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Spatial intratumor heterogeneity in uveal melanoma: Tumor cell subtypes with a presumed invasive potential exhibit a particular epigenetic staining reaction

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ABSTRACT

Cancer evolves from a combination of genetic and epigenetic abnormalities resulting in aberrant gene expression profiles as well as altered epigenomic patterns. Epigenetic alterations such as DNA methylation and histone modification play an important role in tumorigenesis. While in the pathobiology of uveal melanoma (UM) genetic changes have been well characterized, there is growing evidence suggesting that epigenetic changes are also involved. We investigated whether epigenetic modifications (global levels of histone acetylation, DNA methylation, ubiquitination) are detectable in UM tissues compared to healthy controls with respect to inter- and intratumoral heterogeneity. Formalin-fixed paraffin-embedded tissues of primary UM (n = 15), UM metastasis (n = 13), and control choroid (n = 12) were immunohistochemically investigated by two ophthalmic pathologists for global levels of histone acetylation (Histone 3 acetylation, H3Ac; Histone 4 acetylation, H4Ac), DNA methylation (5-methylcytosine, 5-MeC; 5′-hydroxymethylcytosine, 5-hMeC), global ubiquitination (UBC) as well as Ubiquityl-Histone H2A (H2Aub). The nuclear staining intensity of primary tumors, metastases and control choroids was evaluated using a score from 0 to 3, which was multiplied with the percentage of stained cells (score from 0 to 4). The control choroid and the choroid next to the tumor showed a more intense nuclear staining than the primary tumor tissue. The choroid next to the tumor was stained less than the control choroid. The nuclear staining intensity in the tumor was comparable to that in the metastases. The tumor tissue itself often exhibited a heterogeneous staining pattern, as nuclei in the tumor center were less intensely stained than in the periphery. Cells with a presumed invasive potential (extraocular extension, growth along emissary canals) showed also an intense staining reaction. Although no prognostically relevant pattern of global epigentic markers could be identified, our results suggest that epigenetic changes play a role in UM pathogenesis and metastasis. In particular the staining reaction of tumor cell subtypes with a presumed invasive potential warrants further attention. The role of epigenetically relevant interactions with the tumor micromilieu should be further investigated as immune cells are predominantly located in the tumor periphery which showed a different staining intensity than the tumor center. However, as considerable epigenetic diversity exists in primary tumors, studies on biopsy tissue are not recommended for the immunohistochemical investigation of epigenetic markers.

1. Introduction

Cancer is characterized by uncontrolled cell growth with potential for local invasion and metastasis. It is well known that a tumor does not consist of a uniform, clonal cell population and is instead composed of different cell populations which may have certain functions for the tumor micromilieu (McGranahan and Swanton, 2017; Prasetyanti and Medema, 2017).

Recent developments in cancer genomics showed that the combined impact of genetic and epigenetic variants is a fundamental

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characteristic of nearly all human cancers: Epigenetic alterations which comprise altered DNA methylation, histone post-translational modification (methylation and acetylation) and small noncoding RNAs result in an aberrant gene regulation (e.g. by silencing tumor suppressor genes or activation of oncogenes) and play - beside genetic changes - an important role not only in tumorigenesis but also in tumor cell heterogeneity (Wainwright and Scaffidi, 2017). In general, epigenetic regulation of gene expression can be globally (by chromatin remodelling) or locally (by DNA methylation) (Li et al., 2013). Histone acetyltransferase (HAT) activity leads to chromatin remodelling with acetylation of histones resulting in an increased gene transcription via relaxation of the chromatin structure (euchromatin). Histone deacetylase (HDAC) activity reverses this event by removing acetyl groups leading to a densely packed chromatin (heterochromatin) and gene silencing (Moschos et al., 2018). Upregulation of DNA methyltransferases (DNMTs) results in methylation, e.g. of CpG islands in usually non-methylated promotor regions and thus decreased gene transcription (Herman et al., 1996).

Genetic changes (including gene expression profiles) have been well characterized in uveal melanoma (UM) which is the most frequent intraocular primary malignant tumor in Caucasian adults (incidence: 5.1 per million in the United States (Singh et al., 2011)) and have replaced histopathologic characteristics as most important predictors for patients' survival (Landreville et al., 2008). However, there is growing evidence suggesting that epigenetic changes are also involved in the pathobiology of this disease potentially resulting in characteristic altered epigenetic patterns (Sharma et al., 2018).

A Pubmed Search (PubMed Database: https://www.ncbi.nlm.nih.gov) in December 2018 using the term "uveal melanoma AND epigenetic*" revealed 51 published items which have been released between 2003 and 2018. The search term "uveal neoplasms AND epigenetics" (as the MeSH term uveal melanoma was first introduced in 2010), did not reveal further entries. Locus specific epigenetic alterations associated with UM have already been discussed previously (Smolkova et al., 2018), while the global impact of epigenetic alterations has not been studied before.

Evaluation of global levels of epigenetic markers by immunostaining has refined the predictive information on several cancers (Barbisan et al., 2008; Ellinger et al., 2010, 2016; Mosashvilli et al., 2010; Piyathilake et al., 2005). In addition, epigenetic modifiers (e.g. histone deacetylases inhibitors) are available as adjuvant treatment option. However, information on global levels of epigenetic markers is as yet missing for UM.

We therefore investigated whether epigenetic modifications, in particular global levels of histone acetylation (Histone 3 acetylation, H3Ac; Histone 4 acetylation, H4Ac), DNA methylation (5-methylcytosine, 5-MeC; 5′-hydroxymethylcytosine, 5-hMeC) and ubiquitination (global ubiquitination, UBC, as well as H2A-ubiquitination; H2Aub) which have been identified as relevant markers in several other tumor entities are also present in UM primary tumors and metastases in comparison to healthy controls with special emphasis on inter- and intralesional heterogeneity.

2. Material and methods

2.1. Material

Formalin-fixed paraffin-embedded tissue of primary UM (n = 15), UM metastasis (n = 13) and control choroid (n = 12) were studied: Primary UM comprised enucleated eyes (n = 13) as well as tissue from orbital invasion of the primary tumor (n = 2). The patients had a mean age of 71 years (range: 44–84 years) and eight were female/seven were male. Further tumor characteristics are summarized in Table 1. The tumors were graded for T (for size and localization) and G (for cell type) category according to the American Joint Committee on Cancer (AJCC) Cancer Staging Manual (8th edition). Presence of extraocular growth (n = 3), growth along emissary canals (n = 5), and scleral invasion (n = 5) were documented.

Table 1
Demographic and tumor characteristics.

Case	Age	Gender	T category	G category	Special findings
1	63	m	pT4b	G3	Emissary canals
2	52	m	pT3a	G1	Emissary canals
3	81	w	pT3a	G2	Emissary canals
4	79	w	pT3a	G1	Scleral invasion
5	44	w	pT3b	G2	Scleral invasion
6	68	m	pT2c	G3	Extraocular extension
7	80	w	pT2a	G2	Emissary canals
8	81	w	pT4b	G1	Emissary canals
9	72	w	pT2c	G2	Extraocular extension
10	74	w	pT3a	G3	Scleral invasion
11	64	m	pT3a	G2	Scleral invasion
12	80	m	pT3b	G2	Extraocular extension
13	84	m	pT4a	G2	Scleral invasion
14	58	m	N/A	G3	Orbital Recurrence
15	85	w	pT4e	G2	Orbital invasion

Tissue from UM metastases (kindly provided by Melissa Schlitter, tissue bank University Munich) comprised liver metastasis (n = 5), brain metastasis (n = 3), bone metastasis (n = 3), and lung metastasis (n = 2). The patients had a mean age of 58 years (range: 30–74 years) and seven were females.

The choroidal control tissue was obtained from archived calottes of enucleated globes. The enucleations were mostly performed for chronic corneal diseases leading to a blind painful eye. In detail, the underlying reasons for enucleation were perforated corneal ulcer (n = 5), therapy-resistant Fusarium keratitis (n = 1), recurrent corneal graft failure (n = 1), congenital glaucoma (n = 1) as well as a blind painful eye due to scleritis (n = 1) or trauma (n = 3). The patients had a mean age of 65 years (range: 27–95 years) and five were female/seven were male.

The research was conducted in adherence to tenets of the Declaration of Helsinki. Ethic Board Approval of the University of Bonn was obtained.

3. Methods

Formalin-fixed paraffin-embedded tissue of primary UM (n = 13), UM metastasis (n = 15), and control choroid (n = 12) was immunohistochemically stained for Histone 3 acetylation (H3ac), Histone 4 acetylation (H4Ac), DNA methylation (5-methylcytosine (5-MeC) and 5-hydroxymethylcytosine (5-hMeC), global levels of ubiquitination (UBC) as well as Ubiquityl-Histone H2A (H2Aub) [detailed information regarding the antibodies can be found in Table 2]. A detailed description of the immunohistochemical staining procedure can be found in one of our previous publications (Kaiser et al., 2018).

The immunostaining results were analyzed semi-quantitatively by two independent ophthalmic pathologists (MCHC, KUL) who were masked to the sample identity. The nuclear staining intensity of the tumor (and adjacent not affected choroid) resp. metastases and the melanocytes of the control choroids was evaluated by a score from 0 to 3 (0 = no staining; 1 = weak staining; 2 = moderate staining; 3 = strong staining) which was multiplied with the number of stained cells using a score from 0 to 4 (0 = no positive cells; 1 = 1–25% positive cells; 2 = 26–50% positive cells; 3 = 51–75%; 4 = 76–100% positive cells). A mean staining score for each tumor and its adjacent choroid, the metastases and the control choroids was calculated.

Statistical analysis was done by IBM SPSS Statistics 24.0 using non parametric rank sum tests (Mann-Whithney *U*-test, Wilcoxon test). A p value < 0.05 was interpreted as statistically significant.

Table 2
Antibody information.

Antibody	Characteristics	Catalog number	Company	Dilution
Anti-acetyl-Histone H3	polyclonal, rabbit anti-human	06–599	Millipore, Darmstadt, Germany	1:800
Anti-acetyl-Histone H4	polyclonal, rabbit anti-human	06–866	Millipore, Darmstadt, Germany	1:2000
Anti-5-Methylcytosine	monoloncal, mouse anti-human	NBP2-42814	Novus Biologicals, Darmstadt, Germany	1:500
Anti-5-Hydroxymethylcytosine	monoclonal, mouse anti-human	MA5-23525	Thermo Fisher Scientific, Dreieich, Germany	1:150
Anti-ubiquitination	polyclonal, rabbit anti-human	A3207	Abclonal, Woburn, MA, USA	1:700
Anti-Ubiquityl-Histone H2A	polyclonal, rabbit anti-human	8240	Cell signaling, Denver, CO, USA	1:2000

Table 3
Mean value and standard deviation of the investigated tissues for the staining reaction of the investigated antibodies.

Tissue	H3Ac	H4Ac	5-MeC	5-hMeC	UBC	H2Aub
Control choroid	8.3 ± 2.7	11.1 ± 1.4	11.6 ± 0.7	10.0 ± 2.0	8.1 ± 2.7	11.9 ± 0.4
Choroid adjacent to the tumor	4.3 ± 3.4	8.6 ± 3.3	7.8 ± 3.0	7.3 ± 3.1	6.7 ± 3.6	9.3 ± 4.0
Primary tumors	1.4 ± 2.1	4.6 ± 3.6	4.2 ± 3.2	2.0 ± 1.4	2.4 ± 3.1	8.4 ± 3.7
Metastases	1.1 ± 0.9	5.7 ± 2.7	5.8 ± 2.4	2.5 ± 1.0	4.0 ± 3.4	10.2 ± 2.7
Cells with invasive potential	8.6 ± 4.0	11.3 ± 1.3	10.5 ± 1.5	11.4 ± 1.2	9.8 ± 2.5	10.2 ± 2.4

4. Results

4.1. Analysis of the staining reaction in the primary tumors, metastases, and the choroid

The control choroid showed an intense nuclear staining reaction of the choroidal melanocytes for all global markers (H3Ac, H4Ac, 5-MeC, 5-hMeC, UBC) with a mean staining intensity between 8.1 and 11.6 (Table 3, Supplemental Fig. 1). The staining reaction of the inner and outer nuclear layer of the retina served as internal control for the antibody staining reaction. The choroid adjacent to the tumor showed a slightly less nuclear staining intensity (between 4.3 and 8.6) in the melanocytes (Table 3, Fig. 1). The primary tumors displayed a weaker nuclear staining intensity (between 1.4 and 4.6) in the melanocytic tumor cells compared to the control melanocytes (Table 3, Fig. 1). The nuclear staining intensity of the different metastases was between 1.1 and 5.8 (Table 3, Supplemental Fig. 2). The mean staining intensity for the antibody against H2Aub ranged from 8.4 for primary tumors over 9.3 for the adjacent choroid and 10.2 for the metastases up to 11.9 for the control choroid (Table 3).

The staining reaction of the control choroid was statistically significantly more intense than the nuclear staining reaction of the uveal melanomas for all investigated antibodies ($p_{H3Ac} < 0.001$; $p_{H4Ac} < 0.001$; $p_{5\text{-}Me} = < 0.001$; $p_{5\text{-}hMeC} < 0.001$; $p_{UBC} < 0.001$; $p_{H2Aub} = 0.002$; Mann-Whitney *U*-Test). The staining reaction of the choroid adjacent to the tumor was also statistically significantly more intense than the nuclear staining reaction of the uveal melanomas for all investigated antibodies except H2Aub ($p_{H3Ac} = 0.002$; $p_{H4Ac} = 0.028$; $p_{5\text{-}MeC} = 0.019$; $p_{5\text{-}hMeC} = 0.001$; $p_{UBC} = 0.003$; $p_{H2Aub} = 0.414$; Wilcoxon-Test, Fig. 1). However, the staining reaction in the control choroid was more intense than in the choroid next to the tumor for all antibodies except UBC ($p_{H3Ac} = 0.006$; $p_{H4Ac} = 0.016$; $p_{5\text{-}MeC} < 0.001$; $p_{5\text{-}hMeC} = 0.024$; $p_{UBC} = 0.528$; $p_{H2Aub} = 0.012$; Mann-Whitney *U*-Test). There was no statistically significant difference for the staining intensity of the primary tumors compared to the metastases ($p_{H3Ac} = 0.707$; $p_{H4Ac} = 0.255$; $p_{5\text{-}MeC} = 0.145$; $p_{5\text{-}hMeC} = 0.137$; $p_{UBC} = 0.135$; $p_{H2Aub} = 0.194$; Mann-Whitney *U*-Test; Fig. S2).

There was no correlation of the staining intensity with cell type (spindle cells versus epithelioid cells, G category), tumor size (T category) or other parameters such as extraocular extension or vortex vein infiltration.

4.2. Analysis with respect to the heterogenetic staining reaction in the primary tumors

The primary tumor tissue itself exhibited an inhomogeneous staining pattern for all investigated antibodies. In particular the tumor cells in the center were less intensely stained than in the periphery (Fig. 2, Supplemental Fig. 3) showing spatial epigenetic heterogeneity. Despite this common pattern, there were differences with regard to the amount of peripheral cells with an intense staining reaction (which may be attributed to different sections or the immunohistochemical marker). This heterogeneous staining pattern was observed in the primary tumors and larger metastases while small metastases (and peripheral sections of the tumor) showed a more homogeneous and intense staining pattern.

To further evaluate this phenomenon, we analyzed the staining intensity of the tumor cells with obvious invasive potential. These cells were defined by growth along emissary canals (n = 5), vortex vein invasion (n = 0), or extraocular extension (n = 3) which are histologic features associated with a higher risk for metastases. The tumor cells along emissary canals and the ones contributing to extraocular extension showed an intense staining reaction (mean: 10,3; median: 12; comparable to the choroidal melanocytes next to the tumor) for all investigated antibodies (Fig. 3, Table 3). Tumor cells adjacent to or invading the sclera were in some areas intensely stained and exhibited in other areas nearly no staining reaction. Both patterns could be investigated in one primary tumor at different locations.

5. Discussion

Cancer is a heterogeneous disease which is characterized by genetic and epigenetic modifications. Tumor heterogeneity in general can manifest as subpopulations of cells that may have genetic, epigenetic, and/or phenotypic differences. Genetic alterations in cancer are mainly caused by genomic instability (Yao and Dai, 2014). Aberrations in epigenetically controlled gene expression and cell cycle regulation also significantly contribute to cancer. While the impact of certain mutations or chromosomal aberrations can be predictive for the patient outcome in UM, the emergence and the formation of heterogeneous subpopulations of cancer cells may be attributed to epigenetic changes (Litzenburger et al., 2017). Still it is the heterogeneity which causes profound variation and challenges therapeutic strategies.

In our study, we investigated the global levels for Histone 3 and Histone 4 acetylation, DNA methylation and ubiquitination in primary UM, metastases and control choroid with respect to inter- and intratumoral heterogeneity. Histone H2A ubiquitination was also studied

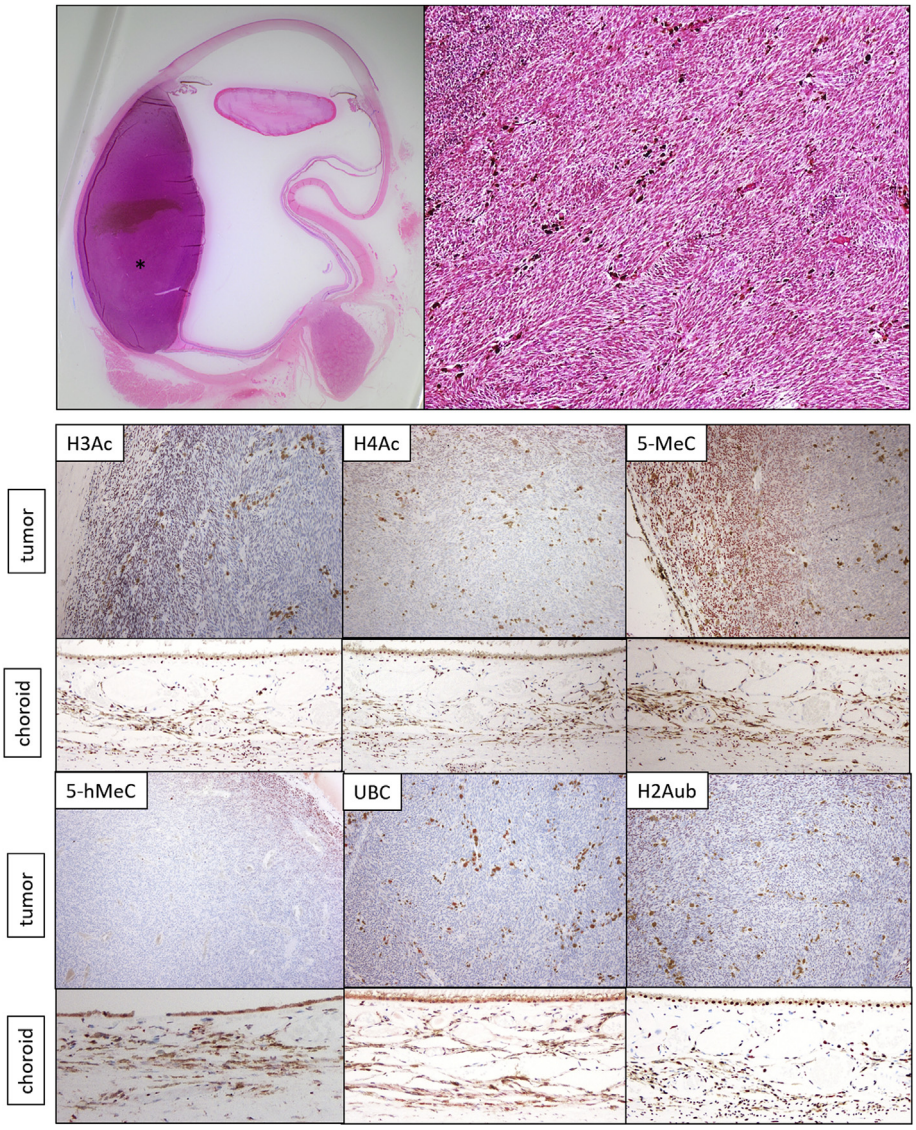

Fig. 1. Uveal melanoma. Overview of a globe with a uveal melanoma (astisk) close to the ciliary body (H&E stain). The immunohistochemical stains for H3Ac, H4 Ac, 5-MeC, 5-hMeC, UBC, and H2Aub shows a weaker nuclear staining reaction in the tumors compared to the adjacent choroid. The periphery of the tumor displays a more intense immunostaining than the center (illustrated for H3Ac, 5-MeC, 5-hMeC).

as it is connected to BRCA1 associated protein-1 (BAP1). BAP1 is a prognostically relevant tumor suppressor gene in UM involved in ubiquitin removal from histone H2A K119 (Sahtoe et al., 2016).

5.1. Differences in the staining intensity of primary tumors/metastasis and control choroid

Significant differences of the immunohistochemical staining intensity for all investigated markers were observed between the choroid (control choroid and choroid adjacent to the UM) and the primary tumors/metastases. This finding suggests a role for epigenetics in uveal

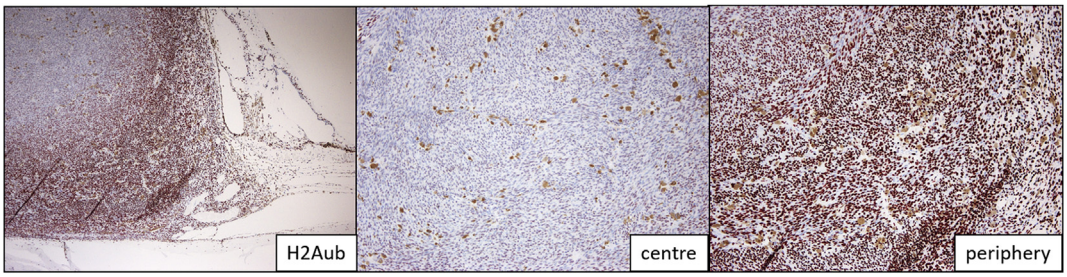

Fig. 2. Intratumoral heterogeneity. Uveal melanoma with nuclear staining reaction for H2Aub. The cells in the center exhibit only a weak staining reaction while most of the peripheral cells are intensely stained.

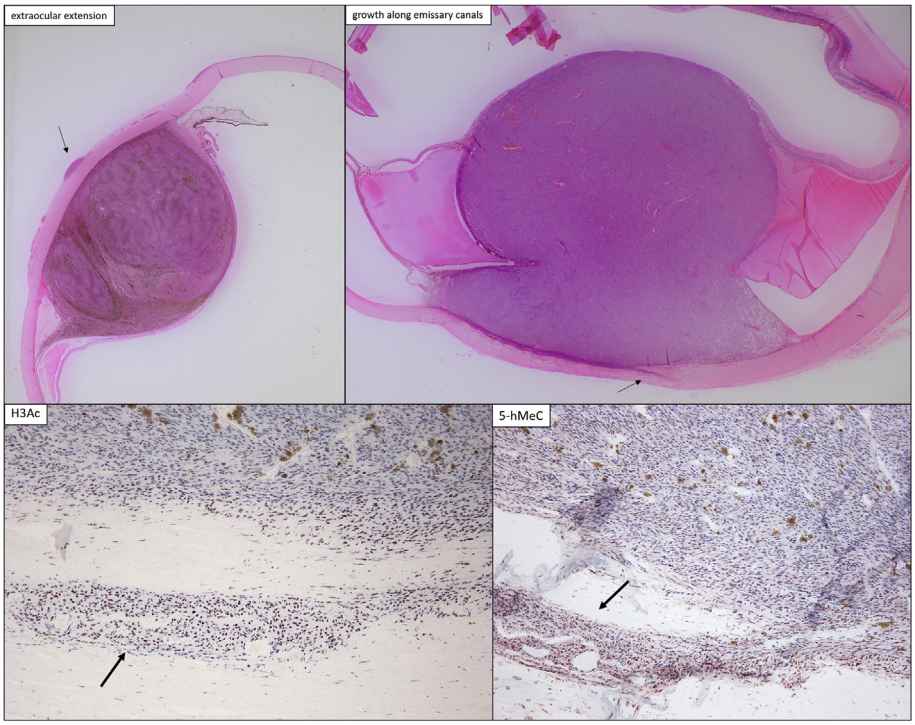

Fig. 3. Cells with a presumed invasive potential. Uveal melanoma with extraocular extension (arrow, H&E). Uveal melanoma with a typical mushroom shape resulting from break through Bruch's membrane and cells growing along emissary canals (arrow, H&E). The cells growing along emissary canals stain intensely positive for all markers, reference pictures for H3Ac and 5-hMeC are shown. The peripheral cells of the primary tumor display also a strong nuclear staining.

melanoma. There was also a lower staining intensity of the choroid adjacent to the primary tumor compared to the control choroid. This may be attributed to the tumor micromilieu within the UM eyes.

Although we identified significant differences between the choroid and the primary tumor/metastases, we were not able to identify a prognostically relevant epigenetic pattern for global markers in this study as it has been described for other tumor entities (Barbisan et al., 2008; Ellinger et al., 2010, 2016; Mosashvilli et al., 2010; Piyathilake et al., 2005). Genetic analysis of the tumor tissue (e.g. gene expression profile, chromosome 3 status and others) already allows for relatively precise information regarding the likelihood of developing metastatic disease in UM (Onken et al., 2004; Prescher et al., 1996). We therefore do not consider epigenetic patterns particularly relevant in this context.

5.2. Spatial epigenetic heterogeneity in UM

A heterogeneic staining pattern for all investigated antibodies in the primary tumors and larger metastases with intense staining of the peripheral tumor cells and a weak staining of large areas (centrewards) of the primary tumor was detected. Scoring of the entire tumor resulted subsequently in a weaker staining of the tumor compared to the control choroid (as discussed above).

When evaluating immunohistochemical stains, staining artifacts have to be considered in the interpretation an unexpected immune reaction. In our case, the peripheral nuclear staining reaction of tumor tissue was accurate and similar to the staining of the choroidal melanocytes. A diffuse “soaking” of the peripheral tumor cells was not observed. The weak staining reaction of the central parts of the tumor was also rated as genuine as the staining reaction increased once peripheral areas of the particular tumor were stained after several step sections (which still harbored a large tumor in diameter and height).

Tumor heterogeneity in UM has been observed before for genetic changes (Dopierala et al., 2010; Lake et al., 2011). Only in rare cases can the discordant gene profile be attributed to different histological features (Miller et al., 2017). In general, genetic tumor heterogeneity is – although present – not a major issue as a tumor biopsy allows for precise evaluation of the genetic risk profile (Bagger et al., 2015). Furthermore, a heterogeneic staining reaction was also observed for other immunohistochemical markers in UM (Donoso et al., 1986; Herwig et al., 2013).

There may be several interpretations of the tumor heterogeneity for epigenetic markers in our study: (1) As UM is a slowly growing tumor (and can grow over several years without being detected), the tumor cells in the center are older and change their epigenetic profile due to an ageing process. (2) UM cells in the center have a different blood and nutrient supply than cells in the periphery. This hypothesis may be supported by the observation that small metastases did not show heterogeneity while larger metastases which have acquired more blood vessels and vascular channels also showed a staining heterogeneity. Pina and co-workers analyzed the composition of blood vessels (mature vessels and neovessels) in UM and detected significant differences in particular for the tumor center in which more neovessels than mature vessels were present (Piña et al., 2009). (3) Tumor cells in the periphery are in close exchange with the tumor microenvironment including immune cells such as macrophages. In addition, macrophages infiltrating the tumor ar e often detected in the tumor periphery (Herwig et al., 2013) and may alter the epigenetic profile of the tumor cells.

As intratumoral epigenetic heterogeneity seems to be more relevant for UM than genetic diversity, it should be further investigated in the future, in particular for its role for metastasis and also with regard to specific epigenetic changes.

5.3. Staining reaction of potentially invasive tumor cells

When evaluating cells with a presumed invasive behavior, i.e. cells which are growing along emissary canals, invading vortex veins or have already led to extrocular extension, these cells showed a very intense staining reaction comparable to cells in the tumor periphery and control choroidal melanocytes for the investigated markers. Invasion of vortex veins, extraocular extension and growth along emissary canals were shown to be associated with an aggressive UM phenotype and a higher risk for metastases (Affeldt et al., 1980; Raoof et al., 2009). Also, the peripheral tumor cells which showed also an intense immunohistochemical staining reaction, may be more “active” than the weakly stained central tumor cells. Our study suggests that these (intensely stained) cells should be further analyzed for specific epigenetic

changes rather than global changes. Furthermore, alterations of the transcriptional status can be expected.

5.4. Age as influencing factor

Age is another influencing factor which has to be considered with respect to epigenetics (in particular for methylation) (Jones et al., 2015). The patients with primary tumors had a similar mean age as the controls (71 versus 65 years) while the patients who developed metastases were younger (mean age: 58 years) than the patients with primary tumors. A direct comparison of the metastases and their primary tumors would be desirable. However, this material is extremely difficult to obtain, in particular as the small and medium-sized tumors are predominantly treated by irradiation and these eyes are – in contrast to large tumors – not primarily enucleated. In addition, although there are differences regarding the age (1) our control group lies in between the two other groups of primary tumors and metastases and (2) the patients with metastases can - in general - not be considered as “young”. As epigenetic changes in ageing are fluent, it is unclear whether there is a crucial impact of age in our cohort. However, there might be a selection bias as some of the enucleated eyes were from patients who survived at least the time span which allowed the tumor to become rather large. (In some cases, the eye was enucleated for other reasons such as extraocular tumor growth).

5.5. Implications on UM biopsies

Finally, as uveal melanoma can be biopsied prior to further treatment (e.g. brachytherapy) or immediately after enucleation of the eye for predicting the risk for metastases by genetic analysis, it may not be advisable - based on our observations - to study biopsy tissue alone for epigenetic changes. The results may not be representative for the entire tumor. Multiple biopsies, in particular of representative areas in the periphery (and potentially invasive cells), are difficult to obtain as the tumor is small (in comparison to malignancies in other organs) and transretinal biopsies are associated with a risk for hemorrhages. Therefore, in contrast to the established use of tumor biopsies for the genetic classification of UM, analysis of the entire tumor (after enucleation or transscleral resection) may be more advisable for the study of epigenetic changes.

6. Conclusions

Our results suggest a considerable role of epigenetic changes in UM pathogenesis and metastasis formation. As epigenetic heterogeneity was observed in primary tumors, biopsy tissue should be avoided for the investigation of epigenetic markers in UM. Epigenetically relveant interactions with the tumor micromilieu as well as the epigenetic characteristics of cells with a presumed invasive potential should be further investigated as consequence from the herein observed spatial intratumoral heterogeneity for global epigenetic markers.

Our results suggest a considerable role of epigenetic changes in UM pathogenesis and metastasis formation. As epigenetic heterogeneity was observed in primary tumors, biopsy tissue should be avoided for the investigation of epigenetic markers in UM. However, based on our findings of spatial intratumoral heterogeneity for global epigenetic markers, epigenetically relevant interactions with the tumor micromilieu as well as the epigenetic characteristics of cells with a presumed invasive potential should be further investigated.

Founding

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Author contributions

Martina C. Herwig-Carl and Amit Sharma were mainly involved in conceptualization of this study.

Martina C. Herwig-Carl and Karin U. Loeffler performed the histopathologic analysis of the specimens.

Anna Melissa Schlitter contributed material of metastases including patient data.

Tobias Höller did the statistical analysis.

Martina C. Herwig-Carl prepared the original draft. Amit Sharma, Karin U. Loeffler and Frank G. Holz were involved in critically reviewing the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.exer.2019.04.001.

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