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DETECTION OF CIRCULATING TUMOUR CELLS IN PROSTATIC CANCER PATIENTS USING POLYMERASE CHAIN REACTION

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Abstract: Cancer of the prostate is recognized as one of the principal medical problems facing the male population. Worldwide, prostate cancer is the second most common cancer in males after lung cancer. It accounts for 13% of all cancers in males worldwide. The incidence of prostate cancer in the Middle East, including Egypt, has been reported to be lower than the western world. However, in the last decade, increased diagnosis of prostate cancer is noted by urologists in their clinical practice. This might reflect increased awareness, improved diagnostic tools or increased incidence. Both genetic and environmental factors play a role in the evolution of prostate cancer. The development of metastasis is a complex, multi-staged process in which the hematogenous spreading of tumour cells is considered to be an intermediate and essential step for the spread of the disease beyond the prostate. Thus circulating tumour cells (CTCs), also known as the 'leukemic phase of solid tumours', constitute the hematogenous route of metastasis, and are of utmost clinical importance during the metastatic cascade for the establishment of distant metastasis. The aim of this study was to determine the presence of hematogenous neoplastic cells in patients with prostate cancer. Reverse transcription (RT) polymerase chain reaction (PCR) of prostate- specific antigen (PSA) mRNA was used to detect the presence of circulating tumour cells in clinic-pathologically proven prostatic cancer patients, 56% of them were proven to have locally malignant tumour and 44% were diagnosed with metastatic prostate cancer. 26.3 % of patients with locally malignant tumour were positive for CTCs and 80% of patients with metastatic disease were positive for CTCs. There was a statistically significant positive correlation between CTCs and t- PSA, Gleason score and imaging studies. We recommend screening for CTCs as a routine in all newly diagnosed non metastatic prostate cancer patients for the early detection of metastasis and improved preoperative staging modalities for patients with localized prostate cancer, a need for improved predictive markers to facilitate treatment selection and to monitor the effect of treatment.

Key words: prostate cancer, prostate specific antigen, PCR and circulating tumour cells.

INTRODUCTION

Cancer of the prostate is now recognized as one of the principal medical problems facing the male population. Worldwide, prostate cancer is the second most common cancer in males after lung cancer. The incidence of prostate cancer in the Middle East, including Egypt, has been reported to be lower than the western world. However, in the last decade, increased diagnosis of prostate cancer is noted by urologists in their clinical practice. In Egypt in the year 2008, approximately 1,661men were diagnosed with prostate cancer with a rate of 6.6 per 100,000 and 1,283 men were expected to die from this disease with a rate of 5.1 per 100,000 and this statistics proved comparable to the GLOBOCAN 2008 database.[1]

Both genetic and environmental factors play a role in the evolution of prostate cancer.[2] Although the specific causes of prostate cancer initiation and progression are not yet known, classical and molecular epidemiological studies have identified a number of potential risk factors associated with the development of prostate cancer which include: hereditary factors like linkage to hereditary prostate cancer gene 1 (HPC1)[3], polymorphism in vitamin D receptor gene[4], 5α-reductase type 2 gene[5], Type 2 3β-hydroxysteroid dehydrogenase gene[6] and androgen receptor gene[7]. Many genes have been implicated in the development of prostate cancer like Prostate Cancer gene 3(PCA3), α-methylacyl-CoA racemase (AMACR) and TMPRSS2: ERG fusion gene[8].

The main diagnostic tools used to look for evidence of prostate cancer include DRE, serum concentration of PSA and transrectal ultrasonography (TRUS).[9] The measurement of serum PSA is widely used for the screening and early detection of prostate cancer.[9,10]

The development of metastasis is a complex, multi-staged process in which the hematogenous spread of tumour cells is considered to be an intermediate and essential step for the spread of the disease beyond the prostate[11,12]. Thus circulating tumour cells (CTCs), also known as the 'leukemic phase of solid tumours, constitute the hematogenous route of metastasis, and are of utmost clinical importance during the metastatic

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cascade for the establishment of distant metastasis.[13,14] If these CTCs can be traced in the bloodstream of prostate cancer patients, they could potentially provide useful clinical information as regards evaluation of tumour progression, prediction of long-term prognosis, identification of patients who are likely to respond to therapies with curative intent, as well as assessment of possibility for future recurrence. The 'seed-and-soil hypothesis.[15]of the mechanism of bone metastasis proposes that during the metastatic process, specialized subpopulations of highly metastatic cells within the tumour detach from the primary tumour, migrate to the adjacent tissue, adhere to the wall of lymphatic and blood vessels, enter into vessels and disseminate through the systemic vasculature thus avoiding shear force and anchorage independence, ultimately arresting in specific tissues, extravasating and proliferating in a permissive organ.

The principal prostate specific markers used for PCR-based CTCs detection include Prostate-Specific Antigen which is a well-known marker used for reverse transcriptase polymerase chain reaction, due to its well-defined biologic properties and utility as a serum marker.[16] Expression of mRNA for PSA has been shown to be androgen-dependent and to be decreased in more poorly differentiated tumors.[17] This marker has been used primarily until now in successive clinical studies as a tissue-specific target for RT-PCR-based detection protocols and it is the marker used in the current study. Other markers include Prostate-Specific Membrane Antigen which is expressed in all cases of prostate adenocarcinoma, with the greatest extent and intensity observed in the highest grades,[18,19] Prostate Stem Cell Antigen (PSCA) and Cytokeratin 19 (CK19).

Patients and methods:

This study was conducted on 68 newly diagnosed untreated prostatic cancer patients admitted to the Genito-Urinary Surgery Department and the out-patient clinic at Alexandria Main University Hospital. Their ages ranged from 55 to 82 years. Thirty eight of these patients were clinic-pathologically diagnosed as locally malignant and thirty cases were diagnosed as having metastatic prostate cancer. Seventy control subjects were enrolled in this study; they were divided into thirty patients with benign prostatic hyperplasia, twenty patients with prostatitis and twenty healthy volunteers as normal control subjects (normal PSA level and DRE). Patients who had invasive prostatic procedures like needle biopsy, transurethral resection of the prostate (TURP) or open surgery within the last four weeks or those who had prostatic massage within the last 2 days prior to sampling or those who received radiation or hormonal therapy were excluded from the study. Before enrollment in the study all subjects signed an informed written consent and the approval of Medical Ethics Committee of Alexandria Faculty of Medicine was obtained. All patients were subjected to full history taking, complete clinical examination including meticulous rectal examination at least three days before sampling, laboratory investigations included complete blood picture including hemoglobin level, hematocrit, platelet count and total leucocytic count, all were measured by cell counter ADVIA2120 (Siemens Healthcare Diagnostics, USA), serum urea, serum creatinine, serum alanine aminotransferase, aspartate aminotransferase and alkaline phosphatase were measured by chemistry auto analyzer Dimension RXL(Siemens Healthcare Diagnostics, USA). Serum total Prostate specific antigen, free PSA level was measured for patients with total PSA level form 4 to 10ng/ml by automated electrochemilluminescence assay by Advia Centaur (Siemens Healthcare Diagnostics, USA). Imaging studies (computed tomography, transrectal ultrasound, radionucleotide bone scans) used to detect secondaries. Clinical and pathological staging was determined according to the TNM system. The molecular study entailed the detection of circulating tumour cells that express prostate specific antigen in peripheral blood using reverse transcriptase polymerase chain reaction technique [20].

Sampling: Five milliliters of whole blood were collected from each subject by venipuncture from the antecubital vein into three vaccutainer tubes. The first tube was a plain red topped tube for the analysis of the liver functions, kidney functions and PSA level. Blood was left to clot slowly and then the serum was used for the analysis. The two other tubes contained Ethylene Diamine Tetra Acetic acid (EDTA) as an anticoagulant; one was used for complete blood picture analysis and the other was used for the molecular detection of CTCs.

RNA extraction: Total RNA was immediately purified using QIAamp RNA Blood Mini Kit (Qiagen, Germany).

Reverse Transcription: The purified RNA was reverse transcribed to the complementary DNA (cDNA) using the kit provided by Applied Biosystems (AB) supplied by life technologies, California, USA). For each sample, the following quantities were added: 2µl RT buffer, 0.8 µl of dNTP's mix, 2µl of random hexamers, 1µl RT enzyme and 4.2µl nuclease free water. Then 10 µl of a mixture of the above reagents was mixed with 10 µl of purified RNA to give a total volume of 20 µl. PCR program was performed as follows: Incubation at 25°C for 10 minutes then the temperature was raised to 37°C for 120 minutes for the reverse transcriptase enzyme to act then, the temperature was raised to 85°C for 5 seconds to stop the action of the reverse transcriptase

enzyme.The cDNA products were stored at –20°C until the PCR analysis was performed.

Polymerase chain reaction (PCR): The set of primers used were of the following sequences: F 5´-CAC AGC CTG TTT CAT CCT GAA- 3´, R 5´-CCT TGA TCC ACT TCC GGT AAT-3´. These sequences span the exons 2-4 of the cDNA that codes for PSA. They were previously published by Sardi et al [20] and yielded a 532-bp PCR product. PCR-GOLD Master-Mix Beads ready to use were used with Catalogue number was 10020-96. (Bioron, Germany). Master-Mix Beads consist of: 1 U Taq DNA polymerase, 200µM dNTP (dATP, dCTP, dGTP, dTTP), 10Mm Tris-HCl (pH 9.0), 30mM KCl and 1.5 mM Mgcl2.To each PCR tube the following were added: 50 ng (6µl) of cDNA, 10 picomole (2µl) of forward primer, 10 picomole(2µl) of reverse primer, 10 µl nuclease free water to give a total volume of 20 µl. The lyophilized pellet was mixed by vortexing. The PCR amplification cycles were then carried out as follows: Initial denaturation at 95°C for 5 min then 25 cycles of denturation at 94°C for 1 min, anealing at 58°C for 1 min and extension at 72°C for 1.5 min then final extension at 72°C for 5 min. The PCR products were visualized using ethidium bromide-stained 2% agarose gel. A 100 bp DNA Ladder (Fermentas, Germany) was used for sizing of the double stranded DNA fragment on the agarose gel by running it on the gel alongside the PCR products. Five volumes of the amplicon were mixed with one volume of loading dye and the mixture was slowly loaded into the slots of the gel. The gel was electrophoresed for 15 to 20 minutes. DNA bands were visualized by ultraviolet Tran illumination and band analysis was done using Dolphin gel documentation system. (21)

Statistical analysis:

Data was analyzed using SPSS software package version 18.0 (SPSS, Chicago, IL, USA). Qualitative data was analyzed using Fisher's Exact and Monte Carlo which was applied to compare different groups and was expressed in frequency and percent. Quantitative data was either parametric or non-parametric. Parametric data for the normally distributed data was analyzed using student t-test to compare between two groups while F-test (ANOVA) was used to compare the three groups. Parametric quantitative data was expressed using mean and standard deviation. Non parametric data for the none normally distributed data was analyzed using Mann Whitney test for comparing two groups while for more than two groups Kruskal Wallis test was applied. Non parametric quantitative data was expressed using median and range. P value was assumed to be significant at 0.05. The performance of the test was assessed using Receiver Operating Characteristics or (ROC) analysis.

RESULTS

The studied groups were divided into the following five groups:

- **Group I:** Thirty patients with metastatic prostate cancer according to clinico-pathological staging.
- **Group II:** Thirty eight patients with non-metastatic prostate cancer according to clinico-pathological staging.
- **Group III:** Thirty patients with benign prostatic hyperplasia according to histo-pathological records.
- **Group IV:** Twenty patients with prostatitis.
- **Group V:** Twenty healthy volunteers as normal control subjects.

As shown in table (1) there was a statistically significant difference in the t-PSA value among the five studied groups (p <0.001), the highest value was shown in group I metastatic while the lowest value was shown in the control group V.

	Metastatic (n = 30)	Non-metastatic (n= 38)	BPH (n = 30)	Prostatitis (n = 20)	Controls (n= 20)	χ^2 (p)
t-PSA (ng/ml)						
N: < 4 ng/ml						
Range	18.0 – 915.0	4.9 – 52.0	4.4 – 25.0	4.2 – 15.0	0.10 – 1.20	51.250* (<0.001)
Median	150.0	15.2	6.5	6.15	0.85	
Z_1(p)		4.093* (<0.001)	4.480* (<0.001)	4.160* (<0.001)	4.162* (<0.001)	
Z_2(p)			2.411* (0.016)	3.213* (0.001)	4.360* (<0.001)	
Z_3(p)				1.137 (0.255)	4.162* (<0.001)	
Z_4(p)					3.782* (<0.001)	

As shown in table (2) that in the t-PSA range of (4-10 ng/ml), the median value of f-PSA was 0.5 for prostate cancer group and 2.35 for BPH group. The median value of f/t PSA was 0.1 for prostate cancer and 0.38 for BPH. There was a statistically significant difference in the f-PSA and f/t PSA between prostate cancer and BPH with p values of 0.007 and 0.005 respectively.

	Prostate cancer (n = 68)	BPH (n = 30)	Z (p)
t- PSA			
Range	4.90 – 9.50	4.40 – 9.20	0.354 (0.723)
Median	6.35	6.05	
f- PSA			
Range	0.49 – 1.50	1.32 – 4.0	2.690* (0.007)
Median	0.54	2.35	
f/t PSA			
Range	0.08 – 0.15	0.25 – 0.45	2.828* (0.005)
Median	0.10	0.38	

Table (3) showed that there was a statistically significant difference between the metastatic and the non-metastatic groups as regards the Gleason's score ($p < 0.001$).

	Metastatic (n = 30)	Non-metastatic (n= 38)	t (p)
Gleason's score			
Range	7.0 – 10.0	5.0 – 8.0	5.236*
Mean ± SD	8.20 ± 1.01	6.37 ± 1.01	(<0.001)

Table (4) showed the distribution of the different group as regards the clinicopathological staging; as for the metastatic group, 66.7% were of stage T4, 26.7% were of stage T3b, and 6.7% were of stage T3a. As for the non-metastatic group, 42.2% were of stage T3a, 26.3% were of stage T3b, 10.5% were of stages T2a, T2b and T2cThere was a statistically significant difference between the metastatic and the non-metastatic groups in the clinical staging ($p< 0.001$).

Stage	Metastatic (N 30)		Non-metastatic (N 38)		FEp
	No.	%	No.	%	
T4	20	66.7	0	0.0	<0.001*
T3b	8	26.7	10	26.3	1.000
T3a	2	6.6	16	42.2	0.047*
T2c	0	0.0	4	10.5	0.492
T2b	0	0.0	4	10.5	0.492
T2a	0	0.0	4	10.5	0.492
MCp	<0.001*				

Table (5), showed that the CTCs were present in 80% (24/30) of the metastatic group and in 26.3% (10/38) of the non-metastatic group. In the BPH, prostatitis and control groups, CTCs were absent. There was a statistically significant difference in the CTCs between the metastatic and the non-metastatic groups with a p value of 0.005.

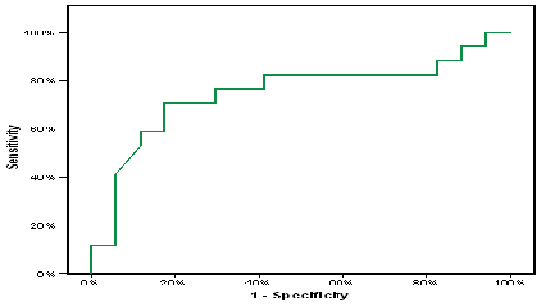
CTCs	Metastatic (n = 30)		Non-metastatic (n= 38)		BPH (n= 30)		Prostatitis (n = 20)		Controls (n= 20)		MCP
	No.	%	No.	%	No.	%	No.	%	No.	%	
Negative	6	20	28	73.7	30	100	20	100	20	100	<0.001*
Positive	24	80	10	26.3	0	0.0	0	0.0	0	0.0	
FEp	0.005*										

Table (6) showed that there was a statistically significant correlation between the CTCs and the t-PSA in the whole prostate cancer patients group ($p= 0.029$). The t-PSA was significantly higher in patients who were positive for CTCs (median= 100 ng/ml) than in those who were negative for CTCs (median= 18.46 ng/ml).

t –PSA	CTCs		Z (p)
	Negative (n = 34)	Positive (n = 34)	
Range	4.9 – 520.0	5.4 – 915.0	2.187* (0.029)
Median	18.46	100.0	

In order to determine a cut off value for the t-PSA at which we could start searching for CTCs, a ROC curve was constructed. It was found that at a t-PSA value of 36.5 ng/ml showed the highest sensitivity (76.47%) and the highest specificity (70.59%). The AUC was 0.747.

t-PSA (ng/ml)	Sensitivity (%)	Specificity (%)
28	76.47	58.8
34.5	76.47	64.7
36.5	76.47	70.59
43.7	70.6	70.59
50.7	70.6	76.5



	AUC	p
t-PSA	**0.747***	**0.014**

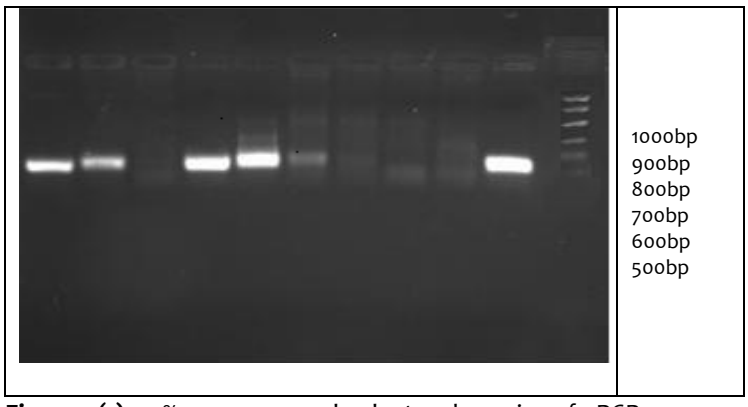


Figure (1): 2% agarose gel electrophoresis of PCR products.

Lanes 1-5: Cases with metastatic prostate cancer. Where lanes 1,2,4,5 show bands for PSA gene at 532 bp.
Lane 3: a case with metastatic prostate cancer, no PSA gene band is seen a532bp.
Lanes 6-10: cases with non-metastatic prostate cancer.
Lane 10: a case with non-metastatic prostate cancer, a band for PSA gene is seen at 532bp.
Lane 11: 100-1000bp molecular weight marker (Fermentas, Germany).

Table (7): Cut off value of t-PSA at which CTCs could be detected

		CTCs		Sensitivity	Specificity	PPV	NPV	Accuracy
		Negative	positive					
t-PSA (36.5) ng/ml	Negative	24	8	76.47	70.59	72.22	75.00	73.53
	positive	10	26					

DISCUSSION

The widespread introduction of prostate specific antigen (PSA) screening in the late 1980s favored an increased proportion of prostate cancer diagnosed at locally malignant stages, which, after being cured, has an overall good prognosis.[22,23]

However, 25 to 40% subset of patients with locally confined disease, despite appropriate surgical and pharmacological treatments, would have recurrence due to local disease progression and/or the development of distant metastases.[24,25]

The group of patients with an initial diagnosis of locally confined malignancy which has an adverse outcome cannot be identified at diagnosis with computed tomography or radionucleotide bone scans, although micrometastatic foci may already be present. This means that the frequency of detection of distant metastasis by routine clinical analysis is not concordant with the frequency of disease recurrence after definitive local therapy.[26] for this reason, there is an urgent need for improved preoperative staging modalities for patients with localized prostate cancer, a need for improved predictive markers to facilitate treatment selection and to monitor the effect of treatment.

Elevated serum levels of PSA is currently the most powerful and widely used prostate cancer screening test.[27] However, the utility of serum PSA test is limited because of high false-positive rates and up to 75% of patients with elevated serum PSA may not have cancer. Also, Gleason score, cannot be easily assessed repeatedly over the course of the disease because it is an invasive procedure. In addition to that, bone scans offer only limited information on changes in the disease.[29] Thus the early detection of CTCs seems to be a promising approach with significant clinical implications, such as classification of prostate cancer patients with clinically localized disease into prognostic groups, which may influence therapeutic decisions. [30]

The present study aimed to detect the CTCs in peripheral blood of prostate cancer patients using reverse transcriptase polymerase chain reaction (RT-PCR).

In the present study all patients had their t-PSA, f-PSA and their f/t PSA ratio was calculated. The median value of t-PSA was 150ng/ml ranging from18ng/ml to 915ng/ml for the metastatic group, and was15.2ng/ml ranging from 4.9ng/ml to 52ng/ml for the non-metastatic group. These results were statistically significant with a p value of <0.001. There was a statistically significant positive relation between t-PSA and CTCs in the whole prostatic cancer patients (metastatic and non-metastatic) groups with a p value of 0.014. Similar results of Goodman et al found.[31] They found that t-PSA, LDH and prostatic acid phosphatase all correlated positively with CTCs with a p value of < 0.001. *Zambon et al*[32] stated that the most sensitive index of prostate cancer was t-PSA (70%), and the most specific was f/t PSA ratio (80%). However *Ennis et al*[33] found that there was no relationship between clinical stage, pretreatment PSA, Gleason score and RT-PCR positivity. This might be due to the difference in the number of patients enrolled (227 patients in their study versus 19 patients in the current study) or it may be because their study was conducted on only non-metastatic prostate cancer patients or due to the fact that some of their patients were treated pre-operatively with antiandrogens which are known to decrease PSA mRNA expression.[17] Also, *Gao et al* [34] found that there was no statistically significant relation between RT-PCR for PSA-expressing cells and clinical stage (p= 0.722), age (p= 0.485), and serum PSA (p= 0.409). This was discordant with the results of the current study. We found a statistically significant relation between CTCs and age, t-PSA, Gleason's score and clinical staging with p values of < 0.001, 0.014, 0.042 and 0.008 respectively. This could be due to the larger sample size in their study used or the different technique applied (density gradient centrifugation and nested RT-PCR assay).[34]

Because of the overlap in the serum t-PSA in patients with BPH and prostate cancer in the PSA range of 4-10 ng/ml (named the grey zone), any improvement in the accuracy of t-PSA measurement will have a tremendous cost saving potential. The f/t PSA ratio seems to facilitate the differential diagnosis of prostate cancer and BPH in this grey zone.[35] Numerous studies have demonstrated that the percent of f-PSA in serum increases in BPH because the inner portion (transition zone) of the prostate, where BPH usually occurs, contains elevated levels of f-PSA, while in prostate cancer most of the PSA in blood is bound. Therefore,

the lower the ratio of free to total PSA or the percentage of free PSA, the higher the likelihood that the patient has prostate cancer.[35] Free to total PSA ratio (f/t PSA) is used to enhance the specificity of cancer detection[36]. Partin et al[37] reported that f/t PSA in serum more accurately distinguishes prostate cancer from a nonmalignant disease. In the current study, f/t PSA ratio was calculated for prostate cancer and BPH groups whose t-PSA values ranged from 4 to 10ng/ml (4 patients with prostate cancer and 10 patients with BPH). The median value for the prostate cancer patients group was 0.1 ranging from 0.08 to 0.15 while for the BPH group the median value for f/t PSA was 0.38 ranging from 0.25 to 0.45 .These results show that f/t-PSA ratio was higher in BPH than in prostate cancer. These results were in agreement with *Jung et al*[38] who found that f/t-PSA ratio was higher in BPH (0.25) than in prostate cancer (0.12). The difference in the ratio might be due to the increase in the size of the studied population as their study was conducted on 89 patients with BPH and 144 patients with prostate cancer while in the present study they were 30 and 68 respectively.[38]

As regards the detection of CTCs in the present study, For the metastatic group, (24/30) were positive for the CTCs with a percentage of 80%, For the non metastatic group, Ten patients were positive for the CTCs out of 38 patients (10/38) with a percentage of 26.3%, For the BPH, prostatitis and the control groups, no CTCs were detected. It was expected that all patients of the metastatic group would be positive for CTCs. whereas the true state showed 20% were negative for CTCs. This could be due to either that these patients were taking anti androgens, as it is known that expression of mRNA for PSA has been shown to be androgen-dependent [17], or due to the fact that some CTCs might lose their ability to produce PSA. The later hypothesis was explained by *Pfitzenmaier el al* who found that out of 84 patients positive for CTCs only 36 of them expressed PSA. For this reason, they recommended the use of multiple tumour specific markers in the same procedure like PSMA, PSCA and hK2. [39]

In this study, we detected a cut-off value for t-PSA at which we could start detecting for CTCs. For the t-PSA level, the cut off was 36.5 ng/ml with a sensitivity of 76.4%, and a specificity of 70.5% with an AUC of 0.767. This cut off could be of clinical importance; it could guide clinicians for metastasis work up to be done for those patients. However, *Ling Huei et al*[40] found patients with a cut-off value of 13 ng /ml and above, showed hot spots by bone scintigraphy. The sensitivity was 96.43% and specificity 84.09%. This discrepancy in results might be due to the difference in the sample size, as they conducted their study on 101 prostate cancer patients while we conducted this study on 68 patients. This discrepancy might be due to the fact that they did not acquire the t- PSA concentration and bone scintigraphy on the same day.[40]

CTC detection methods have often produced discrepant results, and most reports have failed to show clinical value.[41]Furthermore, the proportions of positive samples in different disease stages remain unclear. Frequencies of blood samples positive for prostate-specific RNAs have ranged from 0% to 81% in clinically localized disease and from 31% to 100% in metastatic disease.[42,43]This widespread inconsistencies in results may result from different methodologies of blood sampling, sample handling, cDNA synthesis, the use of different primer sets, detection and quantification technologies, reaction chemistries, and operator effects.[44]since the early 1990s, the detection of disseminated prostate cancer cells has relied primarily on the use of RT-PCR to detect PSA, prostate-specific membrane antigen, or hK2 RNA in specimens of peripheral blood, bone marrow, or lymph nodes. Several studies have compared between this RT-PCR method and new cell isolation and enrichment methods like immunomagnetic selection, filtration and density gradient centrifugation methods. An example of this is what Pfitzenmaier et al[39] did. They found that 31 out of 165 (19%) prostate cancer patients were positive by RT-PCR for PSA, while 36 out of 183 (20%) prostate cancer patients were positive for PSA expressing CTCs by cell enrichment techniques. In addition to that, *Helo et al*[45] found that RT-PCR and Cell Search CTC results were strongly concordant (85%) and correlated. [45]In contrast to the present study, *Hara et al*[46]used a nested RT-PCR assay combining three prostate-associated molecules, prostate specific antigen (PSA), prostate specific membrane antigen (PSMA), and prostate stem cell antigen (PSCA) to reveal patients with poor prognosis. PSA-, PSMA-, and PSCA-mRNA were detected in 7/58 (12.1%), 12/58 (20.7%), and 8/58 (13.8%) prostate cancer respectively. In the current study an RT-PCR assay was used to detect only PSA mRNA. We were more precise in classifying our patients into metastatic and non-metastatic group with the positive findings of 80% and 26.3% respectively. Their result was only 12.1% although this could be explained by their large sample size as compared to ours or due to the fact that before their enrollment in the study, all of the studied groups received androgen blockage therapy, some of them underwent radical prostatectomy and some with local progression received radiation therapy. From their findings they suggested that PSCA PCR would be the most promising for the molecular staging of prostate cancer. They concluded that RT-PCR is a highly cost-effective and rapid procedure, enabling the molecular staging of prostate cancer with RT-PCR as a diagnostic routine.[46] *Ignatoff et al* [47] used RT-PCR assay in cases of clinically localized prostate cancer. They found that 18 out of 82 patients (22%) with clinically localized prostate cancer

were positive for CTCs. These results were very close to the results of the current study (26.3% for the non-metastatic group). In accordance with our study, they found positive RT-PCR assay results correlated significantly with skeletal metastases and elevated levels of serum PSA. [47]

Therefore it is obvious that different methods of detection had led to variable results in the percentage of CTCs. It should be pointed out that the new method of isolation and concentration of circulating tumor cells by the cell search system (the immunomagnetic separation), as described by *Helo et al*[29] and Gao et al[34] gives us the opportunity to quantify the number of those CTCs and to find a cut off value for their number with respect to correlation with survival outcomes as well as predictive of metastatic disease as *Goodman et al*[31] did. In the present study and the studies conducted by Hara et al[46], *Gao et al*[34], *Ignatoff et al*[47] and *Sardi et al*[20], total RNA was extracted from the peripheral blood, converted it to the complementary DNA and used PSA primers to produce PSA segment of 532bp. This method is qualitative. However, *Yilkoski et al*[48] used quantitative reverse transcription-PCR (QRT-PCR) which appear to offer a more accurate and reliable assessment of metastatic disease and, estimating the correlation between the quantity of RNA and the presence of metastases or further disease progression thus appearing to be more promising in clinical decision making.[48]

This study has its strengths and limitations. Among its strong points is that blood samples were collected prior to medical, radiological or surgical treatment, thus eliminating the effect of drugs on circulating prostate cells levels. The major limitation of the study was its relatively small sample size, which might be the cause of variation between the results of all the above studies. Another limitation of this study was that there was no follow up for the non-metastatic patients who were positive for the CTCs to detect whether and when metastasis might appear clinically.

In conclusion, detection of CTCs is important in the early detection of metastasis in cases of localized prostate cancer. It can influence the selection of therapies in those patients. Patients who are negative for CTCs can be treated with radical treatment only (radical prostatectomy or radical radiotherapy) while those who are positive for CTCs may benefit from adjuvant therapy. Detection of CTCs can also affect the prognosis of the disease and it is useful in follow up during treatment as we need only a peripheral blood sample which is minimally invasive.

Finally, we recommend searching for CTCs to be done as a routine in all newly diagnosed non metastatic prostate cancer patients for the early detection of metastasis. Further follow up for patients with non-metastatic prostate cancer who were positive for the CTCs to determine whether and when distant metastasis may occur. The use of multiple prostate specific markers together, like PSA, PSMA, PSCA and hK2 in detection of CTCs because some CTCs are not PSA expressing cells.

REFERENCES

1. Ferlay J, Shin HR, Bray F, Forman D, Mathers C and Parkin DM. GLOBOCAN 2008, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 10. www.globocan.iarc.fr. Accessed on 21/11/2010.
2. Kolonel LN, Altshuler D, Henderson BE. The multiethnic cohort study: exploring genes, lifestyle and cancer risk. Nat Rev Cancer 2004; 4:519-27.
3. Steinberg GD, Carter BS, Beaty TH, Childs B, Walsh PC. Family history and the risk of prostate cancer. Prostate 1990;17:337-47.
4. Torkko KC, van Bokhoven A, Mai P, Beuten J, et al. VDR and SRD5A2 polymorphisms combine to increase risk for prostate cancer in both non-Hispanic White and Hispanic White men. Clin Cancer Res 2008; 14:3223-9.
5. Hayes VM, Severi G, Padilla EJ, et al. 5alpha-Reductase type 2 gene variant associations with prostate cancer risk, circulating hormone levels and androgenetic alopecia. Int J Cancer 2007; 120:776-80.
6. Dianat SS, Margreiter M, Eckersberger E, et al. Gene polymorphisms and prostate cancer: the evidence. BJU Int 2009;104:1560-72.
7. Mittal RD, Mishra D, Mandhani AK. Role of an androgen receptor gene polymorphism in development of hormone refractory prostate cancer in Indian population. Asian Pac J Cancer Prev 2007; 8:275-8.
8. Quinn D, Henshall S, Sutherland R. Molecular markers of prostate cancer outcome. Eur J Cancer 2005;41:858-87.
9. Song JM, Kim CB, Chung HC, Kane RL. Prostate-specific antigen, digital rectal examination and transrectal ultrasonography: a meta-analysis for this diagnostic triad of prostate cancer in symptomatic korean men. Yonsei Med J 2005; 46:414-24.
10. Divakara M, Daniel P, Donald P. Underutilization of Digital Rectal Examination When Screening for Prostate Cancer. Arch Intern Med 2004;164:313-6.
11. Glaves D. Correlation between circulating cancer cells and incidence of metastases. Br J Cancer 1983; 48:665-73.
12. Jacob K, Sollier C, Jabado N. Circulating tumor cells: detection, molecular profiling and future prospects. Expert Rev Proteomics 2007; 4:741-56.

13. Mocellin S, Keilholz U, Rossi CR, Nitti D. Circulating tumor cells: the 'leukemic phase' of solid cancers. Trends Mol Med 2006; 12:130-9.

14. Poste G, Fidler IJ. The pathogenesis of cancer metastasis. Nature 1980 ;283:139-46.

15. Fidler IJ.The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis revisited. Nat Rev Cancer. 2003 Jun;3(6):453-8.

16. Crawford ED, Abrahamsson PA. PSA-based screening for prostate cancer: how does it compare with other cancer screening tests? Eur Urol 2008; 54:262-73.

17. Henttu P, Liao SS, Vihko P. Androgens up-regulate the human prostate-specific antigen messenger ribonucleic acid (mRNA), but down-regulate the prostatic acid phosphatase mRNA in the LNCaP cell line. Endocrinology 1992; 130:766-72.

18. Silver DA, Pellicer I, Fair WR, Heston WD, Cordon-Cardo C. Prostate-specific membrane antigen expression in normal and malignant human tissues. Clin Cancer Res 1997; 3:81-5.

19. Israeli RS, Powell CT, Corr JG, Fair WR, Heston WD. Expression of the prostate-specific membrane antigen. Cancer Res 1994; 54:1807-11.

20. Sardi I, Piazzini M, Ponchietti R, Fatini C, Di Loro F, Guazzelli R. The use of RT-"nested" PCR of prostate specific antigen to detect hematogenous neoplastic cells in patients with prostate adenocarcinoma. J Mol Med 1997;75:751-7.

21. Aslanidis C, Pfannenschmid F, Schmid Gm Manual of the workshop of the Egyptian assosciation of Clinical chemistry; DNA diagnostics and DNA finger printing. Egypt; December 10-11, 2004.

22. Mettlin CJ, Murphy GP. Why is the prostate cancer death rate declining in the United States? Cancer. 1998; 82:249-51.

23. Lerner SP, Seale-Hawkins C, Carlton CE, Jr., Scardino PT. The risk of dying of prostate cancer in patients with clinically localized disease. J Urol 1991; 146:1040-5.

24. Labrie F, Candas B. Androgen blockade in prostate cancer. Lancet 2000; 356:341-2.

25. Wilson SS, Crawford ED. Screening for prostate cancer: current recommendations. Urol Clin North Am. 2004; 31:219-26.

26. Crawford ED, Bennett CL, Stone NN, et al. Comparison of perspectives on prostate cancer: analyses of survey data. Urology 1997; 50:366-72.

27. Barry MJ. Clinical practice. Prostate-specific-antigen testing for early diagnosis of prostate cancer. N Engl J Med 2001; 344:1373-7.

28. Carroll P, Coley C, McLeod D, et al. Prostate-specific antigen best practice policy--part II: prostate cancer staging and post-treatment follow-up. Urology 2001; 57:225-9.

29. Helo P, Cronin AM, Danila DC, et al. Circulating prostate tumor cells detected by reverse transcription-PCR in men with localized or castration-refractory prostate cancer: concordance with CellSearch assay and association with bone metastases and with survival. Clin Chem 2009; 55:765-73.

30. Mukamel E, Hanna J, deKernion JB. Pitfalls in preoperative staging in prostate cancer. Urology 1987; 30:318-21.

31. Goodman OB, Jr., Fink LM, Symanowski JT, et al. Circulating tumor cells in patients with castration-resistant prostate cancer baseline values and correlation with prognostic factors. Cancer Epidemiol Biomarkers Prev 2009; 18:1904-13.

32. Zambon CF, Basso D, Prayer-Galetti T, et al. Quantitative PSA mRNA determination in blood: a biochemical tool for scoring localized prostate cancer. Clin Biochem 2006; 39:333-8.

33. Ennis RD, Katz AE, de Vries GM, et al. Detection of circulating prostate carcinoma cells via an enhanced reverse transcriptase-polymerase chain reaction assay in patients with early stage prostate carcinoma. Independence from other pretreatment characteristics. Cancer 1997; 79:2402-8.

34. Gao CL, Maheshwari S, Dean RC, et al. Blinded evaluation of reverse transcriptase-polymerase chain reaction prostate-specific antigen peripheral blood assay for molecular staging of prostate cancer. Urology 1999; 53:714-21.

35. Eriksson S, Vehniainen M, Jansen T, et al. Dual-label time-resolved immunofluorometric assay of free and total prostate-specific antigen based on recombinant Fab fragments. Clin Chem 2000; 46:658-66.

36. Carducci MA. Prostate cancer update: advanced disease. Rev Urol 2003; 5: 47-53.

37. Bratt O. Hereditary prostate cancer: clinical aspects. J Urol 2002; 168:906-13.

38. Jung K, Elgeti U, Lein M, et al. Ratio of free or complexed prostate-specific antigen (PSA) to total PSA: which ratio improves differentiation between benign prostatic hyperplasia and prostate cancer? Clin Chem 2000; 46:55-62.

39. Pfitzenmaier J, Ellis W , Hawley S, et al. The detection and isolation of viable prostate-specific antigen positive epithelial cells by enrichment: A comparison to standard prostate-specific antigen reverse transcriptase polymerase chain reaction and its clinical relevance in prostate cancer. Urologic Oncology 2007; 25:214-220.

40. Ling-Huei W, Jainn-Shiun C, Shiou-Ying C, Yuh-Feng W. Predicting Bone Metastasis in Prostate Cancer Patients: Value of Prostate Specific Antigen. Tzu Chi Med J 2008; 20:291-5.

41. Thomas J, Gupta M, Grasso Y, et al. Preoperative combined nested reverse transcriptase polymerase chain reaction for prostate-specific antigen and prostate-specific membrane antigen does not correlate with pathologic stage or biochemical failure in patients with localized prostate cancer undergoing radical prostatectomy. J Clin Oncol 2002; 20:3213-8.

42. de la Taille A, Olsson CA, Katz AE. Molecular staging of prostate cancer: dream or reality? Oncology 1999;13:187-948.

43. Schamhart DH, Maiazza R, Kurth KH. Identification of circulating prostate cancer cells: a challenge to the clinical implementation of molecular biology. Int J Oncol 2005;26:565-77.

44. Rissanen M, Helo P, Vaananen RM, et al. Novel homogenous time-resolved fluorometric RT-PCR assays for quantification of PSA and hK2 mRNAs in blood. Clin Biochem 2007; 40:111-8.

45. Helo P, Cronin AM, Danila DC, et al. Circulating prostate tumor cells detected by reverse transcription-PCR in men with localized or castration-refractory prostate cancer: concordance with CellSearch assay and association with bone metastases and with survival. Clin Chem 2009; 55:765-73.

46. Hara N, Kasahara T, Kawasaki T, et al. Reverse transcription-polymerase chain reaction detection of prostate-specific antigen, prostate-specific membrane antigen, and prostate stem cell antigen in one milliliter of peripheral blood: value for the staging of prostate cancer. Clin Cancer Res 2002;8:1794-9.

47. Ignatoff JM, Oefelein MG, Watkin W, Chmiel JS, Kaul KL. Prostate specific antigen reverse transcriptase-polymerase chain reaction assay in preoperative staging of prostate cancer. J Urol 1997; 158:1870-5.

48. Ylikoski A, Karp M, Pettersson K, Lilja H, Lovgren T. Simultaneous quantification of human glandular kallikrein 2 and prostate-specific antigen mRNAs in peripheral blood from prostate cancer patients. J Mol Diagn 2001;3:111-22.

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