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Detection of Novel Quantitative Trait Loci for Cutaneous Melanoma by Genome-wide Scan in the MeLiM Swine Model

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Short title: Novel melanoma QTLs in pigs.

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Abbreviations: CI: cutaneous invasion; cM: centiMorgan; CM : cutaneous melanoma ; HSA: *Homo sapiens* chromosome; LRT: likelihood ration test; LT: lesion type; MC1R: melanocortin receptor 1; MeLiM : Melanoblastoma-bearing Libechov Minipig; MET:

presence of metastasis; NAM: number of aggressive melanoma; NL: number of lesions; NM: nodular melanoma; PAB: presence of melanoma at birth; QTL: quantitative trait locus; RH: radiation hybrid; SSC: *Sus scrofa* chromosome; SSCP: single-stranded conformational polymorphism; SSM: superficial spreading melanoma; ST: synthetic trait ; ULC: ulceration; CGH: Comparative Genomic Hybridization.

Journal Category: Research Article: Cancer Genetics

Novelty and Impact:

Novel melanoma QTLs were detected in the MeLiM swine model of hereditary cutaneous melanoma and for specific traits including tumour ulceration, presence of tumour at birth, histological tumour type and number of melanomas. An association was also observed between the development of melanoma and one MC1R allele.

The results indicate that MeLiM swine is a model for human multigenic diseases, i.e. melanoma. Several genomic regions contain several QTLs, underlining the interest of these regions. Some of them are new potential candidate regions, therefore the results may have an impact on research in human melanoma genetics.

EMBL Accession Numbers:

MC1R*2 : AM231528

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MC1R*6 : AM231530

ABSTRACT

Human cutaneous melanoma is a complex trait inherited in about 10% of cases. Although two high-risk genes, *CDKN2A* and *CDK4*, and one low risk gene, *MC1R*, have been identified, susceptibility genes remain to be discovered. Here, we attempted to determine new genomic regions linked to melanoma using the pig MeLiM strain which develops hereditary cutaneous melanomas. We applied quantitative trait loci (QTL) mapping method to a significant genome-wide scan performed on 331 backcross pigs derived from this strain. QTLs were detected at chromosome-wide level for a melanoma synthetic trait corresponding to the development of melanoma. The peak positions on *Sus scrofa* chromosomes (SSC) were at 49.4 and 88.0 cM (SSC1), 56.0 cM (SSC13), 86.5 cM (SSC15) and 39.8 cM (SSC17), and, on SSC2, at 16.9 cM, in families derived from F1 males only ($P < 0.05$, except for SSC13, $P < 0.01$). Analysis of 7 precise specific traits revealed highly significant QTLs on SSC10 (ulceration), on SSC12 (presence of melanoma at birth), on SSC13 (lesion type), and on SSC16 and SSC17 (number of aggressive melanomas) at the respective positions 42.0, 95.6, 81.0, 45.3 and 44.8 cM ($P < 0.001$ and $P < 0.05$ respectively at the chromosome- and genome-wide levels). We also showed that MeLiM *MC1R**2 allele, which determines black coat colour in pigs, predisposes significantly to melanoma. Interactions were observed between *MC1R* and markers located on SSC1 ($P < 0.05$). Taken together, these results indicate that MeLiM swine is a model for human multigenic diseases. Comparative mapping revealed human regions of interest to search for new melanoma susceptibility candidates.

INTRODUCTION

Like for most cancers, the aetiology of cutaneous melanoma (CM) is complex, involving heterogeneous, interacting genetic and environmental determinants. Familial melanoma accounts for about 10% of cases. Mutations in two high-risk genes encoding cell-cycle-regulatory proteins have been shown to cause familial CM, including the cyclin-dependent kinase inhibitor 2A (*CDKN2A*) located on the *Homo sapiens* chromosome (HSA) 9p21, and the cyclin-dependent kinase 4 (*CDK4*) located on HSA12q14.¹ *CDKN2A* germline mutations occur in about 40% of families with linkage to 9p21,^{1,2} suggesting the existence of additional gene(s) which predispose to melanoma in this chromosomal area. In the *CDK4* gene, the Arg24Cys or Arg24His mutations affect the domain binding to the p16 protein, thus preventing regulation of the p16-dependent cell cycle. These mutations have only been identified in a few families world-wide.¹ Although mutations in the *BRAF* gene located on HSA7q34 were found in a high proportion of primary melanoma tumours, *BRAF* did not appear to be a gene predisposing to familial melanoma,³ however, germline single nucleotide polymorphisms (SNPs) in *BRAF* were found to be significantly associated with melanoma in German males.⁴ One low-penetrance gene, the melanocortin receptor 1 gene (*MC1R*) which has a key role in human pigmentation, was found to code for melanoma susceptibility variants,⁵ some of which increased the penetrance of *CDKN2A* mutations.⁶⁻⁸ Other low-risk genes have been investigated in a few case-control studies but the results were of low significance.⁹

To date, few genome scans for human melanoma have been reported. The most significant, with high-density coverage of microsatellite markers, was conducted for human CM in families from which *CDKN2A* and *CDK4* involvement was excluded.¹⁰ The results suggested that the human chromosomal region HSA1p22 might contain a novel melanoma susceptibility locus, and this was confirmed in 33 additional pedigrees in the same study.

However, further screening of candidate genes for mutations in this region did not reveal any causal mutation.¹¹ High-penetrance genes may be present in other regions; linkage was reported to 1p36 in North American CM/dysplastic nevi pedigrees,¹² and to 9q21.32 in Danish pedigrees with CM and ocular melanoma.¹³

A genome scan using animal models of spontaneous CM can be of great value, not only to guide the choice of candidate genes and chromosomal regions to be studied in humans, but also to support evidence for the possible associations found with these genes before embarking into functional studies. Although very few animal models of spontaneous melanoma exist, CM is inheritable in three stocks of miniature swine: the Sinclair swine,¹⁴ the Munich Miniature Swine Troll¹⁵ and the Melanoblastoma-bearing Libechov Minipig (MeLiM).^{16,17} In these pigs, tumours arise spontaneously *in utero* or in the first months after birth, and then disseminate mainly in the lymph nodes and occasionally in inner organs, including the lung, liver, spleen, gastrointestinal tract, pancreas and kidney. However, most of these tumours regress spontaneously. As in humans, but contrary to mice, swine precursor melanoma cells originate from the basal layer of the epidermis, so that human and swine melanocytes have a comparable microenvironment. The histopathological characteristics of spontaneously occurring swine tumours were also found to resemble those of human melanomas as well documented in Sinclair and MeLiM breed models.^{17,18} Previous statistical genetic analyses of hereditary melanoma in these models suggested that two or three genes affected tumour formation, although none was identified.^{15,19,20}

In our preliminary study, the MeLiM model was used to initiate a search for predisposing genes, by conducting a preliminary genome-wide scan on 79 backcross pigs (MeLiM x Duroc) deriving from a unique MeLiM boar. Interval mapping analysis provided evidence for linkage to two regions on *Sus scrofa* chromosome (SSC) 1q25 homologous to the human region HSA9p21 and to one region on SSC2p17 homologous to HSA11p15 and

11q13.²¹ The *CDKN2A* gene was mapped to the SSC1q25 region,²² but was excluded as a major susceptibility gene in this MeLiM family by a haplotype study.²³ An association was observed with a MeLiM allele for a marker close to the *MC1R* gene on SSC6p1.5, but the *CDK4* and *BRAF* genes did not appear to be melanoma susceptibility genes in this MeLiM model.²¹

In the present study, we undertook a more extensive genome-wide scan, including a total of 331 back-cross animals issued from 4 MeLiM pigs, to confirm our initial results and to locate additional regions of potential interest. Quantitative trait loci (QTLs) corresponding to the development of melanoma, a synthetic trait (ST) which combined all phenotypic informations, were first detected. Next, 7 specific traits corresponding to precise tumour characteristics were studied, leading to the detection of additional and more significant QTLs. Furthermore, black pigs with the *MC1R*2* allele were found to be significantly predisposed to melanoma. All our findings indicate that the development of melanoma in pigs is a multigenic process and comparative mapping revealed human regions of interest in the search for new melanoma susceptibility candidates.

MATERIALS AND METHODS

Families

The MeLiM pig strain was created and maintained at the Institute of Animal Physiology and Genetics in Libečhov (Czech Republic) and was derived from crossings with several breeds (Hormel, Göttingen, Canadian Landrace, Cornwall, Large White and Vietnamese). Within this strain, cutaneous melanoma appeared spontaneously, and selective breeding led to a closed stock in which melanoma is inherited and occurs, both in utero and postnatally, in 50% of the progeny.¹⁶ The MeLiM strain is a suitable model for human melanoma because of its histopathological similarities. MeLiM animals were imported from

Czech Republic and a french herd was bred at LREG (CEA-INRA) in Jouy-en-Josas, France, for physiological and genetic studies.

As shown in Figure 1, four affected MeLiM pigs (3 females and 1 male) were mated to 5 healthy Duroc pigs (2 females and 3 males). Nine of the resulting affected F1 pigs (6 females and 3 males) were crossed with 26 healthy Duroc pigs (21 females and 5 males) to produce 331 backcross (BC) pigs which included the 79 BC used in our preliminary genome-scan which derived from F1 pigs: 91016, 81014, 81015.²¹ Thirty ml of fresh blood was collected from each animal for lymphocyte isolation.

The F1 and BC families were produced on the experimental farm of Rouillé (INRA, France). Permission for our animal experiments was obtained from the Animal Protection Office of the French Ministry of Agriculture and Forestry (78-16).

Phenotypes

Pigs were classified according to their clinical and histopathological characteristics.¹⁷ Briefly, they were diagnosed by clinical follow up over 6 months, except for the 79 BC used in our preliminary study,²¹ which were only followed up for 3 months. Flat and raised pigmented cutaneous lesions were observed, photographed and recorded, at birth and then once a month. A search for sentinel lymph nodes was performed by palpation. For each piglet of the BC progeny, all raised lesions and each type of flat lesion were histologically analysed according to the human classification, and lesion-bearing animals were autopsied for the detection of inner metastases. All raised lesions were classified as superficial spreading melanoma (SSM) or nodular melanoma (NM), with Clark's level II to V for invasion criteria. Flat lesions were classified as SSM restricted to epidermis, Atypical melanocyte proliferation or Lentigo.

On the basis of these characteristics, a categorical synthetic trait (ST) combining all the clinical and histopathological information on melanoma was defined by 5 categories, from

absence of melanoma (Phenotype I) to very aggressive forms (Phenotype V), as follows: **Phenotype I**: pigs with no pigmented lesions; **Phenotype II**: pigs with only flat lesions which were either histologically benign or could not be histologically analysed because of their large number; **Phenotype III**: pigs bearing small raised lesions with a slow growth phase (SSM or NM –Clark’s levels II to IV) or flat lesions classified as SSM-Clark’s level I; these lesions appear during the first three postnatal months; **Phenotypes IV and V**: pigs with one or more large raised or polypoid lesions, often ulcerated and associated with metastasis; these lesions correspond to deeply invasive melanoma (SSM or NM- Clark’s level IV to V), and mostly develop in utero or during the first month of postnatal life. Some of them exhibited fast growth (Phenotype V, also called Malignant A), and others, slow growth (Phenotype IV or Malignant B).

Note that for phenotypes III to V, some pigs had additional lesions corresponding to a lower grade.

We recorded the trait values for sex and coat colour and for the following specific traits: presence of melanoma at birth (PAB), number of lesions (NL) including all types, number of aggressive Malignant A lesions (NAM), presence of metastasis (MET), defined by 4 categories (no metastasis, adenomegaly detected by palpation, lymph node metastasis confirmed by histology and lymph node metastasis associated with visceral metastasis confirmed by histology), and for the most severe lesion on each animal, histological lesion type (LT) i.e. SSM or NM, cutaneous invasion (CI), graded according to Clark’s levels I to V, and clinical ulceration (ULC). Two traits (PAB and ULC) were binary; the other specific traits displayed a categorical distribution.

Genotyping data

DNA from each animal was extracted from lymphocytes isolated from 10 ml of fresh blood, and the DNA concentration was adjusted to 10ng/μl. In order to cover the entire swine

genome, we selected microsatellite markers from panels for pig genome mapping (INRA Toulouse), and tested them first on the F1 parents to establish the allelic distribution and parental information content. Some genotyping data used for the first genome scan²⁰ were combined with the newly obtained data for linkage analysis, depending on the informativity level of the markers. A total of 153 microsatellite markers was selected (supplementary Table 1) and the average coverage was estimated at ~20 cM.

Microsatellite genotyping

Large-scale genotyping was mainly performed on an automated sequencer (Applied Biosystems 3700) at the CRGS Platform of the Toulouse Genopole, France. PCR reactions were run on a Gene Amp System 9700 thermocycler (Applied Biosystems) in 10µl final volume, containing 2µl fluorescent end-labelled primers (1µM each), 2µl 10×PCR buffer, 1µl dNTP nucleotide mixed solution (20µM for each nucleotide), 2.96µl H₂O, 0.2 U of GoTaq[®] (Promega) and 2µl DNA(10ng/µl). The two µl of DNA solution and 8µl of PCR mix solution were distributed among 384 microplates using a TECAN robot (Genesis 200 x8). Samples were preheated for 5 min at 94°C, run for 35 cycles (94°C for 20 s, 55°C for 30 s and 72°C for 30 s), and heated to 72°C for a final extension step of 5 min. After amplification, PCR products were pooled, and then analysed on the capillary sequencer (Applied Biosystems 3700). PCR fragment size was analysed using GeneScan and Genotyper v3.7 analysis software (Applied Biosystems), and stored in the GEMMA database.²⁴ ³²P polyacrylamide gel was used to complete the genotyping and also to compare allele identification by these two genotyping methods.²¹ Details about methods used in the different families are described in the supplementary Table 1.

Sequencing and SSCP genotyping of *MC1R*

We first sequenced the overlapping fragments from the entire coding region of the *MC1R* gene using the following three pairs of primers derived from the swine *MC1R*

sequence (Gene accession number: AF326520). These primers amplified respectively sequence regions 401-1209 (5'-CAAGGAGCCAGGACCAACT-3' and 5'-AGATGAGCACGTCCATGACA-3'), 1041-1624 (5'-CTGCACTCGCCCATGTACTA-3' and 5'-AGGGAGAGGTGCAGGAAGA-3') and 1446-2125 (5'-GCGGTACTGTACGTCCACAT-3' and 5'-GAGGTCCTGCAGTGAGCAAC-3'). PCRs were performed on 25 ng DNA with 2mM MgCl₂. The optimized PCR conditions were 96°C for 5 min, then (45 sec at 95°C, 45 sec at 61°C, 61°C or 58°C respectively for the 3 pairs of primers, and 90 sec at 72°C) for 35 cycles, and finally, 72°C for 10 min. PCR products were then sequenced according to recommended procedures (Genome Express). After sequence analyses, the PCR-SSCP (single-stranded conformational polymorphism) method was used to detect polymorphisms of codons 17 and 22 on 478 animals (MeLiM, F1 and backcross), using the following primers to amplify sequence region 738-910 (5'-CACCTCTGGGAGCCATGA-3' and 5'-CGTCTGGTTGGTCTGGTT-3'). PCR conditions were 94°C for 5 min, (45 sec at 95°C, 45 sec at 60°C, 45 sec at 72°C) for 35 cycles, and 72°C for 10 min. Seven µl of $\gamma^{33}\text{P}$ labelled PCR products was then mixed with 7 µl of loading buffer (final NaOH: 2M), then heat-denatured at 94°C for 10 min and chilled on ice. Lastly, PCR products were separated on non-denaturing 5% polyacrylamide gel, for 9 hours at 4°C, in 0.5xTBE buffer. The PCR amplification in our families can lead to 2 sizes of fragment, a 173 bp fragment corresponding to alleles *MC1R**2 and *MC1R**4, and a 175 bp fragment corresponding to allele *MC1R**6. Due to the differences in the sequence of these fragments in the 3 *MC1R* alleles, 3 conformational single-strand doublets can be detected after electrophoretic migration, depending on animal genotype.

Radiation Hybrid Mapping

Using the pig-hamster radiation hybrid (RH) panel,²⁵ high-throughput comparative RH mapping focusing on homologous genes between HSA9 and SSC1, was performed with

primers designed to amplify swine genomic DNA according to human gene conservative sequences (Kagoshima University and National Institute of Agrobiological Sciences, Tsukuba, Japan). Information regarding the gene primers used is available on request.

Data Analysis

All data were imported into and managed by the statistical analysis system SAS 8.2, on the AIX2 platform (SAS Institute). Genotyping data not in the mendelian inheritance were discarded. The genetic map was evaluated using CRI-MAP,²⁶ and marker order and positions were compared with sex-averaged maps from the U.S. pig genome databases (www.animalgenome.org/pigs/maps/index.html), and (www.marc.usda.gov/genome/genome.html) and from the Roslin Institute (www.thearkdb.org) database. Marker order and most of the genetic distances between markers were in agreement with the public sex-averaged map, with only slight differences for the 4 marker intervals SW461-SW1466 (SSC2), SW268-S0376, SW1551-S0178 (SSC8) and SW1-SW1349 (SSC9). We therefore used the public map for interval mapping analyses.

In humans, melanoma penetrance might be affected by sex and by skin and hair pigmentation,⁵ and in pigs, by sex and coat colour.²¹ Therefore, the synthetic trait was first analysed as raw data, then after correction for sex and coat colour (PROC GLM/SAS); and lastly for sex, coat colour and *MC1R* genotype. For each specific trait, only the results after correction for sex and coat colour are given. PROC FREQ (SAS) was used to test the effects of sex and coat colour on tumour incidence. The general linear model (PROC GLM/SAS) was used to test the effects of individual markers on melanoma occurrence with a regression model,²⁷ and similarly, to test the effects of paired markers. Finally, the transmission-disequilibrium-test was performed by the Quantitative Trait Disequilibrium test (QTDT)²⁸ to trace the parental alleles associated with susceptibility to melanoma.

Models for QTL Detection

For QTL detection, interval mapping using maximum likelihood method proposed in livestock, comprising a mixture of full and half sib families,^{29,30} was applied to our data. No crossover interference was assumed. All pedigree information was used to determine the transmission phase of marker alleles: the first generation consisted of MeLiM and Duroc individuals, mated to obtain F1 males and females. The backcross generation was produced by mating F1 with new Duroc individuals. Only the most probable F1 genotype was kept in the calculation, and all genotypes of their mates were estimated with a probability >0.1, so that the likelihood was partially linearized within full-sib families. No assumption was made concerning the number of QTL alleles. The model for a given trait determined by one QTL was:

$$y_{ijk} = \mu + \mu_{S_i} + \mu_{D_j} + g_{S_i} + g_{D_j} + e_{ijk}$$

where y_{ijk} is the phenotype of the k th offspring in the family of the i th F1, mated with the j th Duroc ($i = 1, \dots, n; j = 1, \dots, n_j; k = 1, \dots, n_{ij}$), μ is the averaged family value, μ_{S_i} and μ_{D_j} are respectively the family means of i th F1 and its j th mate, g_{S_i} and g_{D_j} are the fixed effects of the QTL genotype of each F1 animal and its mate, respectively (a and $-a$ for homozygotes Q_iQ_i and $q_{ij}q_{ij}$, and d for heterozygote Q_iq_{ij}), and e_{ijk} is the residual random effect, assumed to be normally and independently distributed in the i th family, with a variance of σ_i^2 . In practice, for each F1 individual, the family mean, residual variance and QTL substitution effect were estimated, and for each Duroc individual, only the family mean and QTL substitution effect.

Three hypotheses were tested for a given trait: the usual null hypothesis “no QTL on the chromosome” (H_0), the hypothesis of one QTL on the chromosome (H_1), and the hypothesis of two linked QTLs on the chromosome (H_2). For each trait, H_0 versus H_1 was tested to exclude the absence of QTL from each chromosome. When necessary, H_1 versus H_2

test was performed for particular chromosomal regions. For the two-QTL model, we used the grid-search method, with QTL effects modelled as $g_{S_i} = g_{S_{ip}} + g_{S_{iq}}$ and $g_{D_{ij}} = g_{D_{ijp}} + g_{D_{ijq}}$, where: $g_{S_{ip}}, g_{D_{ijp}}, g_{S_{iq}}, g_{D_{ijq}}$ are fixed QTL effects of F1 animal and its mate at the p and q positions which were considered jointly.

Significance levels were determined empirically by 1,000 Monte Carlo simulations. To take into account missing genotypes, differences in marker density and family structure, only trait values were simulated under the null hypothesis model (normal distribution without QTL). The approximated Bonferroni correction of the type I error test was applied to the chromosome-wide significance levels to obtain the significance levels for genome-wide linkage.³¹ Briefly, the significance level for a specific chromosome is proportional to its contribution (r) to the total autosomal genome length. So, the genome-wide significance level,

$P_{genome-wide} = 1 - (1 - P_{chromosome-wide})^{\frac{1}{r}}$, is used to take account of testing the whole autosomal genome.

In addition, most of the animals in this swine model developed melanoma *in utero* or in the perinatal period, suggesting that some aberrant epigenetic or imprinting effect might be responsible for melanoma. Consequently, QTL detection depending on parental origin (sire *versus* dam) was performed on SSC2, which harbours an imprinting region.

RESULTS

Family study

F1 animals were produced from 4 closely-related affected MeLiM pigs (B143, 7, 9025 and B310) whose origins could be traced back to MeLiM brothers H30 and H10, and healthy Duroc animals (91291D, 75015D, 51761D, 91299D and 270D, Figure 1), so that affected F1 pigs were expected to be heterozygous for potential susceptibility loci. Table 1 shows that, in all, 331 backcross animals (42 families), with 7.9 animals per family (range:

5.0-10.5), were generated by crossing 9 affected F1 pigs with 26 healthy Duroc animals for this genome-wide linkage study. Alleles of melanoma susceptibility loci were expected to segregate in this backcross generation. Thirty per cent of the F1 animals and 17.5% of the backcross animals developed melanoma (aggressive phenotypes IV and V), suggesting a complex melanoma inheritance with incomplete penetrance. The tests on the frequencies (PROC FREQ statistical analysis system, SAS) revealed that the melanoma occurred independently of the sex but not of coat colour as pigs with a black or grey coat developed significantly more melanoma than red pigs ($P < 0.05$).

QTL mapping of the synthetic trait

To search for genetic linkage between genomic regions and melanoma development, the interval mapping method was applied to the genome scan performed with 153 microsatellite markers on 331 backcross animals. First, the single-QTL model was applied to a synthetic trait (ST) corresponding to the development of the tumours, after correction of the phenotypic data for sex and coat colour. QTLs were detected, at a significant chromosome-wide level only, on SSC1 (49.4 and 88.0 cM), 15 (86.5 cM) and 17 (39.8 cM) at a significant level ($P < 0.05$), and on SSC13 (56.0 cM) at a very significant level ($P < 0.01$, Figure 2). On SSC1, a QTL was also detected with raw data ($P < 0.05$), with a maximum likelihood ratio test (LRT) at 96.0 cM, suggesting multiple locus segregation on this chromosome. In addition, a significant QTL was revealed on SSC2 at position 16.9 cM ($P < 0.05$), in families derived from F1 males only. Three additional QTLs were detected at a suggestive chromosome-wide significance on SSC2, 7 and 14 ($P < 0.1$). The maximum LRTs and their positions are shown in Table 2. Significant contributions of F1 individuals to the LRT are indicated, showing only one to three significant QTLs for some families, due to either differences in the informativity of genetic markers and the number of animals in each family or to different locus segregations.

A grid search for two linked QTLs conducted on SSC1 for the synthetic trait, corrected for sex and coat colour, showed a pair of QTLs at 31.0 cM and 87.0 cM, with a very significant LRT (120.4, $P < 0.01$, Figure 3A).

QTL mapping of specific traits

Seven precise specific traits were used with the aim of detecting more significant QTLs underlying different aspects of melanoma development, including clinical ulceration (ULC), presence of melanoma at birth (PAB), histological lesion type (LT), number of aggressive melanomas called “Malignant A” (NAM), presence of metastasis (MET), cutaneous invasion (CI) and the total number of lesions (NL). Application of the single-QTL model to these 7 traits revealed 5 QTLs, respectively on SSC10 (ULC), 12 (PAB), 13 (LT), and 16 and 17 (NAM) at a highly significant chromosome-wide level ($P < 0.001$, Table 3 and Figure 4) and at a significant genome-wide level after approximated Bonferroni correction ($P < 0.05$). Five other QTLs were respectively found on SSC1 (NAM), 14 (LT), 4, 12 and 14 (ULC), at a very significant chromosome-wide level ($P < 0.005$) and a suggestive genome-wide level ($P < 0.1$). For those 7 specific traits, 21 additional QTLs were detected at a significant chromosome-wide level ($P < 0.05$ and $P < 0.01$). Some QTLs for different traits were located close to each other on the same chromosome (e.g. PAB and ULC on SSC12; NAM and CI on SSC1), suggesting that close or pleiotropic gene(s) might segregate for those traits.

***MC1R* and Melanoma Predisposition**

We previously observed an association between melanoma development in our MeLiM breed and the microsatellite S0035 located at 7.5 cM on SSC6, 2-3 cM downstream from the *MC1R* gene.²¹ To test the relationship between *MC1R* and melanoma, we first sequenced this gene on DNA from 6 animals representing the 5 different alleles of the marker S0035 detected in the breed. The sequences of the entire coding regions were compared to the full swine *MC1R* sequence and to the *MC1R* sequence of the European Wild Type Boar (EWB, Table 4).

We found three *MC1R* alleles, *MC1R*2*, *MC1R*4* and *MC1R*6*, as previously described,³² except that in all three, we observed an additional polymorphism in the second codon (Pro2, CCC to CCT). The *MC1R*2* allele corresponded to black animals and to the S0035 alleles 1, 4 and 5, associated with black coat colour and melanoma. As more MeLiM and F1 families than backcross families segregated for black and red coat colour, we genotyped 478 animals for S0035 and *MC1R*, including all available MeLiM, F1 and backcross families. The Quantitative Transmission Disequilibrium test (QTDT) showed highly significant associations with raw data between both *MC1R*2* and S0035-1 alleles and melanoma development ($P=3\times 10^{-6}$ and 7×10^{-5} respectively). However, after stratification for sex and coat colour effects, only *MC1R*2* which contained the Val95Met and Leu102Pro substitutions, was significantly associated with melanoma ($P= 4\times 10^{-2}$), suggesting a direct role for *MC1R* or a close gene in melanoma susceptibility.

The frequency of melanoma according to *MC1R* genotypes (Figure 5) revealed that all the 17 pigs homozygous for the *MC1R*2* allele developed melanoma. Animals heterozygous for this allele also developed more melanoma than the others, mainly when *MC1R*2* was associated with the MeLiM allele *MC1R*6* instead of the Duroc allele *MC1R*4*. These results also suggest that *MC1R* or a closely linked gene plays an important role on the development of melanoma, and that a close linked recessive gene may also cooperate. Application of the single-QTL model to the MeLiM, F1 and backcross families as a whole, for the synthetic trait, revealed a QTL at the *MC1R* position ($P<0.01$) with raw data only, suggesting that pigmentation also affects melanoma development (data not shown).

Furthermore, when the general linear model (GLM) was used to search for 2 marker interactions between *MC1R* and other microsatellite markers over the whole genome, interactions were observed between *MC1R* and the markers S0316 and SW1653 located respectively at 33.4 cM and 49.4 cM on SSC1 ($P<0.05$). When the synthetic trait value was

corrected, not only for sex and coat colour, but also for *MC1R* genotype, the QTL at 49.4 cM on SSC1 was no longer detected, but a new QTL at 84.0 cM was mapped (Figure 6). In addition, application of the two-QTL model to these phenotypic data revealed a pair of QTLs at positions 77.0 cM and 85.0 cM on SSC1 (LRT: 109.0, $P < 0.001$, Figure 3B).

Potential candidates for melanoma susceptibility

We used large-scale comparative mapping to select potential candidate melanoma susceptibility genes in homologous genomic regions between human and swine. As three potential QTL regions might exist on SSC1, which contains the HSA9p21 human counterpart, massive radiation hybrid (RH) mapping was done in swine for the genes lying on HSA9, to establish the relative positions of orthologous genes between HSA9 and SSC1. We found that two distinct regions on SSC1 were related to HSA9p21 (Figure 7). One region located at about 81.0 cM (SSC1q25), which might contain two melanoma QTLs, and the other, located at about 56 cM, which is included in the region displaying significant linkage with ST.

Comparative mapping was then done for other swine QTL regions by scanning the literature and available public genome mapping databases for humans and swine (see Table 5). The human chromosomal regions corresponding to the swine regions surrounding the main QTLs (peak position ± 15 cM), as well as the breakpoints in QTL regions on pig chromosomes 1, 2, 13 and 16, are listed in Table 5. Among the potential candidate genes located in the swine QTL human counterparts, we selected, on the OMIM site, the genes involved in melanocytic neoplasia and other malignancies, tumour progression, cell cycle regulation and DNA repair. As swine melanoma originates from neonatal neural crest-derived melanocytes (NC-M), we also selected genes involved in childhood tumours and in the signalling pathways of melanocyte development connected with the NC-M survival and terminal differentiation. This analysis revealed that the human counterparts of some swine QTL regions such as HSA 9p21 and 9q21, 1p36, 3p25 and 16q24 were previously shown to

be related to melanoma. Similarly, some candidate genes have already been stated or suggested to play a role in human cutaneous melanoma, like the genes *CDKN2A*, *MC1R*, *MITF*, and *TP53*. Lastly, potential candidates may lie on other human regions, mainly on HSA 1q32-q44, 3p26-q22, 5q34-q35 or 18p11, 17p13-q12 and 20p12-q11, for both ST and the different specific traits which are governed by various loci.

DISCUSSION

In the present study, we reported the first significant genome-wide scan for melanoma susceptibility in animals. This study combined the data acquired in our preliminary study²¹ of the MeLiM swine model conducted on 79 backcross pigs with data obtained for 252 additional swine. We therefore increased the number of families, and also paid special attention to the phenotypes, using more precisely defined specific traits in order to improve power of QTL detection.

We detected four significant QTLs for the development of melanoma, defined by a synthetic trait (ST) combining all clinical and histological data, on chromosomes 1, 13, 15 and 17, irrespective of the sex of the affected F1 pigs. They were significant at the chromosome-wide level only, the QTL on SSC13 being the most significant ($P < 0.01$). A fifth QTL was detected on SSC2, when data from affected F1 males only were used. Analysis of the data for 7 more precise traits (PAB, LT, NL, NAM, CI, ULC, and MET) revealed 31 additional QTLs. Five of these QTLs corresponding to 4 different traits (ULC, PAB, LT and NAM) on chromosomes 10, 12, 13, 16 and 17, were highly significant at chromosome-wide level, and reached genome-wide significance.

Compared to our previous analysis for the synthetic trait,²¹ we observed almost the same LRT profile on SSC1 in the present study, whereas the peak detected on SSC2 decreased in significance. The previous association on SSC6 in the *MC1R* region was also

confirmed by the finding that MC1R*2 allele was related to melanoma development. QTLs for ULC were observed on SSC7 (73 cM) and SSC8 (60cM) in the regions where associations were found with melanoma in the preliminary study.

A large number of QTLs was detected here but only 5 reached genome-wide significance. We used our QTLMAP program to analyse a mixture of full-sib and half-sib families.³⁰ This program is designed to analyse continuous normally distributed traits in familial populations. The traits studied here were hardly within the normality hypothesis: synthetic trait (classes I to V) and some specific traits were categorical, and some were binary (PAB and ULC), usually with an unbalanced number of progeny in each class. Specific models have been previously developed within the QTLMAP software to account for such distributions of the data, using data transformations and threshold models.³⁹ However, when the power of these models was compared to that of the usual Gaussian models, the general conclusions agreed for robustness of the Gaussian model. In the present study, data transformations of melanoma traits (not shown) were explored but did not significantly improve the analyses, thus justifying the choice of robust Gaussian models, although this choice may have reduced the general power of the analysis. The relatively small number of highly significant QTLs may also be due to the fact that, as in human models for melanoma occurrence, the traits analysed here are still complex and correspond to various physiological processes, especially the major trait (ST) which is a global symptom, interpreted as the result of numerous interacting and multigenic determinisms, from the time of disease onset to the severe stage. Such traits might therefore be affected by several QTLs. Analyses of traits specific for the different development phases of melanoma indeed led to higher significance, thus refining the individual phenotypes linked to the QTLs detected for ST. In addition, the recent origin of the MeLiM population, a hexahybrid cross obtained in the 1980s, appeared to be a source of the diversity of the haplotypes segregating in the F1 families. This diversity

allows the detection of more QTLs, but might cause the dispersion of heterozygous loci in different families, thus reducing the general power for the detection of individual loci. This reduction might be amplified by differences in family size and marker informativity. This is very similar to the multigenic inheritance observed in human populations exhibiting genetically linked melanoma. To overcome those limits, new genetic markers will be tested in the regions harboring the most significant QTLs and new families will be produced, to obtain new informative recombinations and refine the locations.

Among the 5 most significant QTLs detected in this study, the QTL for ULC on SSC 10 (42 cM) lies in a region homologous to the human 219-241 Mb area on HSA1q41-q44 (Table5). At this time, we did not find any potential candidate on the OMIM site. The QTL for PAB on SSC12 (95.6 cM) was mapped in the SSC12q12-q14 region containing the gene coding for the most common tumour suppressor p53, already suggested as being involved in predisposition to early-onset melanomas in humans.¹ This candidate is in contradiction with the fact that the SSC12q12-q14 region is included in the region of chromosome gain (SSC12q) identified by Comparative Genomic Hybridization (CGH) analysis on both SSM and nodular melanomas in our pigs.⁴⁰ However, this chromosomal gain correlated with the DNA amplification observed on HSA17q in human neuroblastoma, a childhood neoplasm that also arises from neural crest-derived cells.⁴¹ The QTLs for LT and CI observed around 81 cM and 71 cM respectively on SSC13q are in agreement with the recurrent loss of SSC13q36-q49 restricted to nodular melanomas previously observed in our CGH study.⁴⁰ This observation reinforces the idea that this region plays an important role in tumour invasion, since the growth of nodular melanoma, contrary to superficial spreading melanoma, starts both vertically and superficially. Note that the two QTLs may be governed by the same gene. Around 56 cM, also lies the most significant QTL for ST, underlining again the importance of this region for the development of melanomas in our pigs. The human counterpart for the 3

QTLs lies from HSA3p26 to 3q22, and harbours not only the melanoma uveal susceptibility locus UBM2, but also, numerous other potential candidate loci involved in cancer susceptibility, DNA repair, cell cycle regulation and signalling pathways suggested to play a role in melanoma (Table 5). For example, *MITF*, the master melanocyte regulator involved in the regulation of pigment genes and the melanocyte lineage survival, was recently shown to function as an oncogene in primary melanocytes bearing the V600E mutation in *BRAF*, and *MITF* amplification in melanoma was found to correlate with a decrease in patient survival.⁴² Finally, two QTLs highly significant were detected for NAM on SSC16 (45.3 cM) and SSC17 (44.8 cM). The regions harbouring these QTLs on SSC16 and SSC17 correspond respectively to the HSA5q34-q35 (or HSA18p11.31) and to the HSA20p12-q11 regions. Both of them contain potential candidates listed in table 5. Like for SSC13, on SSC17, additional QTLs were also detected in the region between 35.8 cM and 52.8 cM, including QTLs for the development of melanoma, ST and EP, MET and CI, thus underlining the interest of this region and that of the corresponding human region.

The similar LRT profiles observed on SSC1 in our 2 separate studies and the fact that several QTLs lie in a swine region corresponding to several human counterparts displaying linkage with human melanoma (1p36, 9p21 and 9q21), reinforce the confidence in true QTL segregation on this chromosome, despite the low level of significance. The results with the single-QTL model revealed 2 significant areas around 49.4 cM and 88 cM, suggesting the presence of at least 2 QTLs on this chromosome. Then, analysis with the two-QTL model, the search for interactions between *MC1R* and chromosome markers, and the correction for the *MC1R* genotype suggested the existence of at least 3 QTLs on SSC1, one in the 33.4-49.4 cM region interacting with *MC1R*, and two others at positions 77 cM and 85 cM respectively. The overall results on SSC1 are shown on Figure 6. Analysis of specific traits led to a model in which one or both these QTLs might determine the relationship of NAM to tumour

development and that of CI to the invasion, two traits which showed significant QTLs with the single-QTL model. The 77-85 cM region contains the *CDKN2A* gene, but in the affected MeLiM parental strain, this gene was excluded as a major susceptibility gene in the first family by haplotype analysis of the 2 cM area surrounding *CDKN2A*,²³ suggesting the existence of other susceptibility gene(s) in this chromosomal region. The possible presence of additional melanoma suppressor genes around the *CDKN2A* gene has already been suggested in humans, as a large proportion of families without a *CDKN2A* mutation displayed linkage to the 9p21 region. Evidence for the presence of additional tumour suppressor gene(s) in this region was provided both in familial analyses and in tumours, as well as in *in vitro* experiments using microcell hybrids.⁴³⁻⁴⁵ Our comparative mapping, using all available data, suggests that potential tumour susceptibility candidates such as the *MTAP* gene, located at about 82 cM (Figure 7), might be present on SSC1. The human *MTAP* gene is deleted in a subset of carcinomas,⁴⁶ acts as a tumor suppressor in a breast cancer cell line⁴⁷ and also seems to have a role in hepatocellular carcinoma development and invasiveness.⁴⁸ In humans, the survival-related gene *SRG* located at 1p36.22, a region also linked to melanoma in certain human families,¹² controls apoptosis and tumorigenesis.³⁷ This gene, which is probably located at about 79 cM on SSC1 (Table 5), might be a melanoma-susceptibility candidate in both humans and swine.

We found in this study that after stratification for sex and coat colour, the *MC1R**2 allele, which is dominant for black coat colour, was associated with melanoma, suggesting that *MC1R* is directly involved in its development. This result is in agreement with the fact that *MC1R* variants in humans might also increase the risk of melanoma independently of UV exposure and other clinical risk factors linked to pigmentation^{49,50} although the underlying mechanism is still unknown. In human melanocytes, α -MSH *via* its binding to *MC1R* stimulates cAMP formation, pigmentation and proliferation.⁵¹ The swine *MC1R**2 allele

contains the amino acid substitution Val95Met which corresponds to the human substitution Val92Met. In humans, this substitution was first suggested to reduce the binding affinity of MC1R for α -MSH.⁵² However, human melanocytes homozygous for Val92Met have also been shown to increase significantly the response to α -MSH, suggesting that it did not induce a loss of function.⁵³ The *MC1R*2* allele contains also the amino acid substitution Leu102Pro suspected to induce constitutive activation of the *MC1R* receptor in animals with dark coat colour.^{54,55} If that is indeed the case, a constitutive cAMP level might be sufficient to up regulate the genes involved in cell survival *via* the targets of the cAMP signalling pathway in black swine,⁵⁶ and thus explain the association observed in our model between black coat colour or *MC1R*2* and predisposition to melanoma. In humans, the common *MC1R* variants Arg151Cys and Arg160Trp that are highly associated with red hair and fair skin⁵⁷ and confer high melanoma risk, are not “loss of function” receptors. A recent study revealed that they retained considerable signalling capacity,⁵⁸ arguing for a direct role of MC1R independently of pigmentation that might be common in individuals with dark or light complexion. In humans, *MC1R* variants also modify predisposition to melanoma in segregating families with *CDKN2A* mutations.⁶⁻⁸ MC1R interactions detected in our study, with microsatellite markers on SSC1 located at 33.4 cM and 49.4 cM may also increase the effect of the alleles segregating for the QTLs on SSC1. In this study, only one marker, S0644(~81 cM), located within *CDKN2A*, has been genotyped in the MeLiM, F1 and backcross families. Interaction of *MC1R* and this marker was detected in these families (data not shown), however, new markers have to be tested to target the location of the highest interaction. The fact that red pigs also develop melanoma indicates that *MC1R*, or a close gene, is not a major gene, but acts probably as a modifier gene. We indeed cannot rule out the possibility that *MC1R* was a marker of other neighbouring gene(s) that play a role in carcinogenesis or in cell cycle regulation such as *Gas11*, *SNAI3* or *CDK10* (Table 5).

To conclude, we found putative QTLs underlying the development of melanoma in swine. These results confirm and greatly extend those of our preliminary study. Comparative mapping revealed that some of these swine QTL regions are homologous to human regions known to be involved in human cutaneous or uveal melanoma such as 1p36, 3p25, 9p21, 9q21 and 16q24, whereas others correspond to additional regions that should be investigated in human melanoma research, mainly on HSA 1q32-q44, 3p26-q22, 5q34-q35 or 18p11, 17p13-q12 and 20p12-q11. Conventional fine mapping and large-scale tumour expression analyses combined with a candidate approach will be the next steps. The availability of the swine whole genome sequence⁵⁹ expected by the end of 2006 will greatly help to increase the density of markers, including single nucleotide polymorphisms, in the regions of interest, and to improve fine comparative mapping and detection of melanoma susceptibility candidates, both in humans and swine.

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LEGENDS TO FIGURES

Figure 1. Origin of F1 pigs. Asterisks signify that these 4 MeLiM pigs, which can be traced back to MeLiM brothers H10 and H30, were used for production of F1 animals. Filled squares and circles represent affected animals, and empty squares and circles, unaffected animals. Animals with letter D are of Duroc origin.

Figure 2. Likelihood ratio test (LRT) statistic profiles. LRT statistic profiles for QTL detection (1-QTL model) on the Synthetic Trait (raw data and data corrected for sex and coat colour) on SSC1 (A), SSC13 (B), SSC15 (C), SSC17 (D), and SSC2 (E). A significant QTL on SSC 2, after statistical adjustment, was only detected with animals derived from F1 males. Thresholds are indicated at chromosome-wide level. Triangles indicate microsatellite marker positions.

Figure 3. Two-QTL model detection on SSC1, without (A) or with (B) correction for the *MC1R* genotype. A, Arrow indicates that two QTLs at 31 cM and 87 cM are located on SSC1 using the Synthetic Trait corrected for sex and coat colour effect, and without correction on the *MC1R* genotype. B, With further correction for *MC1R* genotypes, one pair of QTLs at 77 cM and 85 cM were mapped. Horizontal X and Y axes: chromosomal genetic positions in centiMorgan. Vertical Z axis: likelihood ratio test value.

Figure 4. Likelihood ratio test statistic profiles of significant QTLs identified for 4 specific traits (1-QTL model). A, Presence of melanoma A at birth: Only one QTL was detected for this trait, at one end of SSC12 and at a 1% chromosome-wide significance level,

which corresponds to 5% genome-wide significance. B, Lesion type: Two genome-wide significant QTLs, at 5% and at a suggestive 10% level, on SSC13 and SSC14, respectively. C, Number of melanoma A tumors: Two 5% genome-wide significant QTLs on SSC16 and SSC17 respectively, and one suggestive 10% genome-wide significant QTL on SSC1. D, Ulceration: One 5% genome-wide significant QTL on SSC10, and three other suggestive 10% genome-wide significant QTLs on SSC4, SSC12, and SSC14 respectively.

Figure 5. Association between the risk of melanoma and the *MC1R* genotype. The figure represents the frequency of melanoma according to *MC1R* genotypes. The synthetic trait value (Y-axis) is plotted against each *MC1R* genotype combination of alleles 2, 4 and 6, as defined in Table 4 (X-axis).

Figure 6. Likelihood ratio test statistic profiles for significant QTLs detected on SSC1 (1-QTL model). The different coloured curves correspond to the different QTLs detected on SSC1. Arrows indicate the significant peak positions for each trait. One asterisk: significant at 5% chromosome-wide level. Two asterisks: 1% chromosome-wide significance. Blue curve: one QTL for Cutaneous Invasion located at 85cM (5% chromosome-wide level). Green curve: a QTL for Tumor Number of Melanoma A located at 78cM, (1% chromosome-wide level). Red dotted curve: a QTL identified for raw data at 96cM (5% chromosome-wide level). Light blue curve: a QTL at 49cM detected after correction for sex and coat color (5% chromosome-wide level). Violet curve: a QTL at 84cM detected when data were further corrected by *MC1R* genotype (5% chromosome-wide level).

Figure 7. Comparative mapping between the HSA9 and QTL locations on SSC1. Massive radiation hybrid mapping in swine of genes lying on HSA9 revealed that genes homologous to HSA9p21 genes are present in two genomic regions on SSC1, at about 56 cM and 80 cM, corresponding to two statistically significant peaks ($P < 0.01$). The vertical dashed and dash-dotted lines represent the significant thresholds at 5% and 10%, respectively.

Table 1. Family structure of F1 pigs with melanoma, backcrossed with healthy Duroc pigs.

F1 pig numbers	Sex	<i>Backcross family</i>		Coat colour	<i>Phenotype classification^a</i>					<i>Sum</i>
		<i>Number of families</i>	<i>Average number of progeny</i>		<i>V</i>	<i>IV</i>	<i>III</i>	<i>II</i>	<i>I</i>	
78	male	5	9.2	Black	0	1	9	3	4	17
				Grey	0	0	3	0	5	8
				Red	0	0	10	2	9	21
96	female	3	5.0	Black	3	2	3	0	0	8
				Grey	0	0	1	2	1	4
				Red	0	0	2	1	0	3
81	female	4	7.3	Black	1	2	8	2	0	13
				Grey	0	1	0	0	6	7
				Red	0	0	1	3	5	9
81014	female	1	9.0	Black	0	2	0	0	1	3
				Grey	0	0	0	1	1	2
				Red	0	0	0	0	4	4
81015	male	8	7.8	Grey	4	2	10	2	2	20
				Red	3	1	6	10	21	41
91016	female	1	9.0	Grey	0	3	3	0	0	6
				Red	0	0	2	0	1	3
29	female	4	10.5	Grey	4	5	6	4	3	22
				Red	2	5	6	1	6	20
79	male	12	7.5	Grey	9	3	17	4	10	43
				Red	2	3	20	8	14	47
84	female	4	7.5	Grey	0	0	6	1	1	8
				Red	0	0	5	3	14	22
Total		42			28	30	118	47	108	331

^a Phenotype I: pigs with no pigmented lesions; Phenotype II: pigs with only flat lesions; Phenotype III: pigs bearing small raised lesions with a slow growth phase (SSM or NM – Clark’s levels II to IV) or flat lesions classified as SSM-Clark’s level I; Phenotypes IV and V: pigs with melanomas; some of them exhibited fast growth (Phenotype V, also called Malignant A), and others, slow growth (Phenotype IV or Malignant B).

Table 2. Interval mapping results for the Synthetic Trait in the backcross population.

SSC	Position (cM)	Nearest marker	Test statistic	P ^a	Significant QTLs at F1 family level									
					78 ♂	96 ♀	81 ♀	14 ♀	15 ♂	16 ♀	29 ♀	79 ♂	84 ♀	
1	49.4	SW1653	84.9	*			*		*					
1	88	SW1020	80.6	*					*					
2	9.9	SW2623	70.5	+					*					
	16.9 ^b	SW2623	51.1	*										
7	4.0	S0025	72.8	+					*					
13	56.0	SW2448	93.0	**										*
14	46.0	SW1709	72.4	+			**							
15	86.5	SW936	79.4	*							*			
17	39.8	SW2441	83.2	*										

^a Chromosome-wide significance (** 1%; * 5%; + 10%). **These QTLs are not significant at genome-wide level after Bonferroni correction.**

^b Analysis of families from F1 males.

Table 3. Quantitative trait loci detection of specific traits

Trait	SSC	Position (cM)	Nearest marker	Test statistic	P ^a
PAB	12	95.6	SWC23	106.2	*** ^b
LT	2	17.9	SW2623	64.2	*
	4	84.0	SW1996	78.1	**
	6	140.5	SW322	65.0	*
	8	22.0	SW905	73.9	*
	9	42.0	SW911	77.3	**
	13	81.0	SW398	93.2	*** ^b
	14	98.0	SW2515	81.6	** ^c
NL	3	42.0	SW487	81.6	*
	7	49.0	LRA1	82.3	*
NAM	1	78.0	SW962	92.3	** ^c
	2	21.9	SWR783	87.9	*
	6	17.5	SW2406	82.5	*
	7	4.0	S0025	92.7	**
	10	54.0	SWC19	87.0	*
	16	45.3	SW1454	103.9	*** ^b
	17	44.8	SW2441	111.0	*** ^b
CI	1	85.0	SW1020	93.9	*
	6	108.5	S0299	89.0	*
	13	71.0	SW520	98.4	**
	17	52.8	SW1920	86.1	*
ULC	2	25.9	SWR783	87.0	*
	4	4.0	S0227	98.1	** ^c
	7	73.0	S0102	86.8	*
	8	60.0	SW1070	85.0	*
	10	42.0	S0351	126.3	*** ^b
	12	95.6	SWC23	95.0	** ^c
	14	34.0	SW1321	94.0	** ^c
MET	18	19.0	SW787	85.8	*
	9	67.0	SW2571	82.0	*
	17	35.8	SW2441	85.1	*

^a Chromosome-wide significance: * 5%, ** 1% and *** 1%.

^b Genome-wide significance: (5%) (after Bonferroni correction).

^c Suggestive genome-wide significance: (10%) (after Bonferroni correction).

Table 4. *MC1R* gene polymorphisms

MC1R alleles were determined by sequencing and compared in MeLiM and Duroc pigs, versus European wild boar (EWB) (23). *MC1R* alleles 2, 4 and 6 in the melanoma herd (MeLiM, F1 and Backcross families) were determined after analysis of PCR amplification of codons 17 and 22 by single-stranded conformational polymorphism (SSCP) method.

<i>MC1R</i> allele	S0035 allele	Coat colour	Breed	Codon									
				2	17	22	95	102	121	124	164	243	
<i>MC1R*1</i>		WT	EWB	CCC Pro	GCG Ala	CGG Arg	GTG Val	CTG Leu	AAT Asn	GAC Asp	GCG Ala	GCG Ala	
<i>MC1R*2</i>	1,4,5	Black	MeLiM	-- T	-- A		A -- Met	- C - Pro	-- C			-- A	
<i>MC1R*6</i>	2	Red	MeLiM	-- T		+ CC				A -- Asn			
<i>MC1R*4</i>	2,3	Red	Duroc	-- T							- T - Val	A -- Thr	

Table 5. Putative candidate genes for MeLiM melanoma selected through swine-human comparative mapping

Quantitative trait loci (QTLs) are shown by chromosome. Only **porcine** chromosome regions bearing at least two QTLs or one highly significant QTL (P<0.001) are shown.

SSC	Trait ^a	Marker and QTL Positions (cM) ^b	Nearest Markers or Genes	Swine Cytogenetic Band ^c	Human Homologous Cytogenetic Band ^d	Human Physical Position (Mb) ^e	Potential Candidate Genes ^f			Functional involvement
							Gene	MIM	Name	
I		23.5	SW317		6q25.1	149				
					6q24.1	146.6	<i>GRM1</i>	604473	<i>Glutamate receptor metabotropic 1</i>	Melanocytic neoplasia
					6q24.2	145.4				
		ST²	29	SW1332						
			31*							
			43.5	S008	1p22-p23	6q23.2	131.9			
		ST¹, EP	49.4*	SW1653		6q12	65.67			
			55.8	SW781	1p13	9p21.2	27.15			
			~55.8	TEK (Fig.7)		9p21.2	27.15			
			58.5	SW1430		6q14.1	81.1			
			60.2	SW1668		18	43			
			67.6	SW2185		6q14.1	81			
			67.6	SWR702		15	44-54 ⁱ			
			73	S0331		18q22.3	67.8			
		ST², NAM	78**							
			79.4	SW2073		15q22.2	60.9			
			EST(AR021B04)		1p36.22	10.5 ⁱ				
					1p36.22		<i>SRG</i>	Yuan et al. 2005	<i>Survival-related gene</i>	Novel cell survival gene controlling apoptosis and tumorigenesis
		79.4	SW1846		14q21-q22	40-53 ⁱ				
		~81	CDKN2A	1q25	9p21.3	21.96	<i>CDKN2A</i>	600160	<i>Cyclin-dependent kinase inhibitor 2A</i>	Melanocytic neoplasia
		81	SW780	1q21-q27	9p22	19.9				
		82.4	SW216							
		~82.4	MTAP1 (Fig.7)		9p21.3	21.85	<i>MTAP1</i>	156540	<i>Methylthioadenosine phosphorylase</i>	Inactivation in several carcinomas
		83.2	S0142		9p22.3	16.3				
		83.7	SW501							

2	ST ² , CI ST ¹	~83.7	TYRP1 (Fig.7)		9p23	12.69					
		85*									
		93.9	SW1462		9q21.2	76.8					
					11p15.5			<i>WT2</i>	194071	<i>Multiple tumour-associated chromosome region 1</i>	Wilms tumours
					11p15.5	0.52		<i>HRAS</i>	190020	<i>V-HA-RAS Harvey rat sarcoma viral oncogene homolog</i>	Bladder cancer Few mutations in human melanoma
					11p15.5	1.97		<i>H19</i>	103280	<i>H19 gene</i>	Imprinting disrupted in childhood and adult cancers
		1.1	IGF2 (near SWC9)	2p17	11p15.5	2.1		<i>IGF2</i>	147470	<i>Insulin-like growth factor 2</i>	Imprinting disrupted in childhood and adult cancers
					11p15.4	2.86		<i>CDKN1C</i>	600856	<i>Cyclin dependent kinase inhibitor 1C</i>	Beckwith-Wiedemann syndrome
		9.8	SW2623	2p17	20q11.22-q12 ^h						
					20q11.22	31.73		<i>E2F1</i>	189971	<i>E2F transcription factor-1</i>	Cell cycle regulation, tumour suppressor in mice
				11q12.2	60.8		<i>DDB1</i>	600045	<i>DNA damage-binding protein 1</i>	DNA repair	
	ST ¹ ST ¹ (males) LT	9.9+ 16.9* 17.9*									
		19.2	SW256	2p14-p17	11q13 ^h						
					11q13.1	64.33		<i>MEN1</i>	131100	<i>Multiple endocrine neoplasia type 1</i>	Endocrine neoplasia Few mutations in human melanoma
					11q13.1	65.17		<i>SIP1</i>	602180	<i>Signal-induced proliferation associated gene 1</i>	Tumour metastasis candidate
					11q13.2	65.86		<i>BRMS1</i>	606259	<i>Breast cancer metastasis suppressor1</i>	Breast cancer metastasis suppressor
					11q13.2	66.9		<i>RAD9A</i>	603761	<i>RAD9A</i>	DNA repair
					11q13.3	69.17		<i>CCND1</i>	168461	<i>Cyclin D1</i>	Cell cycle progression, amplification or overexpression in several cancers
	NAM	21.9*									
		24	SWR783		11q12-q13 ^h						
	ULC	25.9*									
		31.2	SW2545	2p16	11q12.1	58.6					
6	ST ¹	4.5 * ^g	MC1R	6p15	16q24.3	88.5		<i>MC1R</i>	155555	<i>Melanocortin 1 receptor</i>	Genetic risk factor for melanoma and non-melanoma skin cancer.
					16q24.3	88.3		<i>CDK10</i>	603464	<i>Cyclin-dependent kinase 10</i>	Cell cycle regulation
					16q24.3	88.2		<i>FANCA</i>	607139	<i>Fanconi anaemia,</i>	DNA repair

					16q24.3	87.5	<i>GAS11</i>	605178	<i>complementation group A Growth arrest-specific 11</i>	Expressed in mice during growth arrest
					16q24.3	87.2	<i>SNAI3</i>	Katoh et al 2003	<i>Snail homolog 3, Drosophila</i>	Transcriptional repressor family implicated in carcinogenesis and embryogenesis.
	NAM	17.5* 21.4	SW2406	6p1.4	16q23.3	80.6				
7	ST¹ NAM	4.0 4.0+ 4.0**	S0025	7p13						
			EDN1	7p12-p13	6p24.1	12.4	<i>EDN1</i>	131240	<i>Endothelin 1</i>	Mitogenic and melanogenic factor Regulation of melanoma invasion Promoters
10	ULC	42 42**** 43	S0351		1q44	241.2				
			SW2491	10p14	1q41	219.4				
	NAM	50.5 54* 56	SWC19	10p11-p12	1q32.1	196.9				
			S0366	10cen						
12	PAB ULC	95.6**** 95.6** 95.8	TP53	12q12-q14	17p13.1	7.5	<i>TP53</i>	191170	<i>Tumour protein p53, Li-Fraumeni syndrome</i>	Cell cycle regulation DNA repair Predisposition to early –onset melanoma
			SWC23	12q13	17p13.1-q12 17q11.2	7.8-29 26.55	<i>NF1</i>	162200	<i>Neurofibromatosis type I</i>	Cases of melanoma in NF1 patients
13			MITF	13q23-q24	3p14.2-p14.1	69.95	<i>MITF</i>	156845	<i>Microphthalmia-associated transcription factor</i>	NC-M survival and terminal differentiation (amplified in melanoma)
		55.3	SW2412	13q23-q24	3p25.1	134.9				
					3p25-p22		<i>OVCAS1</i>	607893	<i>Ovarian cancer, epithelial, susceptibility to</i>	Ovarian cancer susceptibility loci
					3p25.3	8.94	<i>RAD18</i>	605256	<i>RAD18 homolog, S. cerevisiae</i>	DNA repair
					3p25.3	9.77	<i>OGG1</i>	601982	<i>8-oxoguanine DNA glycosylase</i>	DNA repair

				3p25.3	10.16	<i>VHL</i>	608537	<i>von Hippel-Lindau tumour suppressor</i>	Predisposition to several neoplasia
				3p25.3	10.08	<i>FANCD2</i>	227646	<i>Fanconi anaemia, complementation group D2</i>	DNA repair
		13q23-q26		3p25.2	12.64	<i>RAF1</i>	164760	<i>v-raf-1 murine leukaemia viral oncogene homolog 1</i>	RAS/MAPK pathway
				3p25.2		<i>UBM2</i>	606661	<i>Melanoma, uveal, susceptibility to, 2</i>	Uveal melanoma susceptibility Locus
				3p25.1	13.86	<i>WNT7A</i>	601570	<i>Wingless-type MMTV integration site family, member 7A</i>	WNT-β-catenin pathway
		XPC	13q31-q32	3p25.1	14.17	<i>XPC</i>	278720	<i>Xeroderma pigmentosum, complementation group C</i>	DNA repair
				3p25		<i>ST11</i>	602011	<i>Suppression of tumorigenicity 11, pancreas</i>	Pancreatic endocrine tumour Suppressor
ST¹	56**								
	56.4	SW2448	13q31	3p25.1	14.4				
		TF	13q31	3q22.1	134.96				
	56.4	SWC22	13q32	3q22.1	134.3				
		TFDP2	13q32	3q23	143.22	<i>TFDP2</i>	602169	<i>Transcription factor Dp-2, E2F dimerization partner 2</i>	Cell cycle regulation-Interaction with E2F transcription factor
	61.1	SW960		3p26.1	5.2				
	69.6	SW2007	13q41	3q13.31	115.4				
CI	71**								
	71.7	SW968		3p12.3	78.7				
	79.3	SW955		3q13.11	104.6				
LT	81***								
15		GDF8	15q23	2q32.2	190.7				
				2q33	189.4	<i>DIRC1</i>	606423	<i>Disrupted in renal carcinoma 1</i>	Disrupted in familial clear cell renal cancer
				2q33.1	201.9	<i>CASP8</i>	601763	<i>Caspase 8</i>	Loss leads to neuroblastoma metastasis
				2q33	202.7	<i>FZD7</i>	603410	<i>Frizzled homolog 7, Drosophila</i>	Family of receptors for WNT proteins WNT-β-catenin pathway
	76	SW1945		2q33.2	204.7				
				2q33.3	208	<i>CREB1</i>	123810	<i>cAMP responsive element binding protein 1</i>	Major nuclear target of the cAMP Pathway
				2q33.3	208.5	<i>FZD5</i>	601723	<i>Frizzled homolog 5, Drosophila</i>	WNT-β-catenin pathway

				2q35	215.4	<i>BARD1</i>	601593	<i>BRCA1-associated RING domain 1</i>	Potentially involved in breast cancer predisposition	
				2q35	216.8	<i>XRCC5</i>	194364	<i>X-ray repair complementing defective repair in Chinese hamster cells 5</i>	DNA repair	
ST¹, EP	81.1	SW2083	15q24							
	86.5*									
	88.5	SW396	15q25	2q35	219.6					
				2q35	219.56	<i>WNT6</i>	604663	<i>Wingless-type MMTV integration site family member 6</i>	Overexpressed in cervical and colorectal cancer cell lines	
		PAX3	15q25	2q36.1	222.9	<i>PAX3</i>	606597	<i>Paired box gene 3</i>	Melanocyte terminal differentiation	
16		S0390	16q21							
	40.1	SW81	16q21							
				5q35.2	176.4	<i>FGFR4</i>	134935	<i>Fibroblast growth factor receptor 4</i>	Cell motility and cancer progression	
				5q35.1	172.1	<i>DUSP1</i>	600714	<i>Dual specificity phosphatase 1</i>	Inhibition of MAPK activation	
				5q35.1	170.8	<i>FGF18</i>	603726	<i>Fibroblast growth factor 18</i>	Cell mitogenesis and survival	
		44.2	SW977	5q35.1	169.7					
NAM	44.8	SW1454	18p11.31	4.4						
	45.3****									
	46.9	SW262		5q34	161.1					
	55.7	SW2517	16q22							
17	32	S0296	17q13-q14	20p12.3	6.7					
				20p12.3	5.04	<i>PCNA</i>	176740	<i>Proliferating cell nuclear antigen</i>	Cofactor for DNA polymerase delta Fidelity of mammalian DNA replication	
		40.6	SW2441	17q12-q14	20p12.1	16.54				
	MET	35.8*								
ST¹	39.8*									
EP	41.8**									
NAM	44.8****									
CI	52.8*									
				20q11.22	31.7	<i>E2F1</i>	189971	<i>Transcription factor E2F1</i>	Cell cycle regulation, tumour suppressor in mice	
	56.4	SW1920		20q11.22	32.9					

^a Traits are defined below, except for ST¹ and ST², which are synthetic traits respectively analysed by the one-QTL and two-QTL models.

NAM, number of aggressive melanoma A tumour; CI, cutaneous invasion; LT, histological lesion type; ULC, ulceration; PAB, presence of melanoma at birth; MET, metastasis; EP, extreme phenotype (I and IV+V only, data not shown).

^b QTL positions are in bold type, as well as their significance level (see Tables 2 and 3). Marker positions were retrieved from the MARC/USDA site. The following genes were located on the swine RH map: TEK, MTAP1 and TYRP1 (Fig. 7), CDKN2A (22), MC1R (20) and IGF2 (3'UTR contains the microsatellite marker SWC9 at 1.1 cM).³³

^c The cytogenetic positions of the swine genes and markers are derived from the INRA sites (<http://www.toulouse.inra.fr/lgc/pig/cyto/cyto.htm>) or the multispecies comparable table (<http://locus.jouy.inra.fr/lgbc/multisp/multispecietable.htm>).

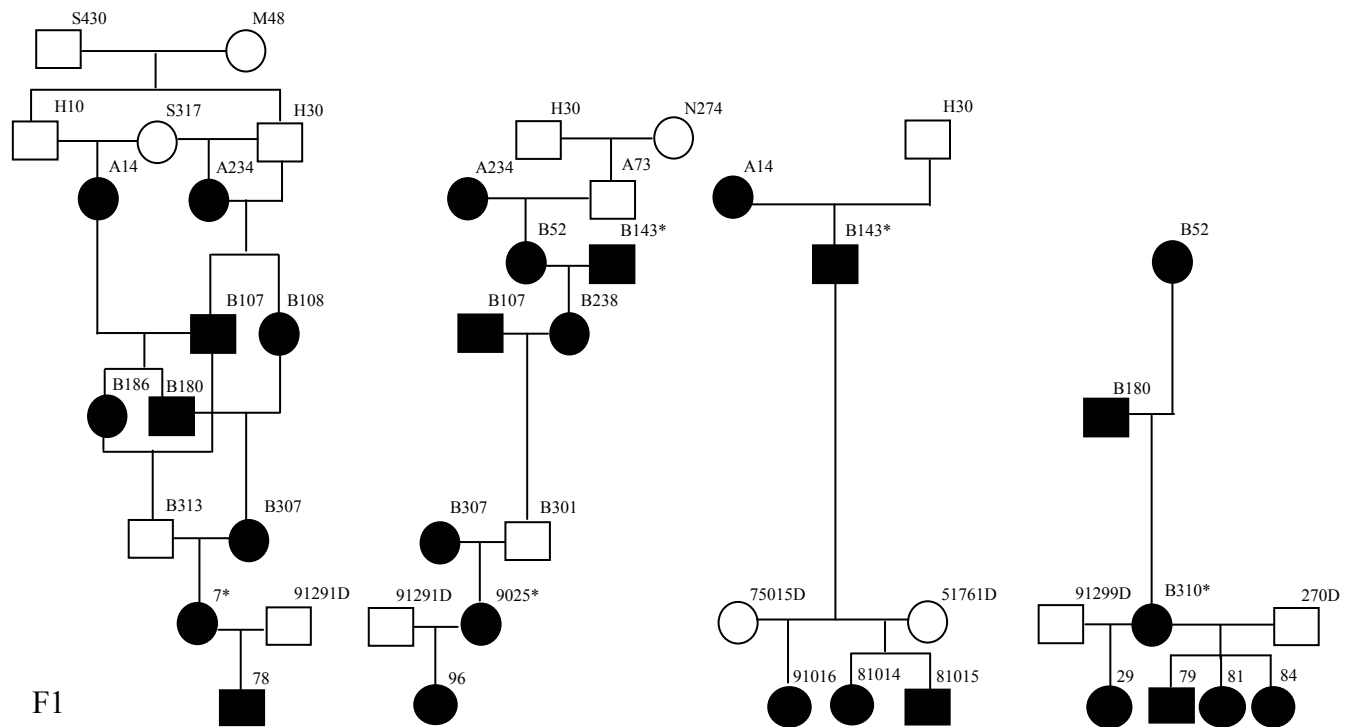
^d Data for human cytogenetics were isolated from the Ensembl database, after the position in Mb had first been determined by Robic et al.,³⁴ or retrieved from Rattink et al.^{35, h}

^e Corresponding HSA location in Mb derived from Robic et al.³⁴ (http://w3.toulouse.inra.fr/lgc/pig/msat/SSC_table.htm) or from the Ensembl Human Genome Browser (http://www.ensembl.org/Homo_sapiens/index.html), and from Demeure et al.^{36, i}

^f Potential candidate genes and their disease involvement were derived from the OMIM site (<http://www.ncbi.nlm.nih.gov/Omim/>), except for SRG and SNAI3, which were respectively described by Yuan et al.³⁷ and Katoh et al.³⁸

^g This QTL was mapped using all available animals, including the MeLiM, F1 and Backcross families (data not shown).

Figure 1



F1

Figure 2

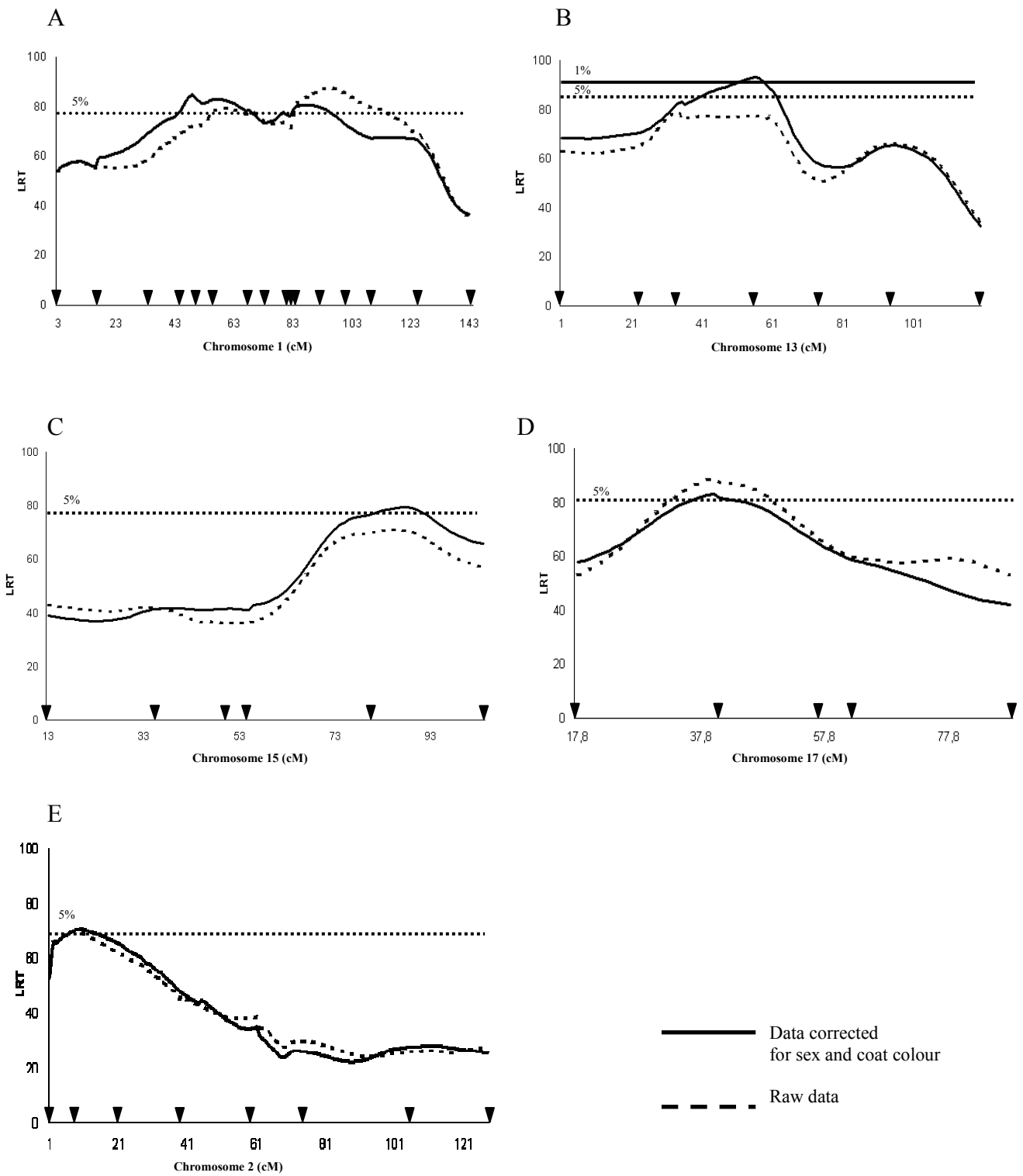


Figure 3

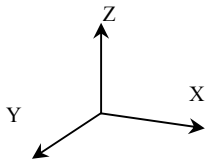
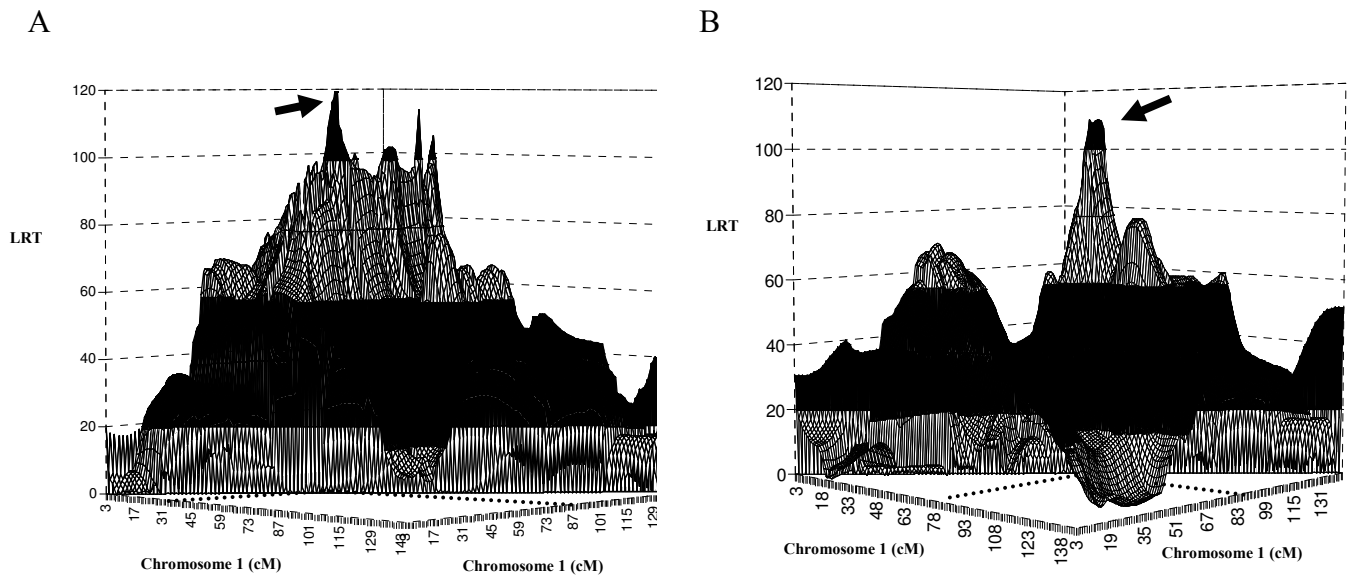
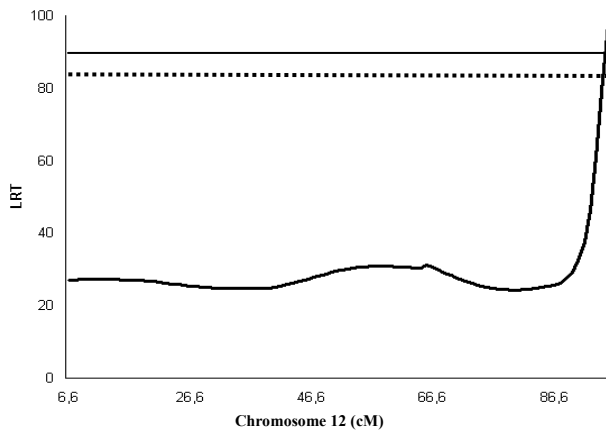
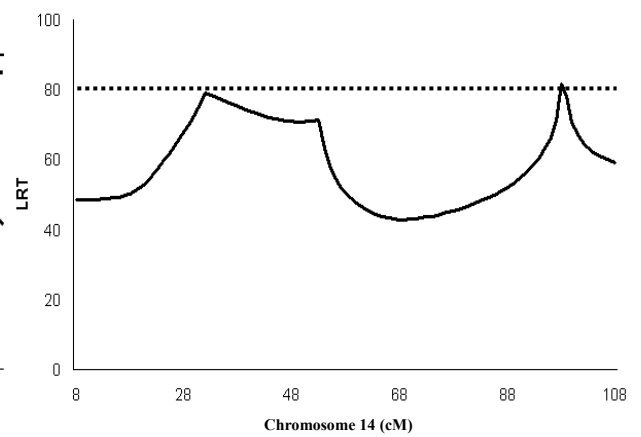
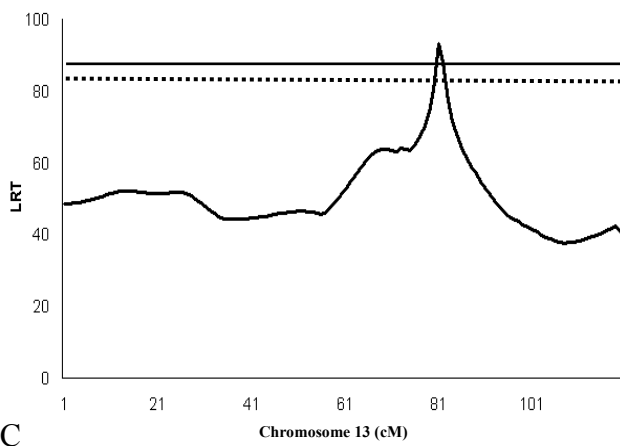


Figure 4

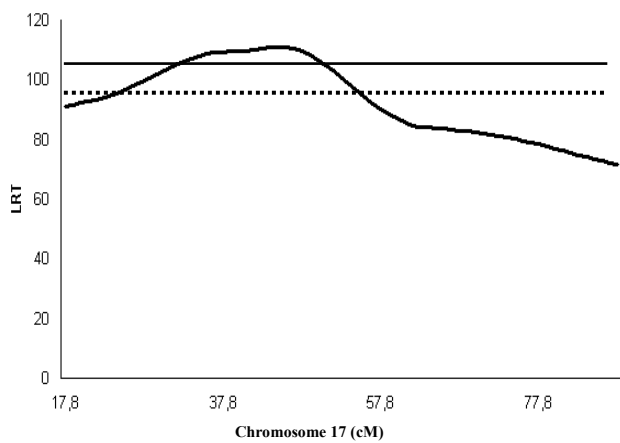
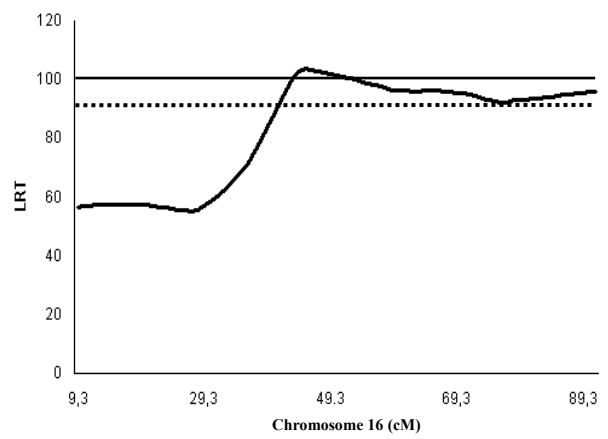
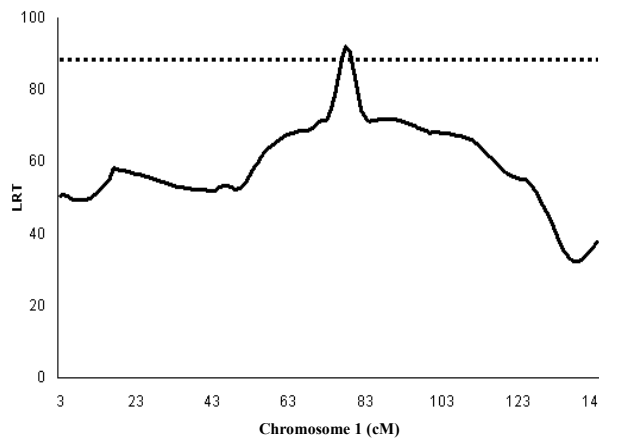
A



B



C



D

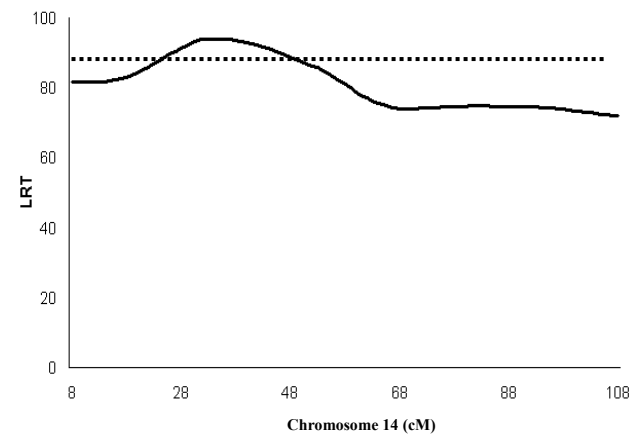
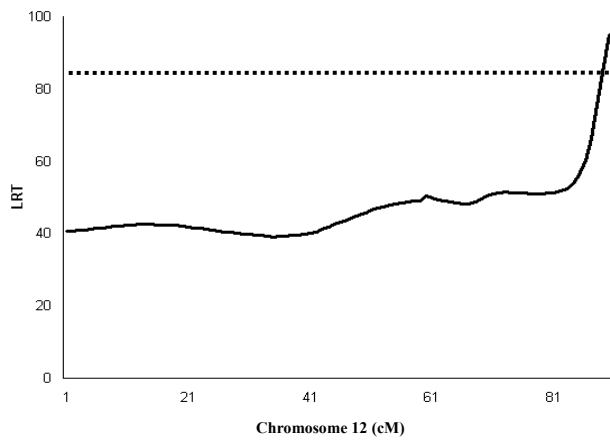
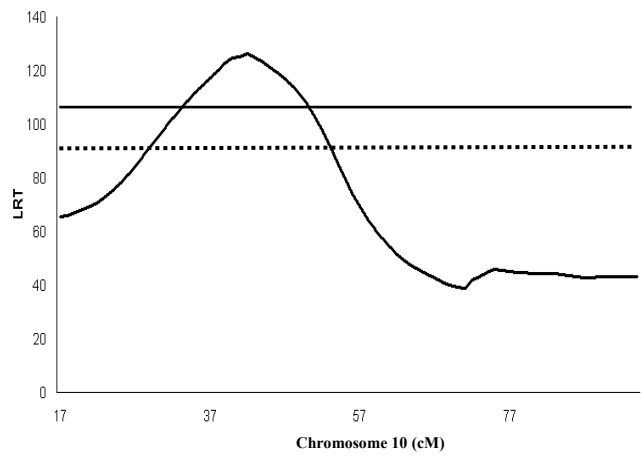
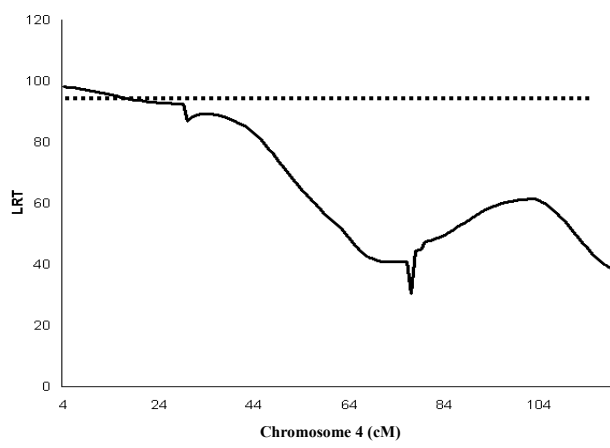


Figure 5

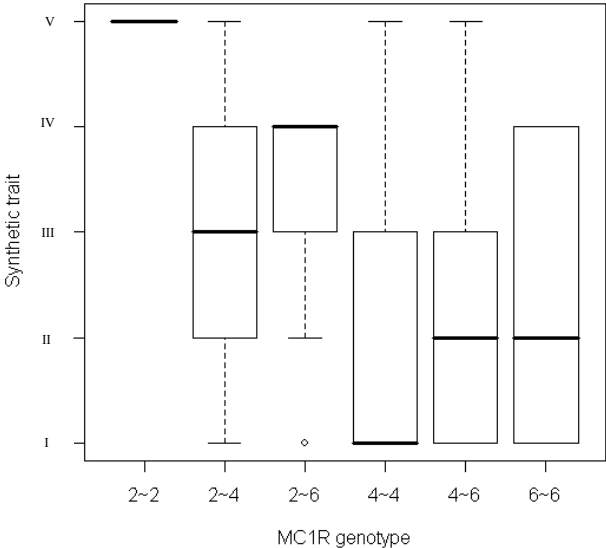


Figure 6

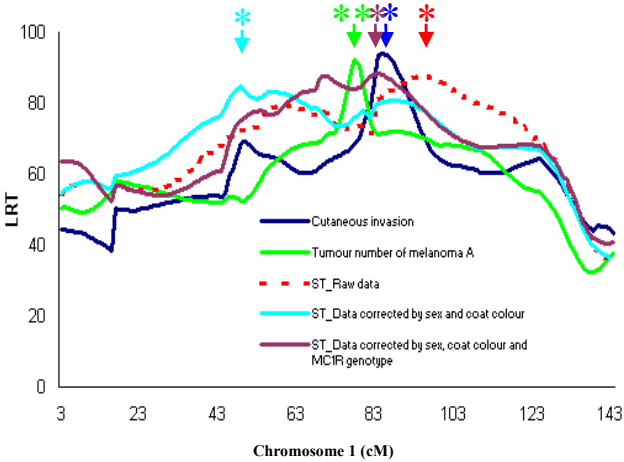
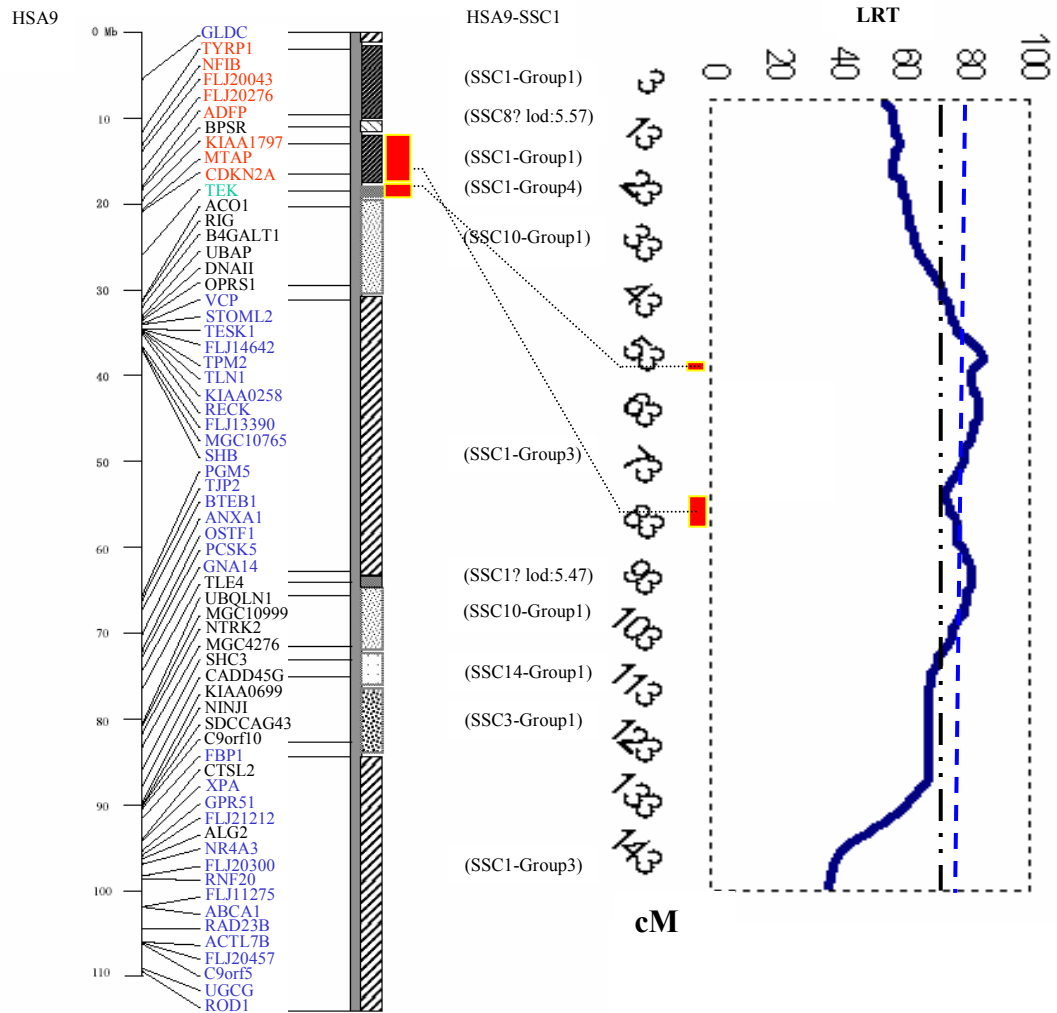


Figure 7



Supplementary Table 1. Markers used in linkage analysis and their genotyping methods

Altogether, 153 markers were selected for linkage analysis according to their informativity level in the families, from a total number of 244 markers used for the genome scan. Nearest markers to QTL positions listed in Tables 2 and 3 were selected from the total 244 markers. Families in the preliminary study derived from the following F1 pigs: 91016, 81014, 81015. Three methods were used for marker genotyping: PE3700, Applied Biosystems 3700; P33, $\gamma^{33}\text{P}$ polyacrylamide gel electrophoresis; PE377, Applied Biosystems 377; (-), not genotyped (absence of informativity). Most markers were derived from public databases (www.animalgenome.org/pigs/maps/index.html) (www.marc.usda.gov/genome/genome.html) (www.thearkdb.org), except S0644 and S0654,²⁰ UMNp948 (accession number: AY285616), MCS565A10A and CBGR1 (Denis Milan, personal communication), MC1R (this work).

Chromosome	Position (cM)	Marker	Families in preliminary study	New families
1	3	SW2184	-	PE3700
1	3	SW1824	P33	-
1	16,4	SW1515	P33	PE3700
1	33,4	S0316	-	PE3700
1	44	S0008	PE377	PE3700
1	49,4	SW1653	PE3700	PE3700
1	55,8	SW781	P33	P33
1	68	SW2185	PE377	PE3700
1	73	S0331	PE3700	PE3700
1	80,5	SW962	P33	PE3700
1	~81	S0644	P33	P33
1	81	SW780	P33	PE3700
1	83,7	SW1020	PE3700	PE3700
1	93,9	SW1462	P33	PE3700
1	100,8	UMNp948	P33	P33
1	110,5	SW1957	P33	P33
1	125	MCS565A10A	PE3700	PE3700
1	144	SW2512	P33	PE3700
2	0,9	SW2443	PE377	PE3700
2	10	SW2623	PE377	PE3700
2	24	SWR783	PE377	PE3700
2	42	SW461	-	PE3700
2	42	SW1201	PE377	-
2	61	SW1026	PE377	-
2	62	SW1466	-	PE3700
2	77	SW1517	-	PE3700
2	79	S0226	PE377	-
2	81	SW1695	-	PE3700
2	107	SW1844	P33	P33
2	114	SWR345	-	PE3700
2	128	SWR308	PE3700	PE3700
3	0	APR22	P33	P33
3	4	TFMS	P33	P33
3	17	SW2429	-	PE3700
3	17	SW72	PE377	-
3	42	SW487	PE377	PE3700
3	60,5	S0164	P33	-
3	61	SW1978	-	PE3700

3	83	SW1693	-	PE3700
3	90,6	SWR201	P33	-
3	91	SW314	-	PE3700
3	109	SW1327	-	PE3700
3	110	S0397	PE377	-
3	129	SW2532	PE3700	PE3700
4	4	S0227	PE377	-
4	5	SW480	-	PE3700
4	30	SW2547	PE377	PE3700
4	42	S0001	PE377	-
4	44	SW188	-	PE3700
4	62	SW1475	-	PE3700
4	62	SW839	PE377	-
4	77	SW1996	-	PE3700
4	78	S0073	PE377	-
4	102,8	SW2435	P33	PE3700
4	120	S0097	PE377	-
4	121	SW1461	-	PE3700
5	0	ACR11384	P33	P33
5	20	SW491	-	P33
5	39	SW1482	PE377	P33
5	72	S0092	-	PE3700
5	72	SW2425	PE377	-
5	108	S0018	PE3700	PE3700
5	133	SW378	PE377	PE3700
6	4,5	MC1R	P33	P33
6	7,5	S0035	P33	P33
6	21	SW2406	PE377	PE3700
6	46	SW2525	PE3700	PE3700
6	71	SW1067	PE377	P33
6	89	SW316	PE377	P33
6	109	S0299	PE3700	PE3700
6	116	S0121	PE377	PE3700
6	150	SW322	PE377	PE3700
7	4	S0025	P33	P33
7	30	S0064	PE3700	PE3700
7	59.4	S0654 (LRA1)	PE377	PE3700
7	60	TNFB	PE377	PE3700
7	70	S0102	PE3700	PE3700
7	83	SW1418	P33	P33
7	109	SW1083	PE3700	PE3700
7	135	S0101	PE377	-
7	136	CBGR1	-	PE3700
7	155	SW764	PE377	-
7	157	SW1380	-	PE3700
8	1	SW2410	PE377	PE3700
8	20	SW905	PE377	PE3700
8	27	SW268	PE3700	PE3700
8	47	S0376	PE377	PE3700
8	56	SW1070	-	PE3700
8	56,7	SW29	P33	-
8	74	S0442	PE3700	PE3700
8	96	S0144	PE3700	PE3700
8	127	S0178	PE377	PE3700

9	11	SW21	PE377	PE3700
9	32	SW911	PE377	PE3700
9	52	SW2401	PE377	P33
9	73	SW2571	PE3700	PE3700
9	82	SW1	PE3700	PE3700
9	100,5	S0295	P33	PE3700
9	126,1	SW2116	P33	P33
10	17	SW249	PE377	-
10	20	SW767	-	PE3700
10	42	S0351	-	PE3700
10	43	SW2491	P33	-
10	67	SW1041	PE3700	PE3700
10	95	SW951	PE377	PE3700
11	9	SW1460	PE3700	PE3700
11	44	SW151	PE3700	PE3700
11	64	S0230	PE3700	PE3700
11	85	SW2413	PE3700	PE3700
12	6,6	S0143	P33	P33
12	40	SW1307	PE377	P33
12	65	SW2559	PE3700	PE3700
12	80	S0090	PE377	PE3700
12	96	SW60	P33	P33
13	1	S0282	-	PE3700
13	1	S0219	PE377	-
13	23	SWR428	PE3700	PE3700
13	35	SW344	PE3700	PE3700
13	56,4	SW2448	P33	-
13	57	SW2450	-	PE3700
13	75	SW520	PE3700	PE3700
13	96	SW1056	PE3700	PE3700
13	126,2	S0291	P33	P33
14	8	SW857	PE377	-
14	9	SW1631	-	PE3700
14	36	SW1321	P33	P33
14	53,2	SW2593	P33	P33
14	69	SW77	PE3700	PE3700
14	108	SW2515	PE377	PE3700
15	12,5	SW2072	P33	PE3700
15	37	SWR283	-	PE3700
15	38	SW1118	P33	-
15	50,7	SW159	P33	P33
15	79,4	SW936	PE377	PE3700
15	104	SW1119	PE377	P33
16	9,3	SW742	P33	-
16	17	SW2411	-	PE3700
16	27,6	SW1645	P33	P33
16	55,7	SW2517	P33	P33
16	92	S0061	PE377	-
16	93	S0105	-	PE3700
17	17,8	SWR1004	P33	PE3700
17	40,6	SW2441	P33	P33
17	62	SW1031	PE3700	PE3700
17	88,7	S0332	P33	P33
18	1	SW1808	-	PE3700

18	2	SW2540	PE377	-
18	31,6	SW787	P33	PE3700
18	45,2	S0120	P33	PE3700
XY	11,9	SW980	P33	PE3700
X	58	SW259	PE3700	PE3700
X	97	S0511	PE3700	PE3700
X	114	S0022	PE3700	PE3700
