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Evaluation of *CGB, GNRH1, MET* and *KRT19* Genes Expression Profile as a Circulating Tumor Cells Marker in Blood of Cancer Patients

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Abstract

The aim of this study was to evaluate human chorionic gonadotropin beta subunit (*CGB*), gonadotropin releasing hormone type 1 (*GNRH1*), hepatocyte growth factor receptor (*MET*) and keratin 19 (*KRT19*) genes expression profile as a circulating tumor cells (CTC) marker in blood of cancer patients. Expression level of studied genes was assessed in peripheral blood of 122 patients with of different type of cancers and 86 healthy volunteers using reverse transcription real time quantitative polymerase chain reaction (RT-qPCR). The result of the experiments showed that *in blood of cancer patients the* level of *MET* transcripts *showed positive correlation with KRT19 and negative correlation with GNRH1*. In the control group negative correlation between *CGB* and *GNRH1* was documented. What is more the level of *CGB, MET* and *KRT19* expression was significantly higher in blood of cancer patients. Even though the analysis proved that studied genes are expressed in blood of both cancer patients and healthy volunteers, their expression level was is highly heterogeneous. In order to interpret the results, the obtained data was log-transformed and fitted to multiplied normal distribution model using the maximal likelihood method.

The results of this analysis showed elevated expression of *CGB, MET* and *KRT19* together with extremely high levels of *GNRH1* in blood of cancer patients which might indicate the presence of circulating tumor cells and increased risk of metasis.

Keywords: Circulating tumor cells; Chorionic gonadotropin beta subunit; Human gonadotropin releasing hormone type 1; Hepatocyte growth factor receptor; Keratin 19; Quantitative PCR

Abbreviations:

PBS: Phosphate Buffered Saline Buffer; CTC: Circulating Tumor Cells; *CGB*: Human Chorionic Gonadotropin Beta Subunit; *GNRH1*: Gonadotropin Releasing Hormone Type 1; *MET*: Hepatocyte Growth Factor Receptor; KRT8: Keratin 8; KRT18: Keratin 18; *KRT19*: Keratin 19; RT-PCR: Reverse Transcription Polymerase Chain Reaction; HGF: Hepatocyte Growth Factor; RT-Qpcr: Reverse Transcription Real Time Quantitative Polymerase Chain Reaction; HPRT: Hypoxanthine-Guanine Phosphoribosyltransferase; Epcam: Epithelial Cell Adhesion Molecule; MUC1: Mucin 1, Cell Surface Associated; PSA: Prostate-Specific Antigen; CEA: Carcinoembryonic Antigen

Background

Despite advances in early diagnostics and therapeutic strategies cancer remains a leading cause of death [1]. At present there are no accurate non-invasive tests that enable cancer staging, assessment of its progression or identifying patients at high risk for metasis and most carcinomas can only be treated by surgical removal of the tumor mass.

It has been demonstrated that in patients who have undergone complete removal of the primary tumor, the spread of tumor cells in their peripheral blood, referred to as circulating tumor cells (CTC), may be present [2]. These cells could be responsible for the later development of metasis [2-4].

Circulating tumor cells detection appears to be a promising, minimally invasive diagnostic and prognostic tool for patients with cancer. CTC presence in peripheral blood of cancer patients may be used in recurrence risk assessment, monitoring of response to treatment, follow up studies as well as development of novel therapeutic agents [5,6].

The technical challenge in CTC detection and characterization is their extremely low occurrence in blood, estimated to be one cancer cell per 105-7 mononuclear cells [7]. Due to technical difficulties, the clinical significance of CTC has not yet been well established. Conventional Methods of cancer cells recognition within tissues and body fluids are unable to accurately identify the spread of micrometastatic disease [5]. Methodological limitations could be one of the reasons for CTC suboptimal capture and their low detection rate in cancer patients' blood. Adjustment of flow cytometry, magnetic cell separation and dielectrophoresis has been suggested to increase CTC detection yield in peripheral blood [8,9]. A potentially more sensitive molecular approach for CTC identification is the reverse transcription polymerase chain reaction (RT-PCR) employing a variety of marker transcripts as targets. Especially multimarker RT-PCR assays have been successfully used to detect micrometasis in blood and tissues of cancer patients [5,8,10].

In this study four mRNA markers: human chorionic gonadotropin beta subunit (*CGB*), gonadotropin releasing hormone type 1 (*GNRH1*), hepatocyte growth factor receptor (*MET*) and keratin 19 (*KRT19*) were assessed for their ability to become markers of tumor cells circulating in peripheral blood of patients with cancers of different origin.

We have previously demonstrated that human chorionic gonadotropin beta subunit is a valuable marker of CTC in gynecological cancers [11]. Free CGB was showed to play an important role in growth, invasion and metasis of tumors of various origins [12-15]. What is more we documented that the expression of *CGB* in endometrial cancer as well as endometrial atypical hyperplasia is accompanied by the expression of gonadotropin releasing-hormone type 1, which physiologically stimulates the expression and secretion of gonadotropins. Our group and others have also previously showed the involvement of both *GNRH1* and *CGB* in the development and growth of tumors [11,16,17]. The aforementioned study demonstrated that assessment of *CGB* and *GNRH1* expression level in blood may be useful for indicating the *MET*astatic spread of tumor cells of gynecological cancer patients [11].

In order to detect CTC, in addition to *CGB* and *GNRH1*, we decided to evaluate *MET* expression. *MET* mRNA, normally expressed in cells of epithelial origin, encodes a cell surface receptor to which hepatocyte growth factor (HGF) binds and regulates cellular functions. Its abnormal activation occurs in many types of cancers, what correlates with disease progression, poor prognosis, neoangiogenesis and metasis [18,19]. It was also demonstrated that proliferation of epithelial cells in ovary is associated with induction of HGF expression by chorionic gonadotropin (CG) [20]. The level of *MET* expression was used in CTC detection before and similarly to CG it was showed to correlate with tumor stage [21,22].

Among other CTC biomarkers the cytokeratin family member – *KRT19* has been most frequently used in clinical studies [23,24]. Detection of *KRT19* mRNA-positive cells was showed to be an independent predictive and prognostic factor of poor clinical outcome [5,25,26]. Cytokeratin genes are known to be expressed in epithelial cells, such as CTC, but not in blood cells. Moreover, immunocytochemical detection of KRT8, KRT18, and *KRT19* is routinely used in cancer diagnosis [27]. Thus, during this study KRT19 expression was used as a marker of epithelial cells.

All selected markers: *CGB, GNRH1, MET* and *KRT19* were successfully used in cancer spread detection before [28]. In this study we decided to combine them in one diagnostic panel and test their ability to detect CTC in peripheral blood of patients with cancers of different origin.

The results of the experiments showed that all analyzed genes may be active in blood of both cancer patients and healthy volunteers. The expression level of *CGB, MET* and *KRT19* was however significantly higher in blood of cancer patients. Moreover t*he expression of MET gene positively correlated with KRT19 and negatively with GNRH1* i*n cancer patients' blood, while i*n control group negative correlation between *CGB* and *GNRH1* was documented.

The log-transformation of raw data fitted to multiplied normal distribution model using the maximal likelihood method showed that analyzed genes expression is heterogeneous and more than one subpopulation can be distinguished within defined group.

Methods

The study was approved by ethics review board of Poznan University of Medical Sciences and all patients participated after written informed consent.

The peripheral blood samples of cancer patients were obtained at the Great Poland Cancer Center, Poznan and at the Department of Gynecologic Oncology, Poznan University of Medical Sciences in 2008–2012.

The study group consisted of 122 patients with different types of malignances: gynecological (n=55), head and neck (n=30), breast (n=13), digestive system (n=13), lung (n=5), prostate (n=4), pancreas (n=2). All blood specimens were collected before surgical procedures.

Since we intended to find universal markers, independent of tumor origin, histological subtype and grading of studied cancers were not taken into consideration.

The control group included blood samples obtained from 86 healthy volunteers.

Sample collection and RNA isolation were conducted as described previously[11].

cDNA synthesis and qPCR

cDNA library was constructed as described previously [11].

To assess the expression level of *CGB* [all genes encoding beta subunit of the hormone were analyzes in one assay; GenBank: *CGB3*/9– NM_000737; *CGB5* – NM_033043; *CGB6*/7 – NM_033142; *CGB8* – NM_033183; *CGB1* – NM_033377; CGB2 – NM_033378], *GNRH1* [GenBank: NM_000825.3], *MET* [GenBank: NM_000245.2 and NM_001127500.1], *KRT19* [GenBank: NM_002276.4] and housekeeping gene – HPRT [Human HPRT Gene Assay Cat. No. 05 046 157 001 (Roche Diagnostics)] quantitative PCR (qPCR) with sequence specific primers, TaqMan hydrolysis probes and LightCycler® TaqMan® Master Kit was performed. TaqMan hydrolysis probes for examined genes and HPRT gene assay were designed using ProbeFinder software version 2.44 and purchased from the collection of Universal Probe Library (Roche Diagnostics).

qPCR was performed using the TaqMan Master Reagents Kit (Roche Diagnostics). Experiments were performed according to the delivered protocol. PCR efficiencies were calculated from the standard curves, which were generated using serial dilutions of cDNA library constructed from endometrial cancer (Adenocarcinoma endometrioides G2). Relative expression of analyzed genes was normalized against phosphoribosylotransferase (HPRT) housekeeping gene (Human HPRT Gene Assay, Roche Diagnostics).

Hydrolysis probes and primers used in the experiments are described in Table 1.

Each reaction sets involved template control (NTC, negative control).

Assays were designed to avoid amplification of DNA. All experiments were performed in triplicates, using independently synthesized cDNA and qPCR data was assembled using the LightCycler software 4.05 dedicated for the LightCycler 2.0 (Roche Diagnostics).

Gene	TaqMan probe No.	forward primer 5' 3'	Reverse primer 5' 3'
CGB*	#71 (Cat. No. 04688945001)	TACTGCCCCACCATGACC	CACGGCGTAGGAGACCAC
GNRH1	#29 (Cat. No. 04687612001)	GACCTGAAAGGAGCTCTGGA	CTTCTGGCCCAATGGATTTA
MET	#67 (Cat. No. 04688660001)	CGTCAACAAAAACAATGTGAGAT	AAACTCTGTTCGATATTCATCACG
KRT19	#71 (Cat. No. 04688945001)	GCCACTACTACACGACCATCC	CAAACTTGGTTCGGAAGTCAT
HPRT	Human HPRT Gene Assay, Cat. No. 05 046 157 001 (Roche Diagnostics)		
*The nucleotide sequences of primers were designed to enable amplification of all genes encoding beta subunit of human chorionic gonadotropin (CGB1-9).			

Table 1: Primers and TaqMan hydrolysis probes used in real time qPCR.

Data collection and statistical analysis

CGB, KRT19, MET and *GNRH1* concentration ratios were log-transformed to achieve normal data distribution and presented as the logarithm to the base ten. Kruskal-Wallis test was performed and differences in expression between blood of cancer patients and volunteers were considered to be statistically significant if P-value was lower than 0.05.

In order to distinguish populations with homogeneous genes' expression the maximal likelihood method for one- and multiplied normal distribution was used and several subpopulations within analyzed groups were distinguished. To assess the improvement of quality of the fit, the outcome of this analysis was tested with F-test. Model of higher complicity (with greater number of subpopulations) was selected only if statistical significance of improvement ($P<0.05$) was achieved. Additional verification of correctness of the chosen model was performed using Kolmogorov-Smirnov test. P value found in all cases was $P>0.7$.

Associations between studied genes expression were evaluated using Spearman's rank correlation coefficient.

Results

The expression of four genes: *CGB, KRT19, MET* and *GNRH1* was evaluated using RT-qPCR in peripheral blood of cancer patients as well as in blood of a control group consisting of healthy volunteers. Due to the nature of real time PCR measurement raw data was log-transformed.

The results of the study demonstrated that the analyzed genes may be transcriptionally active in both cancer patients' and healthy volunteers' mononuclear blood cells (Table 2). Despite the fact that not all samples showed the presence of the genes' transcripts the level of *CGB, KRT19* and *MET* expression assessed for group of cancer patients was higher than the one noted in healthy volunteers. The differences in *CGB, KRT19* and *MET* expression between cancer patients and healthy volunteers was found to be statistically significant ($P<0.001$ for *CGB*, $P=0.008$ for *KRT19* and $P<0.001$ for *MET*). The difference of expression levels between these groups for *GNRH1* gene was not statistically significant.

Co*rrelation of* expression levels *of the analyzed genes* between studied groups using Spearman test *showed that in blood of cancer patients the* level of transcripts
of MET correlates with KRT19 (r=0.41) as well as with GNRH1 (r=-0.27).In the control group consisting of blood of healthy volunteers correlation between CGB and *GNRH1* (r=-0.28) was also documented. All correlations were statistically significant ($p<0.05$).

Gene	Cancer patients	Healthy volunteers
CGB	90%	70%
KRT19	25%	36%
MET	65%	57%
GNRH1	99%	92%

Table 2: Percentage of cancer patients and healthy volunteers showing expression of analyzed genes in blood.

The log-transformed values of studied genes expression were analyzed against the existence of potential subpopulations varying in genes expression. The results proved that the model assuming the existence of more than one group with normal distribution is significantly better for describing heterogeneous expression of the analyzed genes (Table 3).

Log-transformed RT-qPCR results of *CGB* expression in blood of both cancer patients and healthy volunteer were fitted into two subpopulations (Table 3 and Figure 1). *CGB* expression levels characterizing cancer patients subpopulations were ten and hundred fold higher than corresponding distributions found in the control group (Table 3 and Figure 1).

Evaluation of *KRT19* transcripts level revealed the presence of three normal data distributions within both analyzed groups. The subpopulations characterized by the lowest gene's expression in cancer patients and control group showed very similar expression level of *KRT19*. The other two subpopulations distinguished in cancer patients group demonstrated ten fold higher *KRT19* level than the two distributions noted in the control group (Table 3 and Figure 2).

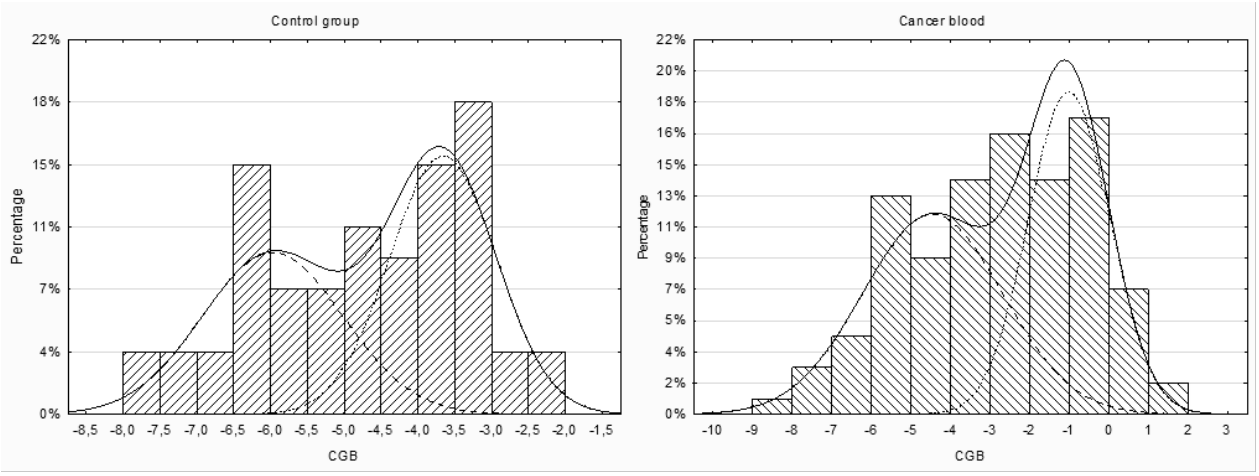

Figure 1: Relative expression level of *CGB* in blood of healthy volunteers and cancer patients. Results obtained in RT-qPCR assays were log-transformed using the logarithm to the base 10 to achieve normal distribution of data. The use of maximal likelihood method allowed distinguishing two normal distribution of *CGB* expression results in both the control (left plot) and cancer patients (right plot) group.

		I subpopulation			II subpopulation			III subpopulation		
		%	mean $log_{10}Cr$	SD	%	mean $log_{10}Cr$	SD	%	mean $log_{10}Cr$	SD
Blood of cancer patients	*CGB*	52	-4.45	1.76	48	-1.00	1.02			
	KRT19	12	-11.68	1.63	67	-4.69	1.92	21	0.41	0.74
	CMET	18	-8.52	1.50	82	-2.27	1.09			
	GNRH1	36	0.73	0.13	60	0.96	0.72	4	8.27	0.47
Blood of healthy volunteers	*CGB*	45	-5.97	0.95	55	-3.66	0.72			
	KRT19	16	-11.89	1.57	74	-5.97	1.03	10	-0.68	0.50
	CMET	9	-4.74	0.46	49	-1.58	0.77	42	-1.44	0.16
	GNRH1	75	0.93	0.26	25	0.96	0.78			
log10Cr – the logarithm to the base 10 of Cr (concentration ratio).										

Table 3: The distributions of *CGB, KRT19, CMET* and *GNRH* gene expression within studied groups.

Relative expression level of MET was categorized into two normal distributions in blood of cancer patients, and into three distributions in healthy volunteers group (Table 3 and Figure 3). The highest expression of MET distinguished 42% of healthy volunteers' blood samples while the lowest gene's level of transcripts was observed in cancer patients' bloods.

Detailed analysis of *GNRH1* expression allowed categorizing the results into three normal distributions in cancer patients and two subpopulations in the control group (Table 3 and Figure 4). Both subpopulations found among healthy volunteers were characterized by almost equal mean of *GNRH1* expression. The second subpopulation of the control group's results showed a level of *GNRH1*, which matched one of the distributions distinguished for cancer patients. Still the subpopulation showing the highest *GNRH1* gene level of transcripts was representative for cancer patients only. This mean expression level was 108 fold higher than the one observed in the control group (Table 3 and Figure 4).

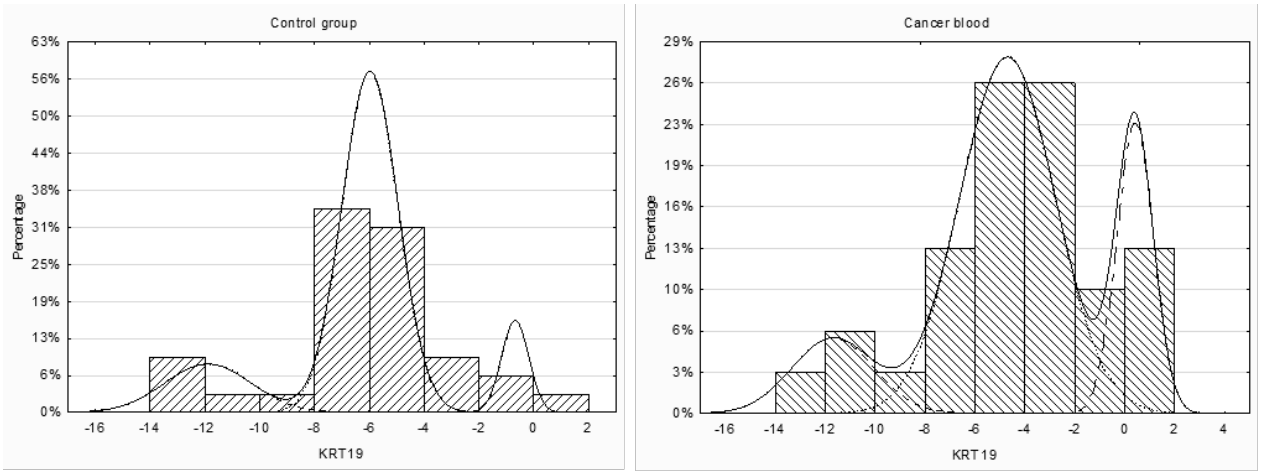

Figure 2: Relative expression level of *KRT19* in blood of healthy volunteers and cancer patients. Results obtained in RT-qPCR assays were log-transformed using the logarithm to the base 10 to achieve normal distribution of data. The use of maximal likelihood method allowed distinguishing three normal distributions of *KRT19* expression results in both the control (left plot) and cancer patients (right plot) group.

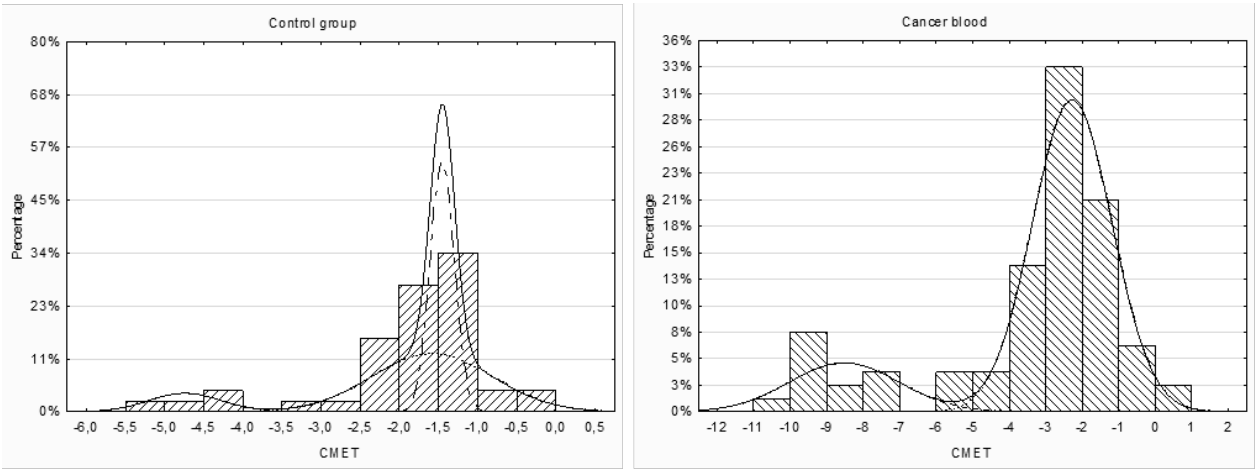

Figure 3: Relative expression level of MET in blood of healthy volunteers and cancer patients. Results obtained in RT-qPCR assays were log transformed using the logarithm to the base 10 to achieve normal distribution of data. The use of maximal likelihood method allowed distinguishing three and two subpopulations with normal distributions of MET expression results in blood of healthy volunteers (left plot) and cancer patients (right plot), respectively.

Discussion

When spreading tumor cells are found in peripheral blood they are referred to as circulating tumor cells (CTC) [2]. CTC may be present not only in pre-operative state but also in patients who underwent complete removal of the primary tumor. These cells could be responsible for the later development of *Metasis* [2,3]. Thus, detection of CTC in peripheral blood may be important for both diagnosis and treatment of cancer patients [5,29-31].

The most widely used assays for CTC identification are *mRNA-based strategies* targeting specific mRNAs. Especially multimarker reverse transcription polymerase chain reaction assays have been successfully used to detect micrometasis in blood and tissues of cancer patients [5,8,10]. Therefore in this study four different mRNA markers: human chorionic gonadotropin beta subunit (*CGB*), gonadotropin releasing hormone type 1 (*GNRH1*), hepatocyte growth factor receptor (*MET*) and keratin 19 (*KRT19*) were combined into one diagnostic panel in order to assess their ability to detect tumor cells in peripheral blood of cancer patients.

The results of the study demonstrated that all analyzed genes: *CGB, MET, GNRH1* and *KRT19* may be active in peripheral blood of both cancer patients and healthy volunteers. The expression level of three genes – CGB, KRT19 and *MET* differed significantly between studied (non-subdivided) groups and was higher in case of cancer patient. The expression of studied genes was however heterogeneous and for that reason the model assuming presence of more than one normal distribution and conversions of RT-qPCR data were applied.

In case of *CGB* analysis the maximal likelihood method used fitted both patients and volunteers group into two subpopulations. Mean expression level of *CGB* found in cancer patients was over tenfold higher than the one noted in the control group. The increase of *CGB* expression levels may suggest the presence of CTC.

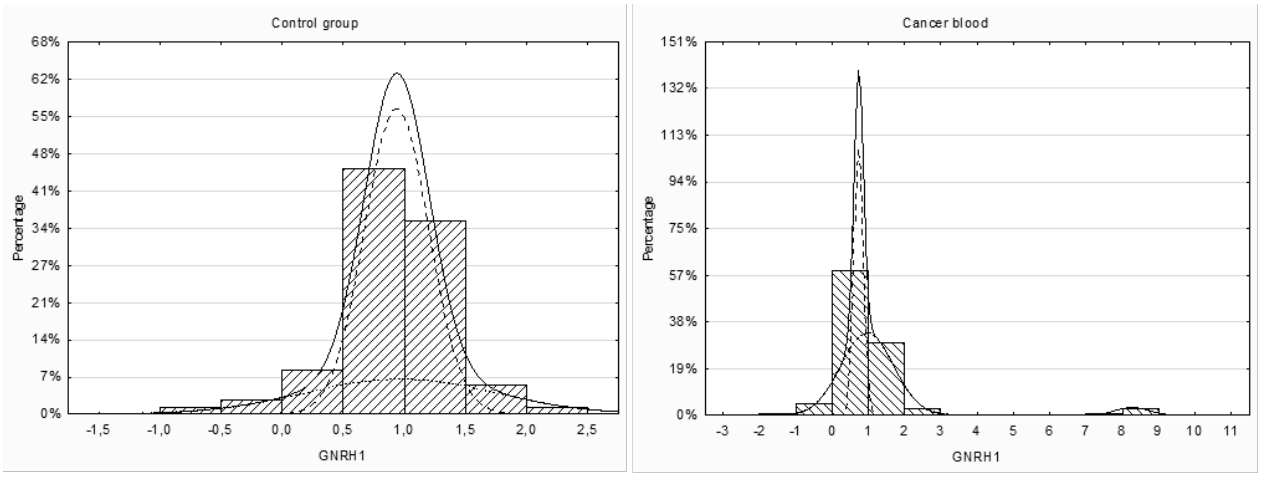

Figure 4: Relative expression level of *GNRH1* in blood of healthy volunteers and cancer patients. Results obtained in RT-qPCR were log-transformed using the logarithm to the base 10. The use of maximal likelihood method allowed distinguishing two normal distribution of *GNRH1* results in both healthy volunteers (left plot) and cancer patients (right plot) group.

Detailed analysis of *GNRH1* expression showed that the level of gene's transcripts in blood of cancer patients and healthy volunteers is similar. However in blood of cancer patients one of the three distinguished subpopulations showed the maximal *GNRH1* gene expression noted for all participants. Similarly, as in *CGB* expression evaluation, it could be presumed that high *GNRH1* gene activity may manifest the spread of cancer cells and can be useful in order to recognize CTC.

High level of *GNRH1* transcripts in cancer patients was not related to high *CGB* expression as it might have been expected. On the contrary weak negative correlation between *GNRH1* and *CGB* was found in the control group. Thus, it could be suggested that cancerogenesis is characterized by deregulation of *CGB* synthesis by *GNRH1*.

Previously we showed that assessment of CGB and GNRH1 expression level in blood may be useful for indicating the metastatic spread of tumor cells of gynecological cancers [11]. The results of the present work show that *CGB* and *GNRH1* genes activity characterizes also blood of patients with non-gynecological tumors of various origins. However, in the former study, the levels of these genes expression in gynecological cancer patients' blood were much higher than the ones observed in the control group. This allowed distinguishing a critical value of *CGB* expression, indicating the metastatic spread of tumor cells. In the present study a critical value for the studied genes could not have been established since the results of *CGB* and *GNRH1* expression in blood of cancer patients and healthy volunteers were partially overlapping.

Analysis of *MET* expression results demonstrated the presence of gene's transcripts in both cancer patients' and healthy volunteers' blood. Surprisingly the highest gene's expression was found in the subpopulation, comprising 42% of healthy volunteers.

Contradictory to our results *MET* receptor has been shown to be frequently overexpressed in a number of epithelial–derived tumor types. It is also overexpressed in stromal and interstitial cell–derived tumors [19,32]. Its expression has been associated with tumor progression whereas inhibition of *MET* signaling in orthotopic and xenografts mouse models has been shown to block primary mammary tumor growth and metastatic spread [33,34].

Study of *KRT19*expressionin cancer patients' and healthy volunteers' blood demonstrated that in both studied groups the results fit into the three normal distributions model. The subpopulations with the lowest and average gene expression levels were however overlapping. Still the maximal expression of *KRT19* distinguished cancer patients subpopulation only.

For molecular CTC detection most researchers turned to RT-PCR assays to target especially epithelium–specific or organ-specific mRNA species, such as cytokeratin 19 and 20. In fact, *KRT19* mRNA has been the most frequently used marker in clinical studies of breast cancer [23,24]. On the other hand false positivity in *KRT19* RT-PCR studies including: illegitimate transcription, haematological disorders, the presence of pseudogenes has been reported. It also has to be kept in mind that during tumor progression, a subpopulation of cancer cells of the primary tumor acquires a mesenchymal–like migratory phenotype [24,35] and during this so called epithelial–to–mesenchymal transition cells lose their epithelial properties because of EpCAM and cytokeratins downregulation [2,36,37].

Still the statistically significant correlation *between MET* and *KRT19* expression levels in *blood of cancer patients was found to be the strongest one in this study. Since correlation between these genes was not noted in the control group, it can be assumed* that *analysis of this relationship might be helpful in CTC identification.*

The fact that transcripts of all studied genes were found in blood of healthy volunteers may be surprising, however it was documented previously that many genes including *KRT18, KRT19, KRT20, MUC1, PSA*, and *CEA* are also expressed at low levels in normal blood. Therefore RT-qPCR assays with validated cut-off values are required

to overcome this problem [6,23,38]. Still, quantification of markers present in both tumor and non-tumor cells allowing the discrimination of authentic CTC may be a challenge. Even though the results of this study demonstrated that expression level of three genes – *CGB, KRT19* and *MET* differs significantly between studied groups and is higher in case of cancer patients, a cut-off value was not established. It was related to the fact that in many cases the expression level of analyzed genes in blood of healthy volunteers and cancer patients was similar. Since in the present study sequence specific primers and hydrolysis probes were used, the possibility of obtaining false-positive results in RT-qPCR experiments can be excluded. The level of analyzed genes' transcriptsnoted in healthy volunteers' blood is probably related to high sensitivity of real time PCR, which allows detection of a single copy of a gene transcript in cells. In fact, as shown by many other investigators, most of molecular targets used in RT-PCR assays lack the requisite specificity due to illegitimate expression of the analyzed genes in non-target hematopoietic cells [28].

The results of the present study demonstrated that even a multimarker system does not eliminate problems associated with tumor heterogeneity, clonal selection, as well as variable expression of individual mRNA markers during tumor progression. The raw data, obtained during analyses of *CGB, GNRH1, MET* and *KRT19* were log-transformed and were fitted to multiplied normal distribution model. This proved that expression levels of analyzed genes were heterogeneous. In all cases more than one population was distinguished within both cancer patients and healthy volunteers group.

CTC detection based on the expression of specific markers is believed to be a valuable tool for detection of tumor spreading in cancer patients, but as our study shows it requires using both specific markers and detailed statistical analysis. Even though, for certain types of tumors of the same origin, RT-qPCR assay based on specific mRNA might improve diagnosis and treatment of cancer patients it cannot serve as the only method of cancer cells spread detection. Thus, RT-PCR techniques are still not applicable in clinical practice. Further research which will help to develop newmethodsdetecting tumor cancer cells circulating in blood is needed to characterize and validate CTC as important markers of an ongoing cancerogenesis.

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