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Anatomic site-specific patterns of gene copy number gains in skin, mucosal, and uveal melanomas detected by fluorescence in situ hybridization

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Abstract To assess the differences between melanomas of different location and different etiology, 372 malignant melanomas were brought in a tissue microarray format. The collection included 23 acral and 118 non-acral skin melanomas, 9 mucosal melanomas, 100 uveal melanomas, and 122 melanoma metastases. Fluorescence in situ hybridization (FISH) was used to assess copy number changes of the cyclin D1 (CCND1), MDM2, c-myc (MYC), and HER2 genes. FISH analysis revealed distinct differences between melanomas from different locations. CCND1 amplifications were detected in skin melanomas from sites with chronic sun exposure (6 of 32 cases), acral melanomas (4 of 17 cases), and mucosal melanomas (one of ten cases) but not in uveal melanomas. High-level MDM2 amplifications were exclusively present in acral melanomas (2 of 19 cases). MYC copy number gains were detected in 32 of 71 uveal melanomas, five of eight mucosal melanomas, and 6 of 67 melanomas from sites with intermittent sun exposure but not in acral melanomas nor melanomas from sites with chronic sun exposure. Alterations of the MYC gene were associated with advanced tumor stage. There were no high-level HER2 amplifications. Site-specific genetic and epigenetic features may impact the response of melanomas to various anti-cancer drugs and should be considered in future studies on the molecular pathogenesis of malignant melanomas.

Keywords Melanoma · Fluorescence in situ hybridization · Tissue array · Amplification

Introduction

There is considerable hope that a new generation of anti-cancer drugs targeting specific genes or molecular pathways may be more efficient in the future. The introduction of trastuzumab (Herceptin), a humanized antibody for the treatment of HER2-positive metastatic breast cancer [9, 29], has pointed out the potential of molecular-targeted therapy. As the results of systemic treatment with classical cytostatic drugs in melanoma patients presenting distant metastases have been discouraging with only a minority of these patients living beyond 1 year [1]. The discovery of frequent mutations of the BRAF gene in malignant melanomas [13] and the subsequent development of BRAF antagonists [7] have raised new hope. If individual molecular features should be targeted in cancer cells, melanoma subgroups with different molecular signatures may be clinically important. It is interesting that there is increasing evidence for distinct molecular characteristics of melanoma subtypes depending on the localization and different levels of sun exposure of the primary tumor [3, 5, 11, 12, 33, 37, 39, 42]. It is tempting to speculate that such differences may be due to a site-specific etiology of melanoma. For example, mutations of the BRAF gene have been shown to be preferentially present in melanomas from sites with intermittent sun exposure [12, 24] and acral melanomas [33] but were nearly inexistent in tumors arising in sites of chronic sun-induced damage, mucosal melanomas [15], and uveal melanomas [10, 14]. Gene amplifications were found to be significantly more frequent in acral and mucosal melanomas than in melanomas from non-acral skin [3, 12]. In addition to the site-specific differences in the degree of genomic instability, there is increasing evidence of distinct patterns of genetic alterations depending on the localization of the primary tumor [12, 41].

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To collect more information on possible site-specific molecular differences, we determined the frequency of amplifications of the proto-oncogenes cyclin D1 (CCND1), MDM2, c-myc (MYC), and HER2 in melanomas from different localizations including mucosal and uveal melanomas. For the purpose of this study, we selected a tissue microarray format which allowed us to analyze a total of 372 melanomas on one tissue slide under highly standardized experimental conditions.

Materials and methods

Tumors

Formalin-fixed and paraffin-embedded tissues from 141 primary skin melanomas, 100 uveal melanomas, 9 mucosal melanomas, and 122 metastases (70 lymph node and 52 hematogenous) were brought in a tissue microarray (TMA) format [21]. Primary tumors and corresponding metastases were available from 16 patients and more than one metastasis from 12 patients. Among the 141 primary skin melanomas, there were 37 melanomas located in areas of chronic sun exposure (27 in the head/neck and 10 in the forearm), 78 melanomas located in areas of intermittent sun exposure (45 in the thorax, 8 in the upper arm, and 25 in the legs), 2 anal melanomas (no sun exposure), 23 acral melanomas, and 1 melanoma of unknown location. All melanomas from sites with chronic sun exposure (head/neck and forearm) had marked solar elastosis microscopically. All cases were classified according to the pTNM classification (UJCC, 6th edition, 2003). The stages of skin and primary mucosal tumors were pTis in 6, pT1a in 32, pT1b in 3, pT2a in 27, pT2b in 4, pT3a in 23, pT3b in 14, pT4a in 12, and pT4b in 29. The subtypes of the primary tumors were as follows: superficial spreading (82), nodular (46), lentigo maligna (5), and acral lentiginous (17). The pT stages of the uveal melanomas were pT1a in 3, pT1b in 1, pT2a in 25, pT2b in 27, pT2c in 6, pT3 in 15, and pT4 in 20. pT stage could not be determined in three uveal melanomas. Histologically, a spindle-cell type was present in 50, an epitheloid type in 13, and a mixed type in 37 cases of uveal melanomas. All slides from all tumors were reviewed by one of two pathologists (K.G. for non-uveal melanomas and P.M. for uveal melanomas) to define the histological tumor type and the pathological stage.

A semiautomated arraying instrument was used for TMA construction as described [36]. A representative tissue cylinder of the vertical growth phase was taken for the tissue array from primary tumors in the vertical growth phase (119) and of the radial growth phase from primary tumors in the radial growth phase (22). Of the 25 skin melanomas and 40 uveal melanomas, two tissue cores from the same tumor block were placed in the tissue array.

Tissue samples were collected during 1985–2002 at our institution. The use of the archival material and data in research was approved by the Ethics Committee of the University Hospital Basel.

Fluorescence in situ hybridization

A set of 4- μ m TMA sections was used for two-color fluorescence in situ hybridization (FISH). For the proteolytic slide pretreatment, a commercial kit was utilized (Paraffin pretreatment reagent kit, Vysis, Downers Grove, IL, USA). Spectrum Orange-labeled CCND1, MDM2, MYC, and HER2 were used in combination with Spectrum Green-labeled chromosomes 11, 12, 8, and 17 centromeric probes as a reference. All locus-specific probes were obtained from Vysis, IL, USA. Before hybridization, the sections were deparaffinized, air dried, and dehydrated in 70, 85, and 100% ethanol followed by denaturation for 5 min at 74°C in 70% formamide–2 \times SSC solution. After overnight hybridization at 37°C in a humidified chamber, slides were washed and counterstained with 0.2 μ M DAPI in an anti-fade solution. For each tumor, the predominant gene and centromere copy numbers were estimated. A tumor was considered amplified if the ratio of gene probe to centromere probe was ≥ 2.0 .

Statistical analysis

Contingency table analysis and chi-square tests were used to study frequency comparisons of nominal categorized variables.

Results

FISH analysis was successful for CCND1 in 293 (79%), for MDM2 in 301 (81%), for MYC in 271 (73%), and HER2 in 322 (87%) out of the 372 melanoma samples. Non-informative samples were mainly caused by insufficient hybridization, too high background, or absence of tumor cells on arrayed tissues.

CCND1

CCND1 amplifications were found in 8% of 120 primary skin and mucosal melanomas and in 11.2% of 107 metastases from skin melanomas but in none of the 83 uveal melanomas. Among the 21 amplified cases, there were eight tumors with high-level amplifications showing >10 CCND1 signals per tumor cell (Fig. 1). The associations with localization, metastases, and stage are shown in Table 1. It is interesting that CCND1 amplification was significantly more frequent in acral melanomas and melanomas of the forearm and the head and neck (chronic sun exposure) than in melanomas of the trunk, the legs, and the upper arms (intermittent sun exposure, $p=0.001$). All melanomas from chronically sun-exposed sites with CCND1 amplifications showed moderate to severe solar elastosis. As lentigo maligna melanomas arise in chronically sun-damaged skin, CCND1 amplifications were more frequent in this subtype than in superficial spreading or nodular melanomas.

MYC

An increase of MYC gene copies was frequently observed in uveal (45.1% of 71) and mucosal (62.5% of 8) melanomas but only rarely in melanomas from sites with intermittent sun exposure (9% of 67) and not from sites with chronic sun exposure or acral sites. In most cases with increased MYC signals, there were only low-level MYC gene copy number gains with the ratio of MYC copies as compared to centromere 8 being 2:1. Clear-cut high-level MYC amplifications with an average ratio of MYC signals per centromere 8 above 5 and the signals occurring in distinct clusters was found in four tumor samples: a nodular melanoma of the upper arm, a nodular melanoma of the elbow, an esophageal melanoma, and a subcutaneous metastasis of a substernal primary. The esophageal melanoma was the only tumor in this study showing high-level amplifications of two genes (CCND1 in addition to MYC).

There was an association between MYC copy number increase and increased tumor stage when all stages were compared ($p=0.018$). MYC alterations were significantly more frequent in melanomas from the nodular than the superficial spreading type ($p=0.002$).

MDM2

MDM2 amplifications were found in 3.6% of 139 primary tumors of the skin but not in metastases nor uveal melanomas. Two of five amplified tumors had high-level amplifications (>10 MDM2 copies per cell, Fig. 1). Both were acral melanomas (Table 1).

HER2

One primary tumor from the back skin (Breslow 0.98 mm, pT2) showed a low-level amplification of the HER2 gene (two centromere and five HER2 copies per cell). All the

other 322 interpretable primary melanomas and metastases showed no HER2 amplifications.

Results of multiple samples from one patient

For CCND1, MDM2, and HER2, all multiple samples from the same patient showed a consistent result. Among these multiple samples, there were four concordant amplifications of the CCND1 gene [multiple metastases (2 \times), samples of the same tumor block (1 \times), and a primary tumor of the esophagus with its lymph node metastasis].

The evaluation of the MYC gene revealed some discrepant results in multiple samples of the same patient. In five uveal melanomas and one skin melanoma, one tissue cylinder had low-level MYC gene copy number gains while the other cylinder of the same tumor block showed a normal MYC copy number as a result of intratumoral heterogeneity. The primary tumor of an esophageal melanoma but not its corresponding lymph node metastasis had a high-level MYC amplification. In another 45 cases with multiple tissue cylinders from one patient, the results of MYC copy numbers of all samples were congruent. Of these 45 cases, 24 had an increased MYC copy number.

Discussion

In this study, the analysis of 372 melanoma tissue samples revealed significant molecular differences between tumors of different sites.

FISH was used to investigate gene amplifications of CCND1, MDM2, MYC, and HER-2. Among these genes, high-level amplifications of CCND1 were most frequent. It is interesting that CCND1 amplification was significantly more frequent in locations with chronic sun exposure (head/neck and forearms) than in locations with intermittent sun exposure (trunk, legs, and upper arms), raising the

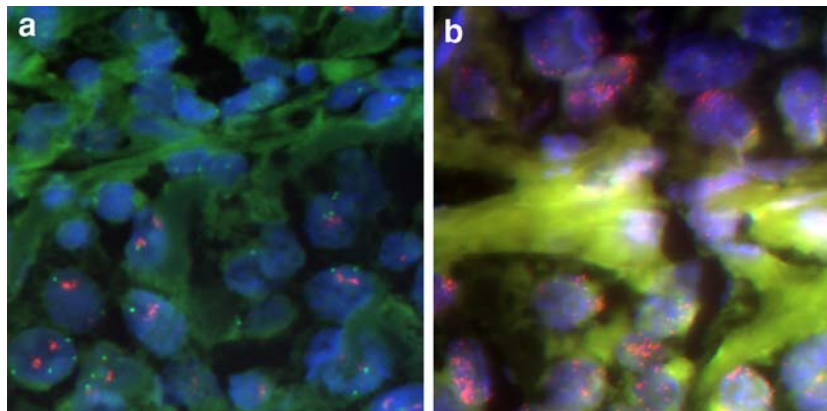


Fig. 1 **a** CCND1 amplification in a lentigo maligna melanoma. Hybridization was done with probes for centromere 11 (green) and CCND1 (red). Large tumor cells in the lower half of the image contain one or two irregularly formed clusters consisting of many closely packed red CCND1 signals and up to four centromere 11

signals. $\times 640$ magnification. **b** MDM2 high-level amplification in acral lentiginous melanoma. Hybridization was done with probes for centromere 12 (green) and MDM2 (red). Large tumor cells with several clusters of MDM2 signals. $\times 640$ magnification

Table 1 Molecular findings, tumor phenotype, stage, and location

		CCND1 (%)	MDM2 (%)	MYC (%)	HER2 (%)
Localization of primary tumor	Uvea	0/83	0/59	32/71 (45.1)	0/78
	Intermittent sun exposure	0/65	3/72 (4.8)	6/67 (9)	1/66 (1.5)
	Chronic sun exposure	6/32 (18.8) ^a	0/32	0/24	0/33
	Acral	4/17 (23.5) ^b	2/19 (10.5)	0/9	0/19
	Mucosa	1/10 (10)	0/8	5/8 (62.5)	0/9
pT (skin primary tumors)	pT1	1/24 (4.2)	3/27 (11.1)	0/25	1/21 (4.8)
	pT2	2/25 (8.0)	0/26	1/25 (4)	0/28
	pT3	3/32 (9.4)	1/34 (2.9)	4/29 (13.8)	0/33
	pT4	5/33 (15.2)	1/32 (3.1)	6/23 (26.1) ^c	0/34
Metastases	Lymph node	8/65 (12.3)	0/60	3/92	0/66
	Distant	5/43 (11.6) ^d	0/46	3/92	0/43
Growth phase	Radial	0/18	1/17 (5.9)	0/17	0/21
	Vertical	11/101 (10.9)	4/107 (3.7)	7/92 (12.0)	1/102 (1.0)
Histologic subtype	Superficial spreading	3/70 (4.3)	3/71 (4.2)	2/67 (6.0)	1/71 (1.4)
	Nodular	3/39 (7.7)	0/37	7/31 (22.6) ^e	0/38
	Lentigo maligna	2/4 (50)	0/5	0/3	0/5
	Acral lentiginous	3/12 (25)	2/13 (15.4)	0/8	0/14

Tumors with CCND1, MDM2, MYC, and HER2 gene probe to centromere probe ratio ≥ 2.0 detected by FISH. Percentages are indicated in brackets

^aSite of chronic sun exposure (head/neck and forearm) vs site of intermittent sun exposure (upper arm, leg, and trunk), $p < 0.0001$

^bAcral vs non-acral skin, $p = 0.02$

^cMYC copy number increases when all stages are compared, $p = 0.018$

^dPrimary tumors vs metastases, $p < 0.0001$

^eNodular vs superficial spreading type, $p = 0.002$

possibility that CCND1 activation could be particularly important for melanoma development in sites with chronic sun exposure. Our overall frequency and site distribution of CCND1 amplifications in primary skin melanomas is consistent with previous data [12, 34].

The presence of frequent CCND1 amplifications in acral melanomas could be explained by an overall higher level of genetic instability of these tumors involving other molecular pathways than in classical skin melanoma [5, 12].

Our mucosal melanoma with CCND1 amplification had originated from the esophagus where CCND1 amplification occurs at a particularly high frequency in carcinomas as well [35]. A review of our case confirmed the melanoma diagnosis, however. In the same tumor, we additionally detected a high-level MYC amplification illustrating the higher degree of genetic instability in mucosal melanomas [12].

MDM2 was only amplified in five melanomas including two high-level amplifications. Both high-level amplifications were seen in acral melanomas. This finding and the overall low number of MDM2 amplifications are consistent with previous data from Bastian et al. [3]. They found 12q14 copy number gains in 5 of 22 acral melanomas but none in 108 non-acral skin melanomas using CGH and MDM2 amplifications in 1 of 15 acral melanomas by FISH.

Amplifications in acral melanomas but not in non-acral melanomas have been shown to be present in the in situ portion and in the invasive areas at mostly similar levels [2, 3]. It was concluded that the amplifications arise very early during tumor progression in acral melanomas, suggesting

that acral melanomas develop a specific amplification-permissive defect early in tumorigenesis unique to the pathogenesis of this tumor type [3, 5]. In most cancers, gene amplifications occur frequently at a late stage and are clearly associated with tumor progression though the mechanisms through which amplifications are generated are largely unknown [23]. The fact that amplifications, in general, occur late in tumorigenesis may explain why amplifications are rather frequent in mucosal melanomas [12] which are usually diagnosed at a late stage.

The unique molecular features in uveal melanomas are not unexpected due to their special location. The chromosomes 1, 3, 6, and 8 are consistently affected by non-random aberrations mainly in the form of chromosomal gains and losses [18, 38]. Additional copies of the long arm of chromosome 8 either by translocation or in the form of an isochromosome i(8q) have been shown to correlate with reduced survival [25]. Parrella et al. [27] had described MYC amplification in 14 of 43 uveal melanomas. These amplifications were of borderline nature typically showing twice as many MYC copies as compared to centromere 8 in FISH analysis. Twice as many gene copies as centromere copies typically reflect a duplication of a large stretch of DNA (e.g., isochromosome formation) rather than a gene-specific amplification event as has been shown in studies of uveal and skin [22, 39] melanomas. By CGH analysis, over-representation of the long arm of chromosome 8 has been reported in 38% of primary melanomas [4]. The poor prognosis associated with extra copies of chromosome 8q may point to the presence of several oncogenes in this region. Ehlers et al. [16] suggest that the DDEF1 gene

located at 8q24 rather than MYC which is also located at 8q24 may function as an oncogene during uveal melanoma progression. The fact that the copy number alterations of the MYC gene are more common in advanced tumors of the nodular subtype has been shown before in studies with limited case numbers [22, 40]. Blocking the expression of MYC with liposomal MYC antisense oligodeoxynucleotides has been reported to inhibit tumor growth and metastases in an in vivo melanoma model [28]. Hopefully, such an approach will finally provide a targeted therapy for selected patients with advanced melanomas.

One skin melanoma showed HER2 amplification according to our definition. This case had two centromere and five HER2 signals which do not constitute a classical amplification. Persons et al. [30] have also reported one HER2 amplification in 40 advanced melanomas. It is not surprising that several studies have proposed HER2 overexpression in up to 80% of melanomas [6, 26] as IHC can yield highly variable results under non-standardized conditions. More recent IHC studies using Food and Drug Administration-approved reagents have described inexistence [17] or a very low rate of detectable HER2 expression (0.9–3%) [19, 31] (own unpublished findings). Altogether, these data strongly argue against a possible therapeutic application of Herceptin in malignant melanoma.

The hypothesis of distinct genetic alterations between melanomas arising in different sites is also supported by the results of recent studies on the frequency of BRAF mutations. BRAF mutations have been observed preferentially in melanomas from skin without chronic sun-induced damage [12] but only rarely or not at all in acral melanomas, melanomas from chronically sun-exposed skin, and mucosal and uveal melanomas [10, 15, 24, 33]. The comparison of aberration frequencies in CGH analysis of melanomas with different sun exposure patterns revealed significant differences between the subgroups defined by location [12, 34]. Altogether, there is increasing evidence for site-specific molecular pathways of melanoma development.

As we know from H&E morphology, malignant melanomas often show considerable intratumoral heterogeneity. On several occasions, this heterogeneity has also been observed at the molecular level [4, 8, 32]. TMAs provide a method for the rapid and simultaneous analysis of up to 1,000 tissue samples in a single experiment under highly standardized conditions. They are not intended for making clinical diagnoses of individual cases but to screen for molecular alterations. The validity of TMA analysis has been shown by comparisons with whole-section analysis in breast, prostate, and brain cancer [20].

In a limited number of cases, we had analyzed more than one sample from the same patient revealing a low number of discordant results. It is interesting that, in one case, a high-level MYC amplification was present in the esophageal primary tumor but not in its lymph node metastasis. A more detailed study of intratumoral heterogeneity will be especially important for the evaluation of potential therapeutic targets that should be present throughout the whole

tumor and in its metastases if targeted therapy is to be effective. For that purpose, whole-section slides or several samples of different areas within a given tumor, e.g., of the vertical and radial growth phases, will have to be analyzed.

Due to their genetic characteristics, melanomas may respond differently to various cytotoxic and biologic cancer therapies depending on their body location and the presence or absence of chronic sun-induced damage. The latter can roughly be estimated microscopically by the degree of solar elastosis. In future studies that investigate the molecular pathogenesis of malignant melanomas and in clinical trials with new drug targets, the genetic diversity of melanomas from different sites should be taken into account.

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