Mesenchymal and stemness transdifferentiation via in-vitro infection of T24 cell line with Klebsiella pneumonia

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Abstract
Klebsiella pneumoniae has been found in the urinary tract of some bladder cancer patients. Bacterial presence within tumor tissue may affect the tumor-microenvironment and consequently influence cancer behavior, development, and treatment response. This study investigated mesenchymal and stemness transdifferentiation of bladder cancer cell line due to environmental stress of K. pneumoniae. Cultures of urothelial bladder cancer cell line (T24) were infected with K. pneumoniae with different multiplicity of infection (MOI) for two and four days. Transdifferentiation-associated features were morphologically assessed. Moreover, transdifferentiation markers were estimated using Q-PCR and immunohistochemistry. Q-PCR data showed an increase in mesenchymal transdifferentiation traits; vimentin expression was upregulated, and cytokeratin19 expression downregulated significantly (P<0.001) compared with controls, which were emphasized by immunohistochemistry results. Moreover, stemness transdifferentiation markers expression increased significantly (P<0.001). The heterogeneous tumor cell population may be altered by bacterial infection, which improves cancer cells' migration and self-renewal ability. Thus, bacteria may be engaged in cancer progression and metastases.

Keywords: Bacterial infection, Bladder cancer, Epithelial-mesenchymal transition, K. pneumonia, Stemness transdifferentiation.

Introduction
Transdifferentiation is an epigenetic process by which a given cell type acquires phenotypic traits of another cell type in place of its own. At the molecular level, it causes a change in the expression level of master genes that distinguish the two cell types during normal development1. It occurs naturally during tissue regeneration, while recently, the transdifferentiation ability of malignant tumor cells has been reported2, 3. Recent studies have pointed out the role of tumor cells transdifferentiation in mediating drug resistance and tumor progression4. For instance, tumor epithelial cells undergo mesenchymal transdifferentiation or epithelial-mesenchymal transition (EMT)5, which provides cancer cells with the plasticity and migration ability required for dissemination, invasion, and metastasis. During EMT, epithelial cells reduce certain epithelial molecules' expression while increasing the expression of mesenchymal cell ones6. Moreover, stemness transdifferentiation empowers tumor plasticity and differentiation ability into different tumor cell types1. Stemness transdifferentiation gives rise to cancer stem cells (CSCs), a small tumor subpopulation. Cells with CSCs traits are implicated in dormancy and drug resistance of tumor cells7. Bladder cancer (BC) is a common type of cancer8. Primary bladder tumor can be successfully controlled, while after being developed and metastasized to a distant organ, it would be too hard to eradicate the disease8. The dominant urinary
tract infection UTI bacteria are E. coli and K. Pneumoniae\textsuperscript{10, 11}. Several reports revealed that E. coli, followed by K. Pneumoniae, are the most common uropathogenic bacteria, which infect bladder cancer patients\textsuperscript{10, 12-14}. Tumor leads to poor immunity, so bacteria find their way to inhibit tumor tissue\textsuperscript{15}. That raises the importance of studying the relationship between tumors and bacterial refugees. Interestingly, this relationship is found to be complicated, and it affects -in different manners- tumor development. In some cases, bacteria may drain the required nutrients for tumor cells metabolism resulting in an anti-tumor effect. For instance, Salmonella spp. can pervade tumor and may retard neoplasm growth or completely clear tumor\textsuperscript{16}. On the other hand, bacteria may play as cancer allies, either during carcinogenesis or during cancer progression and development \textsuperscript{17, 18}. For instance, bladder cancer risk increases in case of a previous bladder infection. Moreover, cancer metastasis to the lung increases by acute bacterial infection, and H. pylori has been involved in the development of gastric cancer\textsuperscript{16}. This study aims to estimate the potential of bacterial infection in mesenchymal and stemness transdifferentiation induction. That was achieved by \textit{in vitro} investigation of K. pneumoniae impact on EMT and stemness-related markers.

**Materials and methods:**

**Bacterial identification**

\textit{K. pneumoniae} has been isolated from urine samples of bladder tumor patients. Bacteria were sub-cultured in C.L.E.D agar media (Oxoid, England). After that, the isolated bacteria were identified biochemically using VITEK 2 (Biomérieux), a fully automated system that performs bacterial identification and antibiotic susceptibility testing.

**In-vitro infection**

The cell line T24 are epithelial cells that originate from urothelial cell carcinoma. In vitro infection, T24 cell line was performed by previous work\textsuperscript{15}. The T24 cells were inoculated with the isolated \textit{K. pneumoniae} bacteria at different MOI. Then, cells were washed after inoculation for two hours and incubated for two and four days. T24 cells without inoculation with any bacteria were cultured in the same conditions as a control.

**Detection of morphological changes**

Morphological changes have been detected before and after four days of infection (at MOI 20:1) with Olympus inverted microscope to estimate the EMT process.

**Gene expression analysis**

Gene expression analysis was performed for epithelial and mesenchymal markers CK19 and vimentin, respectively. RNAeasy Plus Mini Kit (Qiagen GmbH, Hilden, Germany) was used for total RNA extraction. High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, USA) was used for cDNA synthesis. Designing oligonucleotide primers for genes was performed by a previous work\textsuperscript{15}. PCR thermal cycler (CFX96 Real-Time System, Bio-Rad, USA) was used for template amplification. Samples normalization was done using Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and the gene expression fold changes were calculated relative to the control.

**Immunohistochemistry**

Immunohistochemistry assay was performed to detect changes in EMT markers due to bacterial infection. After cultured cells trypsinization, cells were fixed and permeabilized, followed by blocking non-specific binding using bovine serum albumin. Then, the cells were incubated overnight with an anti-CK19 monoclonal antibody (1:100, Genemed, USA) and a mouse anti-human vimentin monoclonal antibody (1:500, Sigma Aldrich, USA). The percentage of positively stained cells was estimated using the light microscope (CX31RTSF; Olympus, Tokyo, Japan). CK19 and vimentin-positive cells are calculated by counting positively and negatively stained cells in five different microscopic fields. These fields were chosen randomly from each sample of three separated experiments.

**Statistical analysis**

Statistical Package for Social Sciences (SPSS) 26 (Chicago, IL, USA) was used for statistical analysis. Mean ± SEM was used to express values. Kolmogorov-Smirnov test (K-S test) was done to test data distribution. An independent sample T-test was done to estimate the significance of changes resulting from infection regardless of both duration of infection (DOI) and MOI. One-way ANOVA was done to assess significance among variables concerning MOI and DOI. Moreover, post-hoc Dunnett's test compared experimental data sets with control. Pearson test (r) was performed to test the correlation between gene expressions. Finally, if P values were less than 0.01, the statistical significances were considered.

**Results**

Excellent identification of \textit{K. pneumonia} isolates was performed using VITEK 2 (Biomérieux) with VITEK bionumber...
Mesenchymal transdifferentiation assessment

Microscopic examination revealed that T24 cells infected with *K. pneumonia* possess an elongated shape and lack intercellular contacts. On the other hand, a clear cell-cell adhesion and more epithelial polygonal morphology have been detected for the control T24 cells. This newly rearranged cell shape refers to EMT and mesenchymal transdifferentiation of T24 cells (Fig. 1 a, b).

The gene expression profile of the infected T24 cells was representative of mesenchymal transdifferentiation. The control T24 cells showed the highest CK19 expression level and the lowest vimentin expression level (Fig.1 c). Due to infection, the CK19 gene mean transcription level decreased to (0.5 ± 0.03-fold), while vimentin elevated to (6.5 ± 1.1 folds). Comparing the mean expression of the two genes between infected T24 cells and control revealed a significant difference at P-value ≤ 0.001. Changes in both genes due to bacteria showed a significant correlation (r: -0.89, f: P<0.001).

The CK19 highest transcription level (0.8-fold relative to control) because of infection was found at low MOIs on the second day. Then, on the fourth day, the transcription level declined to the lowest level at higher MOI (20:1) and became less than 0.5-fold. Concerning MOI and DOI, the CK19 transcription level revealed a significant difference at a p-value ≤ 0.001. While at the second day after infection at low MOIs, the lowest level of vimentin transcription was detected, reducing it to two-folds on the fourth day. Concerning DOI and MOI, the transcription level of vimentin revealed significant differences at P-value ≤ 0.01 and P-value ≤ 0.001, respectively, as shown in Fig.1 e.

Figure 1. Impact of *K. pneumoniae* infection on mesenchymal transdifferentiation at morphological and gene expression levels.

a. shows microscopic images for control T24 cells.
b. shows microscopic images for infected T24 cells on the fourth day. The arrows refer to the EMT morphological changes after bacterial stimulation.
c. shows the transcription level of CK19 and vimentin genes before and after the infection. ** independent t-test was (p ≤ 0.001).
d, e. show the transcription level CK19 and vimentin genes in infected T24 cells at different MOIs and DOIs, compared to control. #, ## refers to the P values resulting from one-way ANOVA (p≤0.01) or (p≤0.001) concerning DOIs and MOIs, respectively. * **means that the P values resulting from Post Hoc Dunnett t (2-sided) were (p ≤ 0.01) or (p ≤ 0.001), for each MOI and DOI.
compared with control. 2nd n = 12, 4th-day n = 12, each MOI n = 6 and control n = 6. c.

IHC results showed that the mean percentage of T24 cells positively stained with CK19 decreased from 82% to 64% (Fig. 2. a, b). In addition, the mean rate of T24 cells positively stained with vimentin increased from 48% to 70% (Fig. 2. c, d).

Figure 2. IHC images show the impact of K. pneumoniae infection on mesenchymal transdifferentiation related proteins.

a, b: illustrates the IHC microscopic images of T24 cells positively stained with CK19 at zero and 4th day of infection.

c, d: illustrated the IHC microscopic images of T24 cells positively stained with vimentin at zero and 4th day of infection.

Stemness transdifferentiation estimation

On the other side, stemness transdifferentiation was evaluated by measuring CD44, SOX2, NANOG, and OCT4 transcription levels. Overall, the highest mean transcription levels of CD44 (3 ± 0.2 folds), SOX2 (2.6 ± 0.2 folds), NANOG (12.3 ± 1.6 folds), and OCT4 (2.6 ± 0.2 folds) relative to control have been recorded for infected T24 cells (Fig. 3.a). Comparing the mean transcription level for the previous four genes between infected and non-infected T24 cells revealed significant differences at P-value ≤ 0.001.

After two days of infection, stemness markers slightly upregulated up to 2-folds relative to control, in the case of CD44, SOX2, and OCT4 (Fig. 3.b, c &d). While in the fourth day of infection at higher MOIs, the expression elevated approximately to 4-folds. Comparing the relative transcription level of the four genes with control concerning DOI and MOI resulted in significant differences at P-value ≤ 0.001. NANOG followed the same expression pattern, with a slight difference as after two days of infection, it upregulated gradually up to 10-folds (Fig. 3. e). While after four days of infection and at higher MOI, it increased up to 25-folds. Concerning DOI and MOI, NANOG transcription levels showed significant differences (P-value ≤ 0.01 and P-value ≤ 0.001), respectively.
Figure 3. The impact of K. pneumoniae infection on stemness transdifferentiation at the gene expression level.

(a) illustrates the transcription levels of CD44, SOX2, NANOG, and OCT4 in infected T24 cells and control. **Refers to the obtained significance at (p ≤ 0.001) by independent t-test. Infected T24 n = 24, control T24 n = 6.

(b, c, d and e) show the T24 cells transcription level of CD44, SOX2, NANOG, and OCT4 at different DOIs and MOIs, compared to control. # and ## Refer to the obtained significant differences in genes transcription level one-way ANOVA at (p ≤ 0.01) and (p ≤ 0.001) concerning the infection DOI and MOI, respectively. * and ** Refer to that the P values resulting from Post Hoc Dunnett t (2-sided) were (p ≤ 0.01) or (p ≤ 0.001), for each MOI and DOI, compared with control. 2nd n = 12, 4th day n = 12, each MOI n = 6, and control n = 6.

Discussion

Transdifferentiation means losing the original phenotype of a fully differentiated cell due to specific factors, and the cell starts to acquire a different cell phenotype1. Cancer cells possess this ability by which they acquire new traits that enforce their ability to replicate, migrate, and metastasize. The tumor environment plays a crucial role in regulating cancer cell transdifferentiation. Recently, bacteria have been recognized as tumor inhabitants, and their presence within the tumor influences the tumor environment and may affect tumor behavior. Thus, understanding this effect may help better understand cancer progression and help therapy development. K. pneumoniae is a Gram-negative bacterium causing several infections due to its ability to colonize different tissues, including the urinary tract, lungs, and skin wounds19. Moreover, a previous study pointed out the presence of K. pneumoniae in the urine of bladder cancer patients12. This study investigated the ability of K. pneumoniae infection to stimulate bladder cancer cellular transdifferentiation

In the current study, it was first investigated K. pneumonia ability to trigger mesenchymal transdifferentiation. The main characteristic of mesenchymal transdifferentiation is increased mesenchymal genes in advance of epithelial genes. The transcription level of CK19 was significantly downregulated due to K. pneumoniae infection. CK19 is one of the cytokeratin subtypes, and cytokeratins are the main structural protein forming the cytoskeleton of epithelial cells20. In fact, it has been recognized as a transitional bladder carcinoma diagnostic marker21. Moreover, it was revealed that the knockdown of CK19 enhances cancer traits like increased cell ability to proliferate and migrate and develop drug resistance. At the same time, its overexpression led to significant attenuation of cancer properties21. On the other side, this study reveals that the vimentin
level was significantly upregulated due to *K. pneumoniae* infection. Vimentin is the primary mesenchymal cell cytoskeletal component, and it maintains its integrity\textsuperscript{22}. Therefore, vimentin is often used as a marker of mesenchymal-derived cancers. However, it can also be expressed in epithelial cells undergoing mesenchymal transdifferentiation during normal or metastatic progression\textsuperscript{22}. It has a crucial role in changing morphology, motility, and adhesion that occurs during the EMT\textsuperscript{19}. It was previously reported that the silencing of the vimentin gene enhances mesenchymal cells to adopt epithelial shapes\textsuperscript{23}. The current study suggests that bacteria trigger the mesenchymal transdifferentiation process through epithelial marker downregulation and mesenchymal marker upregulation. Downregulation of epithelial markers has been mentioned previously compared with those without infection\textsuperscript{24}. This result agrees with a previous study that reported the ability of *K. pneumoniae* to increase mesenchymal traits in airway epithelial cells\textsuperscript{19}. Moreover, S. Song et al.\textsuperscript{16} illustrated mesenchymal transdifferentiation induced by periodontal pathogens through different pathways differing according to bacterial species in oral squamous carcinoma. Besides that, microbial-induced persistent chronic inflammation leads to the induction of mesenchymal transdifferentiation in the lungs and intestine\textsuperscript{23}. This may be attributed to bacterial modulation of transforming growth factor\textbeta{} (TGF\textbeta{}), which stimulates signaling pathways targeting EMT transcription factors downstream\textsuperscript{6,24}. Stemness transdifferentiation engages a molecular network related to tumor progression\textsuperscript{25}.\textsuperscript{26}. This study pointed out that *k. pneumoniae* infection increased CD44 transcription levels. CD44 is a transmembrane glycoprotein that exists on embryonic stem cells and other cells\textsuperscript{27}. The main ligand for CD44 is hyaluronic acid (HA), expressed by stromal and cancer cells. The results of this study agree with a previous study that reported the role of CD44 in reducing inflammation and increasing bacterial dissemination\textsuperscript{28}. Another study pointed out that the expression of CD44 by urotheelial cells facilitates urinary tract bacterial infection and invasion\textsuperscript{29}. Moreover, CD44 may be regarded as a binding site for bacteria. It could bind to HA adherent to CD44 on urotheelial cells, which will help bacteria migrate through epithelial cells. In addition, Van der Windt et al.\textsuperscript{30} pointed out that CD44 absence affects the host's response and reduces *K. pneumonia* dissemination. Furthermore, CD44 is considered a marker and critical regulator of stem cell and CSCs pluripotency, as it helps in self-renewal, tumor initiation, and metastasis\textsuperscript{30}. Upregulation of transcription levels of stemness markers SOX2, NANOG, and OCT4 was reported in this study, which also confirmed stemness transdifferentiation due to bacterial stimulation. These three markers play an important role in regulating self-renewal and maintaining pluripotency of stem cells. Moreover, their expression is very important for cancer pathogenesis\textsuperscript{31}, as they control cancer cells' stemness transdifferentiation. Previous research reported that SOX2 silencing decreases the proliferative, migrative, invasive, and tumorigenic potential of cancer cells\textsuperscript{31}. Moreover, upregulation of NANOG was associated with tumor metastasis and poor prognosis in various human malignancies. In bladder cancer, it was reported that increased expression of NANOG was associated with an increase in pathological grade\textsuperscript{32}. Research has documented OCT4 detection in tumor cells and tissues, thus indicating its enrichment in a subpopulation of undifferentiated tumor-initiating cells\textsuperscript{33}. OCT4 upregulation is associated with tumorigenesis, tumor recurrence, and therapy resistance\textsuperscript{32}. Atlasi et al.\textsuperscript{33} reported that OCT4 was detected in most tissue bladder tumors in their study\textsuperscript{34}. Higher OCT4 expression in bladder cancer was related to the higher tumor grade, progression, and treatment modality\textsuperscript{35}. Therefore, SOX2, NANOG, and OCT4 make a strong transcription regulatory network, facilitating cell pluripotency and self-renewal. These three genes also act as activators of other genes involved in self-renewal and differentiation inhibition\textsuperscript{36}. Moreover, they have been overexpressed in aggressive cancers, including MIBC\textsuperscript{35}. To some extent, our finding agrees with a previous study on colon cancer as it reported stemness modulation due to intestinal bacteria\textsuperscript{37}. In addition, another study that reported the role of *Mycobacterium leprae* infection on cell differentiation program gradually shut down and reprogrammed adult Schwann cells to stem cell-like cells\textsuperscript{38}. Conclusion: *K. pneumoniae* infection to the bladder cancer cell line altered the heterogeneous cell population of the tumor. Cancer cells underwent mesenchymal transdifferentiation, which induces tumor cell migration and progression. Moreover, cancer stemness features increased because of bacterial infection, which influences the self-renewal ability of cancer cells. Thus, bacteria may be engaged in cancer progression and metastases.
Authors’ declaration:
- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are mine ours. Besides, the Figures and images, which are not mine ours, have been given the permission for republication attached with the manuscript.
- Ethical Clearance: The authors declare that sample collection was done according to ethical committee approval from Mansoura University (RP/41).

Authors Contribution:
R A ER., S O. and M M. Z conceived and designed the study. Materials preparation, data collection and analysis were performed by R AER, M M. Z. The first author (R A ER) wrote the initial draft of the manuscript and S O, K F. EY. and Maha G.
Haggag. commented on the initial version of the manuscript. Salama Ouf read and approved the final manuscript.

References:
التحول التماثلي للخلايا الظهارية للخلايا الجذعية البكتيرية مرتبط بخلايا سرطان المثانة بالعملية التكاثرية البكتيرية.

الخلاصة:
- البنى الدقيقة للخلايا التكاثرية البكتيرية من خلال التجربة الورمية يمكن أن يؤدي إلى تطور الخلايا السرطانية.
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الخلايا السرطائية: الخلايا السرطانية، الخلايا السرطانية، الخلايا السرطانية، الخلايا السرطانية، الخلايا السرطانية، الخلايا السرطانية، الخلايا السرطانية، الخلايا السرطانية، الخلايا السرطانية، الخلايا السرطانية، الخلايا السرطانية، الخلايا السرطانية.