 weeks post tumor transplantation by $CO_2$ inhalation and euthanasia conformed to American Veterinary Medical Association Guideline on Euthanasia.

### 2.2. Cell culture

The UROtsa parent cells and the six independent $As^{+3}$-transformed isolates were cultured in 75 $cm^2$ tissue culture flasks in Dulbeco's modified Eagle's medium (DMEM) supplemented with 5% vol/vol fetal bovine serum as described previously (Rossi et al., 2001; Sens et al., 2004). Since the $As^{+3}$-transformed cells have not been cloned, they are being referred to as isolates in the study. The cells were subcultured at a 1:4 ratio using trypsin-EDTA and the cultures were fed fresh growth medium every three days. To determine the effects of epidermal growth factor (EGF), the cells were grown to confluency in 25 $cm^2$ flasks as described above. At confluence, EGF (10 ng/ml) was added to the cells for various time periods and the cells were harvested.

### 2.3. Real-time analysis of KRT 6A mRNA expression

Keratin 6a mRNA expression was assessed with real-time reverse transcription polymerase chain reaction (RT-PCR) using primers that were developed using Oligo 6.0 software. The sequences of the upper and lower primer of KRT6A along with the product sizes are as follows: sense: CTAAAGTGCGTCTGCTA antisense: TGGGTGCTCAGATGGTATA (product size, 184 bp). Total RNA was purified from the cells lines and 0.1 μg was subjected to cDNA synthesis using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA) in a total volume of 20 μl. Real-time RT-PCR was performed using the SYBR Green kit (Bio-Rad Laboratories) with 2 μl cDNA and 0.2 μM primers in a total volume of 20 μl in an iCycler iQ real-time detection system (Bio-Rad Laboratories). Amplification was monitored by SYBR Green fluorescence. Cycling parameters consisted of denaturation at 95 °C for 15 s, annealing at 55 °C for 45 s, and extension at 72 °C, which gave optimal amplification efficiency. The levels of keratin 6a were determined by serial standards. The resulting levels were normalized to the change in β-actin expression assessed by the same assay using the primers, sense: CGACAACGGCTCCGGCATGT and antisense: TGCCGTGCTCGATGGGGTACT, giving a product size of 194 base pairs and with the cycling parameters of annealing/extension at 62 °C for 45 s and denaturation at 95 °C for 15 s.

### 2.4. Western blot analysis

Cells were rinsed twice with cold phosphate-buffered saline and were incubated with 1× Radio-immunoassay Precipitation Assay (RIPA) lysis buffer supplemented with PMSF, protease inhibitor cocktail and sodium orthovandate (Santa Cruz Biotechnology, Dallas, TX) for 5 min on ice. Following incubation, the cells were scrapped and transferred to a conical tube. The cell suspension was sonicated and the lysate was centrifuged to remove cellular debris. The protein concentration was determined using the bicinchoninic protein assay (Pierce Chemical Co., Rockford, IL). Total cellular protein (20 μg) was separated on a 12.5% SDS-polyacrylamide electrophoresis gel and transferred to a hybond-P polyvinylidene difluoride membrane (Amersham Biosciences, Piscataway, NJ). Membranes were blocked in Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBS-T) and 5% (wt/vol) nonfat dry milk for 1 h at room temperature. After blocking, the membranes were probed overnight with the primary antibody diluted in buffer containing 5% bovine serum albumin. The primary antibodies against KRT6 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) whereas the antibody against β-actin was purchased from Abcam Inc. (Cambridge, MA). The KRT6 antibody does not distinguish between the isoforms and recognizes proteins made by the KRT6A, KRT6B and KRT6C genes. Hence, the protein is referred to as KRT6 in the manuscript. The antibodies against EGFR, p-EGFR, Extracellular Signal-Regulated Kinases 1 and 2 (ERK1/2), p-ERK1/2, c-Jun N-terminal kinase (JNK), p-JNK, AKT serine/theonine kinase (AKT) and p-AKT were purchased from Cell Signaling Technology (Beverly, MA). After incubation with the primary antibody, the blots were washed 3 times with TBS-T, and the membranes were incubated with the anti-mouse or anti-rabbit secondary antibody (1:2000) for 1 h. The blots were visualized using the Phototope-HRP Western blot detection system (Cell Signaling Technology).

### 2.5. Determination of MAPK activation by EGF in UROtsa parent and transformed cells

UROtsa parent and transformed cells were grown to confluence in serum containing medium, following which the cells were incubated with DMEM without serum for 24 h. The cells were then exposed to

Please cite this article as: Slusser-Nore, A., et al., The expression of keratin 6 is regulated by the activation of the ERK1/2 pathway in arsenite transformed human urothelial cells, Toxicol. Appl. Pharmacol. (2017), http://dx.doi.org/10.1016/j.taap.2017.05.007