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A relationship between the polymorphism in the coding and 5' regions of the porcine *MyoD* genes and microstructure traits of *longissimus lumborum* muscle

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The *MyoD* family consists of four structurally related genes, products of which control the processes of myogenesis. The objective of the study was to analyse the relations existing between selected polymorphisms in the coding and 5' flanking regions of the porcine *MYOD1, MYF5* and *MYOG* genes and microstructure of *longissimus lumborum* (LL) muscle. Torhyb Line [(Polish Large White × Polish Landrace) × Pietrain] fatteners were used (n=115). The diameter (μm) and content (%) of slow-twitch oxidative (STO), fast-twitch oxidative (FTO), fast-twitch glycolytic (FTG) and pathological fibres was determined. The relation between *MyoD* genotypes and microstructure characteristics was analysed in a group of 93 fatteners of the *CC* or *CT* genotype at *locus RYR1*. The pigs of *TT* genotype at *RYR1 locus* were excluded due to significant effect identified of that genotype on the muscle microstructure. Sex appeared to have no significant effect on the muscle microstructure traits. The significant effect on LL microstructure was identified regarding the *locus MYOD1*. The *C*489*T* transition affected the diameter of FTO fibres, and the transversion *G*566*C* affected the content of fibres FTO and FTG in a bundle. The mutation *G*566*C* may be interesting for further studies, because it changes amino acid sequence from arginine to proline. In this study a significant effect of the *MYF5* and *MYOG* genotype on the LL muscle microstructure was not identified.

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One of the most important traits in farm animals which are improved are muscle deposition and meat quality. These are quantitative, polygenic traits affected by genetic and environmental factors [Karlsson *et al*. 1999]. An increase in lean meat content of carcass may be caused by hypertrophy or hyperplasia. Hypertrophy is an increase in myofibre diameter and leads to increase of muscle mass. Hyperplasia is an increase in the number of myofibers in prenatal period by multiple mitotic division of original muscle cells. The number of these divisions is correlated with the number of adult muscle cell, which is determined in prenatal period [Koohmaraie *et al*. 1995, Grobet *et al*. 1997].

Skeletal muscles consist of different fibre types, which are identified on the basis of enzymatic reactions. These methods allow to distinguish the slow-twitch high oxidative (STO), fast-twitch oxidative (FTO) and fast-twitch glycolytic (FTG) (or fast-twitch low oxidative) – Karlsson *et al*. [1999]. Domestic pigs are selected on the basis of rapid growth ability. Their skeletal muscles contain more of fast glycolytic, white muscle fibres than those of pigs growing slowly [Karlsson *et al*. 1999].

Several candidate genes are indispensable in the processes of muscle development. The myostatin gene (*MSTN*) was identified in 1997 in mice by McPherron *et al*. [1997] who were able to demonstrate that knock-out of *MSTN* caused high growth in mice as a result of the higher muscle mass deposition. Myostatin is a member of TGF-β superfamily of growth and differentiation factors, and controls the development of skeletal muscles as a negative regulator during muscle growth [McPherron *et al*. 1997]. In myostatin gene several mutations were reported responsible for muscular hypertrophy in cattle [Grobet *et al*. 1997, Smith *et al*. 2000] and meat quality [Kobolák and Gócza 2002].

Calpastatin is endogenous inhibitor of calpains. The calpain-calpastatin system plays an important role in postnatal growth of skeletal muscle. The active calpain is indispensable for fusion of myoblasts proliferation and cell growth [Goll *et al*. 1998]. A relationship between genotypes at the porcine *loci MSTN* and *CAST* and microstructure traits of *longissimus lumborum* (LL) muscle have been reported by Kłosowska *et al*. [2005].

In the last years interesting for muscle development are genes of the *MyoD* family, which consists of four structurally related genes: *MYOG* (*myogenin*), *MYOD1* (*MYF3*), *MYF5* and *MYF6* (*MRF4*) – Te Pas and Visscher [1994]. The products of these genes are specific transcription factors with the bHLH domain, and participate in muscle development from commitment and proliferation through fibre formation up to their postnatal maturation and function. The expression of *MyoD* genes takes place exclusively in skeletal muscles [Te Pas and Visscher 1994]. The *MYOD1* and *MYF5* genes are expressed in early period of myogenesis, during proliferation of myoblasts, while *MYOG* and *MYF6* control the processes of myoblasts differentiation [Buckingham 1994]. Postnatal expression of *MYOD1*, *MYF5* and *MYOG* is characteristic of satellite cells [Koishi *et al*. 1995]. It has been shown that in adult

rat the *MYOD1* gene was expressed in white muscles, while *MYOG* in red muscles [Hughes *et al*. 1993]. The studies performed on mice showed that knock-out of the *MyoD* genes caused the pathological changes in differentiation of muscle, skeleton and ribs [Braun *et al*. 1992].

The effect of several mutations has been demonstrated in regulatory, coding and non-coding regions of the porcine *MyoD* genes on the growth rate and carcass traits [Te Pas *et al*. 1999ab, Cieślak *et al*. 2000, 2002, Kurył *et al*. 2002, Urbański *et al*. 2005, 2006, Wyszyńska-Koko *et al*. 2006, Anton *et al*. 2006, Verner *et al*. 2007].

Earlier, some of the authors of this study showed, for the first time, a significant relation between polymorphisms identified in the non-coding regions of the *MyoD* genes and muscle microstructure traits [Kłosowska *et al*. 2004]. It was concluded that further studies were needed in order to find the causal mutations in the coding or regulatory regions of *MyoD* family. Several polymorphisms were found in coding and 5' flanking regions of *MYOD1* and *MYF5* genes [Urbański and Kurył 2004ab] as well as in *MYOG* gene [Wyszyńska-Koko and Kurył 2005].

The aim of the present study was to evaluate an association between above mentioned polymorphisms in the coding and 5' regions of the porcine *MYOD1*, *MYF5* and *MYOG* genes and microstructure of LL muscle.

Material and methods

A total of 115 Torhyb Line crossbred fatteners were studied [(Polish Large White × Polish Landrace) × Pietrain]. The animals were fattened and slaughtered at about 105 kg live body weight at a private farm of northwestern Poland. Both maintenance and feeding were similar for all animals and remained in accordance with feeding standards.

Samples of about 5 × 5 × 15 mm were excized from the middle part (between 4th and 5th rib) of *longissimus lumborum* (LL) muscle about 45 min *post mortem*. The samples were frozen immediately in liquid nitrogen, stored up to the time of analysis, cut in a cryostat into 10 µm thick slides and, in order to identify muscle fibre types – slow-twitch oxidative (STO), fast-twitch oxidative (FTO) and fast-twitch glycolytic (FTG) – subjected to a double reaction for activity of NADH-TR oxidoreductase and myofibrillar ATPase [Wegner *et al*. 1993]. From each animal 10 bundles, each containing 440-550 muscle fibres, were randomly selected for an evaluation of proportions between muscle fibre types. All fibres within a bundle were counted and measured. In order to determine the various degenerative characteristics of the muscle, the slides were stained according to van Gieson [Dubovitz *et al*. 1973], and an evaluation was made of the proportion of various pathological changes. Mean diameters (µm) of all fibres of the same type were evaluated using a LEICA Q500MC image analysis system. The total number of fibres was calculated per mm^2 of muscle.

Genomic DNA was isolated from blood samples according to Kawasaki [1990]. The *RYR1*/*Hin*PI genotypes were identified using a sequence of primers after Kamiński *et al*. [2001]. PCR-RFLP polymorphism of the *MyoD* family genes was determined according to the following procedures: *MYOD1*/*Bss*SI – in exon 1 [Urbański and Kurył 2004a]; *MYF5*/*Hin*PI – in the 5' region [Urbański and Kurył 2004b]; *MYOG*/*Mae*III – in exon 1 [Wyszyńska-Koko and Kurył 2005]. Polymorphism at *locus RYR1* was defined in all animals. Polymorphism at the porcine *MyoD loci* and the relation between the genotype at these *loci* and microstructure of LL muscle were evaluated for 93 pigs of genotype *CC* or *CT* at *locus RYR1*. The animals of genotype *TT* at this *locus* were excluded from further considerations.

A relationship between *MyoD* genotypes and the LL muscle microstructure traits was evaluated using the least square method of the GLM procedure in the SAS 8.2 statistical package of 2001. The linear model included fixed effects of the sex and genotype. Body weight at slaughter was included as covariates. The following model was applied:

$$Y_{ijklm} = \mu + S_i + H_j + M_k + \beta\,(BWS_{ijkl} - BWS) + e_{ijklm}$$

where:

μ – overall mean;

S_i – effect of i-th sex (i = male; female);

H_j – effect of j-th *RYR1* genotype (j = *CC*, *CT*);

M_k – effect of k-th genotype at the *MYOD1*, *MYF5* and *MYOG loci*;

$\beta(BWS_{ijkl} - BWS)$ – linear regression for body weight at slaughter;

e_{ijklm} – random error.

Results and discussion

Table 1 shows the frequency of genotypes at the *loci MYOD1*, *MYF5* and *MYOG* concerning the mutations in the coding and 5' regions of these genes identified earlier by Urbański and Kurył [2004ab] and Wyszyńska-Koko and Kurył [2005]. The *RYR1* genotype was also defined in tested crossbreds because of its known effect on the muscle microstructure in pigs [Essen-Gustavsson *et al*. 1992, Fiedler *et al* 1999].

The frequency of genotype *CC* at *locus MYOD1* (transversion *G*566*C*) and *TT* at *locus MYF5* (transition *C*613*T*) appeared very low (1.2% and 3.2%, respectively). A similar distribution of these genotypes has been observed in another breeds and lines of pigs [Urbański *et al*. 2005, 2006]. One of the genotypes – homozygote *TT* – at the *MYOG locus* (mutation *C*84*T*) was not present in animals tested in this study. The absence of genotype *TT* at *locus MYOG* has also been noticed in the other pig breeds kept in Poland [Wyszyńska-Koko and Kurył, 2005].

Table 1. Frequency of *RYR1*, *MYOD1*, *MYF5* and *MYOG* genotypes in Torhyb Line fatteners [(Polish Large White × Polish Landrace) ×Pietrian]

Locus	Mutation	Genotype	N	%
RYR1	*C*1843*T*	*CC*	35	30.43
		CT	58	50.43
		TT	22	19.14
MYOD1	*C*489*T*	*CC*	32	34.4
		CT	50	53.8
		TT	13	11.8
	*G*566*C*	*GG*	81	87.0
		GC	11	11.8
		CC	3	1.2
MYF5	*C*613*T*	*CC*	45	48.4
		CT	45	48.4
		TT	3	3.2
MYOG	*C*84*T*	*CC*	81	87.0
		CT	12	13.0

N – number of animals.

Table 2. Least squares means (LSM) and their standard errors (SE) for diameter of STO, FTO and FTG fibres, and content of normal and pathological fibres in *longissimus lumborum* muscle as related to *RYR1 locus* genotype in Torhyb Line fatteners [(Polish Large White × Polish Landrace) × Pietrain]

Trait	Genotype					
	CC		*CT*		*TT*	
	LSM	SE	LSM	SE	LSM	SE
Diameter of fibres (μm)						
STO	**46.1^{A}**	**0.9**	**48.2^{AB}**	**0.8**	**50.5^{B}**	**1.3**
FTO	**45.0^{a}**	**1.1**	**48.3^{ab}**	**0.9**	**48.6^{b}**	**1.4**
FTG	**60.7^{a}**	**1.2**	**63.6^{b}**	**1.0**	**62.8^{ab}**	**1.6**
Content of fibres in a bundle (%)						
STO	16.4	0.8	16.6	0.7	18.1	1.2
FTO	16.5	0.9	16.5	0.9	16.9	1.4
FTG	67.1	1.3	66.9	1.0	65.0	1.9
pathological fibres	**9.8^{A}**	**0.9**	**9.2^{A}**	**0.8**	**14.2^{B}**	**1.4**
giant fibres	**1.6^{A}**	**0.4**	**1.2^{A}**	**0.3**	**5.8^{B}**	**0.6**
angular fibres	0.5	0.3	0.9	0.3	0.5	0.5
Number of fibres/mm^2	**189.7^{Aa}**	**5.3**	**177.53^{a}**	**4.4**	**157.8^{Bb}**	**8.1**

$^{aA...}$Within rows means bearing different superscripts differ significantly at: small letters – P≤0.05; capitals – P≤0.01.

The microstructure of the LL muscle was characterized in a total of 115 Torhyb Line crossbred fatteners. Sex appeared to have no significant effect on the muscle microstructure traits what was also shown earlier by Kłosowska *et al*. [2004]. The effect of *RYR1* genotype on the microstructure traits of LL muscle in the crossbred

fatteners considered in the present study is shown in Table 2. The genotype at the *RYR1 locus* showed a significant or highly significant effect on the diameter of all types of muscle fibres in question, content of pathological fibres in a bundle (including giant fibres) and number of fibres per area unit. Due to significant differences in a value of muscle microstructure characteristics observed between *CC* and *CT* genotypes on one side and genotypes *TT* on the other, the latter were excluded from a further statistical analysis. Thus, in analysis of association between genotypes at the porcine *loci MyoD* and LL microstructure traits only 93 animals were considered (Table 3 and 4).

Table 3. Least squares means (LSM) and their standard errors (SE) for STO, FTO and FTG fibre diameters and number of fibres per mm^2 in *longissimus lumborum* muscle in as related to *MyoD locus* genotypes in in Torhyb Line fatteners [(Polish Large White × Polish Landrace) × Pietrain]

Gene	Mutation	Genotype	Fibre diameter (μm)						Number of fibres/mm^2	
			STO		FTO		FTG			
			LSM	SE	LSM	SE	LSM	SE	LSM	SE
MYOD1	*C*489*T*	*CC*	48.78	1.24	**47.18^{ab}**	**1.3**	63.1	1.43	174.47	6.43
		CT	48.93	0.92	**45.92^{a}**	**0.96**	61.81	1.06	179.36	4.76
		TT	51.15	2.03	**50.89^{b}**	**2.12**	62.41	2.33	164.91	10.5
	*G*566*C*	*GG*	49.29	0.85	46.65	0.9	62.22	0.97	177.9	4.41
		GC	47.83	1.93	46.56	2.07	62.55	2.22	170.6	10.06
		CC	47.9	5.69	52.57	6.08	58.91	6.52	171.76	29.5
MYF5	*C*613*T*	*CC*	49.39	1.06	47.57	1.16	64.04	1.25	171.94	5.96
		CT	48.02	1.03	46.31	1.12	61.13	1.22	180.08	5.80
		TT	49.72	2.51	48.21	2.74	63.52	2.96	176.9	14.24
MYOG	*C*84*T*	*CC*	48.94	0.87	46.39	0.93	62.26	1.04	177.38	4.84
		CT	49.86	1.73	48.2	1.84	62.96	2.05	169.66	9.54

$^{aA,\ldots}$Within columns means bearing different superscripts differ significantly at P≤0.05.

Table 4. Least squares means (LSM) and their standard errors SE) for content (%) of STO, FTO, FTG and pathologically changed fibres in *longissimus lumborum* muscle as related to *MyoD locus* genotypes in Torhyb Line fatteners [(Polish Large White × Polish Landrace) × Pietrain]

Gene	Muta-tion	Geno-type	Fibres content (%)									
			STO		FTO		FTG		Giant		Angular	
			LSM	SE	LSM	SE	LSM	SE	LSM	SE	LSM	SE
MYOD1	*C*489*T*	*CC*	16.32	1.07	18.14	1.33	65.54	1.77	2.72	0.53	0.75	0.44
		CT	17.38	0.80	15.24	0.98	67.38	1.31	2.22	0.39	1.20	0.32
		TT	16.13	1.74	14.37	2.17	69.49	2.88	3.01	0.86	0.52	0.72
	*G*566*C*	*GG*	16.81	0.73	**17.22^{A}**	**0.82**	**65.97^{a}**	**1.17**	2.46	0.36	0.44	2.01
		GC	17.79	1.66	**9.55^{B}**	**1.93**	**72.65^{b}**	**2.67**	2.26	0.82	0.63	0.69
		CC	17.89	4.90	**8.94^{AB}**	**5.69**	**73.17^{ab}**	**7.80**	2.11	0.41	1.03	0.30
MYF5	*C*613*T*	*CC*	18.48	0.94	15.73	1.18	65.79	1.54	2.27	0.41	1.21	0.40
		CT	16.25	0.91	15.49	1.15	68.26	1.49	1.82	0.40	0.88	0.38
		TT	19.24	2.23	17.18	2.80	63.58	3.64	1.20	0.98	1.46	0.98
MYOG	*C*84*T*	*CC*	16.93	0.74	16.06	0.88	67.0	1.18	2.33	0.34	0.83	0.29
		CT	17.32	1.46	14.86	1.74	67.81	2.33	2.46	0.67	1.50	0.58

$^{aA,\ldots}$Within columns means bearing different superscripts differ significantly at: small letters – P≤0.05; capitals – P≤0.01.

The results presented here do not show any significant effect of the *MYOG* genotype on the LL microstructure traits. One may suggest that the absence of one of the homozygous genotypes (*TT*), and a high frequency of the homozygotes *CC* (87%) affected the results of statistical analysis. Non-significant was also the effect of *MYOG* gene polymorphism on carcass traits in pigs as reported by Wyszyńska-Koko *et al*. [2006]. Earlier Kłosowska *et al*. (2004) studied the effect of mutation localized in 3' end of the *MYOG* gene on muscle microstructure characteristics. They also failed to identify one of the homozygous genotypes at this *locus* in analysed breeds and relations between *MYOG* genotype and muscle microstructure traits were not found significant.

A significant effect was observed of genotype at the *locus MYOD1* (transition *C*489*T* identified with enzyme *Bss*SI) on the diameter of FTO fibres (Tab. 3). Animals of genotype *TT* at this *locus* demonstrated the highest, while the heterozygotes – the lowest value of the trait. The second mutation localized in exon 1 of the *MYOD1* gene – transversion *G*566*C* – affected the content of fast-twitch oxidative (FTO) and fast-twitch glycolytic (FTG) fibres. The highest content of FTO fibres was characteristic for animals with genotype *GG*, however the muscle of *CC* animals showed the highest content of fibres FTG (Tab. 4). The transversion *G*566*C* is located in exon 1 of the *MYOD1* gene and its effect on the microstructure of muscle may be interesting for a further studies. This mutation changes amino acid sequence from arginine to proline (*Arg*→*Pro*). The effect of proline upon protein folding is due to its ring structure. Moreover, proline disturbs the formation of α-helix because of the absence of a hydrogen atom in its amide groups which could compose a hydrogen bond. Moreover, both point mutations were identified in exon 1 of the *MYOD1* gene upstream the bHLH domain, within the Cys/His-rich region located between amino acids 62 and 101 [Tapscott *et al*. 1988]. This region may represent a metal-binding domain. Such domain may mediate protein-protein interactions and is an important component of many regulatory proteins [Frankel and Pabo 1988]. These point mutations influencing the content and diameter of FTO and FTG fibres also affect several carcass traits in Polish Large White, Polish Landrace and Line 990 pigs [Urbański *et al*. 2005, 2006].

Kłosowska *et al*. [2004] analysed the relation between polymorphism in intron 1 of the *MYOD1* gene and muscle microstructure. They reported the association of this polymorphism with the content of FTG fibres. However, due to its localization (on intron) the mutation was not considered to date as a causal mutation.

In the present study a significant effect of the *MYF5* genotype on the muscle microstructure was not demonstrated. This do not corroborate the results presented by Kłosowska *et al*. [2004] who reported a significant relationhip between genotype at *locus MYF5* (polymorphisms identified with enzymes *Dde*I and *Hinf*I in introns 2 and 1, respectively) and the proportion of FTO fibres in a bundle. Moreover, they observed differentiation in the percentage of FTG fibres in a bundle as related to the *MYF5*/*Dde*I genotype. Maybe, a low number of pigs of genotype *TT* (three animals only) affected the results of statistical analysis.

The polymorphisms of the *MyoD loci* reported in the present study had no significant effect on the proportion of giant and angular fibres in a fibres' bundles of LL muscle. On the contrary, the study by Kłosowska *et al*. [2004] showed a significant effect of polymorphism in intron 1 of the *MYF5* gene (identified with *Hinf*I enzyme) on the proportion of angular fibres.

Summarizing the results presented here one may conclude that the mutations in exon 1 of the porcine *MYOD1* gene (*C*489*T* and *G*566*C*) should be considered in further analyses as a causal mutations for variation in diameter and proportion of fibres of different metabolic types in *longissimus lumborum* muscle of pigs.

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Zależność między polimorfizmem w rejonach kodujących i 5' flankujących genów rodziny *MyoD* a mikrostrukturą mięśnia *longissimus lumborum* świń

S t r e s z c z e n i e

Analizowano wpływ zidentyfikowanych uprzednio mutacji w rejonach kodujących i 5' flankujących genów *MYOD1*, *MYF5* i *MYOG* na mikrostrukturę mięśnia *longissimus lumborum* (LL). Łącznie analizą objęto 115 tuczników linii Torhyb [Pietrain × (wielka biała polska × polska biała zwisłoucha)]. Badania zależności między genotypami w *loci MyoD* a mikrostrukturą LL oparto na materiale uzyskanym od 93 zwierząt o genotypach *CC* i *CT* względem *locus RYR*. Z analizy wykluczono zwierzęta o genotypie *TT*, bowiem wartość większości cech mikrostruktury ich LL różniła się istotnie od odpowiadających im wartości stwierdzonych wśród zwierząt o pozostałych dwóch genotypach *RYR1*. Nie stwierdzono istotnego wpływu płci na mikrostrukturę badanego mięśnia. Określono średnicę i udział (% w sumie włókien) następujących rodzajów włókien: wolno kurczliwych oksydacyjnych (STO), szybko kurczliwych oksydacyjnych (FTO) i szybko kurczliwych glikolitycznych (FTG) oraz udział włókien patologicznie zmienionych. Istotne zależności zaobserwowano tylko względem mutacji zidentyfikowanych w eksonie 1 genu *MYOD1*. Tranzycja *C*489*T* okazała się istotnie związana ze średnicą włókien FTO, natomiast transwersja *G*566*C* – z zawartością włókien FTO i FTG. Z występowaniem transwersji *G*566*C* wiąże się zamiana argininy na prolinę, co z racji znanego modyfikującego wpływu proliny na strukturę przestrzenną α-helisy może być interesujące z punktu widzenia przyszłych badań. W prezentowanych badaniach własnych nie stwierdzono istotnych zależności między genotypem *MYF5* i *MYOG* a mikrostrukturą mięśnia LL.