MOLECULAR AND GENETIC PREDICTORS OF IMPAIRED REPARATIVE REGENERATION OF LONG BONES

Bezsmertnyi Y. O., Shevchuk V.I.,
Branitsky O. Y., Bondarenko D.V., Bezsmertna H. V.

Scientific Research Institute of Invalid Rehabilitation on the base of Vinnitsa Pirogov National Medical University, Vinnitsa, Ukraine

Introduction. The osteoinductive potential of the body and the activity of bone resorption / biosynthesis processes at the time of injury are determined by numerous factors, among which the most important are age, sex, the presence of metabolic disorders, immunological status, obliterating vascular diseases, etc [1, 6, 8]. In recent years, it has been established that hyperhomocysteinemia (HHcy) is one of the factors of vascular lesions and thrombosis, which is associated with the risk of osteoporosis, osteoporotic fractures and adversely affects reparative osteogenesis [1, 7, 12]. The toxic effect of high levels of homocysteine (Hcy) on bone tissue is mainly associated with activation of bone demineralization, collagen degradation, oxidative stress development, hypomelylation, genital destabilization and chemical modification of proteins [3, 4, 9, 10].

In the experimental conditions it was found [4, 6] that the negative effect of high levels of Hcy on the musculoskeletal system was largely realized through vascular mechanisms, by proatherogenic damage to the peripheral vessels, a violation of the vascular output of nitric oxide and increased fibroblastic expression of the transforming factor growth - β 1 (TGF-β1). Clinical studies have also proved [4, 12] that the violation of reparative osteogenesis of long bones, which leads to the formation of bone nonunion, is associated with metabolic disorders, namely: HHcy, atherogenic dyslipidemia, high levels of inflammatory mediators, and an imbalance in the nitric oxide system. It was established that the prevalence of these metabolic disorders as well as the mutations of the gene of the enzyme exchange of Hcy - methylene tetrahydrofolate reductase (MTHFR C677T) and the promoter of the synthase nitric oxide gene (eNOS T786C) is significantly higher than those with consolidated fractures [5, 11]. In this case, HHcy, atherogenic dyslipidemia, inflammatory syndrome, pathogenic genotypes MTHFR 677-TT and eNOS 786-CC, disorders of endothelial function prevail among patients with acute types of bone nonunion. However, it remains unclear which of the identified metabolic and genetic

factors can be used in predicting disorders of reparative osteogenesis and the formation of bone nonunion of long bones.

Aim of the research. The purpose of the study: based on the methods of statistical analysis and forecasting was determined the independent metabolic and molecular genetic predictors of the violation of reparative osteogenesis and the formation of bone nonunion of long bones.

Object and methods. The main monitoring group was 153 (26.11%) of 586 examined patients with bone nonunion of long bones at the level of diaphyseal who did not have established objective and iatrogenic factors of the disorders of reparative osteogenesis. The average age was 40.3±0.93 years. Male persons were - 118 (77,2%), female - 35 (22,8%). Duration of the disease from 7.5 to 126 months. According to the clinical-radiological characteristics of the bone nonunion, the normoplastic type is diagnosed in 27 (17,65%), hyperplastic – in 24 (15,69%), hypoplastic – in 50 (32,68%), atrophic – in 52 (33,98 %) of patients. Metabolic disorders in the form of HHcy were diagnosed in 125 (21.33%) patients, including its combination with dyslipidemia – in 61 (10.41%) and aberrant levels of interleukin-6 in 39 (6.65%) patients. Signs of dyslipidemia without an increase in the level of Hcy were found in 28 (4.78%) people.

Patients evaluated the mineral density of bone tissue (T-index of densitometry of the heel bone of the affected and contralateral limbs), local osteoporosis (ΔKI) and determined the thickness of the intima-media complex (TIM) of the carotid, femoral and shoulder arteries. The levels of total Hcy, interleukin-6, total cholesterol, low density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C), triglycerides (TG) of the transforming growth factor beta 1 (TGF-β1), osteocalcin, cartilage oligomeric matrix protein (COMP), C-terminal propeptide of type I collagen (CICP), pyridinoline cross-linking were tested by immunoenzymatic methods in accordance to the manufacturer's instructions on the STAT FAX 303 / PLUS analyzer. Polymorphism of the genes of the enzymes Hcy-MTHFR C677T and the endothelial synthase nitrogen monooxide (eNOS T786C) was studied by polymerase chain reaction.

The statistical analysis of the material was carried out using standard methods using the application package "MS Excel XP" and "Statistica SPSS 10.0 for Windows" (license number 305147890). Evaluated mean, standard errors, authenticity of differences. To estimate the intergroup difference, the parametric t-criterion of the Student was used with the relationships between the indicators determined - a correlation analysis of Pierson, when comparing the frequency of changes - Fisher's criterion. Reliable considered the

difference at p <0.05. The risk of the formation of bone nonunion, depending on genetic determinants and metabolic factors was estimated, using the odds ratio (OR) and the 95% confidence interval (CI) was calculated [2]. To determine the metabolic predictors of the formation of adverse structural and functional changes in bone tissue and disorders of reparative osteogenesis, a one-factor dispersion and multiple linear regression analysis with a standardized coefficient β were determined.

Results of the research. At the first stage, we conducted a correlation analysis between the indicators of structural and functional status of bone tissue and markers of metabolic disorders and endothelial dysfunction (Table 1).

Table 1

Correlation coefficients between markers of bone metabolism and the content of homocysteine, lipids and inflammatory mediators, endothelial function in patients with false joints, n = 153 (r)

Characteristics	**Hcy**	**TC**	**HDL-C**	**CRP**	**IL-6**	**TIM femoral artery**	**TGF-β1**
Hcy	-	0.34^{*}	0.38^{*}	0.42^{***}	0.51^{***}	0.51^{***}	-0.24^{*}
TGF-β1	-0.24^{*}	-0.15	-0.13	-0.14	-0.15	-0.19	-
Osteocalcin	-0.35^{**}	-0.18	0.16	-0.24^{*}	-0.24^{*}	-0.18	0.62^{***}
CICP	-0.37^{**}	-0.19	0.18	-0.26^{*}	-0.24^{*}	-0.18	0.60^{***}
Oxiprolin	0.43^{***}	0.24^{*}	-0.19	0.35^{**}	0.37^{**}	0.28^{*}	-0.40^{***}
Pyridinolin	0.47^{***}	0.28^{*}	-0.21	0.27^{*}	0.38^{**}	0.29^{*}	-0.57^{***}
COMP	0.41^{***}	0.16	0.17	0.29^{*}	0.36^{**}	0.20	-0.29^{*}
GAG	0.47^{***}	0.17	-0.18	0.38^{**}	0.34^{**}	0.19	-0.57^{***}
T-score healthy limb	0.42^{***}	0.37^{**}	-0.25^{*}	0.18	0.30^{**}	0.31^{**}	-0.28^{*}
T-score affected limb	0.50^{***}	0.41^{***}	-0.31^{**}	0.25^{*}	0.35^{**}	0.30^{**}	-0.39^{**}
ΔKI (n_{KI}=96)	0.48^{**}	0.41^{***}	-0.32	0.33^{**}	0.45^{***}	0.31^{**}	-0.31^{**}

Notes: * - p <0.05; ** - p <0.01, *** - p <0.001

The most important independent determinants of the metabolic status of bone tissue were the content of TGF-β1 and Hcy in serum, which recorded the highest modulus correlation coefficients of biosynthesis markers and bone resorption. Thus, between the contents of Hcy and the

content of osteocalcin and CICP in the blood serum, the mean power of the inverse correlation bonds (r = -0.35 and -0.37) and the more direct correlations with the markers of bone and cartilage degradation-content pyridinoline, oxyproline, COMP and GAG (r = 0.47, 0.43, 0.41 and 0.47).

The results of the correlation analysis revealed additional evidence that the negative effect of Hcy on bone tissue can be mediated through vascular factors. Evidence of this is the reliable linkages of the Hcy levels with levels of total cholesterol, HDL-C, CRP, interleukin-6 and TIM of the femoral artery, on the one hand, and the existence of reliable links between the latter and markers of the metabolic state of the bone on the other. To a greater extent, the effect of proatherogenic and proinflammatory factors is realized through the strengthening of bone resorption processes, since it is precise with the level of oxyproline and pyridinoline that there is a significant direct correlation between the levels of total cholesterol (r = 0.24 and 0.28), interleukin-6 (r = 0.37 and 0.38) and TIM of the femoral artery (r = 0.28 and 0.29). Mediators of inflammation also weakly inversely correlated with the level of markers of bone biosynthesis - osteocalcin and CICP (r = -0.24 -0.26).

Conversely directed and with larger association modules were found between the content of TGF-β1 in serum and markers of the metabolic state of bone tissue. The content of TGF-β1 was directly correlated with the content of osteocalcin and CICP (r = 0.62 and 0.60) and vice versa - containing pyridinoline, oxyproline and GAG (r = -0.57, -0.40 and -0.57). The relative independence of TGF-β1 as a factor of disturbances of reparative osteogenesis, whose action is not mediated through vascular mechanisms, shows a weak inverse correlation with the level of Hcy (r = -0.24) and the absence of reliable connections with the level of total cholesterol, HDL-C, CRP, interleukin-6 and TIM femoral arteries (r = -0.13-0.19).

It was established that from the metabolic factors, the largest modulators of communication with the markers of systemic and local osteoporosis were recorded at the level of Hcy in serum (r = 0.42-0.50). At the same time, proatherrogenic markers, inflammatory mediators, and TGF-β1 levels showed medium-mediated ligaments, mainly with indices of local osteoporosis. For example, the correlation coefficients of total cholesterol, interleukin-6 and TGF-β1 with T-index of healthy limb were 0.37, 0.30, -0.28, with the T-score of the affected limb 0.41, 0.35, - 0.39, and with the index ΔKI 0.41, 0.45, -0.31, respectively.

Correlation analysis, as you know, allows you to establish only the force and direction of communication between the two variables, but does not allow the establishment of a causal relationship. In order to identify independent predictors of reparative osteogenesis disorders that would allow estimating the risk of forming done nonunion and predict their formation according to a certain clinical-radiological type, at the next stage, the relative risk (OR) was determined and the method of multiple linear regression analysiswas utilized.

The analysis of the chance ratios showed (Table 2) that HHcy, hypercholesterolemia and an increase in the content of interleukin-6 proved to be significant factors risk violations of reparative osteogenesis. Thus, with the damage of long tubular bones in the presence of high levels of Hcy (above 15 μmol / l), the risk of false joints formation increases by almost 5 times (OR = 4.92, 95% CI: 2.13-11.4), and with the presence of high levels of total cholesterol (above 6.1 mmol / l) of interleukin-6 (above 9 ng / l) - more than tripled.

Table 2

Relative risk of forming bone nonunion of long bones with metabolic disorders and polymorphism of the MTHFR C677T and eNOS T786C genes

Risk factor	**Risk of false joint formation (p <0,05)**	
	OR	**95% CI**
Hyperhomocysteinemia (> 15 mmol / L)	4.92	2.13-11.4
Hypercholesterolemia (> 6.1 mmol / L)	3.90	1.60-9.47
High levels of interleukin-6 (> 9 ng / l)	3.29	1.36-8.01
Genotype MTHFR 677-CT	1.22	0.60-2.44
Genotype MTHFR 677-TT	2.05	0.70-5.98
Genotype eNOS 786-TC	1.21	0.60-2.44
Genotype eNOS 786-CC	2.55	0.77-8.38
Genotype 677-T MTHFR + 786-C eNOS	2.10	0.85-5.13

Notes: OR relative to consolidated fractures.

Genetic factors also significantly increased the risk of forming bone nonunion, but less significant than the metabolic disturbances associated with them. In this case, only the homozygous carrier of pathological alleles 677-T MTHFR and 786-C eNOS, as well as their association in hetero- and

homozygous variants should be considered as risk factors for the disorders of reparative osteogenesis. Thus, in the presence of a homozygous mutation variant of the gene MTHFR (genotype 677-TT), the risk of reparative osteogenesis increased bya half and in the presence of a homozygous variant of the mutation of the eNOS gene promoter (786-CC genotype), it was 2.5-fold. At the same time, heterozygous mutation variants practically did not increase the chances of forming false joints (OR = 1.22 and 1.21), however, with the association of polymorphisms "677-T MTHFR + 786-C eNOS" in homo- and heterozygous variants, the risk of violations bone repair increases twice (OR = 2.10. 95% CI: 0.85-5.13).

The results of the analysis of the chances ratios are shown in the Table 3, allow to assert that in the presence of HHcy the risk of formation of hypoplastic or atrophic types of bone nonunion increases 6 times (OR = 6.11, 95% CI: 1.94-19.3), in the presence of hypercholesterolemia and inflammatory syndrome – 3-5 times (OR = 3.37 and 4.72, respectively).

Table 3

Relative risk of formation of bone nonunion of long bones of hypoplastic and atrophic types with metabolic disorders and polymorphism of MTHFR C677T and eNOS T786C genes

Risk factor	**Risk of bone nonunion formation (p <0,05)**	
	OR	**95% CI**
Hyperhomocysteinemia (> 15 mmol / l)	6.11	1.94-19.3
Hypercholesterolemia (> 6.1 mmol / L)	3.37	1.23-9.24
High levels of interleukin-6 (> 9 ng / l)	4.72	1.44-15.9
Low TGF-β1 (<14 ng / ml)	6.31	1.53-26.0
GenotypeMTHFR 677-TT	2.47	0.61-10.1
Genotype eNOS 786-CC	2.38	0.58-9.88

Notes: OR relative to the normoplastic type.

Another important risk factor for the formation of non-vital types bone nonunion showed a level of TGF-β1 <14.0 ng / ml (P5 value of practically healthy subjects). Under this condition, the chances of formation of hypoplastic or atrophic types increase more than 6 times (OR = 6.31, 95% CI: 1.53-26.0). With the presence of 677-TT MTHFR or 786-SS eNOS genotypes, the risk of developing bone disorientationon avital type increases more than twice.

In order to identify independent predictors of reparative osteogenesis disorders that would allow them to predict their formation, according to a certain clinical-radiological type, to predict the prevalence of systemic or local loss of bone mass, we applied the method of multiple linear regression analysis. The content of Hcy, lipids, CRP, interleukin-6, TGF-β1, osteocalcin, CICP, pyridinolinum, oxyproline, COMP and GAG in serum was selected as possible predictors (regressors). Since in biological models there is a high intercollearnarity of regressors, we used the model of their turn-by-turn (Forward) in the regression equation, that is, at each step, the most informative parameters that had a high modulus partial correlation coefficient were included at each step and increased the determination coefficient of the linear regression equation. For regression analysis, we assigned the conditional ranges to the clinical-radiological types: the congenital type (normal or hyperplastic) - 3 (the largest bone mass of the affected segment), hypoplastic - 2, atrophic - 1 (the smallest bone mass of the affected segment).

It was established that the most significant independent predictors of the clinical-radiological type of the bone nonunion are the content of the CICP (characterizing the process of synthesis of collagen type I in bone tissue), TGF-β1 (bone and cartilage remodeling regulator), and pyridinolin (bone resorption marker) in serum - coefficients the regression $\beta = 0.366$, 0.313 and -0.310, respectively. The statistical characteristic of the model, including these predictors, is given in Table 4. It is established that non-standardized regression coefficients (B) of the model are significant and reliable ($t > 4.5$, $p = 0.000$), which made it possible to create a regression equation Y (Xi), which describes the mathematical relationship between the dependent variable (clinico- X-ray type of bone nonunion) and independent, selected during the analysis, predictors. Taking into account the value of Fisher's criterion and its significance (121.22, $p = 0.000$), as well as the coefficient of multiple determination (df), we can assume that this model of influence of these predictors is sufficiently informative and statistically reliable.

The most significant independent predictors of systemic decline in bone mineral density were the concentration of Hcy and TGF-β1 in serum - the regression coefficients $\beta = 0.428$ and -0.291, respectively (Table 5). Non-standardized regression coefficients (B) of the model are significant and reliable ($t > 4.5$, $p = 0.000$), which allowed to create a regression equation Y (Xi), which describes the mathematical relationship between the dependent variable (T-index of healthy limb) and independent, selected in

the process of analysis, predictors. Given the value of Fisher's criterion and its significance (36.65, p = 0.000), as well as the coefficient of multiple determination (df), we can assume that this model of influence of these predictors is sufficiently informative and statistically reliable.

Table 4

Characteristics of independent predictors
of avital types of bone nonunion

Indicators		**B**	**B**	**Standard Error B**	**t**		**P**
Constant			0.748	0.310	2.414		0.017
X_1 (CICP)		0.366	0.012	0.002	5.027		0.000
X_2 (TGF-β1)		0.313	0.063	0.012	5.241		0.000
X_3 (Pyridinolin)		-0.310	-0.065	0.014	-4.678		0.000
Regression equation: $Y = 0.748 + 0.012 \cdot X_1 + 0.063 \cdot X_2 - 0.065 \cdot X_3$.							
Regression statistics		Dispersion analysis (ANOVA)					
Multiplicity R	0.863	Indicator	df	SS	MS	F	P
Multiplicity R^2	0.744	Regression	3	55.186	18.395	121.22	0.000
Adjusted R^2	0.738	Remaining	125	18.969	0.152		
Standard error	0.398	Total	128	74.155			

Notes: Dependent variable Y: Clinical and X-ray type of bone nonunion.

It was established that the independent predictors of loss of bone mass, mainly in the affected segment could be considered the level of Hcy, cholesterol and interleukin-6 in serum. The statistical characteristic of the model is presented in Table 6. Taking into account the values of the standardized regression coefficients of HC, cholesterol and interleukin-6 (β = 0.242, 0.258 and 0.248), the significance of these predictors in this model can be assumed to be equivalent. Non-standardized regression coefficients (B) of the model are significant and reliable (t> 2.0, p <0.05), which made it possible to create a regression equation Y (Xi) and describe the mathematical relationship between the dependent variable (ΔKI) and the independent selected in the process of analysis, predictors. Taking into account the size of Fisher's criterion and its significance (15.22, p = 0.000), as well as the coefficient of multiple determination (df), we can assume that this model is sufficiently informative and statistically reliable.

Table 5

Characteristics of independent predictors of systemic decrease in bone mineral density in patients with bone nonunion

Indicators		**B**	**B**	**Standard Error B**	**t**		**P**
Constant			1.067	0.265	4.033		0.000
X_1 (Homocysteine)		0.428	0.098	0.016	6.202		0.000
X_2 (TGF-β1)		-0.291	-0.022	0.005	-4.223		0.000
Regression equation: $Y = 1{,}067 + 0{,}098 \cdot X_1 - 0{,}022 \cdot X_2$.							
Regression statistics		Dispersion analysis (ANOVA)					
Multiplicity R	0.573	Indicator	df	SS	MS	F	P
Multiplicity R^2	0.328	Reg.	2	22.975	11.488	36.65	0.000
Adjusted R^2	0.319	Remaining	150	47.014	0.313		
Standard error	0.559	Total	152	69.989			

Note. Dependent variable Y: T-score of healthy limb.

Table 6

Characteristics of independent predictors of local reduction of bone mineral density in patients with bone nonunion

Indicators		**B**	**B**	**Standard Error B**	**t**		**P**
Constant			-5.802	3.004	-1.931		0.057
X_1 (Homocysteine)		0.242	0.401	0.177	2.270		0.026
X_2(Cholesterol)		0.258	1.473	0.526	2.799		0.006
X_3 (Interleukin-6)		0.248	0.510	0.210	2.423		0.017
Regression equation: $Y = -5.802 + 0.401 \cdot X_1 + 1.473 \cdot X_2 + 0.510 \cdot X_3$.							
Regression statistics		Dispersion analysis (ANOVA)					
Multiplicity R	0.576	Indicator	df	SS	MS	F	P
Multiplicity R^2	0.360	Reg	3	824.62	274.87	15.22	0.000
Adjusted R^2	0.310	Remaining	92	1662.03	18.06		
Standard error	4.183	Total	95	2486.65			

Notes: Dependent variable Y: ΔKI.

Conclusions

The violation of reparative osteogenesis is associated with the formation of an adverse metabolic, proinflammatory and vascular pattern, which to a certain extent is determined by the polymorphism of the genes of the enzymes exchange of Hcy and nitric oxide. This pathogenetic pattern

is based on the formation of HHcy, dyslipidemia and cytokine imbalance, which determine the direction of violations of reparative osteogenesis.

The association of high levels of Hcy, cholesterol and interleukin-6 and low levels of TGF-β1 and CICP in serum, it is highly likely that the formation of avital types of bone nonunion, characterized by inhibition of collagen formation, increased bone resorption and demineralization processes, should be expected. At the same time, with the association of aberrant levels of Hcy, lipids and inflammatory mediators with normal or high levels of TGF-β1 and CICP in the blood serum, the type of disorders of reparative osteogenesis will prevail. Disturbance of endothelial function of blood vessels is also one of the factors that determines the high probability of development of hypoplastic and atrophic types of bone nonunion.

The genetic determinants of the course of reparative osteogenesis in a hypoplastic and atrophic type are the homozygous carrier of mutations MTHFR C677T or eNOS T786S, as well as a combination of both polymorphisms.

Key words: predictors, reparative osteogenesis, bone nonunion, hyperhomocysteinemia, gene polymorphism, cytokines, dyslipidemia.

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✉ Bezsmertnyi Yurii
bezsmertnyiyurii@gmail.com
✆ (Viber, Telegram, WhatsUp):
+380972815160