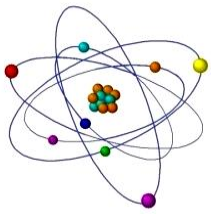
DEVELOPMENT OF THE COMPLEX CYTO- AND MOLECULAR GENETIC MARKERS FOR PREDICTING OF COMPLICATION OF RADIOTHERAPY



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ABSTRACT

Prediction, prevention and treatment of early and especially late adverse effects of radiation therapy is an urgent task in modern biology and medicine. The aim of the present study was to evaluate the informativeness and clinical efficacy of cyto- and molecular-genetic characteristics of genome instability in terms of diagnosis and prognosis of radiogenic complications. 30 oncologic patients with larynx cancer (stage 2 end 3) undergoing the radiotherapy were investigated. The irradiation was carried out on a linear accelerator: 2Gy/fraction, total dose 70 Gy, Leucocytes, dicentric chromosomes, level of micronuclei in buccal (MnB) and erythrocytes and level of DNA-Cometbefore irradiation, after receiving half and total dose were investigated. Exploratory and confirmatory data analyses methods (K-mean Clustering, ANOVA, Xi^2,) were used. To identify the optimal predictors of the risk of complications, a Binary Logistic Regression analysis and Receiver operating characteristic (ROC) curve analysis was performed. After receiving a half-dose of irradiation a statistically significant *changes of all characteristics is observed in patients ($F \geq 9.21$; $p<0.001$). Clustering patients by the amplitude of radio-induced deviations from the background level (level before irradiation) of characteristics revealed groups of low and high risk of complications.It was confirmed that there is a causal relationship between the stage of the disease and the risk of complications, although its statistical significance is close to the threshold value ($\chi 2 = 8.22$, $p = 0.051$), which indicates that the stage of the disease is not the only factor that determines the risk of complications.Analysis of the cyto and molecular-genetic profile of the complication risk revealed a statistically significant relationship between it and the frequency of dicentric chromosomes, level of micronuclei in erythrocytes and buccal cells ($F \geq 5$; $p \leq 0.05$). The levels of leukocytes and DNA comets depend on both the stage and the level of risk ($F \geq 5$; $p \leq 0.05$). To determine the optimal predictors of the risk of complications, a binary logistic regression analysis was performed. It was found that the strongest predictors are the background level of DNA comets (regression coefficient = 0.89 ± 0.41; $p = 0.029$) and the micronuclei level in buccal cells (regression coefficient = 21.92 ± 8.86; $p = 0.013$). Using the Receiver Operating Characteristic analysis, revealed the high clinical efficacy of the criterion (AUC = 0.93), and the optimal Cutoff_level (0,5), providing Sensitivity = 0.866 and Specifity = 0.875.*

Our results suggest that levelof*the of DNA comets together with level of MnB*before treatment can be used to predict the risk of developing *complication* after*radiotherapy.*

Key words: radiotherapy, complications, micronuclei, DNA-comets

INTRODUCTION

Cancer is the second leading cause of morbidity and mortality worldwide, and is responsible for about 10 million deaths per year. Globally, about 1 in 6 deaths is due to cancer.Even though there are no specific drugsdesigned for it, radiotherapy (RT) is the main treatmentfor cancer and over 60% of cancer cases require radiation therapy. Modern cancer therapy has successfully cured many cancers and converted a terminal illness to chronic disease.However, like any therapeutic intervention, it is also characterized by complications that can be conditionally divided into early and late complications[1,2].The most common are short term side effects – results from destruction of some stem cells in the compartment of rapidly dividing epithelial tissues (skin, gut, oral mucosa, bone marrow, sclera and hair follicle). These side effects tend to be short-term and mild, most side effects go away after treatment. But some continue, come back, or develop later. These are

called long-term or late effects. One possible late effect is the development of a second cancer. This is a new type of cancer that develops because of the original cancer treatment. The risk of this late effect is low. And the risk is often smaller than the benefit of treating the first cancer (https://*www.cancer.org/treatment*).

In the last two decades late non-cancer radiogenic effects are considered as a subject of a special attention, which is explained by the level of health risks associated with them, and the growth rate of the population at this risk. These effects, previously called deterministic effects, are now referred as tissue reactions because it is increasingly recognized that some of these effects are not determined solely at the time of irradiation but can be modified after radiation exposure.

In 2011 the International Commission on Radiological Protection approved a Statement on Tissue Reactions, suggesting that there are some tissue reaction effects, with very late manifestation. For the lens of the eye and for circulatory system, the threshold in absorbed dose is now considered to be 0.5 Gy. Most commonlate tissue effects observed in response to radiationtreatment are pericardial disease (acute pericarditis, delayed pericarditis, pericardial effusion, and constrictive pericarditis), radiation-induced atherosclerosis, myocardial and endocardial disease (pancarditis, cardiomyopathy), valvular disease and conduction disturbances [3-6]. If the early effects are temporary and disappear after a few weeks, the late effects tend to be irreversible and progressive in severity.Cardiotoxicity related to thoracic and mediastinal radiation therapy of cancer survivors remains the most common cause of morbidity and mortality [4-5]. Childhood cancer survivors represent a unique, particularly high-risk group, for example, if the radiotherapy for breast cancer was associated with an absolute risk increase of 76.4 (95%CI 36.8-130.5) cases of coronary heart disease and 125.5 (95%CI 98.8-157.9) cases of cardiac death per 100 000 person-years [5], then in survived childhood cancers patients the relative risk of severe cardiac disease at the age of 40 is 1.9 at a cardiac radiation dose of 1–5 Gy and 19.5–75.2 at a dose > 15 Gy [6-7].

Until recently, the classical model for explaining early and late radiogenic complications was the target cell hypothesis, according to which the severity of the complications mainly reflected tissue depletion resulting from direct destruction of putative target cells, leading to subsequent functional deficits[8].

Current understanding of the possible mechanisms of long-term complications, caused by radiation, include senescence to endothelial cells due to DNA damage and oxidative stress, Increased levels of IL-1, IL-6, IL-8, and TNF-α as well as reactive oxygen species [9-11], pro-inflammatory cell recruitment, thrombin, platelet activation [12], metabolic and immunologic alteration [13],the formation of persistent nonresolved inflammation [14]. The product of the above pathways is accelerated atherosclerosis, fibrin deposition, intimal thickening, lipid accumulation, inflammation, and thrombosis. The spectrum of coronary artery disease (CAD), cardiomyopathy, pericardial disease, valvular disease, and conduction abnormalities are collectively described as radiation-induced heart disease [15].

Modern approaches to solving this problem are based on the creation of models for the early stratification of the risk of complications, which will allow the identification of patients at high risk, the development of modified methods of radiation therapy and preventive treatment for them [7,15].

In this respect, the genomic instability is considered as a one of the most promising models, which allows to describe on a single hypothetical basis, both early and late radio-induced effects [16].Research is focused on the search for genes and gene products that determine the response to DNA damage, DNA repair, apoptosis and, most importantly, systemic effects due to the excitation of inflammatory "danger" signals and activation of the innate immune/autoimmune response, find ways of predicting thosepatients likely to suffer with long-term side effects, and develop new approaches for their amelioration [17-20].

The main limitation of genome-wide associative studies (GWAS) is due to the methodological difficulties in establishing a causal relationship between the genotype and phenotype of the patient and the risk of developing long-term complications in multigenic and multifactorial studies, and their relatively high cost [21].

Our approach, which aims to develop inexpensive, simple, clinical practice-oriented criteria for assessment of radiogenic health risk, and which includes experimental, clinical, and population-based studies, focuses on the development of complex risk predictors based on the integral characteristics of a number of functional systems of the body [22-28].

The aim of the present study was to evaluate the informativeness and clinical efficacy ofcyto-and molecular-genetic characteristics of genome instability in terms of diagnosis and prognosis of radiogenic complications.

MATERIALS AND METHODS

Source of Patients and data colection: 30 oncologic patients – 10 women and 20 men (mean age) with larynx cancer, undergoing the radiotherapy taking into account the stage of the disease were investigated. All patients were treated with a fractional radiotherapy. The irradiation was carried out on a linear accelerator with the following level: 2Gy/fraction, total dose (70 Gy). Hematologic and genetic (leucocytes, chromosomal aberration, between them dicentric chromosomes, micronuclei level in buccal mucosa cells and erythrocytes, DNAstrand break damage investigated by comet assay)analyses were carried out in dynamic - before irradiation, during half receiving dose and after finishing the course.

Statistical Methods. Exploratory and confirmatory data analyses methods (K-mean Clustering, ANOVA, Xi^2), were used to reveal the statistical significance of the difference between the mean values of the parameters.

To identify the optimal predictors of the risk of complications, a Binary Logistic Regression analysis was performed:

$$P(+) = \frac{EXP(Z)}{1+EXP(Z)} (1)$$

where P (+) is the probability that a particular patient with specific characteristics falls into the category of severe patients. Only linear combinations of individual parameters were considered at this stage:

$$Z = b_0 + b_1 X_1 + b_2 X_2 + \ldots + b_n X_n \quad (2)$$

Where $\{\boldsymbol{X_i}\}_{\boldsymbol{i=1}}^{\boldsymbol{n}}$- values of n indicators of rescaled and re-centered in relation to physiological norm

$$X_i = \frac{X_i' - \overline{X'}}{STD(X')} (3)$$

To assessment of clinical effectiveness of the complex risk predictors, receiver operating characteristic (ROC) curve analysis was performed.For primary data processing, analysis and graphical visualization MATLAB R 2020 and STATISTICA-12 mathematical software packages were used.

RESULTS AND DISCUTION

After receiving half-dose of radiation, statistically significant changes ($F > 9$; $p < 0.001$) of all characteristics was observed in comparison with thear background values. A fundamentally different regularity of genetic disorders was detected after receiving total dose; statistically significant increase ($F > 9$; $p < 0.001$) of dicentric chromosomes, DNA comets, and and level of micronucleincy in buccal

cells is observed, although the number of leukocyte count is unchanged (F> 9; p <0.001), and the frequency of micronuclei in erythrocytes is significantly reduced (F> 9). ; p <0.001). (Fig.1, Tab.1)

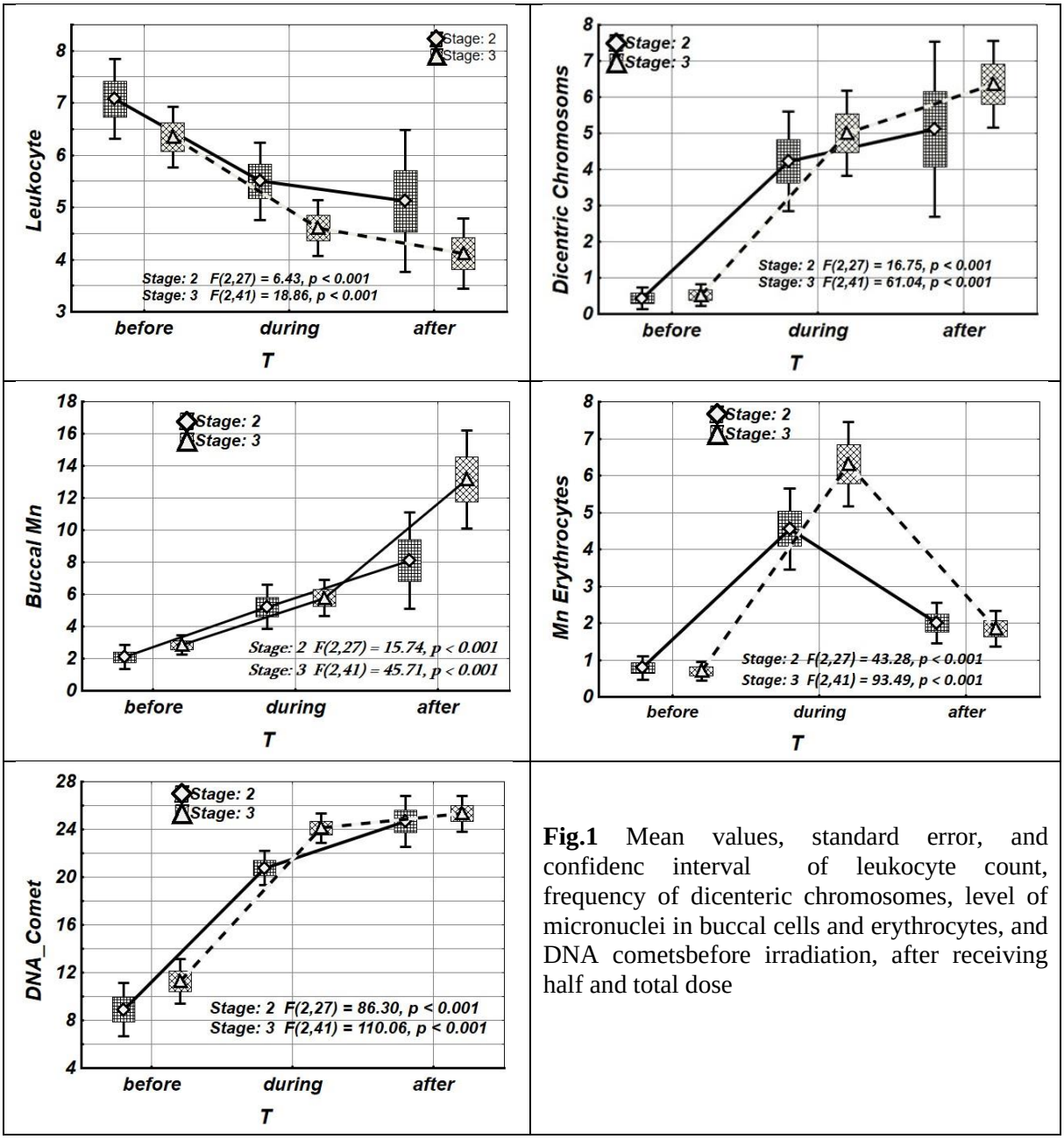

Fig.1 Mean values, standard error, and confidenc interval of leukocyte count, frequency of dicenteric chromosomes, level of micronuclei in buccal cells and erythrocytes, and DNA cometsbefore irradiation, after receiving half and total dose

The postradiation dynamics of the study characteristics presented in Fig.1 Tab.1 as a whole coincide with the existing representations about the processesin proliferating cell populations [28] developed after total irradiation, although the effects of local fractional irradiation are also clear. This is reflected in the nonlinear dose-dependence of quantitative and structural (genetic) changes in proliferating cell populations, as well as in the specificity of dose-dependent changes in different proliferating cell populations, which probably depends on the one hand on te amount of the absorbeddose andtheir radiosensitivity, and on the other hand on the length of their life cycle and the specifics ofeliminationof damaged oraged cells. With regard to the effects associated with the stage of cancer, this clearly indicates a close relationship of the severity of radiological changes with various modifying factors during radiation therapy. The presented regularities clearly indicate the methodological difficulties of prognosys the severity ofradiological complications under fractionated,local irradiation, which are

Tab.1 Dependence of postradiation dynamics of research characteristics on the stage of the disease

N	***Statistics***	***F; p***	***F; p***	***F; p***	***F; p***
	Stage Group	***Stage2***	***Stage2***	***Stage 3***	***Stage 3***
	Period	***Before÷During***	***During ÷After***	***Before÷During***	***During ÷After***
1.	Leucocytes	F=9.21; p<0.001	F=0.14; p=0,71	F=13.57; p<0,001	F=0.99; p=0,32
2.	Dicentrics	F=21.59; p<0.001	F=4.10; p=0.06	F=10.99; p=0.001	F=28.90;p<0,001
3.	Mn_Buccal	F=23.49; p<0.001	F=0,1; p=0,8	F= 17.80; p<0,001	F=28.90; p<0,001
4.	Mn-Erythro	F=76.41; p<0.001	F= 23.60; p<0,001	F=37.18;p<0,001	F=1,20; p=0,30
5.	DNA-Comet	F=84.07; p<0,001	F=12.28; p=0,003	F=125.50; p<0,001	F=1.89; p=0.18

related to the systemicity of effects, and require a complex approach to their solution.

Clustering of half-dose irradiated patients for values of their characteristic (K-mean clustering) revealed two groups of patients: 1) high level of leukocytes, low micronucleilevel in buccal cells and erythrocytes, low level of DNA-comet (low risk of complication), 2) low level of leukocytes, high level of micronuclei and DNA-comet (high risk of complication), (Fig.2). Given that chromosomal aberrations (dicentrics) and leukocytes are classic markers of the severity of acute radiation pathology, with a high degree of credibility we can assume that the identified groups of patients can be considered as high and low risk groups in terms of development of radiological complications. (It should be noted that in about 15% of patients in the high-risk group developed a side effect during or immediately aftertherapy).

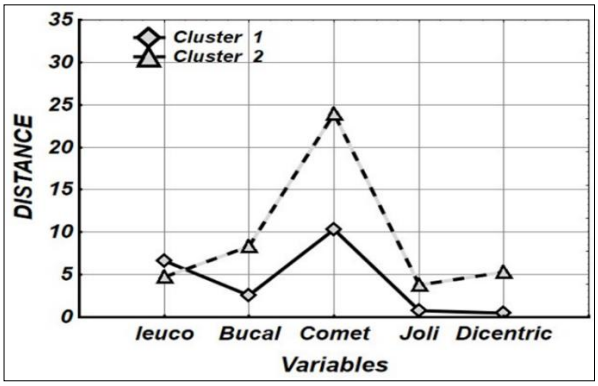

Fig. 2. Cluster means of normalized characteristics in patients of low (solid line) end high risk of complication (dashed line)

Expected and observed frequencies of high and low risk patients were analyzed to determine the possible causal relationship between disease stage and complication risk in the study cohort.It was confirmed that there is a causal relationship between the stage of the disease and the risk of complications, although its statistical significance is close to the threshold value ($\chi 2 = 8.22$, $p = 0.051$), which indicates that the stage of the disease is not the only factor that determines the risk of complications (Fig. 3). Analyze of the cyto- and molecular-genetic profile of complication risk factors have revealed that groups of patients with high and low risk of complications after receiving half-dose of irradiation are statistically significantly different in the frequency of dicentric chromosomes, micronuclei level in erythrocytesand buccal cells**(F≥ 5; p≤ 0,05)**.

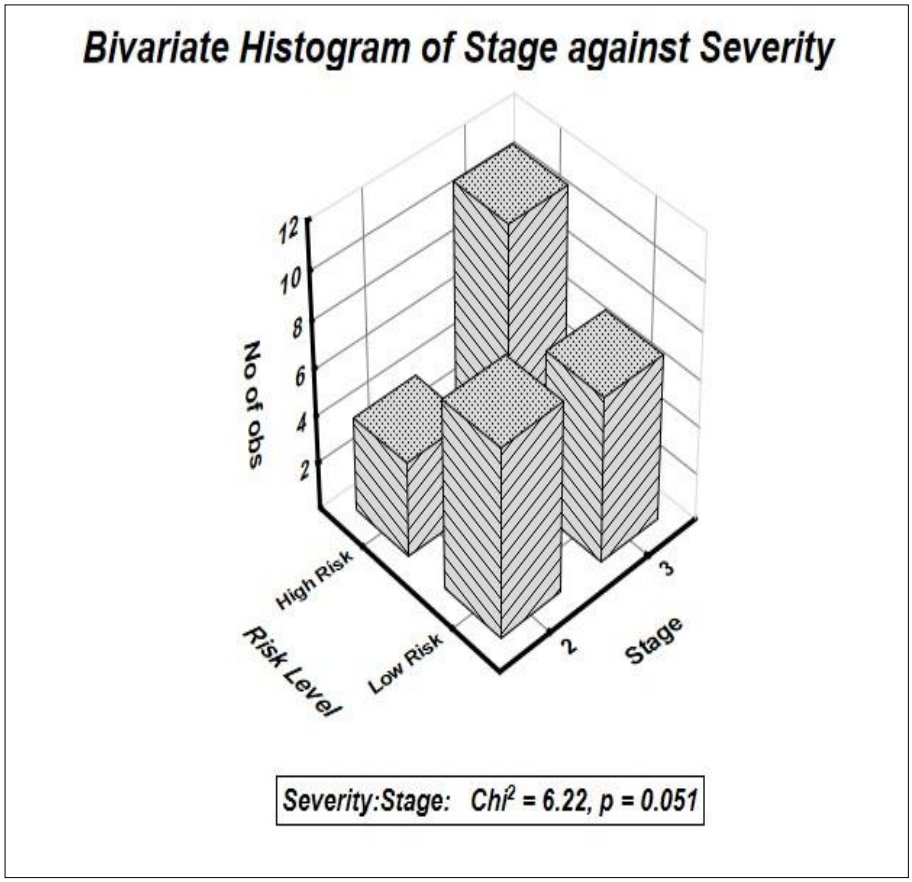

Fig.3. Patients distributions according to the stage of the disease and the risk of complications.

The influence of cancer stage on the values of these characteristics is not revealed (F≤ 2; p≥ 0,3). Leukocyte and DNA comet levels depend on both stage and risk level (F≥ 5; p≤ 0,05), (Fig.4, Tab.2).
The results clearly indicate the multifactorial nature of complication risk, the mechanisms of which we find impossible to analyze in the present article, although it is clear that the complexity of complication risk predictors is necessary.In view of the abovemultivariable logistic regression analysis was performed to develop a complex cyto end molecular-genetic predictor of risk of complication. At this stage of thestudy we were looking for a multivariate discriminant function in the form of a linear combination of the background frequencies of DNA comets of micro-nucleated buccal cells:

$$\mathbf{logit(y) \sim A_0 + A1 \times Mn + A_2 \times DNA\_Comet,}$$

Was shown, theth Linear function with regression coefficientsA_0=21.92±8.86 (p=0.013),$_1$=1.68±0.70 (p=0.017), A_2=0.89±0.41 (p=0.029)significantly predicts high-risk patients groups(Tab.3**).**
The clinical performance of the complex prognostic criterion and the optimal cut-of value were assessed using the receiver operating characteristic (ROC) curve, which includes all possible decision thresholds from the result of the diagnostic test.As knownROC curves are a useful tool in the assessment of the performance of a diagnostic test over the range of possible values of a predictorvariable.

N	*Stage Group*	*Stage2*	*Stage 3*	*High Risk*	*Low Risk*
	Risk group	*High Risk÷Low Risk*	*High Risk÷Low Risk*	*Stage2÷ Stage3*	*Stage2÷Stage3*
1.	**Leucocytes**	F=8,2; p=0,025	F=1,2; p=0,26	F=0.65;p=0,26	F=6,3; p=0,02
1.	**Dicentrics**	F=5,3; p=0,04	F=53; p=0,002	F=0,64; p=0,82	F=1,46; p=0,44
2.	**Mn_Buccal**	F=16,8; p=0,008	F=17,8; p=0,002	F=0,17; p=0,80	F=0,10;p=0,85
3.	**MnErythro-**	F=9,0; p=0,02	F=5,0; p=0,03	F=7,3; p=0,015	F=1,50; p=0,23
4.	**DNA-Comets**	F=7,0; p=0,033	F=31,0; p=0,001	F=11,0; p=0,01	F=29,0; p=0,001

Tab. 2. Statistical significance of the influence of disease stage and complication risk on the level of leukocytes, the quantity of dicentric chromosomes, DNA comets, micronuclei in buccal cells and erythrocytesafter receiving half-dose of irradiation

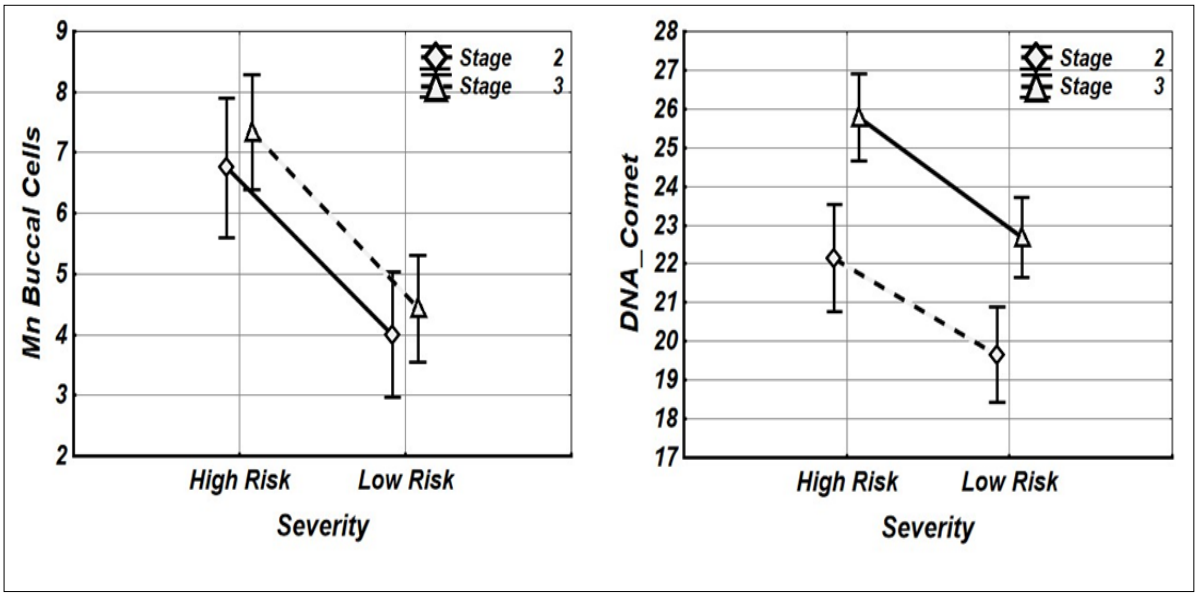

Fig.4. The background levels of micronucle in buccal cells and DNA comets in different risk and stage patient groups.

Logistic Regression, Binomial Distribution				
N	**Indicator**	**Estimate**	**SE**	**pValue**
1.	Intercept	-21.92	8.86	0.013
2.	Micronuclei	1.68	0.70	0.017
3.	DNA-Comet	0.89	0.41	0.029

Tab.3 Regression coefficients, Standard Error and p-value of multivariable linear discriminant function, for predicting the high-risk patients groups

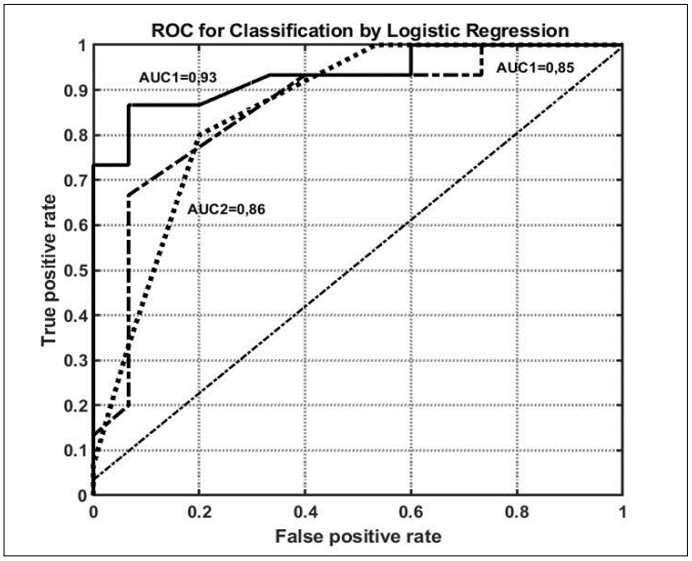

Fig.5: receiver operating characteristic curve (ROC) of MnB level (dotted line), DNA-Comet (dashdotted line) and its linear function(solid line) in predicting cases with highrisk, and the area under ROC curve (AUC) of the studied parameters in predicting cases with high complication risk

The area under the ROC curve of complex complication risk predictors (AUC = 0.93) indicates its high clinical efficacy compared to the individual characteristics of MnB (AUC = 0.86) and DNA-Comet (AUC = 0.85) (Fig.5).

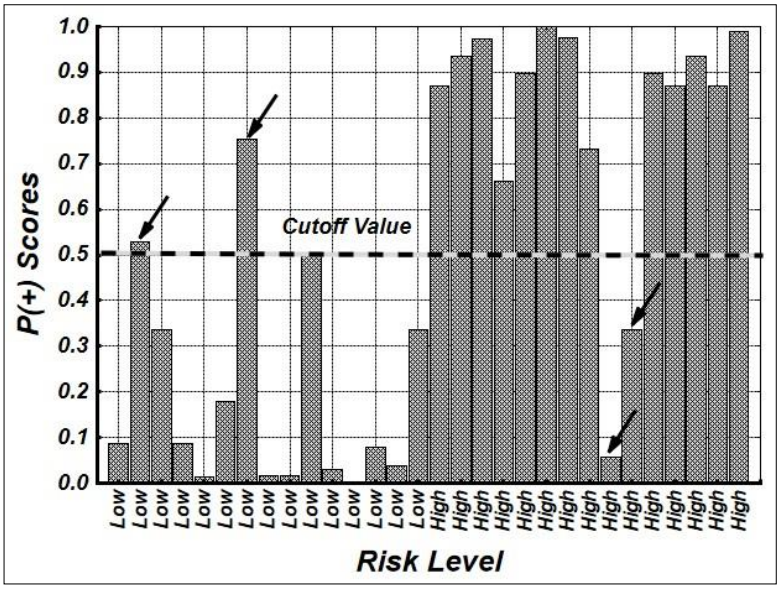

Fig. 6. Values of Logit function (P(+)) in low and high risk group patients and Cutoff value (dotted line). Arrows indicate incorrectly identified cases.

Figure 3 presents the values of complex discriminant function (P(+)) in low (0) and high (1) risk patients. Cut-of value P(+)=0,5 (dotted line) ensures the sensitivity of the criterion = 0.866 (86,6% of high risk patients are correctly identified) and specificity = 0.875(87,5% of low risk patients are correctly identified.

Limitation

We do not consider appropriate to generalize the adopted complex criteria to the whole population of larynx cancer patients, due to the limited sample size of a study cohort. However, the perspectives of the discussed approach are clear.

Conclusion

The results showed a high informative value of the complex criterion for assessing the risk of radiogenic complications of radiotherapy, developed on the basis of the DNA-comets and MnB levels before treatment, the clinical efficacy of the criterion was also confirmed in a limited cohort of cancer patients, but, how effective it will be for predicting Late tissue effects of radiotherapy is a matter for further investigation.

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