# Inheritance of malignant melanoma in the MeLiM strain of miniature pigs

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**ABSTRACT**: Selective breeding of miniature pigs bearing cutaneous tumours resulted in the establishment of the MeLiM strain with hereditary malignant melanoma. The inheritance of melanoma was tested in a two-generation kindred comprising 456 progeny from 78 litters. Melanomas were recognisable visually as black-pigmented lesions on the skin. Their size, shape, number, progression and metastatic spreading varied widely. All possible melanoma forms known in human melanoma, i.e. pigmented naevi, dysplastic naevi, superficial spreading melanoma and nodular malignant melanoma, were found in the MeLiM pigs. Because the occurrence of nodular malignant melanoma segregated in all recorded litters we have included only this form in the genetic analysis. The tumours were nodular with exophytic growth over the skin surface. All showed similar histopathological features, vertical growth to muscle fascia and high metastatic activity. We hypothesize, on the basis of segregation ratios obtained from various mating types, that the mode of inheritance of nodular melanoma in the MeLiM strain is probably controlled by three genes.

**Keywords**: heredity of porcine melanoblastoma; animal model; cutaneous tumour

Malignant melanoma (melanoblastoma) is a cutaneous tumour arising by neoplastic transformation of melanocytes. The frequency of malignant melamoma in humans has grown during the last few decades and it is a highly lethal form of cancer due to common metastatic spreading and poor response to radio or chemotherapy (Fine, 1992; Rigel et al., 1996; Bajetta et al., 2002; Li and McClay, 2002). On the other hand, immunotherapy has shown more promise in malignant melanoma than in most other tumours. Thus, the establishment of a suitable animal model would be an extremely important tool for developing new therapeutic strategies.

Melanoma similar to human type has been described to occur infrequently in darkly coloured pigs, such as Duroc and Hampshire breeds (Hordinsky et al., 1985). An inheritable form has also been described in the Hormel miniature pig strain. A strain of Hormel miniature pigs, known as Sinclair pigs (Oxenhandler et al., 1979; Hook et al., 1982) were founders of a herd that was bred to have a high incidence of spontaneous melanomas. One herd of these Sinclair Melanoma Pigs has been maintained at the Experimental Farm of the College of Veterinary Medicine, Texas A&M University (Blangero et al., 1996; these pigs have been moved to the University

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of Nevada at Reno, Dr. Craig Beattie) and another herd at the Comparative Medicine Research Farm in the University of Missouri-Columbia (Misfeldt and Grimm, 1994; these pigs have been moved to the Sinclair Research Center, Dr. Guy Bouchard). The Sinclair swine model is very useful due to a reliable tumour incidence and it has many features in common with its human counterparts (Hook et al., 1982; Das Gupta et al., 1989).

Genetic studies conducted in Sinclair pigs have suggested an inheritance controlled by two genes. One locus was shown to be associated with the swine major histocompatibility complex (SLA) and a second was a dominant independent autosomal locus (Tissot et al., 1993; Blangero et al., 1996). This second locus has been postulated to be lethal, in utero, when homozygous for the mutant allele but it still remains to be molecularly identified and cytogenetically mapped (Misfeldt and Grimm, 1994; Blangero et al., 1996). A similar pig model of melanoma was established in Germany. A double- or triple-recessive gene model was published for melanocytic lesions in cross-breeding between German Landrace and the Munich Miniature Swine (Munchener Miniaturschwein Troll) because back-crosses produced non-mendelian segregation (Wanke et al., 1988; Muller et al., 1995, 2000). The authors recorded mostly a benign course of melanoma development or they observed a melanocyte hyperplasia similar to human junctional naevi. Only 7.1% or 3.6% of the animals from an $F_1$ generation suffered from a malignant type of melanoma respectively (Muller et al., 1995, 2000).

A new melanoma-bearing strain of miniature pig was developed by selective breeding and has been designated by the acronym MeLiM (**Me**lanoma-bearing **Li**bechov **M**inipigs) (Horak et al., 1999). The MeLiM minipigs are a very suitable animal model for human melanoma due to numerous histopathological (Boisgard et al., 2003; Vincent-Naulleau et al., 2004) and biochemical similarities (Borovansky et al., 2003). Comparing the melanomas in the MeLiM strain and the Sinclair strain or the Munich Troll line, some resemblance exists, such as the occurrence of melanoma at birth and multiple gene inheritance (Hruban et al., 1998; Horak et al., 1999). Unlike the Sinclair pigs, the melanoma in the MeLiM strain is more aggressive (Fortyn et al., 1998). The course of melanoma development in about 1/3 cases is lethal compared to a benign course in some Sinclair pigs, where a complete or near complete regression of the tumours was described (Oxenhandler et al., 1982; Misfeldt and Grimm, 1994; Green et al., 1994). The Sinclair strain and the MeLiM strain have some common breed origin (the Hormel breed) but the MeLiM strain was developed by crossing with five other breeds or strains until it was finally bred as a closed stock. Therefore, as inbreeding has increased, there has been a rise of melanoma frequency and severity.

The melanoma in animals of the MeLiM strain is curable by the devitalization of a tumour by a simple surgical technique. Devitalization of one of multiple tumours is sufficient for successful therapy. The remaining skin melanomas as well as all metastatic lesions (proved by laparotomy in 72 animals) disappear after several months (Horak et al., 1999). Both spontaneous (Oxenhandler et al., 1979; Misfeldt and Grimm, 1994) and devitalization-induced tumour regressions (Horak et al., 1999) are accompanied by partial or almost total depigmentation. The mechanism of melanoma regression is still unknown. Our preliminary results show an increased expression of heat shock proteins followed by tumour infiltration by T lymphocytes (Horak et al., 2003), which suggests that cell-mediated anti-tumour immunity is probably responsible for melanoma regression induced by devitalization in the MeLiM minipigs. Sinclair miniature pigs show a similar spontaneous regression associated with both tumour infiltration by lymphocytes and anti-melanoma antibodies (Cui et al., 1995). Some MeLiM animals healed of melanoma by devitalization were selected for breeding to clarify the mode of inheritance in this porcine cancer model.

## MATERIAL AND METHODS

### Animals

The miniature pigs are housed in the Institute of Animal Physiology and Genetics of the Academy of Sciences of the Czech Republic, Libechov. The experimental herd of laboratory pigs was founded by importation of 5 animals of the Hormel strain from the USA in 1967. These animals were cross-bred for porcine blood group studies with several other breeds or strains: Landrace, Large White, Cornwall, Vietnamese pigs and miniature pigs of the Gottingen origin. Different cross-breeding produced more than 2000 descendants without any signs of melanoma. Nevertheless, a few black piglets with melanoma had occurred in this genetically

heterogeneous population by 1989. They originated from mating two male brothers with four related sows. These parents had no visible skin tumours. The MeLiM strain was established using selective breeding for melanoma. A permanent survival of affected pigs was achieved by devitalization therapy. This surgical procedure is based on tumour ischaemization using overlapping stitches in the form of mattress sutures conducted under the tumour base. After tightening the sutures, the tumour is not dissected but left *in situ*. Disintegration of melanoma cells and gradual transformation of the tumour into fibrous tissue was recorded at different intervals after the treatment. The regression of devitalized melanoma as well as all other intact (non-treated) skin tumours and organ metastases have been histologically documented. No relapses were recorded after healing (Horak et al., 1999). Immunological mechanisms responsible for this unique anti-tumour effect are under study.

### Breeding and inbreeding

A genealogical tree of the MeLiM pigs is very complicated. It can be expressed in a simplified form by contribution of ancestral breeds in percentage ("blood portion"). In the $F_1$ progeny of crosses of melanoma-bearing with non-melanoma pigs the following breed sharing was recorded: Hormel strain 34–53%, Vietnamese pig 10–18%, Landrace 3–7%, Cornwall 1–6%, Large White 0–6% and Gottingen breed 19–34% (but it was developed by cross-breeding of Hormel and Vietnamese pigs, so the percentage of Gottingen pigs does not represent a distinct genetic contribution). These percentages fluctuated from one family to another. The intensity of inbreeding was checked by classic Wright's formula (Nicholas, 1987). The coefficient of inbreeding (Wright's $F$ value) varied from $F = 0.02–0.10$ in families where strong inbreeding had been avoided, to $F = 0.14–0.31$ after mating of half-siblings. The highest $F$ value was reached in 4 litters ($n = 22$) from brother by sister matings ($F = 0.53–0.64$).

### Macroscopic and histological description

Melanoma tumours are recognisable visually as black-pigmented lesions on the skin. An assessment of tumour incidences and characteristics were pursued from birth to at least four months. Melanoma forms recorded in human melanoma, i.e. pigmented naevi, dysplastic naevi, superficial spreading melanoma and nodular malignant melanoma, were found in the MeLiM pigs. Moreover, all human melanoma stages classified according to Clark et al. (1969), that describes a tumour progression characterised by histogenesis and tumour behaviour (horizontal or vertical spreading, formation of metastases), were seen in porcine melanomas. At the beginning of the study we hoped to assess all forms and stages of melanoma, however, this could not be done because the size, number and localisation in different body regions varied extremely. No simple and exact quantifying criteria could be used. Because the occurrence of nodular melanoma segregated in all recorded litters ($n = 78$) we have evaluated only this form of melanoma. The nodular formations are simply scored macroscopically but the findings were confirmed by histology in 72 pigs from all melanoma bearers ($n = 181$). The tumours included in the genetic analysis are nodular, proliferate over the skin surface (exophytic growth, tuberous shape), they have vertical growth to muscle fascia and they show high metastatic activity thus corresponding to the level IV and V according to the Clark's classification for human melanoma (Clark et al., 1969). The explorative laparotomy for presence of metastases as well as "a second look" after healing was conducted in all histologically documented cases ($n = 72$).

## RESULTS AND DISCUSSION

All studied MeLiM pigs had completely black, rusty or rusty-brown coat colour (bristles) with variable intensity of skin pigmentation. In addition, very limited white pigmentation (such as either a small white forehead badge or short white "socks" on forelimbs) was found in several animals. Nodular melanomas were observed in 181 pigs out of at total of 456 progeny of the MeLiM strain. The melanoma-bearing animals were fully coloured with the exception of two piglets that showed the limited white pigmentation. The shape of nodular melanomas was sometimes irregularly spherical but mostly oval with the size from 1 cm to 10 cm with variable protuberance over the skin surface (about 1–3 cm). They were occasionally solitary but mostly multiple (Figure 1). Nodular melanomas were present already at birth or they developed shortly thereafter. Nevertheless, they showed very

Figure l. Miniature piglet with several large exophytic nodular melanomas (age 1 month)

similar histopathological features without any clear variability. Melanoma cells were darkly pigmented (brown or black) infiltrating densely the dermis so that the skin morphology was totally destroyed. Vertical spreading of melanoma cells into the reticular dermis (equivalent to Clark's level IV) or into the subcutaneous tissue (equivalent to Clark's level V) was generally observed. All these findings confirm the deeply invasive character of the porcine nodular melanoma that corresponds to human nodular melanoma.

We have analysed all possible combinations of mating of affected, non-affected, related and unrelated animals. The results are summarized in Table 1. The MeLiM founder animals had no visible skin melanoma but the nodular tumours appeared in their progeny (Table 1 – mating type [1]M– × M–). Segregation of affected and non-affected animals in this mating type (14 litters with 90 offspring) was 1 : 4. All earlier mating combinations including outbreeding of the founder animals (mating outside the MeLiM strain) did not produce any melanoma-affected animals. Mating of affected MeLiM pigs with non-affected animals descending from the same herd (Table 1 – mating type [2]M+ × M–; 4 litters with 23 offspring), gave also segregation near to 1 : 4. Another mating comprising the MeLiM affected pigs that were mated with two non-affected and absolutely unrelated animals (hybrids of the European wild boar and Vietnamese pigs), resulted in segregation 1 : 4, too (Table 1 – mating type [3]M+ × M–; 3 litters with 27 offspring). When we look at

Table 1. The segregation of nodular melanomas in different mating types

| Phenotypes of parents (mating type) | Number of offspring | | | | *$\chi^2$ | | | |
|---|---|---|---|---|---|---|---|---|
| | Litters | Progeny | **M+ | ***M– | $\chi^2_1$ | $\chi^2_2$ | $\chi^2_3$ | $\chi^2_4$ |
| [1]M– × M– | 14 | 90 | 16 | 74 | 37.37 | 2.49 | 0.27 | **0.08** |
| [2]M+ × M– | 4 | 23 | 4 | 19 | 9.78 | 0.71 | 0.10 | **0.01** |
| [3]M+ × M– | 3 | 27 | 5 | 22 | 10.70 | 6.27 | 0.21 | **0.02** |
| [4]M+ × M+ | 53 | 294 | 143 | 151 | **0.22** | 87.63 | 150.70 | 216.40 |
| [5]M+ × M+ | 4 | 22 | 11 | 11 | **0.00** | 2.71 | 7.33 | 12.37 |

*$\chi^2$ = calculated for expected segregations: $\chi^2_1$ = 1 : 1, $\chi^2_2$ = 1 : 2, $\chi^2_3$ = 1 : 3, $\chi^2_4$ = 1 : 4

**M+ = animals bearing nodular melanoma

***M– = animals without nodular melanoma

[1]M = mating of founder animals without nodular melanoma

[2]M = mating of melanoma-bearing and non-affected animals from the MeLiM strain

[3]M = mating of melanoma-bearing MeLiM pigs with totally unrelated animals without melanoma

[4]M = mating of melanoma-bearing MeLiM pigs (mostly half-siblings)

[5]M = mating of melanoma-bearing MeLiM pigs (inbred siblings)

mating of both affected parents, segregation ratio is 1 : 1 (Table 1 – mating type [4]M+ × M+; 53 litters with 294 offspring). All piglets from the mating combination brother x sister both having nodular melanomas (Table 1 – mating type [5]M+ × M+; 4 litters with 22 offspring) bore some melanocytic skin lesions and exactly a half of them ($n = 11$) developed nodular melanoma.

The following hypotheses for the mode of inheritance of nodular melanoma in the MeLiM strain were considered and some can be ruled out on the basis of the observed segregation ratios. A single dominant or recessive gene inheritance can be ruled out. No skin melanoma in the MeLiM founder animals and appearance of the nodular tumours in their progeny (M+ = 18%) rules out a single dominant inheritance. If the trait were a simple recessive gene then breeding two parents with melanoma would result in all offspring having melanoma. However, only 49% tumour-bearing piglets were observed from this mating. Similar to the Sinclair pigs, multiple recessive genes are also ruled out by the finding that multiple generations of M+ × M+ MeLiM animals does not result in 100% M+ offspring. This also makes it highly unlikely that we have a combination of recessive and dominant genetic loci, since successive inbreeding would eventually result in both loci being homozygous. Therefore, there must be a mechanism of forced heterozygosity for the dominant gene. This is very common for tumour suppressor genes, where inheritance of two copies of the tumour suppressor gene results in abnormal cellular growth and death in utero. Thus no animals that live to birth are homozygotes.

A two-gene inheritance with one recessive and one dominant homozygous lethal gene comes closer to the observed data. In this model the founders did not have melanoma because they were heteozygous for the recessive gene and one or more of the parents had the dominant gene. In this case the offspring would have an incidence of melanoma form 12.5%–19%. Since both parents would be obligate heterozygotes for the recessive gene, the probability of a homozygous offspring would be 25%. The dominant gene could be heterozygous in one parent, or in both parents. The probability of an offspring with at least one copy of the dominant gene would be 50% or 75%, respectively. Thus the probability of having both would be 12.5% and 19%, respectively. The observed incidence was 18%.

In this model mating two melanoma-bearing pigs would result in a 66% incidence of melanoma in the offspring. The melanoma-bearing parents would be obligate homozygotes for the recessive gene and obligate heterozygotes for the dominant gene. All offspring would be obligate homozygotes for the recessive gene and they would be ¼ no dominant gene, ½ heterozygotes and ¼ homozygous for the dominant gene but since the homozygotes would die in utero the observed ratio M+ : M– would be 2 : 1. The observed incidence was slightly lower at 49%.

The mating of one parent with melanoma to one without would result in an incidence ranging from 0% to 50%. The melanoma-bearing parent would be an obligate homozygote for the recessive gene and an obligate heterozygote for the dominant gene. The parent without melanoma could be homozygous for the recessive gene, heterozygous or the recessive gene could be absent. This would result in an incidence of 50%, 25% or 0%. This assumes that the dominant gene is absent in this parent. If that parent has the dominant gene but does not have melanoma because the recessive gene is heterozygous the incidence would be 33%. Thus the incidence would vary depending on the incidence of the recessive gene in the non-melanoma bearing population. The observed frequency was 19%. This well within the expected range, however, with the extensive inbreeding of this herd we would expect a fairly high incidence of the recessive gene, which may still reflect some incomplete penetrance of the tumour trait.

The lower than expected incidence of melanoma in the M+ × M+ matings, raises the possibility of a third factor acting to lower the penetrance of the tumour trait. This may be a third gene or it could be a stochastic factor such as the probability of somatic mutation of the wild type copy of a tumour suppressor gene. In the Sinclair pig model, it has been suggested that there is such a factor that is linked to the inheritance of a particular SLA haplotype (Tissot et al., 1989).

In summary, we hypothesize from the above results that the mode of inheritance of nodular melanoma in the MeLiM strain is probably controlled by three genes that take part in a neoplastic process. Thus, melanoma inheritance in this model and in the Sinclair pigs is very similar each other. It corresponds to a large genetic contribution from Hormel breeding stock in both strains. However, classic hybridization experiments cannot give a definitive and exact answer for the mode of porcine melanoma inheritance. The only one way is a molecular analysis of suspected genes (tumour

suppressors, protooncogenes, genes which regulate apoptosis etc.). This objective can be reached by the use of genetic maps that are sufficiently saturated by molecular markers. The mapping approach seems to be realistic due to availability of advanced pig maps at the present time (Hu et al., 2001; Rink et al., 2002; Rohrer et al., 2002). Particular scanning of the MeLiM genome by microsatellite analysis is in progress (in CEA-INRA, Laboratoire de Radiobiologie et d'Etude du Genome, Jouy-en-Josas, France) with aim to reveal melanoma predisposition genes. The first results published recently revealed five chromosomal regions on porcine chromosomes 1, 2, 6, 7 and 8 that are potentially involved in predisposition to melanoma (Geffrotin et al., 2004). Moreover, they excluded the CDKN2A gene, which is involved in a proportion of human familial melanoma, as a candidate for melanoma susceptibility in the MeLiM animals (Le Chalony et al., 2003).

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