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CYFRA 21-1 in Non-Small Cell Lung Cancer – Standardisation and Application during Diagnosis

Radomir Pavićević[1,2], Gordana Bubanović[1], Ana Franjević[1], Dinko Stančić-Rokotov[2] and Miroslav Samaržija[3]

[1] Cancer Genetics Laboratory, Primary Reference Laboratory for Clinical Application of Lung Tumour Markers, University Hospital for Pulmonary Diseases »Jordanovac«, Zagreb, Croatia
[2] Clinic of Thoracic Surgery, University Hospital for Pulmonary Diseases »Jordanovac«, Zagreb, Croatia
[3] Pulmonary Clinic, University Hospital for Pulmonary Diseases »Jordanovac«, Zagreb, Croatia

A B S T R A C T

There is no ideal tumour marker at present. The clinical application of CYFRA 21-1 is possible once a thorough standardisation process is carried out. Standardisation is achieved by determining the reference range in asymptomatic population, benign and malignant lung diseases, and benign and malignant diseases of other organs. Furthermore, it depends on knowledge of research population characteristics, patient medical histories and individual diagnostic procedure results, the size of research target samples and the clinically defined control groups. The cut-off level of CYFRA 21-1 for non-small cell lung cancer (NSCLC) is 1.72 ng/mL in the Croatian population. It is based on the clinically applicable sensitivity of 78% and specificity of 95% in benign lung diseases. The cut-off value is verified by clinical findings. For clinicians the level of CYFRA 21-1 is an early sign of NSCLC in relation to all the benign lung diseases and all the benign diseases of other organs, of which it was confirmed that they can influence the above level, provided that NSCLC is verified using standard diagnostic methods. The level of CYFRA 21-1 is also influenced by the time of sampling in relation to other diagnostic invasive procedures. The marker is clinically applicable if clinical findings verify it; otherwise, it is useless. This research has involved 343 healthy persons, 474 patients with a benign disease and 4440 patients with a malignant disease, 2453 of whom suffer from NSCLC. The sensitivity of CYFRA 21-1 in NSCLC is 78%, in squamous cell lung cancer (SQC) 84.6%, in adenocarcinomas (AD) 74.3% and in large cell lung cancer (LCC) 75.3%. The level of CYFRA 21-1 differs significantly between healthy persons, benign and malignant diseases ($p<10^{-3}$). There are differences between the three histological types of NSCLC ($p<10^{-6}$) and according to T and N ($p<10^{-3}$). The level of CYFRA 21-1 prompts clinicians to repeat the clinical procedure during diagnosis, and helps to detect the disease earlier and implement treatment in NSCLC. We have achieved high concordance between marker findings and clinical diagnostic.

Key words: *CYFRA 21-1, tumour marker, standardisation, non-small cell lung cancer, contribution to diagnosis*

Introduction

In the last few decades lung cancer has become the leading cause of death from cancer by comparison with breast, pancreas, prostate and colon cancer taken together.[1] Moreover, almost 90% of lung tumours are carcinomas. The survival rate generally depends on individual histological types, the stage of the disease and individual treatment durations. Lung cancer incidence data indicates that small cell lung cancer (SCLC) comprises approximately 20% of cases, large cell lung cancer (LCC) and non-differentiated carcinomas 9%, and that the rate of both squamous cell lung cancer (SQC) and adenocarcinomas (AD) is gender-related. More specifically, 44% of lung cancers in males are SQC, while 28% are AD. In females, however, 25% are SQC and 42% AD[2]. The latest data published in 2005 reveal 2988 newly diagnosed cases of lung cancer in the Republic of Croatia, of which 2329 are diagnosed in males and 659 in females of all ages[3]. According to the International Agency for Research of Cancer (IARC) which compares the mortality rates in fifty countries, in 2002 Croatia's lung cancer mor-

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tality rate amounted to 65.3 per 100.000 in males (fourth place) and 9.7 per 100.000 in females[4]. Our experience shows that the percentage of AD has been growing in the last few years.

Modern approaches to diagnostics and treatment (e.g. surgery, oncological treatments and immunotherapy) have not stopped the disease in the last decades, although they do remain the patients' only support. Today's clinical practice is strengthened by insights from the field of molecular biology; namely, patients of the same gender and age group, exposed to the same etiological factors and suffering from the same histological type of tumour in the same stage have completely different disease courses and survival rates. Furthermore, research into DNA mutations implies various types of changes in multiple loci[5,6]. Lung cancer is both a polygenetically determined disease and a multifactorial disorder[7–10].

Tumour markers are the product of tumourous or normal cells, which are used to study their biological characteristics.

Human variation is the interaction of genetic and ecological factors. Genetic studies of complex traits enable researchers to collect specific data on human variability within the framework of biomedical studies, and data on complex phenotypes[11]. Furthermore, such studies contribute to the quality of population specific databases with referential values necessary for direct medical care. In 1997 an interdisciplinary team of clinicians and molecular biologists was formed in the Cancer Genetics Laboratory of the Jordanovac University Hospital for Pulmonary Diseases in Zagreb, Croatia. Their mission was to make molecular knowledge of lung tumour characteristics clinically applicable. Their first task was to introduce tumour markers to lung cancer treatment as a clinical procedure equal to the others. The then available data of other studies indicated that CYFRA 21-1 was a useful marker in NSCLC[12–19]. The application of the cut-off value suggested by the producer failed to produce a clinically satisfactory sensitivity in the Croatian population. This, obviously, required the standardisation of markers because determining the reference range is based on diagnostic sensitivity and specificity in respect of the medical conditions of analysis, the available diagnostic data on patients, total sample size,[20] population characteristics and the differences between well defined groups.

Cytokeratins are the building units of the cytoskeleton that remain intact during the transformation of normal cells into tumourous cells, which allows their use as tumour markers[21]. Their biological activity changes in tumour cells. Namely, modifications, such as phosphorylation, glycosylation and transglutamination, start affecting the N- and C- terminal parts of protein molecules, and lead to the increase in the solubility and reorganisation of their filaments, which includes the process of apoptosis[22–24]. The significance of apoptosis in the degradation of cytokeratins and the origin of soluble fragments in circulation remains unclear at this point. Cytokeratins are insoluble in the cytoskeleton while soluble in circulation, where they are detectable as partially degraded fragments of individual proteins that make up small complexes – apoptotic bodies[25] or they become large polymeric protein complexes amplifying apoptotic signals.[26] Cytokeratin half-life in circulation ranges between 10 and 15 hours depending on the size of fragments. On the other hand, the half-life of cytokeratin 19 is 12 hours. The most frequent mechanisms that participate in the emergence of soluble cytokeratins are proteolytic degradation of cytokeratins in dying cells, abnormal mitosis, the overproduction of monomeric cytokeratin polypeptides from proliferating cells, apoptosis and/or neovascularisation[27].

The aim of this study was to standardise the CYFRA 21-1 marker in Croatian population, which would help clinicians detect NSCLC earlier and in differential diagnosis.

Subjects and Methods

Between December 1997 and June 2007 CYFRA 21-1 levels in the blood of over 27000 patients were measured in the Cancer Genetics Laboratory of the Jordanovac University Hospital for Pulmonary Diseases in Zagreb. This research involved 4914 patients and 343 healthy individuals. The patient samples were collected during standard clinical diagnostics preceding treatment, while strictly adhering to the ethical principles of medical research. The subjects were sorted into 27 groups. There were 2381 patients with NSCLC (Table 1), of whom 2050 (stages IA–IIIAN2) were surgically treated after diagnosis, including stage IIIA patients receiving neoadjuvant therapy. The NSCLC group comprises three histological types of lung cancer – SQC, AD and LCC – and 72 patients with non-differentiated NSCLC, used in the construction of the ROC curve. There were 80 carcinoid tumours, 169 SCLC, 474 patients with benign diseases and 1738 patients with malignant diseases of other organs (Table 2). The healthy individuals had no proof of suffering either from a benign or malignant disease.

All NSCLC patients underwent radiological analysis, computed tomography (CT) of the thorax and abdomen, while patients with an AD larger than 1 cm also underwent a CT scan of the head and magnetic resonance imaging (MRI) if judged necessary. Furthermore, in bronchoscopy, catheter aspirations of the upper airway were performed to obtain antibiograms, as well as brushing and tumour biopsy or transbronchial aspirations. A small number of subjects underwent transthoracic needle biopsy due to the above methods having been insufficient. The nodal status (N) was determined in 85% of patients using CT. With the purpose of assessing N2 (>1.5 cm) in the last few years mediastinoscopy and video-assisted thoracoscopy (VATS) were also performed, with which operability was also assessed. In 7% of patients with ground glass opacities (GGOs) the diagnosis of primary lung cancer was made by means of diagnostic minithoracotomy performed simultaneously with the surgical procedure. The assessment of bone metastases in the advanced stages of the disease was done by scinthigraphy. The

TABLE 1
LUNG CANCER PATIENT CHARACTERISTICS

Variables		Number (%)		
Gender	Males	1784 (76.4)		
	Females	597 (23.6)		
Age (mean years ± SD)	Males	65 ± 9		
	Females	63 ± 10		
		SQC	AD	LCC
Stage and tumour histology	IA	116 (9.3)	160 (17.2)	10 (4.9)
	IB	562 (44.9)	366 (39.4)	68 (33.7)
	IIBT2	83 (6.6)	60 (6.5)	20 (9.9)
	IIBT3	92 (7.4)	62 (6.7)	23 (11.4)
	IIIAN1	34 (2.7)	18 (1.9)	9 (4.5)
	IIIAN2	182 (14.6)	149 (16)	36 (17.8)
	IIIB	46 (3.7)	34 (3.7)	6 (2.9)
	IV	135 (10.8)	80 (8.6)	30 (14.8)
	All	1250 (100)	929 (100)	202 (100)
Surgery	BID	154 (7.5)		
	BSD	64 (3.1)		
	LID	299 (14.6)		
	LIS	453 (22.1)		
	LSD	373 (18.2)		
	LSS	380 (18.5)		
	PD	111 (5.4)		
	PS	173 (8.4)		
	RLM	43 (2.1)		
	All	2050 (100)		

histological classification of lung tumours was carried out according to the current tumour classification of the World Health Organization (WHO) and the International Association for the Study of Lung Cancer (IASLC).[2,28] The surgical pathologic staging (pTNM) was done after examining the total removed tissue according to the TNM classification of malignant tumours,[29] and following the guidelines of the Union Internationale Contre le Cancre and the American Joint Committee on Cancer.[30,31]

From December 1997 to June 2000 the analysis was first conducted on the ES-300 system by enzyme-linked immunoassay using the commercial kit Enzymun-test CYFRA 21-1 (Boehringer Mannheim GmbH, Germany). CYFRA 21-1 was then measured on the Elecsys 2010 system by electrochemiluminescence immunoassay (Roche Diagnostics, Germany). In doing the analyses, the manufacturers' instructions and recommendations were strictly followed. Joining the results obtained through the two methods is considered methodologically correct because the results correlate once compared.

The cut-off value was determined using the ROC curve with the help of MedCalc v. 9.2.1.0 (MedCalc Software, Belgium). The statistical analysis was conducted with the help of Statistica 6.0 software package (Statsoft, USA). Since marker levels were not distributed normally, the results are presented as medians and 25th and 75th percentiles. In addition, nonparametric methods were also used, and the differences are considered significant if $p<0.05$. For the purpose of analysing the differences between two independent groups the Mann-Whitney U-test was used, while for more than two independent groups the Kruskal-Wallis ANOVA was used. For the separation of NSCLC in solitary lung nodules and metastases a discriminant analysis was employed.

Results

The sensitivity and specificity of CYFRA 21-1 were determined on the basis of the level measured while suspecting lung cancer or making diagnosis in 2990 subjects from the reference population consisting of 343 healthy subjects, 164 patients with benign diseases of the lung, and 2453 NSCLC patients (1250 SQC, 929 AD, 202 LCC and 72 non-differentiated NSCLC). With the aim of ultimately determining the optimal upper level of the reference range (cut-off) the ROC curve was used. In healthy

TABLE 2
CYFRA 21-1 LEVEL IN PATIENTS WITH BENIGN AND MALIGNANT DISEASES OTHER THAN SQUAMOUS CELL LUNG CARCINOMAS, LUNG ADENOCARCINOMAS AND LARGE CELL LUNG CARCINOMAS

Group	N	Median (ng/mL)	25th	75th	95th
Healthy persons	343	0.88	0.69	1.21	1.49
Benign diseases of the lung	164	1.12	0.77	1.39	1.72
Benign diseases of other organs	30	1.60	1.02	1.60	2.10
Pleural empyema	51	1.15	0.80	1.62	4.32
Benign lung and mediastinal tumours	153	1.19	0.83	1.42	1.91
Benign breast diseases	31	0.88	0.74	1.01	1.59
Severe septic condition	45	1.98	1.46	4.88	10.07
Larynx cancer	161	1.98	1.49	4.43	8.61
Urinary bladder cancer	132	0.91	0.77	3.73	6.43
Cancer of the prostate	64	1.42	1.12	1.58	2.78
Renal cancer	112	1.23	0.94	1.54	1.89
Colon cancer	102	1.23	1.06	1.45	1.81
Sarcoma	33	1.20	1.01	1.34	2.06
Melanoma	31	1.29	0.89	1.72	2.21
Seminoma	150	1.08	0.77	1.43	1.95
Thyroid carcinoma	51	1.43	1.06	2.23	3.01
Cancer of the ovary and uterus	100	1.59	1.12	1.94	3.43
Breast cancer	102	2.25	1.11	3.15	12.51
Esophageal cancer	98	1.86	1.32	2.74	12.84
Mesothelioma	37	1.66	1.12	2.73	17.37
Mediastinal cancer	59	1.29	1.03	1.59	3.65
Metastases to the lung	289	1.10	0.81	1.36	1.63
Lymphoproliferative tumours	217	1.00	0.74	1.33	1.72
Carcinoid tumour	80	1.22	0.95	1.78	4.34
Small cell lung cancer	169	2.64	1.56	4.23	16.68
Undifferentiated NSCLC	72	2.75	1.62	4.46	18.50

NSCLC – non-small cell lung cancer, 25th – 25th percentile, 75th – 75th percentile, 95th – 95th percentile

individuals, the CYFRA 21-1 median was 0.88 ng/mL and the 95th percentile 1.49 ng/mL, while in benign lung diseases the median was 1.12 ng/mL and the 95th percentile 1.72 ng/mL. Subjects with benign diseases of the lung were used for the calculation of the specificity in relation to subjects with NSCLC who were used for determining the sensitivity. Figures 1a–f presents the values of the ROC curves of each groups. The cut-off value of CYFRA 21-1 in NSCLC of 1.72 ng/mL was selected at 95% of the specificity of benign lung diseases. In other words, this means that it distinguishes healthy individuals and those with a benign lung disease from subjects who suffer from a malignant lung disease well. The sensitivity of CYFRA 21-1 in NSCLC was 78%, 84.6% in SQC, 74.3% in AD and 75.3% in LCC. According to the disease stages and overall, the highest sensitivity was observed in SQC patients (Figure 2).

The difference between two measurements of CYFRA 21-1 during diagnostics (3–9 weeks) of NSCLC possesses a much greater discriminatory power than single measurements. It is possible to separate NSCLC from benign lung lesions, lung metastases and Hodgkin's disease with an accuracy of 91% using the second measurement of CYFRA 21-1 and the difference between the two measurements. A canonical analysis was also carried out, with the help of which the discriminant functions were obtained (Eigen value=0.8223, canonical R=0.6718, Wilks lambda=0.5487, $X^2=256.859$, df=2, $p=1\times10^{-4}$). The discriminant function of the difference between the two measurements was –0.9756 and of the second measurement of CYFRA 21-1 –0.8181. This suggests that the difference between the two measurements is highly significant, as well as that the contribution of the second measurement is substantial.

The serum level of CYFRA 21-1 differs significantly between all the 26 groups of patients and the group of healthy individuals (Kruskal-Wallis ANOVA, $p<10^{-3}$). The highest median was observed in NSCLC (3.08 ng/mL) and in non-differentiated NSCLC (2.75 ng/mL), followed by SCLC (2.64 ng/mL). In benign diseases the median ranges between 0.87 ng/mL and 1.19 ng/mL with the exception of 1.98 ng/mL in severe septic conditions. In the groups of other malignant diseases the median values of CYFRA 21-1 range between 0.91 ng/mL in urinary

bladder cancer and 2.25 ng/mL in breast cancer (Table 2). The median value of CYFRA 21-1 in healthy subjects is 0.88 ng/mL without any significant difference between males and females.

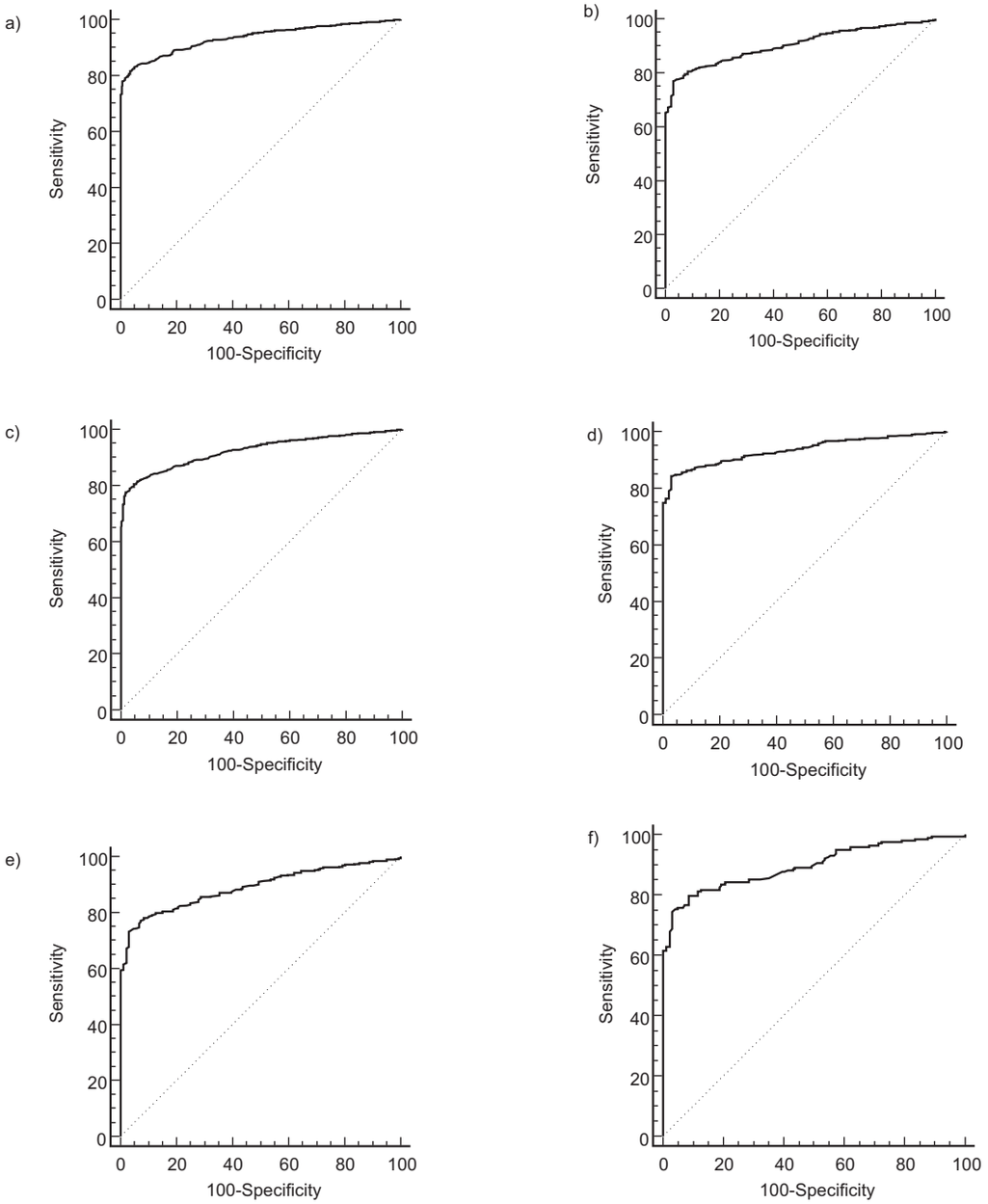

Fig. 1. Receiver operating characteristics (ROC) curves comparing the level of CYFRA 21-1 in persons without malignant diseases(specificity) and non-small cell lung carcinoma patients (sensitivity). a) Healthy and non-small cell lung carcinoma patients (n=343, 2453). The area under the ROC curve was 0.935, standard error 0.005, 95% confidence interval 0.925 to 0.943, significance level p (area=0.5) 0.0001. b) Patients with benign diseases of the lung and non-small cell lung carcinoma patients (n=164, 2453). The area under the ROC curve was 0.903, standard error 0.008, 95% confidence interval 0.891 to 0.914, significance level p (area=0.5) 0.0001. c) Healthy, patients with benign diseases of the lung (n=507) and non-small cell lung carcinoma patients (n=2453). The area under the ROC curve was 0.924, standard error 0.005, 95% confidence interval 0.914 to 0.934, significance level p (area=0.5) 0.0001. d) Patients with benign diseases of the lung and squamous cell lung carcinoma (n=164,1250). The area under the ROC curve was 0.934, standard error 0.007, 95% confidence interval 0.920 to 0.947, significance level p (area=0.5) 0.0001. e) Patients with benign diseases of the lung and lung adenocarcinoma (n=164,929). The area under the ROC curve was 0.889, standard error 0.010, 95% confidence interval 0.869 to 0.900, significance level p (area=0.5) 0.0001. f) Patients with benign diseases of the lung and large cell lung carcinoma (n=164,202).The area under the ROC curve was 0.897, standard error 0.016, 95% confidence interval 0.861 to 0.926, significance level p (area=0.5) 0.0001.

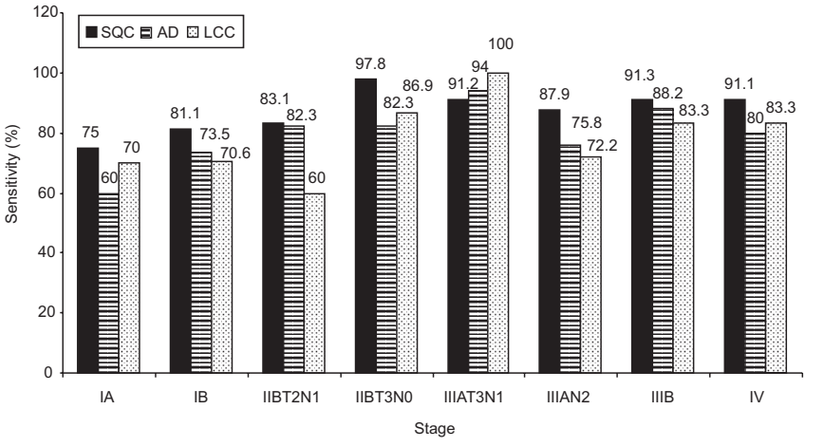

Fig. 2. Sensitivity of CYFRA 21-1 by histological type and stage of non-small cell lung cancer. SQC – squamous cell carcinomas, AD – adenocarcinomas, LCC – large cell carcinomas.

During diagnostics the median and interquartile range of CYFRA 21-1 in SQC were 3.86 ng/mL and 2.16–10.29 ng/mL, in AD 2.51 ng/mL and 1.70–4.48 ng/mL, and in LCC 2.77 ng/mL and 1.74–5.80 ng/mL. CYFRA 21-1 levels differ between the various histological types of lung cancer (Kruskal-Wallis ANOVA, $p<10^{-3}$), with values in SQC higher than in AD and LCC. Moreover, there are significant differences between SQC and AD, as well as between SQC and LCC (Mann-Whitney U-test, $p<10^{-6}$, $p=1\times10^{-6}$), but there are no differences between AD and LCC. CYFRA 21-1 median values and interquartile ranges increase according to stage in SQC, AD and LCC (Table 3). According to Kruskal-Wallis ANOVA, the level increase depending on stage is significant (SQC and AD $p<10^{-3}$, LCC $p<10^{-4}$) (Figures 3–5). This difference is more pronounced between stages IA and IB in all the groups in respect of the other stages. Finally, the difference between all the stages is greatest in SQC (Table 4).

The level of CYFRA 21-1 differs significantly according to the T and N stages (from the TNM classification) between SQC, AD and LCC (Kruskal-Wallis ANOVA, $p<10^{-3}$) (Table 5).

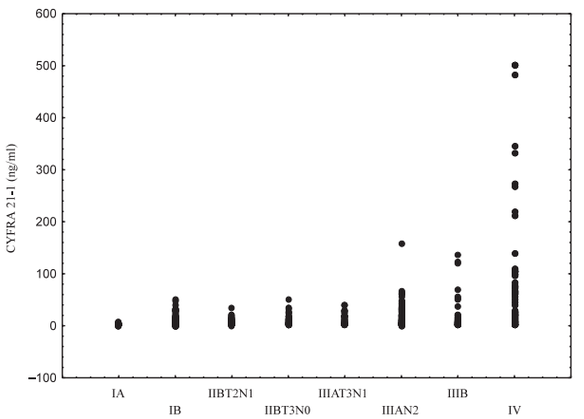

Fig. 3. CYFRA 21-1 serum level distribution in 1250 patients with squamous cell lung carcinoma by stage (Kruskal-Wallis ANOVA, $p<10^{-3}$).

TABLE 3
CYFRA 21-1 DISTRIBUTION IN NON-SMALL CELL LUNG CANCER

	SQC				AD				LCC			
Stage	N	Median (ng/mL)	25th	75th	N	Median (ng/mL)	25th	75th	N	Median (ng/mL)	25th	75th
IA	116	2.11	1.69	2.59	160	1.85	1.39	2.19	10	2.35	1.72	3.3
IB	562	3.3	1.94	5.4	366	2.48	1.68	3.43	68	2.06	1.55	3.13
IIBT2N1	83	4.36	2.05	8.66	60	3.39	2.15	5.12	20	2.47	1.38	4.09
IIBT3N0	92	6.18	3.52	11.68	62	3.35	2.14	7.68	23	3.21	1.97	6.59
IIIAT3N1	34	9.36	3.76	18.13	18	7.94	2.78	13.4	9	3.03	2.37	9.4
IIIAN2	182	6.18	2.74	17.2	149	3.5	1.82	9.17	36	2.92	1.65	5.19
IIIB	46	6.64	2.53	15.31	34	5.24	2.49	12.82	6	3.13	2.83	6.68
IV	135	39.8	11.05	98.7	80	6.5	2	27.54	30	8.55	4.5	32.34

SQC – squamous cell carcinomas, AD – adenocarcinomas, LCC – large cell carcinomas, 25th – 25 th percentile, 75 th – 75 th percentile

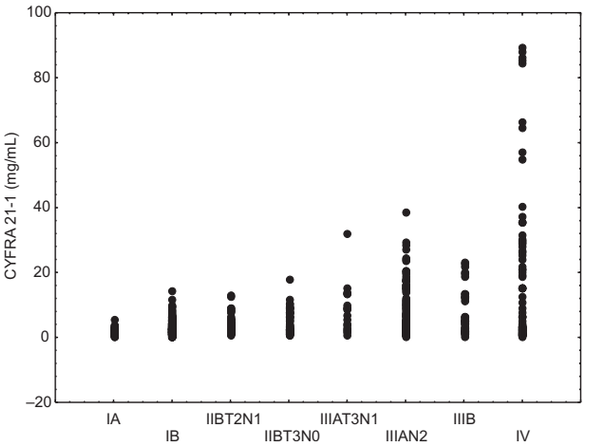

Fig. 4. CYFRA 21-1 serum level distribution in 929 patients with lung adenocarcinoma by stage (Kruskal-Wallis ANOVA, $p<10^{-3}$).

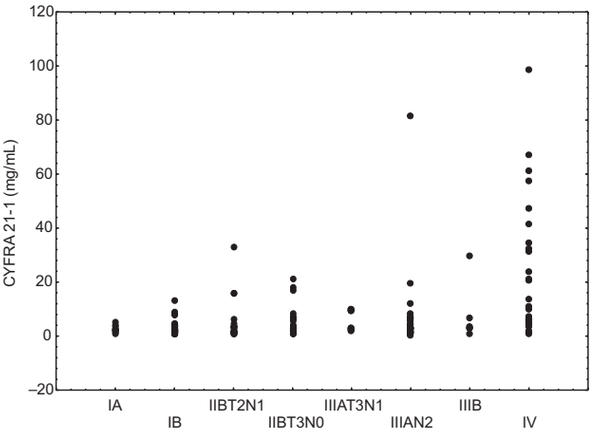

Fig. 5. CYFRA 21-1 serum level distribution in 202 patients with large cell lung carcinoma by stage (Kruskal-Wallis ANOVA, $p<10^{-4}$).

TABLE 4
STATISTICAL DIFFEENCE OF CYFRA 21-1 LEVELS BETWEEN THE STAGES OF SQUAMOUS CELL LUNG CARCINOMAS, LUNG ADENOCARCINOMAS AND LARGE CELL LUNG CARCINOMAS TESTED BY MANN-WHITNEY U-TEST

Stage	Stage	p SQC	p AD	p LCC
IA	IB	$<10^{-6}$	$<10^{-6}$	NS
	IIBT2N1	$<10^{-6}$	$<10^{-6}$	NS
	IIBT3N0	$<10^{-6}$	$<10^{-6}$	NS
	IIIAT3N1	$<10^{-6}$	$<10^{-6}$	NS
	IIIAN2	$<10^{-6}$	$<10^{-6}$	NS
	IIIB	$<10^{-6}$	$<10^{-6}$	NS
	IV	$<10^{-6}$	$<10^{-6}$	1.6×10^{-3}
IB	IIBT2N1	1.5×10^{-2}	1.3×10^{-4}	NS
	IIBT3N0	$<10^{-6}$	1.5×10^{-4}	9.4×10^{-3}
	IIIAT3N1	1×10^{-6}	2.3×10^{-5}	5.3×10^{-3}
	IIIAN2	$<10^{-6}$	$<10^{-6}$	2.6×10^{-2}
	IIIB	7.8×10^{-5}	2×10^{-6}	NS
	IV	$<10^{-2}$	$<10^{-6}$	$<10^{-6}$
IIBT2N1	IIBT3N0	4.4×10^{-3}	NS	NS
	IIIAT3N1	1.4×10^{-3}	8.6×10^{-3}	NS
	IIIAN2	3×10^{-3}	NS	NS
	IIIB	4×10^{-2}	1.7×10^{-2}	NS
	IV	$<10^{-6}$	1×10^{-2}	1×10^{-3}
IIBT3N0	IIIAT3N1	NS	2.4×10^{-2}	NS
	IIIAN2	NS	NS	NS
	IIIB	NS	4.9×10^{-2}	NS
	IV	$<10^{-6}$	8.4×10^{-3}	7.1×10^{-3}
IIIAT3N1	IIIAN2	NS	NS	NS
	IIIB	NS	NS	NS
	IV	6×10^{-6}	NS	NS
IIIAN2	IIIB	NS	NS	NS
	IV	$<10^{-6}$	5.3×10^{-3}	3.7×10^{-4}
IIIB	IV	1×10^{-6}	NS	NS

SQC – squamous cell carcinomas, AD – adenocarcinomas and LCC – large cell carcinomas, NS – nonsignificant

TABLE 5
STATISTICAL DIFFEENCE OF CYFRA 21-1 LEVELS BETWEEN THE T AND N FROM TNM CLASSIFICATION IN SQUAMOUS CELL LUNG CARCINOMAS, LUNG ADENOCARCINOMAS AND LARGE CELL LUNG CARCINOMAS TESTED BY MANN-WHITNEY U-TEST

T/N	T/N	p SQC	p AD	p LCC
T1	T2	$<10^{-6}$	$<10^{-6}$	NS
T1	T3	$<10^{-6}$	$<10^{-6}$	1.9×10^{-2}
T1	T4	$<10^{-6}$	$<10^{-6}$	NS
T2	T3	$<10^{-6}$	$<10^{-6}$	3.8×10^{-5}
T2	T4	$<10^{-6}$	$<10^{-6}$	NS
T3	T4	NS	$<10^{-6}$	NS
N0	N1	$<10^{-6}$	$<10^{-6}$	1.4×10^{-4}
N0	N2	$<10^{-2}$	$<10^{-6}$	2.1×10^{-3}
N0	N3	$<10^{-6}$	4×10^{-2}	$<10^{-2}$
N1	N2	2×10^{-6}	4.6×10^{-3}	NS
N1	N3	7.4×10^{-5}	NS	$<10^{-2}$
N2	N3	2.2×10^{-2}	NS	$<10^{-2}$

NS – nonsignificant, SQC – squamous cell carcinomas, AD – adenocarcinomas, LCC – large cell carcinomas

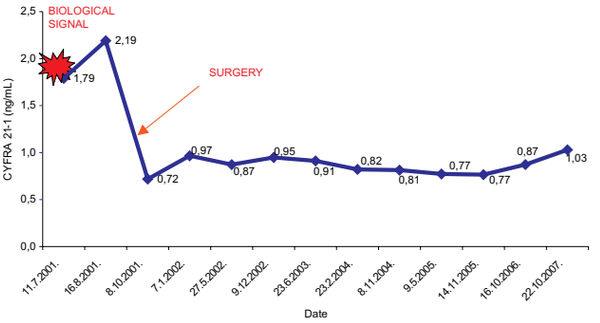

Fig. 6. CYFRA 21-1 level in the longitudinal follow-up of a patient with squamous cell lung carcinoma, stage IB T2N0M0. CYFRA 21-1 level during diagnosis above the referral range of the population suggesting NSCLC (biological signal). This is an example of complete response to therapy and a stable disease.

Discussion

Tumour markers are applicable in clinical practice if they contribute to: risk assessment, early detection, diagnosis, disease monitoring, prognosis, the evaluation of patients' response to therapy, and the prediction of the course of the disease. If a number of the above traits are clinically confirmed, tumour markers can become part of clinical procedure. Markers provide insight into the course of the disease based on their concentration or the number and type of genetic changes detected. As such, they help clinicians to make a diagnosis much earlier and, thus, select the most appropriate modality of treatment. They are not to be viewed as a self-sufficient clinical diagnostic analysis, although their import for the improvement of diagnostics and currently available thera-

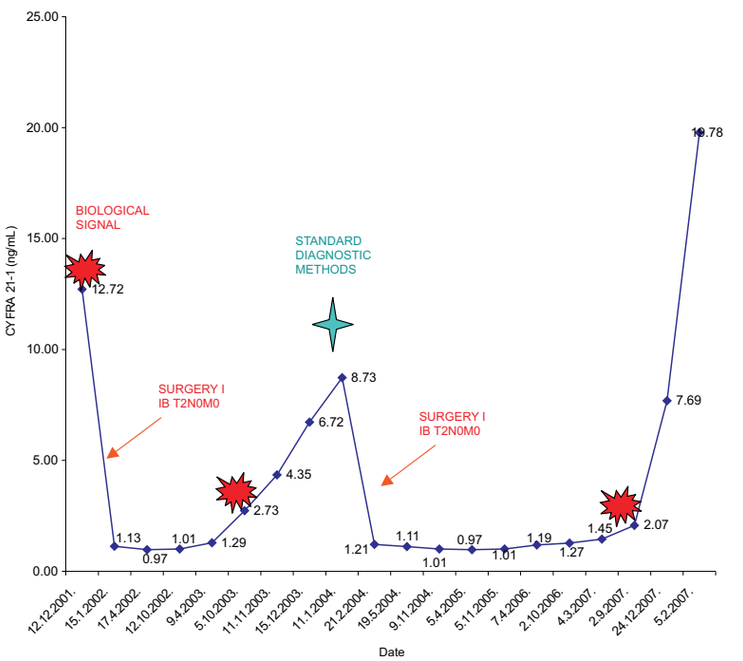

Fig. 7. CYFRA 21-1 level in the longitudinal follow-up of a patient with squamous cell lung carcinoma, stage IB T2N0M0. CYFRA 21-1 level during diagnosis above the referral range of the population suggesting NSCLC (biological signal). In this longitudinal follow-up, the level of CYFRA 21-1 suggest a local relapse 3 months prior the standard clinical methods. Both the operation of the primary tumour and reoperation were performed in the same stage of the disease due to the marker.

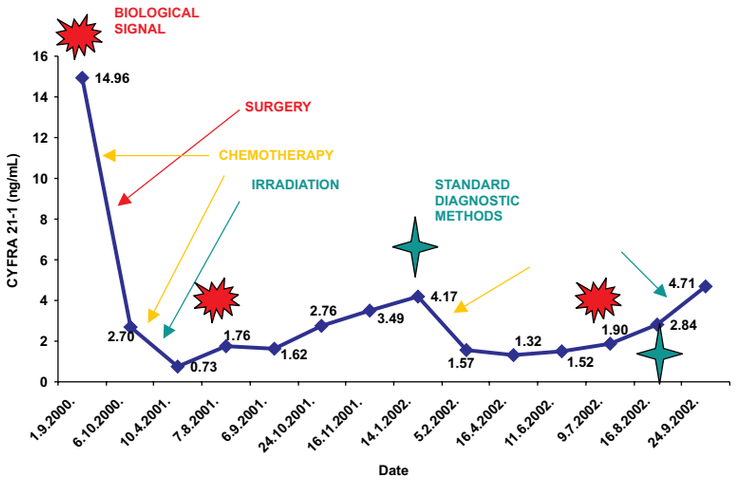

Fig. 8. CYFRA 21-1 level in the longitudinal follow-up of a patient with squamous cell lung carcinoma, stage IIIA T2N2M0, who underwent multimodal treatment. CYFRA 21-1 level during diagnosis above the referral range of the population suggesting advanced NSCLC (biological signal). In this longitudinal follow-up it suggests a local relapse prior to standard clinical method.

pies is tremendous. Marker levels are patient-specific and can be interpreted only with the help of clinical data. If interpreted well by a skilled clinician, markers have a diagnostic and prognostic significance, and can help to guide checkup times and therapy selection. With the purpose of applying markers in clinical practice, it is essential to define all population characteristics that either do or may potentially influence their level or dynamics in healthy individuals, and the sample size involved in the research, as well as to clinically define the control groups of primarily benign lung diseases that are expected to have a dynamics different from the dynamics of the healthy population. This is the basis for determining the reference range. Respecting this marker standardisation procedure, the first standardisation of serum markers for lung cancer in Croatia was done in 1998. Markers have been included in clinical procedure improving currently available treatment methods (diagnostics, surgery, oncology) ever since. Moreover, the approach to patients has been individualised through evaluating all the anamnestic and diagnostic parameters that can influence marker levels. They are defined on a marker referral (as part of standardised laboratory documentation). This allows an earlier diagnosis to be suggested based on the marker.

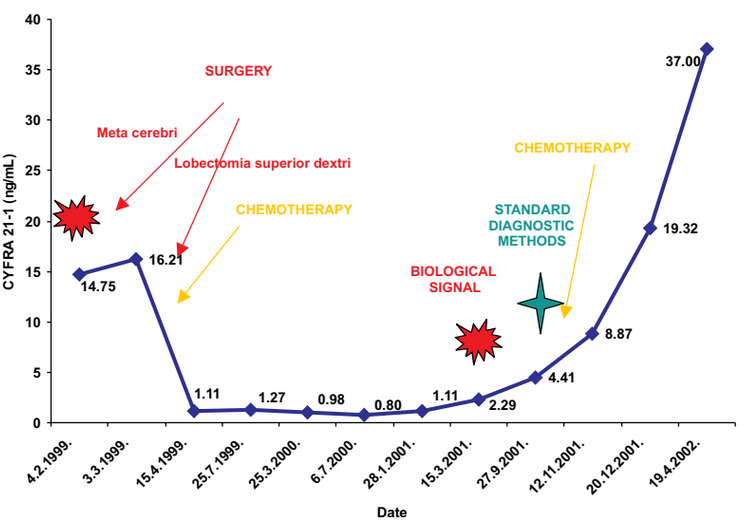

Fig. 9. CYFRA 21-1 level in the longitudinal follow-up of a patient with lung adenocarcinoma in stage IV T2N0M1 underwent a multimodal treatment. CYFRA 21-1 level during diagnosis above the referral range of the population suggesting advanced NSCLC (biological signal). The level of CYFRA 21-1 decreased to the reference range of the population only after the surgical removal of the primary lung cancer. The biological signal of CYFRA 21-1 in this longitudinal follow-up suggests a local relapse before standard clinical methods.

The selection of patients who require marker analysis is done by internists-pulmonologists; further marker measurements are recommended by laboratory physicians based on the initial results. Laboratory findings consist of a tumour marker value and an interpretation of the same assessing both the »biological activity of the tumour« and the patient's clinical status (i.e. stable disease, partial response to therapy and progressive disease). Being familiar with marker dynamics in the healthy population and subjects with benign pulmonary diseases and diseases of other organs has facilitated the elimination of the existing shortcomings of marker application in clinical practice.

The clinically applicable CYFRA 21-1 reference range of 0–1.72 ng/mL reflects the difference between the marker levels of the reference groups researched: healthy individuals, individuals with benign diseases of the lung that influence marker levels and in whom the absence of a malignant disease has been clinically confirmed, and patients with NSCLC, confirmed by standard clinical diagnostic methods. The reference group of benign pulmonary diseases in relation to NSCLC was defined and used with 95% specificity for determining the cut-off value of 1.72 ng/mL. This was continuously confirmed between 1998 and 2007[32–38]. In addition, higher sensitivity was achieved by the implementation of an individual approach to marker level interpretation. First and foremost, knowledge of the influence of population was considered in every patient. The second most important point is information on the patient either currently suffering or having previously suffered from a disease that can influence marker dynamics (such as renal insufficiency, sepsis, hepatic insufficiency, pleural empyema, hepatorenal syndrome, urinary bladder cancer, thyroid carcinoma, colon cancer, esophageal cancer, breast cancer, larynx cancer, etc.). The cut-off selected for the Croatian population enables clinicians to make an earlier diagnosis on the basis of repeating diagnostic procedures. This is further verified by our patient sample, most of whom are in stage I. This approach resulted in the standardisation of markers for lung cancer, its bone metastasis and mesothelioma: CYFRA 21-1, NSE, ProGRP, ICTP and SMRP[32–43]. Data interpretation in clinical laboratories is a systemic and comparative process of evaluating numerous influences and their measured value. The findings are a sum of results, but also a suggestion for further measurements and the decision-making process[20]. This is why our marker value interpretation closely corresponds to clinical tests and opinions. In other words, the tumour marker value before therapy contributes to earlier detection and diagnosis. Value dynamics in longitudinal follow-ups provides insight into the biological activities of the tumour in which the marker with its biological inception and/or degradation participates either with or without the influence of therapy (Figures 6–9). If there is no biological activity in the tumour, then there is no biological pathway that influences its level, which is, clinically, an indicator of the stability of the disease. This study shows the first part of the results of the ten years of the application of the CYFRA 21-1 tumour marker in suggesting diagnostics and earlier detection. In other words, the marker was not used as a self-sufficient diagnostic procedure.

The high sensitivity of CYFRA 21-1 in our results confirms its contribution to the diagnosis of lung cancer in general and NSCLC in particular. Similar results have also been reported by other authors[13,27,44–50]. In contrast to these authors, however, our study brings a NSCLC group much larger in number, type and tumour stage. So-far studies on the clinical contribution of CYFRA 21-1 vary in sensitivity and specificity depending on the selected cut-off level ranging between 1.5 ng/mL and 30 ng/mL[12–14,17,18,44,46,47,49,51–56].

From 1998 the cut-off was continuously controlled on a growing number of samples revealing its dynamics in other tumours and benign diseases, the dynamics of which differs from the dynamics of NSCLC. Besides the already known, more benign diseases have been included in the sample of which it has been discovered that they influence marker dynamics[13,27,57,58]. More than half of the patients are in stage I. Their CYFRA 21-1 sensitivity ranges between 60 and 81.1% and is in accord with our so-far results, which have clinically been verified[32–40]. The use of the cut-off of 1.72 ng/mL with 95% specificity facilitates the earlier detection of a larger number of NSCLCs. In order to analyse sensitivity, tumour size and extent, NSCLC patients were grouped according to the TNM classification (IA, IB, IIBT2N1, IIBT3N0, IIIAT3N1, IIIAN2, IIIB, IV). At present, there is no published study of such a detailed sensitivity analysis. There are some papers on some of the TNM stages varying in sensitivity, i.e. from 17% to 89%[12,16,49,52,54,59]. The lowest values are recorded in LCC and AD and the highest in SQC, which corresponds to the results of this study. What first explains the above variation in other authors is the following: small groups and NSCLC not being divided into subtypes or stages, i.e. a non-representative patient sample lacking some stages from I to IV, which are normally included in routine measurements of the NSCLC marker. The second important reason is the selected cut-off, which depends on the reference group within the reference population, most often including benign lung diseases. Typical specificity is then 95%[13,14,16,44,49,51–53,59–62]. Specificities range between 14% and 100% depending on the reference population[12,14,54,55,56]. The sensitivity and specificity results of this study have been confirmed by clinical diagnostic methods.

The marker has a diagnostic value only if it confirms the clinical diagnosis. This particularly applies to differential diagnosis and to distinguishing NSCLC from other diseases that conceal or coexist with it, or conceal lung metastases originating from other organs (e.g. pleural empyema, SCLC, SQC of the larynx, oesophagus and urinary bladder, and breast cancer). This is the greatest problem with serum markers. With this in mind, we have examined all the benign and malignant diseases that influence marker levels during either short or long periods. The CYFRA 21-1 cut-off for the population of Croatia is the result of a research of its levels in selectively chosen

groups with the aim to separate the diseases that either limit or influence its increased levels. For the purpose of determining the cut-off, subjects with benign pulmonary diseases and diseases of other organs as well as with NSCLC were included. Groups of subjects who at first exhibited a mild to moderate increase in their CYFRA 21-1 levels, which dropped either in repeated measurements (within 3 to 9 weeks) or during treatment (of hemoptysis, pulmonary pneumopathy with severe cough, empyema, severe extrinsic asthma, advanced COPD, septic conditions, advanced hepatorenal syndrome, etc.) were not included in the research. In addition, measurements taken during invasive procedures (i.e. bronchoscopy, oesophagoscopy, drainage, diagnostic VATS, mediastinoscopy and thoracotomy) were also excluded. Any invasive procedure on the bronchial tree leads to an increase in CYFRA 21-1 levels, which is the result of the injured epithelium's release that lasts 14 days at the most. For instance, in postpneumonic empyema the CYFRA 21-1 level is up to twenty times higher than the cut-off value before drainage and therapy; within a few days, following drainage and therapy, the CYFRA 21-1 level drops down to the referential range of the population. Similarly, in late postpneumonectomy empyema following lung cancer surgery, the CYFRA 21-1 level is again up to twenty times higher than the cut-off value, indicating a possible, yet inexistent relapse; after drainage and an antibiotic therapy it drops down to the referential range of the population within ten days. This is why the control groups of healthy individuals and subjects with benign pulmonary diseases were carefully selected, which is best substantiated by ROC curves, as well as by clinical confirmations of early diagnoses. The development of malignant diseases of other organs can result in an excessive release of cytokeratin 19 into the circulation. In some diseases the CYFRA 21-1 level is mildly increased, but clinically useless due primarily to an insufficient sensitivity and specificity. Nevertheless, it must not be neglected. Thus, measuring the CYFRA 21-1 level would be highly useful in SCLC, mesothelioma and particularly in larynx, oesophagus, urinary bladder and breast cancer, and endobronchial metastases originating from other organs. Research on its clinical applicability on a larger sample is currently being conducted. The above makes CYFRA 21-1 an unavoidable clinical parameter in making an earlier diagnosis of NSCLC. The sensitivity obtained in our sample is still insufficient to compensate for the deficiencies of clinical diagnosis. Thus, in order to increase the sensitivity, a biodynamic approach was implemented and CYFRA 21-1 levels were measured twice preceding clinical diagnosis (in patients whose CYFRA 21-1 levels were lower than 1.72 ng/mL). It shows a difference in the levels which is greater in stage I NSCLC patients than in healthy subjects, certain benign lung diseases, malignant diseases of other organs and lung metastases originating from other organs. There is no significant statistical difference between the two measurements in lung metastases originating from other organs, while in primary lung cancers there is. This only confirms the existence of biological differences between primary and secondary lung tumours. The difference in the values obtained in two measurements in primary lung tumours shows the greatest discriminatory power in separating NSCLC from other pulmonary nodules[39]. The use of tumour markers has shortened the period between two checkups, which ultimately means that a diagnosis can be made earlier. The increase of CYFRA 21-1 levels between two measurements (within 3 to 9 weeks) in solitary lung nodules confirms the diagnosis and facilitates the earlier treatment of stage IA NSCLC.

How much time should optimally pass between the two measurements is determined by laboratory physicians depending on tumour size and structure, and the level of CYFRA 21-1. With this in mind, close attention was given to tumour type and the structure of tumour mass. In some stages of tumour development, particularly in the development of necroses with colliquation, lower CYFRA 21-1 values were measured, i.e. mostly around the maximum of the reference population. The values within the reference range dominated in stages IA and IB (0.1–1.4 ng/mL), while in stages IB to IV they ranged between 0.3 and 4.7 ng/mL. Accordingly, lower sensitivity has been expected in some patients, which our research confirmed. In such instances, NSCLC was not excluded from the findings. Instead, measurements were repeated within 3 to 9 weeks. What was taken account of was the fact that some patients with central necrosis of lung cancer frequently have isolated metastases in other organs. In addition, in T2N0M1 with cerebral metastases the marker value does not decrease after removal, but only after the surgical removal of the primary lung tumour (Figure 9). The contribution of CYFRA 21-1 to differential diagnosis (i.e. earlier lung cancer diagnosis and metastases separation) cannot be disregarded. The interpretation that writes »tumour biological activity registered« either confirms the clinical diagnosis or warns clinicians to prove the existence of a tumour within a shorter period using standard clinical tests. Tumour markers are not only a contribution to diagnosis, but are also a control mechanism of other diagnostic methods (upcoming paper).

Opinions on the mechanism causing the release of cytokeratin 19 differ greatly. The results of this research support the fact that SQC's larger tumour mass necrotises more centrally and produces less cytokeratin, whose level in the advanced stages of NSCLC does not always appear to be as high as expected. In advanced stages tumours exhaust the host tissue, diminish, release the cytokeratin from the remaining decomposing mass and send signal molecules to other tissues (i.e. metastases). The fact that in samples of lung metastases the sensitivity of CYFRA 21-1 was low supports and confirms the above. Likewise, AD and LCC also contain less cytokeratin 19, which confirms the fact that simple epithelia, such as the bronchial, abound in cytokeratin 19 (besides cytokeratins 7, 8, 18 and 20), which is why the level of CYFRA 21-1 is higher in SQC.

Moreover, our results show that the level of CYFRA 21-1 depends on tumour size (Kruskal-Wallis ANOVA

$<10^{-4}$). However, this does not exclude some patients with high CYFRA 21-1 levels, who have pathohistologically been diagnosed with a lower stage because they have no lymph node metastases. The reason for this should be sought in the correlation between the time of sampling and the time of tumour development. It is important to underline that tumours in patients exposed to the same etiological factors and of the same age have different chronologies and types of mechanism of emergence, development and metastasis, which means that the acquired characteristics of such tumours also differ[7].

The statistical significance of CYFRA 21-1 levels before therapy in stage I SQC and AD verifies that this marker can indeed distinguish between initial and advanced stages. The case is similar in IIB T2N1. In NSCLC the level of CYFRA 21-1 differs significantly between stage IV and the other stages, then between IIB T3N0 and IIIA T3N1, and between IIIA T3N1 and IIIA N2. However, this statistical data cannot be accepted clinically because not all subjects have displayed it (e.g. LCC). It is crucially important for serum marker findings to measure the same before therapy during diagnostic management. It is proof of a diagnosis, assists therapy selection and is the basis for understanding the events in the tumour caused by therapy and the control mechanisms of treatment. In stage IA to IIIA patients, both the tumour and its necrotic mass were surgically removed respecting the surgical algorithm so as to allow oncologists to implement the most efficient adjuvant therapy. Stage IIIA patients without necrosis first underwent a neoadjuvant therapy and were only then surgically treated. Keeping in mind its stage and the total survival rate, surgically treating NSCLC with extremely high levels of CYFRA 21-1 is indeed questionable. Our results show that in over 95% of NSCLC patients (our unpublished data) both the propagation of the disease and the time of death occurred earlier regardless of the modality of treatment. A significantly increased value of CYFRA 21-1 before surgery confirms the diagnosis, but also indicates the size of the tumour mass. Higher levels of CYFRA 21-1 were also obtained in T2–T4. The extent of the tumour (i.e. clinical N in TNM staging) can be distinguished according to our results, but cannot always be clinically confirmed.

The findings of the tumour marker CYFRA 21-1 have significantly contributed to clinical practice facilitating earlier detection, diagnosis, and therapy selection and control in NSCLC patients[32–40,63, 64].

Conclusion

There is no ideal marker. A sufficiently sensitive and specific marker can facilitate an earlier clinical diagnosis to be suggested and the selection of the modality of treatment.

CYFRA 21-1 has been standardised in the Croatian population and has become part of unavoidable clinical procedure in NSCLC patients. The cut-off of 1.72 ng/mL at 95% specificity has been used in clinical application since 1998 (R. Pavićević) and is confirmed on the sample presented.

Measuring CYFRA 21-1 twice before making a clinical diagnosis shows that the level in stage I NSCLCs is higher than in healthy subjects and subjects with certain benign lung diseases. This unusual dynamics of CYFRA 21-1 prompts clinicians to repeat clinical diagnostic procedures in order to confirm the diagnosis.

Furthermore, the measurement of CYFRA 21-1 levels allows the separation of NSCLC from lung metastases originating from other organs, except intraluminal bronchial metastases. However, lung cancer metastases to other organs cannot be verified.

The sensitivity of CYFRA 21-1 is 78% in NSCLC patients (75–97% in SQC, 60–94% in AD and 60–100% in LCC).

An increase in CYFRA 21-1 levels is a signal of biological activity in NSCLC, which prompts clinicians to confirm the disease and make the diagnosis. This is achieved by repeating standard clinical methods.

With the help of CYFRA 21-1 levels, NSCLC can be distinguished according to its histological type, and according to the T and N classification, particularly SQC, and the initial from the advanced stages. This cannot be applied without comparison to clinical findings.

Measuring marker levels before therapy and during diagnostic management is of crucial importance for the interpretation of serum marker dynamics.

Considering that it is a serum marker, CYFRA 21-1 cannot be a screening marker.

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R. Pavičević

Cancer Genetics Laboratory, Primary Reference Laboratory for Clinical Application of Lung Tumour Markers, University Hospital for Pulmonary Diseases »Jordanovac«, Jordanovac 104, 10000 Zagreb, Croatia
e-mail: radomir.pavicevic@zg.t-com.hr

CYFRA 21-1 U KARCINOMU PLUĆA NEMALIH STANICA – STANDARDIZACIJA I PRIMJENA U VRIJEME DIJAGNOZE

S A Ž E T A K

Za sada nema idealnog tumorskog markera. Klinička primjena CYFRA 21-1 moguća je nakon temeljito provedenog postupka standardizacije koju čini određivanje referentnog raspona u asimptomatskoj populaciji, benignim i malignim bolestima pluća, te benignim i malignim bolestima drugih organa. Ovaj postupak ovisi o poznavanju karakteristika is-

traživane populacije, anamnestičkih podataka i nalaza dijagnostičkih postupaka kod svakog pacijenta, veličini ciljano odabranog uzorka i klinički definiranih kontrolnih grupa. Referentna razina (engl. cut-off) CYFRA 21-1 kod karcinoma nemalih stanica pluća (NSCLC) u Hrvatskoj populaciji iznosi 1,72 ng/ml. Bazira se na klinički primjenjivoj senzitivnosti 78% i specifičnosti 95% kod benignih bolesti pluća. Vrijednost referentne razine je potvrđena u kliničkim nalazima. Razina CYFRA 21-1 ranije ukazuje kliničaru na NSCLC u odnosu na sve benigne bolesti pluća i drugih organa za koje smo utvrdili da mogu utjecati na razinu, ukoliko se potvrdi standardnim dijagnostičkim metodama. Na CYFRA 21-1 utječe i vrijeme uzimanja uzoraka u odnosu na druge dijagnostičke invazivne postupke. Klinički primjenjiv marker ima potvrdu u kliničkim nalazima, inače je beskoristan. U ovom radu su obuhvaćene 343 zdrave osobe te pacijenti s 474 benigne bolesti i 4.440 malignoma od čega je 2.453 NSCLC. Senzitivnost CYFRA 21-1 kod NSCLC je 78%, u karcinomu skvamoznih stanica pluća 84,6 %, adenokarcinomu pluća 74,3 % i karcinomu velikih stanica pluća 75,3 %. Razina CYFRA 21-1 se značajno razlikuje između zdravih, benignih i malignih bolesti ($p<10^{-3}$). Razlike postoje između tri histološka tipa NSCLC ($p<10^{-6}$) te T i N između histoloških tipova ($p<10^{-3}$). CYFRA 21-1 upućuje kliničare da ponavljanim postupcima u doba dijagnoze ranije detektiraju bolest i započnu liječenje pacijenata s NSCLC. Naši nalazi markera su u visokom podudaranju s dijagnostikom.