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RESEARCH ARTICLE

EXOSOMAL CAVEOLIN-1 AS A BIOMARKER OF BLADDER CANCER IN EGYPT.

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Abstract

Back ground: Cystoscopic examination and histological evaluation of bladder tissues are considered the gold standard for initial diagnosis of bladder cancer. For decades, researchers explore novel, non-invasive, specific and sensitive biomarkers for preliminary diagnosis and surveillance of bladder carcinoma. Exosomes are nano-sized vesicles present in various biological fluids and encapsulate huge number of biomarkers such as proteins, mRNAs and miRNAs. Caveolin-1(Cav-1) is a major protein of caveolae structure that expressed in a variety of cells and has a fundamental regulatory role in cancer development. Therefore, determination ofexosomal Cav-1 expression in different stages of bladder cancer is a critical concern for clinical diagnosis and prognosis.

Methods: Exosomes Cav-1 was isolated and extracted from urine and serum samples of 79 patients of bladder cancer at different stages (T_0:T_3) and 12 healthy controls.Exosomal Cav-1 expression levels were determined by using Rt-qPCR technique.

Results: Exosomal Cav-1expression levels at different stagesof bladder cancer patients were significant in both urine and serum samples. The current study showed upregulation ofCav-1 amongserum (**4.03, 9.20, 14.25, 26.92folds)** and urine samples (**2.55, 3.64, 8.33, 19.37folds**) comparative to tumor invasiveness (T_0-T_3).Serum Cav-1 is more sensitive (96.2%) than that in urine (86.1%) although urine Cav-1 is more specific(94.3%).

Conclusion: Exosomal Cav-1 expression could be used as a crucialnon-invasive biomarker in diagnosis and prognosis of bladder cancer in serum and urine.

Introduction:-

Bladder cancerratio wasapproximately19% of total incidence of cancers in Egyptand it is most common in males and the second prevalent cancer type in females[1]. Cystoscopy and urine cytology combinationremains the gold standard in initial diagnosis of bladder cancer and follow-up [2].Most of the diagnosed cases are non-muscle invasive bladder cancer andabout 20% of those cases develop muscle-invasive during five years based

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onhistopathological conditions[3]. For that reason, early diagnosis of superficial stages of bladder cancer is extremely essential.

Exosomes are released from both normal and tumor cells, small size vesicles ranging from 40-100nm, and found in large quantities in blood and urine[4, 5]. Exosomes are complex moleculescontain proteins, lipids, RNA, and mRNA species and have characteristic cell surfacemarkers[6, 7]. Exosomes have many biological functionssuch as intracellular communication between living cells without direct contact[8], modulation of immune system response[9],induction of neoangiogenesis through transport of paracrine signaling factors and transportation of mRNA tosurrounding environment[10]. Exosomes have a major role in inducingepithelial to mesenchymal transition (EMT) initiatingmetastasis process because tumor cells lose their polarity and cell-cell adhesion increasing migration and invasion [11].Among exosomal proteins are cytoplasmic proteins (tubulin, actin,annexins, and Rab proteins), signal transduction proteins and heat-shock proteins (Hsp70 and Hsp90)[12]. Tetraspaninfamily (CD9, CD63, CD8, and CD82) isassociated with exosomes and commonly used as exosomal surfacemarkers [13].

Lipid raftsare specialized domains in cell membranes acting as physical platforms for different molecules to manage various signal transduction processes[14, 15].They are associated with development of severalmalignancies. Caveolins are considered as lipid rafts marker, small transmembrane proteins with intracellular domains that undergo extensive oligomerization to form membrane complexes known as caveolae[16].

Caveolae family is invaginated in plasma membrane and involved in transportation of vesicularmolecules to maintainlipid content of plasma membrane[17]. Caveolae proteins play a dynamic role in cell growth, cell protection, and cell function overall [18]. Signal disorder by caveolae proteins may induce pathological conditions as cancer, lipodystrophy, and cardiomyopathy[19].

Caveolin-1(CAV-1) is a key component in lipid rafts localized at plasma membrane has vital roles cell metabolism, cholesterol trafficking, lipid homeostasis, and vesicular transport[20] through direct interaction with cholesterol,therefore, regulates various receptors and signaling molecules within caveolae[21]. CAV-1 was over-expressed in different types of cancers such as liver, colon, breast, kidney, and lung[22]. CAV-1 has been implicated in pathogenesis of oncogenic cell transformation and metastasis[23],However, CAV-1 was downregulated in several sarcomas and adenocarcinoma. CAV-1hasa contrary role of being either a tumor suppressor or an oncogenic depending on cancer type (breast or prostate), tissue of concern (tumor or stroma), and stage of tumor[24], for example,CAV-1 expression in pancreatic cancer may be important for prognosis of the disease[25].

The present study aimed to investigate the expression of exosomal CAV-1 in urine and serum samples at different bladder cancer stages to determine the significance of CAV-1 as non-invasive biomarker in diagnosis and follow-up of bladder cancer disease.

Subjects and Methods:-

The present study was approved by the research ethics committee for experimental and clinical studies at the Faculty of Pharmacy, Future University, Cairo, Egypt (No. of protocol: REC-FPSPI-2/17).Samples were collected frombladder cancer patients who attended atcystoscopic unit in the National Cancer Institute (NCI), Cairo University, Egypt. All subjects signed an informed written consent for participation.Patients' demographic data and medical history were obtained from Information and Statistics Department of National Cancer Institute.Subjects included in the study were79 patients(males56, females23) their age ranged between 23 and 88 yearswith mean age 61.83 years and 15 healthy controls (males 10, females5) with no previous history of any urological disorders. Histopathology of patients was confirmed by pathology laboratory atNCI, Cairo, Egypt.

Tumor staging and grading were diagnosed according to tumor, necrosis, and metastasis (TNM)[26].All patients underwent cystoscopy as standard reference for identification of bladder cancer andclassifiedinto four groups according to their stages.Group 1 (T_0) 11patients, group 2 (T_1) 13 patients, group 3 (T_2) 32 patients and group 4 (T_3) 23 patients (table 1).

Table (1):- Demographic data of bladder cancer groups and control.

Category variable	Patients group	Control group
Mean age	61.38	54

(years)				
Gender	Male (%) 56 (70.8)	Female (%) 23 (29.1)	Male (%) 10 (66.7)	Female (%) 5 (33.3)
Smoking	Positive 35 (44.3%)	Negative 44 (55.7%)	Positive 4 (27%)	Negative 11 (73%)
Group size	T_0 11 (14%) T_1 13 (16%) T_2 32 (41%) T_3 23 (29%)		15	

Samples collection and preparation.
Fresh urine and blood samples were collected from each patient in sterile containersand vacutainer tubes respectivelyin the morning between 9:00 and 12:00 am.

Preparation of urine and serum samples
Collected urine and serum samples handling mentioned in a previous study[27].

Methods:-

Total exosomes isolation
Total exosomes isolation reagentsfor urine and serum (catalog no. 4484452 and 4478360 respectively ThermoFisher Scientific Company) wereusedaccording tothe manufacturer's instructions, the reagents were added to urine and serum and incubated for 1 hour at room temperature (RT) and for 30 minutes at 2–8°C. Precipitated exosomes are recovered by standard centrifugation at 10,000g for 1 hour and 10 minutes at2–8°Crespectively. Pellets yield were resuspended in phosphate-buffered saline (PBS) and kept at -80 ºC until RNA extraction.

Total exosomes RNA extraction
Total exosomal RNA was extracted from exosomes using RNA and protein isolation kit (catalog no. 4478545 ThermoFisher Scientific Company) for total exosomes RNA extractionaccording to the manufacturer's instructions.Housekeeping gene (Hs-GAPDH) provided by ThermoFisher Scientific Companywas assayed in each sample to normalize sample to sample variation. Purified RNA dissolved in 50 µl of RNase-free water and stored at -80 °C until analysis.

Reverse transcription.
Total RNA was reversing transcribed usinghigh-capacity complementary DNA (cDNA) kit (catalog no. 4374966 ThermoFisher Scientific Company)according to manufacturer's instructions,the reaction involves 2 µl reverse transcription buffer, 0.8 µl dNTP mix, 2µlRT random primers, 1µl MultiScribe™ reverse transcriptase, 1 µl RNase inhibitor and 13.2 µl RNA template. The produced cDNA stored at -20 °C until analysis.

Expression of exosomal CAV-1 using real-time qPCR technique.
4 µl of cDNA product used as a template in 20µl total reaction volume containing 10 µl of TaqMan® Universal Master Mixkit for RT-PCR analysis, 5µl of RNase free water, and 1µl of CAV-1 primer for amplification readily made by ThermoFisher Company. qPCRwas performed using Qiagen rotor gene Q6 plex real-time PCR open system (Qiagen, Germany). Initial activation at 95 °C for 15 min, followed by 40 cycles of 94°Cfor 15sec and 55 °C for 30 sec and 72 °C for 30 sec. Dataanalyzed with the automatic Ct setting for assigning baseline and threshold for Ct determination. Gene expressions levels were normalized to GAPDH and compared using the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis
The SPSS software system for Windows (version 20; SPSS,Chicago, IL, USA) was utilized for statistical analysis of CAV-1 fold expressions. Non-parametric data were used to measure the diagnostic accuracy of measured parameters. Data presented as mean value ± SEM. Differences between groups considered significant at $P < 0.001$

Results:-

Expression of exosomal CAV-1 in urine samples.

CAV-1 wasexpressed upward in all bladder cancer groups compared with control. CAV-1 levels were slightly increasing in superficial stages (T_0& T_1) while in invasive stages (T_2&T_3) were highly surging (table 2).

Table (2):- Relative expression of exosomal CAV-1in urine samples.

Bladder cancer groups	Sample size	Relative expression of CAV-1
Group 1(T_0)	11	2.55*±0.65
Group 2(T_1)	13	3.64*± 0.67
Group 3(T_2)	32	8.33*±0.85
Group 4(T_3)	23	19.37*±3.19
control	15	1± 0.008

Values were expressed as mean andstandard error mean. *P < 0.001compared with the control group.

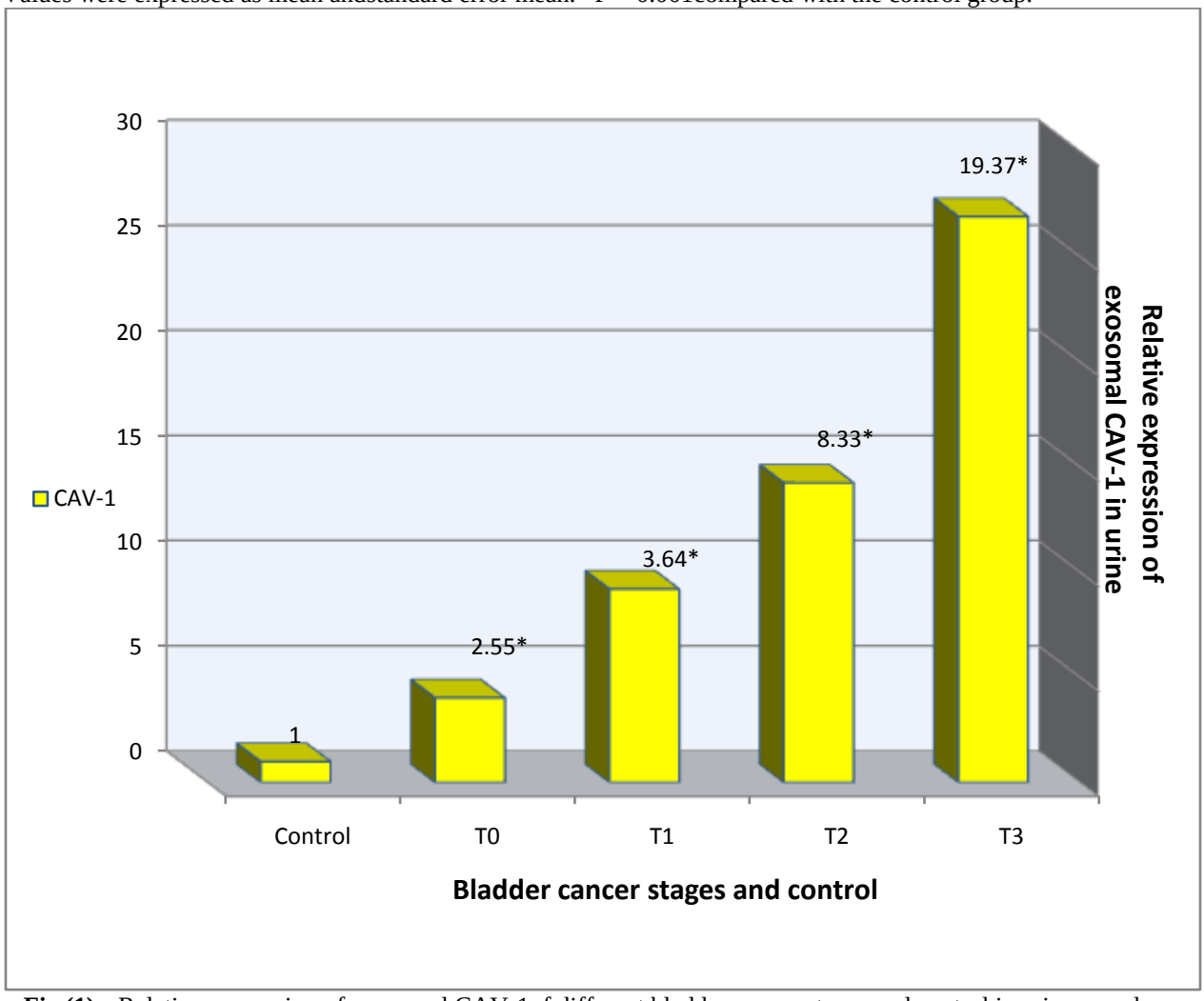

Fig (1):- Relative expression of exosomal CAV-1of different bladder cancer stages and control in urine samples. *P<0.001 compared with the control group.

Expression levels of exosomal CAV-1 in serum samples.

CAV-1 expression levelsin serumwere elevated in all stages of bladder cancer in comparison with control group. In advanced stages, CAV-1 expression levels were obviously elevated.

Table (3):- Expressionof exosomal CAV-1in serum samples.

Groups	Sample size	Relative expression of CAV-1
Group 1(T_0)	11	4.03* ± 0.81
Group 2(T_1)	13	9.20* ± 1.57
Group 3(T_2)	32	14.25* ± 0.93
Group 4(T_3)	23	26.92* ± 2.97
control	15	1 ± 0.009

Values were expressed as mean and standard error mean. *P< 0.001 compared with control group.

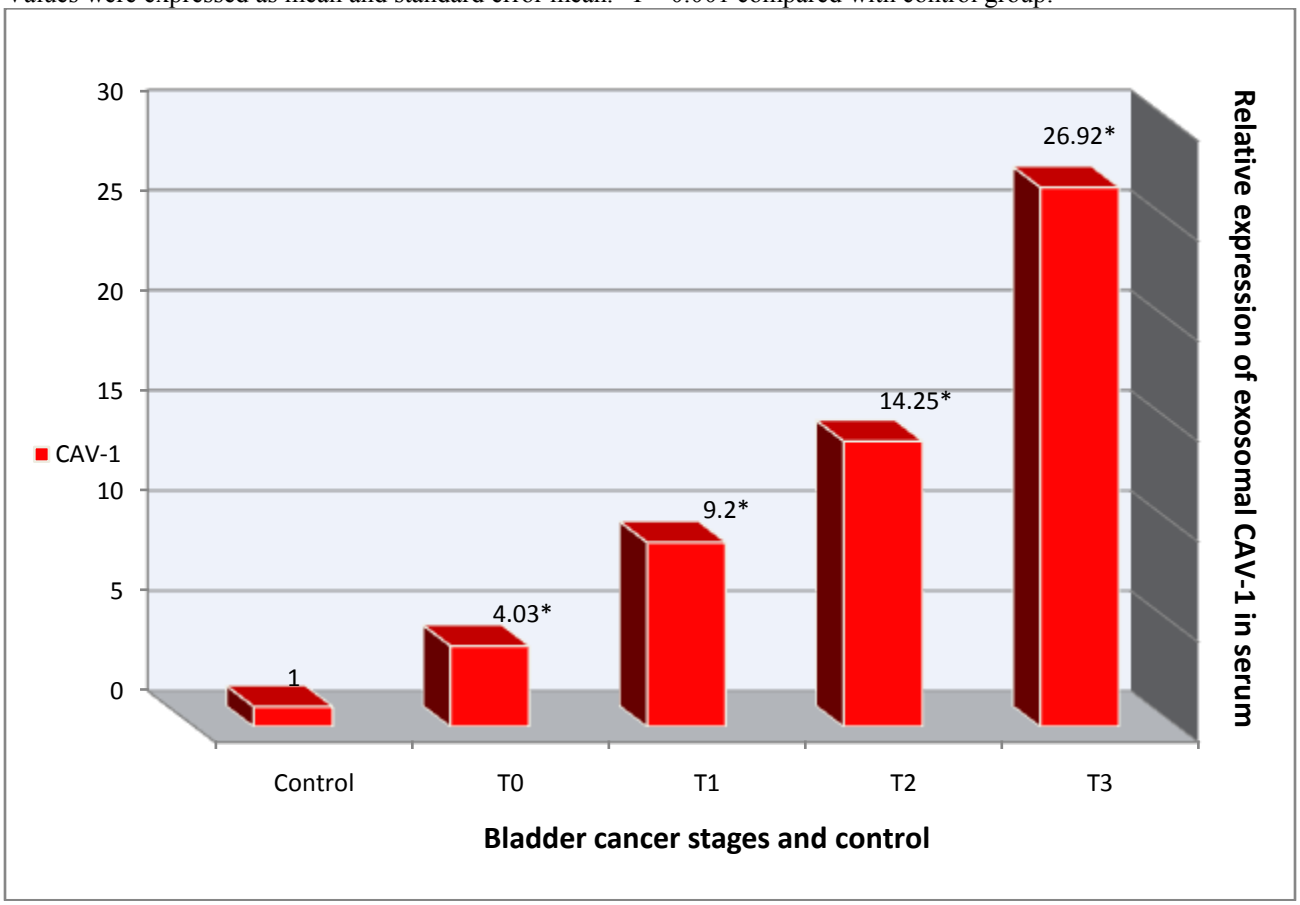

Fig (2):- Relative expression of exosomal CAV-1in serum samples. *P<0.001 compared withcontrol group.

Comparative expression of exosomal CAV-1in urine and serum.

In both biological samples either urine or serum, exosomal CAV-1 expressions were significant among bladder cancer groups. Meanwhile CAV-1 expression levels in advanced stages of bladder cancer were apparently significant. The expression levels in serum samples have a remarkable stepover in urine samples. It was found that serum has more sensitivity (96.2%) than urine (86.1%), while urine has more specificity (94.3%) and serum (93.3%).

Table (4):- Comparative expression of exosomal CAV-1in urine and serum.

Bladder cancer stages	Relativeexpression of CAV-1 in urine	Relative expression of CAV-1 in serum
T_0	2.55 ±0.65	4.03 ± 0.81
T_1	3.64 ± 0.67	9.20 ± 1.57
T_2	8.33 ±0.85	14.25 ± 0.93
T_3	19.37 ±3.19	26.92 ± 2.97

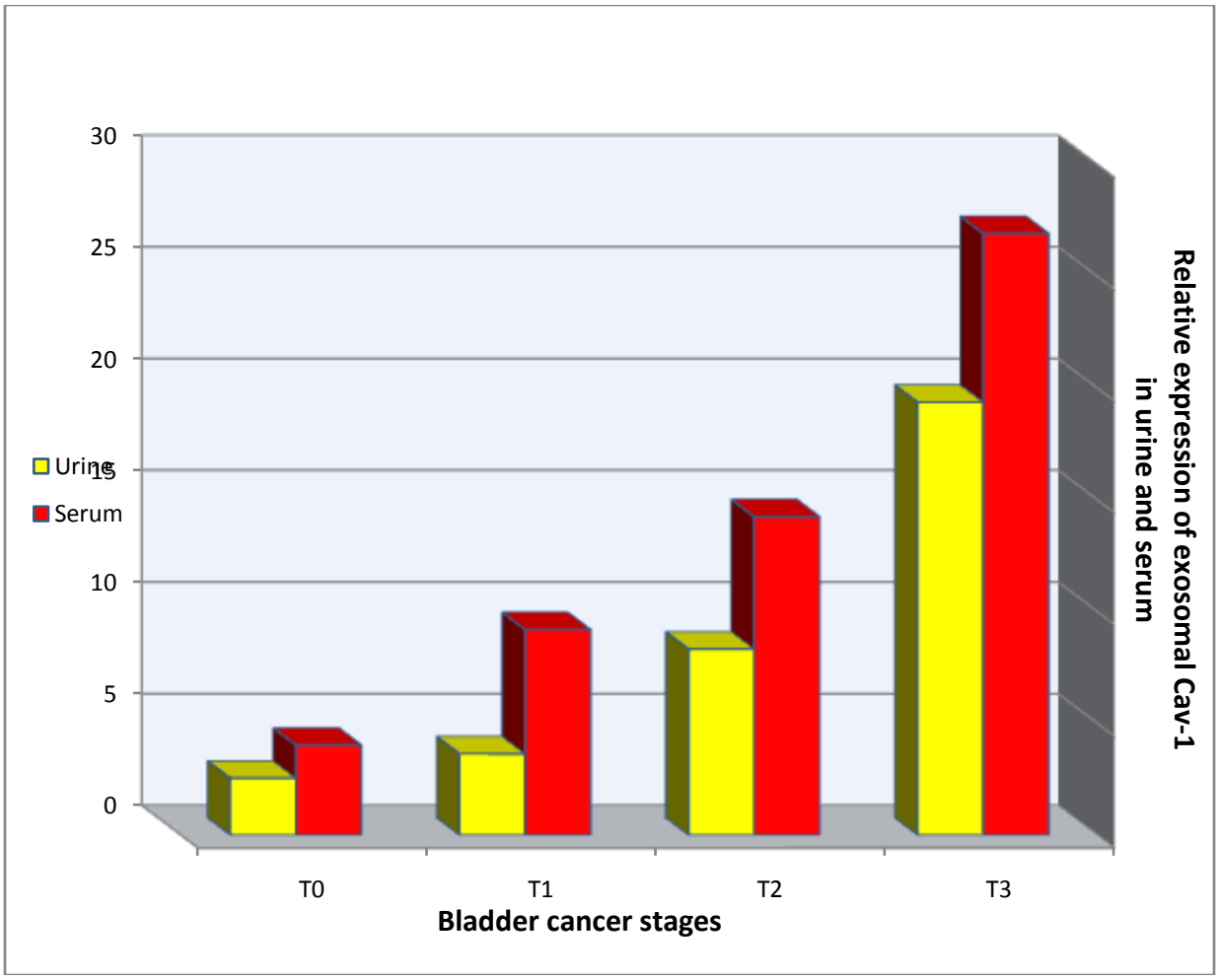

Fig (3):- Comparative expression of exosomal CAV-1inurine and serum.

Discussion:-

Bladder carcinoma is a common malignancy worldwide and is associated with high morbidity and mortality[28].Identification of genetic modifications or proteins associated with molecular events may lead to the discovery of asuccessful methodology for diagnosis, prognosis, and treatment[29]. The previous studies have reported multifunctional and controversial roles of CAV-1 in human cancer progression [30], however, some researchers had attributed variation in CAV-1 expression to stage and type of tumor[22],while others connected that to changes in energy balance which affects CAV-1 level towards or against cancer progression[31].

Application of exosomes in extraction and expression techniques provides significant merits owing to advanced molecular source, non-invasive procedure, and considerable accuracy.

Exosomes reserve extra-protection to their contents indicating that mRNA expressions reflect high accuracy even though circulating mRNA and miRNA are present in a pattern resistant to nucleases [32].

Skog et at., reported that RNA content (mRNA and miRNA) in exosomes has a specific packing technique as exosomes have numerous 100-fold RNA transcripts than original cell and for this reason, exosomes are valuable source of exclusive specific transcripts for malignant cells biomarkers that may be under detection limit in these cells. [33].

The current study has unique prospect for CAV-1 expression estimation because minimal RANases effect and prevention of other factors that might influence CAV-1 expression such as oxidative stress which enhances CAV-1 degradation as reported in a study by Mougeolle et al.[34].On the other hand, CAV-1 is considered one ofcellular

proteins that controlled by ubiquitination and proteasomal degradation pathway [35],therefore,the determination of exosomal CAV-1 expression serves as a potential to getaway of any possible factor that may disturb the expression assessment accuracy. In addition to that the current study was utilizing non-invasive biological samples (urine and serum)to collect and handle.

Exosomal CAV-1 upregulated expressions reported in the present study were remarkable in all bladder cancer stages particularly invasive ones. The levelsofserum exosomal CAV-1 were higher thanin urine amongbladder cancer groups.These results were attributed to presence of large amounts of Tamm-Horsfall Protein (THP) in urine which form polyhydric network that could trap a fraction of exosomes leading to their missing upon isolation[36],in addition to that exosomes derived from serum yield greater amount of RNA than urine according to a study byLi et al., proved that 4 ml serum yielded about 2–10 ng of RNA, however, 10 ml urine yielded approximately 2–4 ng RNA[37].

CAV-1 was found to be overexpressed in many types of cancer which indicatessignificant association between tumor progression and CAV-1 expression in prostate cancer [38], renal cell carcinoma[39],and pancreatic tumor [40]. In a study by Rajjayabunusing tissue samples reported a positive correlation between bladder cancer invasiveness and CAV-1 expression [41].

Principle role of CAV-1in cancer regulation through scaffolding domain interactions with certain proteins such as epidermal growth factor receptor (EGFR), tyrosine kinases and platelet-derived growth factor receptor (PDGFR), phospholipases and G protein-coupled, these interactions control signals sequences involved in cancer [42,43].

Several mechanisms were demonstrated to illustrate key roles of CAV-1 as oncogenic protein in tumor signals and angiogenesis,Liang et al., inspected relationship between CAV-1 and EMT in bladder cancer metastasis through activation of PI3K/AKT/Slug signaling pathway and promoting bladder cancer metastasis[44].Moreover, CAV-1 may initiate cancer progression through regulation of many signaling molecules such as PI3K/AKT, focal adhesion kinase, EGFR, and integrin[45], also may regulate multiple cancer processes such as cellular transformation, cell migration, angiogenesis, invasion, and metastasis[46].

However, in a previous study by Shi et al., It was demonstrated that CAV-1 downregulation supporting cell survival under stress conditions through modulation of autophagy and lysosomes function via lipid rafts disruption. This was considered a substitutive mechanism of CAV-1 and lipid raft inbreast cancer development[47]. Moreover, some studies reported that CAV-1 might act as tumor suppressor as Wiechen et al., utilized inhibitors of DNA methylation and histone deacetylation for suppression CAV-1 in ovarian cancer[48], also Bélanger et al., proved that CAV-1 was downregulated of in lung cancer[49], other study reported that matrix metalloproteinases-2 and 9 (MMP-2 &9) activity was inhibited byCAV-1 in animal model[50]. Furthermore, it was revealed that CAV-1 was downward inmetastatic breast tumor through prevention of Ca^{2+} activated potassium gate activation [51].

The current study illustrated the potential significance of exosomal CAV-1 expressions in urine and serum at different clinical stages of bladder cancer.

In conclusion, CAV-1 may be a useful biomarker fordiagnosis and prognosis of bladder carcinoma because CAV-1 is incorporated in numerous signaling pathways, a firm belief that CAV-1 may be a possible therapeutic target for treatment or preventing invasiveness of bladder cancer.Thestudy's authors recommend more research studies utilizing large number of patientsto validate the results and using blood serum for isolation of exosomes because it is easily reached, comes in contact with all body organs and has nearly stable volume. Although urine is the most popular sample for urinary system disorders and effortless to collect, but the main complexity associated with its fewerexosomes concentration.

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