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Human Genome and Diseases: Review

Can different genetic changes characterize histogenetic subtypes and biologic behavior in sporadic malignant melanoma of the skin?

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Abstract. In sporadic malignant melanoma, different chromosomal regions with nonrandom aberrations have been discovered, including 1p36, 6q, 9p and 10q. First results provide a genetic basis for the concept of primarily vertical, biologically aggressive melanomas and radial growing, mostly benign melanomas. These are mainly represented by nodular melanoma (NM) and early superficial spreading melanoma (SSM), respectively. Deletions in 1p36 could be found only in NMs and metastatic melanoma. Aberrations of chromosome 10 occur predominantly in NMs, whereas deletions on chromosome 9 are more frequent in SSMs. Despite a variety of genes tested, neither a tumor suppressor gene with importance in all malignant melanomas of the skin nor one clearly defining the transition from the radial growth phase to the vertical growth phase has been determined. Nevertheless, the pattern of genetic alterations may soon lead to finding such genes and development of drugs targeting these genes or their products, which would be of great benefit to melanoma patients.

Key words. Sporadic malignant melanoma; RGP; VGP; molecular genetic aberrations.

Introduction

Malignant melanoma is one of the most dangerous cancers, with an increasing incidence all over the world [1]. Therefore, it could become a significant public health problem in the next decades in Europe, as it already is in Australia. Although sporadic cutaneous malignant melanoma accounts for approximately 90% of all melanoma cases, the molecular and genetic background of melanoma development is still subject to investigation and discussion. Cytogenetic and loss of heterozygosity (LOH) studies have revealed chromosomal regions, including 1p, 6, 9p, and 10q [2], that may harbor tumor suppressor genes or oncogenes with importance in melanoma development. As an additional problem, the entity of malignant melanoma comprises various histogenetic subtypes, and it shows two main pathways of tumor progression that may also vary in their genetic background. The radial growth phase (RGP) is mainly represented by early superficial spreading melanoma (SSM) and the vertical growth phase (VGP) by primary nodular melanoma (NM). RGP melanomas are easily recognized and cured. If left intact, they might progress to VGP (secondary NM, also designated as SSM with vertical growth), which is associated with a worse prognosis [3]. Exceptions to this stepwise progression are primary nodular malignant melanomas, which, independent of size and duration, exhibit vertical growth and have the capacity to metastasize [4]. Their in-

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cidence is much lower than that of SSMs. In many reports, both NMs and advanced SSMs with VGP are designated as 'nodular melanomas'. Therefore, evaluation of the literature is obstructed by lack of standarized definitions. One report described a falling incidence of 'nodular melanoma', which is probably due to increased population screening and the early detection of thin SSMs, which are nowadays most often removed before VGP, or 'secondary nodular melanoma', evolves [5]. The designations used by us and others are as follows: SSM is defined by the presence of a microinvasive or in situ RGP (infiltration of the reticular dermis). A VGP tumorigenic compartment may or may not be present as well. NM evolves as a tumor nodule without clinical or histologic evidence of a preexisting nontumorigenic radial growth phase compartment. Here, we must mention that there is a contrary view that denies the existence of different growth phases and their prognostic value [6]. The level of invasion histologically evaluated by the method of Clark [7] represents a biologic model of tumor progression. It can be viewed as a method of defining a stepwise progression of biologic properties associated with an increasing malignant behavior of melanoma cells [8, 9]. As tumors progress from RGP to VGP, the melanoma cells consequently invade the deeper dermal compartments. The anatomic levels of invasion are defined as follows: intraepithelial tumor (Clark level I), extension into the papillary dermis (Clark level II), filling and extending the papillary dermis (Clark level III), invasion of the reticular dermis (Clark level IV) and invasion of subcutaneous fatty tissue (Clark level V). A variety of changes in the expression of certain proteins, for example, increased expression of adhesion receptors and decreased expression of cadherins and integrins, accompany the development from melanocyte over RGP and VGP to metastasis as reviewed by Meyer et al. [10]. On the protein level, integrin alpha v/beta 3 cell adhesion molecule serves as a marker for VGP, but its regulation by genetic changes is not known [10]. It seems logical that changes in biologic property and acquisition of aggressive and destructive tumor growth are reflected by changes on the molecular genetic level as well, which could serve as markers in tumor progression. Many studies of malignant melanoma by cytogenetic analysis [11–13], LOH analysis [14, 15] and first fluorescence in situ hybridization (FISH) analysis [16–22] have been published, and some authors have even concentrated on the detection of specific chromosomal aberration associated with the development of metastases [23–25]. But only a few authors have focused on the difference in the genetic background of these two forms of melanoma progression [26–30]. Therefore, we tried to concentrate our molecular genetic investigations on changes associated with the transition from RGP to VGP and the differences between SSMs and NMs, which, in our opinion, represent two different, unrelated tumors of malignant melanocytes. Beginning with a FISH assay, we were able to demonstrate specific chromosomal aberrations for both tumor types [28], which will be described in the following sections.

Chromosomal region 1p36

The short arm of chromosome 1, especially the region 1p36, is deleted or rearranged in many human cancers, including neuroblastoma, breast cancer and malignant melanoma of the skin [2]. A deletion in 1p36 in malignant melanoma has been shown by a variety of techniques including cytogenetic analysis [11] and LOH analysis [14, 31, 32]. These data indicate that the region 1p36 may harbor one or more tumor suppressor genes with relevance in malignant melanoma. Therefore, we undertook FISH studies to determine the distribution of this deletion in different subtypes and stages of melanoma. We have been able to demonstrate that this region is connected strongly to vertical growth [27, 28, 33]. No deletions of the marker D1Z2 in 1p36.3 could be found in SSMs, but the incidence of this aberration rises with the Clark level in NM, reaching a maximum of 91% in metastatic melanomas derived from NMs or even SSMs. This strongly indicates one or more tumor-relevant genes in this region in which deletions lead to the transformation of tumor cells, such as superficially growing melanocytes to melanocytes showing vertical growth and the capacity to metastasize. One of the advantages of the FISH technique is the possibility to enclose the critical region of a deletion with new panels of DNA probes. So far, we have used yeast artificial chromosome (YAC) DNA probes of the regions 1p36, 1p32, 1p31 and 1p21 in cohybridization with the centromeric DNA probe of chromosome 1 or the subtelomeric probe in D1Z2 [33]. These investigations have revealed that the critical region of deletion in 1p36 is confined to a rather small area near the locus D1Z2. To further determine this area, we analyzed the LOH status of nine highly informative markers in 1p36.3 in our melanoma samples [34]. This study confirmed the connection of a deletion in 1p36 with vertical growth. Retention of constitutional heterozygosity at all informative loci was seen in 80% of SSMs, 19% of NMs and 14% of metastatic melanoma lesions. LOH in 1p36.3 is therefore only a sporadic event in SSMs and involves one locus at maximum. In contrast, LOH in at least one marker from 1p36.3 was found in 77% of NM and 86% of metastatic melanomas. We revealed two different regions of allelic loss, one more telomeric between 1p36.33 and 1p36.32, the other more centromeric between 1p36.32 and 1p36.31 (fig. 1). Our melanoma lesions demonstrated LOH at either one or the other of these regions or showed allelic loss over the whole area covered in our study. These results imply the possibility of two tumor suppressor genes in 1p36

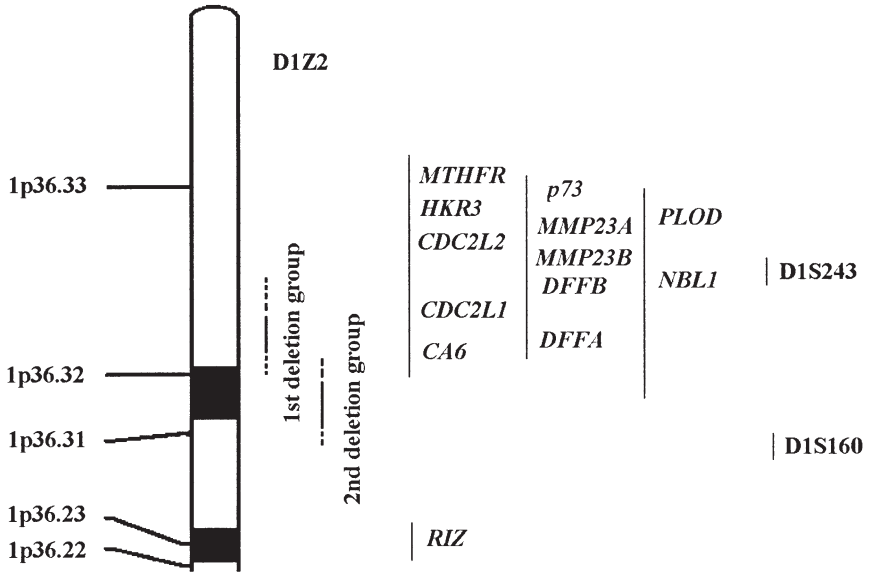

Figure 1. Position of the deleted regions detected in our LOH study [34] at chromosome 1 (1p36.33 to 1p36.31) and a variety of confirmed or proposed genes in this region, according to the Sanger Institute Human Chromosome 1 Web page (http://www.sanger.ac.uk/chr1) and Vance et al. [132].

with importance in malignant melanoma (fig. 1). A comparison of the LOH data with the FISH data concerning D1Z2 revealed a few difficulties. Since the locus D1Z2 investigated by FISH is situated next to the telomere at 1p36.33, we expected at least that all melanoma lesions belonging to the first deletion group would display loss at D1Z2, but two metastases did not. In addition, one metastasis with allelic loss from D1S243 to D1S160 and one NM lesion with losses over the same range had no deletion at D1Z2. In contrast, the majority of tumor samples belonging to the second deletion group and the melanoma lesions without LOH at one of the investigated loci that had also been studied by FISH showed loss of D1Z2. Since we did not find any marker displaying a good rate of heterozygosity in our melanoma samples whose location lies definitely between D1S243 and D1Z2, we could not be sure whether the loss of D1Z2 was confined to just this highly repetitive marker or whether there were other (single-copy) DNA sequences involved also.

A variety of possible candidate tumor suppressor genes have been analyzed for changes in sporadic malignant melanoma. Shortly after its detection in 1997 [35], *p73* was discussed as a tumor suppressor gene for malignant melanoma and other cancers characterized by a deletion in 1p36, but several studies were not able to demonstrate aberrations in *p73* in these tumors [36–41].

Although the *PITSLRE* gene complex [42], which is linked to cell cycle checkpoint regulation [43], has importance in other cancers such as neuroblastoma [44] and non-Hodgkin's lymphoma [45], its impact in sporadic melanoma is questionable, since alterations of the *CDC2L1* gene promoter could only be demonstrated in melanoma cell lines [46] but not in primary tumors or metastases [47]. However, *CDC2L1* may play a role in familial melanoma, since polymorphism of the promoter has been found in members of melanoma-prone families [48].

An additional candidate for a tumor suppressor gene on 1p36 is the retinoblastoma protein-interacting zinc-finger gene *RIZ,* which belongs to the PR (PRDI-BFA-RIZ homology) domain family [49]. Members of this PR domain family are expressed in an unusual yin-yang fashion, with two products differing in the presence or absence of the PR domain. The PR^+ product is underexpressed in cancer cells, whereas the PR^- product is present or overexpressed. In the case of *RIZ*, a loss of *RIZ1* (PR^+ product) expression has been shown, for example, in hepatocellular carcinoma, breast cancer and neuroblastoma [50, 51]. Since *RIZ1* can induce G2/M cell cycle arrest and apoptosis in breast cancer and hepatocellular carcinoma cell lines, it has been supposed as a candidate tumor suppressor gene [50–52]. Recently, *RIZ* was shown to demonstrate a high rate of frameshift mutations in colorectal, gastrointestinal and endometrial cancers [53–55], and forced expression of *RIZ1* in colorectal xenograft tumors with *RIZ1* mutations resulted in cells undergoing apoptosis [56]. In our recent study, however, we could not detect point mutations of *RIZ1* or LOH at this region, but only frameshift mutations in a mononucleotide repeat [the $(A)_9$ tract of exon 8], which are expected to cause a truncated RIZ protein lacking part of the C-terminal region involved in the PR-binding function [57]. In addition, we could not correlate our previous FISH results [28, 33] or LOH data [34] concerning deletions in 1p36 in malignant melanoma with the occurrence of the frameshift mutations in this study. All

frameshift mutations manifest themselves as heterozygous, which is verified by the fact that no LOH of the *RIZ* gene could be detected [57]. In contrast, the same nodular and metastatic melanoma samples displayed a deletion in 1p36 as determined by FISH. Therefore, the described deletion in our tumor samples did not stretch over the *RIZ* gene. Nevertheless, since microsatellite instability is rare in malignant melanoma [58, 59], and the *RIZ* gene is the first gene in which mononucleotide instability in malignant melanoma has been described [57], it may be one of very few targets of inactivation by microsatellite instability pathways in this tumor.

The results concerning the chromosomal region 1p36 are summarized in table 1. To date, approximately 75 genes in 1p36.3 are known, for example, *HKR3*, *MTHFR*, *CA6*, *NBL1*, *MMP23A*, *MMP23B*, *DFFA*, *DFFB*, *PLOD*, and *TNFRSF14*. In addition, at least 25 new genes or open reading frames have been found here (Human Chromosome 1 Map, Sanger Institute, http://www.ensembl.org). Any of these genes with similarities to known tumor suppressor genes may have importance in the shift from RGP to VGP and is worth a mutation analysis in malignant melanoma, but especially any in the two regions of allelic loss detected in our study [34].

Chromosomal region 6q

Cytogenetic [11, 12] and LOH [15] studies have demonstrated the importance of the loss of chromosome 6 in sporadic malignant melanoma and have shown that especially the long arm of chromosome 6 is preferentially lost during progression of the majority of melanomas [24, 60, 61]. The importance of chromosome 6 in melanoma has been confirmed by experimental studies introducing chromosome 6 in melanoma cell lines, thus suppressing metastatic growth [62, 63]. A first candidate for this tumor suppressor gene has been the gene *SOD2* on 6q25, encoding the manganese superoxide dismutase (Mn-SOD) [64]. But despite the potential of MnSOD to suppress the malignant phenotype of melanoma cells [64] and LOH findings at 6q25 [61], the introduction of *SOD2* a metastatic cell clone changed neither metastatic potential nor MnSOD level [65].

Another melanoma metastasis suppressor locus has been mapped to 6q16.3-q23 [66], and the down-regulation of another putative melanoma metastasis suppressor gene, *KiSS1*, has been linked to LOH of this region in cell lines and uncultured melanoma metastasis [67]. Further experiments refining the affected region on chromosome 6 may reveal a new tumor suppressor gene here.

A member of the PMP22/gas3 family of plasma membrane proteins, *THW*, has been proposed as a possible candidate tumor suppressor gene, since in 50% of the few melanoma metastases LOH was found for this gene, but no mutations of *THW* have been detected so far [68]. An investigation of seven dinucleotide markers at 6q in our melanoma panel supports these results, showing LOH mainly at markers surrounding *THW* and mainly in metastases [unpublished data].

Very recently, another putative tumor suppressor gene locus on mouse chromosome 9 was mapped to human chromosome 6q and showed allelic imbalance in osteosarcomas [69]. Here, an analysis of melanoma lesions could be useful to determine the impact of this gene in melanoma pathogenesis. Such an investigation is currently under way in our laboratory.

In addition, the transcription factor *AP-2α* located on chromosome 6p has been shown to play a major role in regulating tumor growth in malignant melanoma, especially concerning the shift from radial growth to vertical growth [70]. Loss of c-kit, a transmembrane tyrosine-protein kinase receptor whose down-regulation has been found to be associated with tumor growth and invasion in melanoma [71], is directly linked to AP-2α expression [70, 72]. In cell lines, transfection of highly metastatic cells (c-kit and AP-2α-negative) with the *AP-2α* gene resulted in induction of c-kit expression in RNA and protein [70, 73]. Re-expression of AP-2α in highly metastatic melanoma cells inhibited their tumor growth and metastatic potential in nude mice, possibly through transactivation of c-kit [72]. But despite results showing that tumor progression in melanoma tumors is linked to AP-2α and c-kit protein loss, a correlation with genetic alterations of *AP-2α* could not be demonstrated [74]. In contrast, we detected that AP-2α in melanoma is probably

Table 1. Summary of literature reporting deletions and analyses of candidate genes in 1p36.

	Reference
Deletion of 1p36 by FISH	
50–53% of NMs	[27, 28, 33]
88–91% of metastatic tumors	[27, 33]
LOH in 1p36	
50% of melanoma cell lines; 18% of metastases	[14]
33% of NMs	[31]
77% of NMs; 86% of metastatic tumors	[34]
Mutation analysis of *p73*	
LOH of *p73* in 6% of primary tumors	[32]
LOH of *p73* in 25% of metastases	[37]
No mutations in melanoma cell lines	[36, 38]
No mutations in primary tumors and metastases	[36, 37]
Mutation analysis of *CDC2L1*	
Promoter mutations in melanoma cell lines	[46]
No mutations in primary tumors and metastases	[47]
Mutations analysis of *RIZ*	
LOH of *RIZ* in 8% of primary tumors	[57]
Frameshift mutations of *RIZ* in 11% of NMs and in 28% of metastatic tumors	[57]
No mutations of *RIZ1* in primary tumors and metastases	[57]

regulated by caspase 6 (located on chromosome 4q25), as has already been described for breast cancer cells [75].

Chromosomal region 9p

The third chromosomal region with importance for sporadic malignant melanoma as shown by cytogenetic, LOH and FISH analysis is the short arm of chromosome 9. Thompson et al. described an underrepresentation of the whole or part of chromosome 9 in 30% [11] of all tumor samples, and Ozisik et al. in 24% [76], while Wolfe et al. showed by FISH an underrepresentation of chromosome 9 in 60% [21] of all tumor sample and Matsuta et al. in 36.5% [19]. In our study, the loss of chromosome 9 was mainly detected in SSM (75% of SSM in Clark level I and II; 38% of SSM in Clark level III and IV) and only to 26% in NM [28].

At chromosome 9p, the gene attracting the highest amount of investigations is *p16/CDKN2A*. It is a very interesting candidate for a tumor suppressor gene in sporadic malignant melanoma, since it has already been shown to be a susceptibility gene in familial melanoma (e. g., reviewed by Pollock and Trent [77]), and alterations of this gene have been found in a variety of cell lines from other tumors, such as esophageal carcinoma, head and neck carcinoma, lung carcinoma, breast carcinoma and osteosarcoma [78–80]. In addition, small homozygous deletions and a low frequency of point mutations have been detected in uncultured tumor cells from esophageal carcinoma, pancreatic adenocarcinoma, non-small-cell lung carcinoma, and head and neck squamous-cell carcinoma [81–85]. An additional important inactivation mechanism of the *p16/CDKN2A* gene is the methylation of the promoter, which is also found in a variety of tumors, for example, head and neck squamous-cell carcinoma and lung, brain, and bladder cancers [86–88]. Furthermore, the p16 protein is a negative regulator of the major growth-control pathway supervising entry of the cell into the cell cycle [89] and thus a likely target of tumor-promoting alterations. In addition, its expression decreases significantly from melanocytes over RGP and VGP to metastatic lesions [90]. In the majority of melanoma cell lines, this gene has been shown to be inactivated by various mechanisms, including DNA mutations and promoter methylation [91]. In uncultured tumors, however, the frequency of *p16/CDKN2A* mutations or promoter methylations is much lower [92, 93]. In our panel of melanoma lesions, no *p16/CDKN2A* mutations could be detected, and the percentage of promoter methylation was lower than 10% [unpublished data]. Therefore, the loss of the p16 protein in melanoma lesions without alterations of the DNA may be due to epigenetic changes. The second product of the *CDKN2A* gene is ARF, another protein with importance in cell cycle control and cell senescence, since it is an activator of p53 [94]. Rare mutations of this gene have been reported in sporadic and familial melanoma [95–97]. Many authors suggest a possible role of the cell cycle control gene *p16/CDKN2A* on chromosome 9p in the progression of melanoma [98, 99]. In contrast, Stone et al. proposed an involvement of this gene mainly in growth regulation and less in tumor progression [100]. A deletion of *p16/CDKN2A* has also been found in benign nevi and normal human melanocytes, which lends doubt to the significance of this gene [101]. Our data support the last-mentioned findings connecting the loss of chromosome 9 to the less aggressive form of malignant melanoma, SSM [28]. The published LOH frequency of 54% on 9p [77] is most probably due to additional tumor suppressor gene(s) mapping in the same region. Up to three regions of common loss have been suggested [102], but no gene with possible tumor suppressor functions has been found so far.

Chromosomal region 10q

Aberrations in the long arm of chromosome 10 or the loss of a whole chromosome 10 have been frequently described in a variety of solid tumors, especially in glioma and malignant melanoma of the skin [2, 11]. In our recent FISH studies, we found a possible correlation between the loss of chromosome 10 and NMs [27, 28]. We detected 35% loss of chromosome 10 in primary tumors and 63% loss in metastases. It has been speculated that the genetic background of this abnormality might be the tumor suppressor gene *PTEN/MMAC1* [103, 104]. First investigations, mostly on melanoma cell lines, revealed several aberrations of this gene [105–107], but the analysis of more primary tumors and metastases limited the importance of *PTEN/MMAC1* to advanced tumor stages [108, 109]. Recently it has been proposed that *PTEN/MMAC1* inactivation may be predominantly important for the propagation of melanoma cells in culture [110], and expression of the PTEN protein was not lost in melanoma samples [111]. Therefore, a reponsibility of this gene at tumor onset or at the shift from radial to vertical growth in melanomas is unlikely. Even if most melanoma samples with *PTEN/MMAC1* mutations also displayed loss of chromosome 10 in our FISH studies [27, 28], the percentages of tumor lesions with loss of chromosome 10 is much higher thus supporting Okami et al. who proposed the existence of another tumor suppressor gene on the long arm of chromosome 10 [112]. This second tumor suppressor gene may be telomeric of *PTEN/MMAC1*, since LOH data demonstrated higher percentages of allelic loss distal of this gene [15, 113]. In addition, another new gene locus on chromosome 10 with an effect on in vivo melanoma growth has been identified [114].

Additional chromosomal regions

There are some other chromosomal regions that may harbor genes with importance in sporadic malignant melanoma. A gain of chromosome 7 has been found by FISH analysis [19], and a polyploidy of chromosome 7 was detected in metastases, correlating with overexpression of the epidermal growth factor receptor (EGFR) [115]. In addition, the serine/threonine kinase BRAF is located on this chromosome. It participates in the mitogen-activated protein kinase (MAPK) pathway, whose activation through both receptor tyrosine kinases and G-protein-coupled receptors has a central role in melanocyte proliferation [116, 117]. Very recently, BRAF mutations were detected in a variety of human cancers, including malignant melanoma [118, 119]. Further investigations by Pollock et al., however, revealed these same mutations in a high percentage of nevi but in lower percentages in subcutaneous and distant organ metastases [120]. They conclude that BRAF activation alone is insufficient for the development of melanoma [120]. An involvement of *BRAF* in familial melanoma could not be detected so far [121].

Another gene involved in the cell cycle is *CCND1* (cyclin D1), located on chromosome 11q13, a region with amplifications in some melanoma lesions [24]. Recent studies demonstrated 11q13 amplifications especially in acral melanomas and to a lesser extent in lentigo malignant melanomas and SSMs, always correlating with overexpressed cyclin D1 protein [122, 123]. We tried to detect amplification of *CCND1* looking at a certain polymorphism as described by Hibberts et al. [124] but found loss of heterozygosity in only a small subset of melanomas [unpublished data].

The last gene discussed here with possible importance in sporadic malignant melanoma is also involved in the cell cycle: the *p27* gene on chromosome 12p13, which is a potent inhibitor of all cyclin-CDK (cyclin-dependent kinase) complexes implicated in the G_1 and S phase [125, 126]. Chromosomal deletions or unbalanced translocations involving 12p13 are frequent events in lymphoid and myeloid malignancies [127, 128] and prostate cancer [129]. However, mutations of this gene could be found neither in leukemias nor in a variety of solid tumors [130, 131]. In our melanomas, 4 out of 20 (20%) SSMs, 13 out of 18 (72%) NMs and 13 out of 16 (81%) metastases showed LOH in at least one of seven microsatellite loci surrounding the *p27* gene, and expression of the p27 protein diminished when comparing melanomas of low pT level, high pT level and metastases [unpublished data]. Nevertheless, we could only demonstrate two different polymorphisms in exon 1 and two mutations, one silent

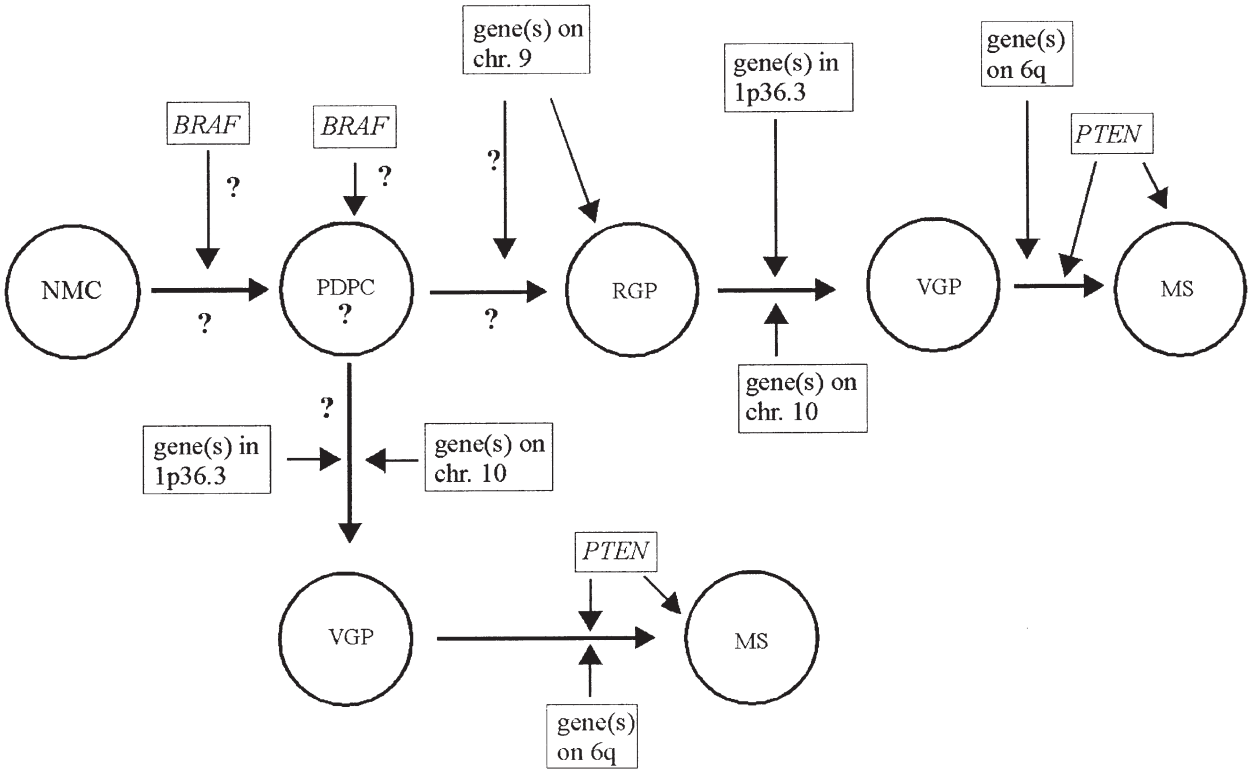

Figure 2. Proposal of a scheme illustrating the effects of important chromosomal regions and genes in initiation and tumor progression of sporadic malignant melanoma. NMC, normal melanocyte; PDPC, possible dysplastic precursor cell; RGP, radial growth phase melanocyte; VGP, vertical growth phase melanocyte; MS, metastatic spread.

and one resulting in an amino acid change in our melanoma samples.

Conclusion

We have attempted to give an overview of genetic alterations characterizing sporadic malignant melanoma of the skin and corresponding putative tumor suppressor genes. In figure 2 we propose a model of melanoma tumorigenesis, indicating possible points of action of the chromosomal regions discussed here in the stepwise malignant transformation of melanoma. In our opinion, the chromosomal site 1p36.3 is the most likely region for genes defining the shift from RGP to VGP, but despite many efforts, no such gene with LOH and mutations in melanoma has been found so far. The long arm of chromosome 6 probably harbors one or more genes with importance for the formation of metastases, but here also the true nature of these genes has not been elucidated. Nevertheless, because the region of allelic loss on 1p36.3 has been clearly limited and a variety of candidate tumor suppressor gene loci on chromosome 6 have been found recently, the detection of important genes in malignant melanoma may happen very soon. This could provide the basis for new therapeutic strategies, such as gene silencing, the development of specific inhibitors derived from these genes or other novel drugs, whose main purpose would be to help combat tumor growth in individuals.

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