Chapter 5

Diagnosis, Histopathologic and Genetic Classification of Uveal Melanoma

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1. Introduction

Uveal melanoma (UM) is the most common cause of primary eye cancer in the western world. During embryogenesis neural crest cells migrate to the neural tract where they develop into melanocytes. Melanomas of the uvea are derived from these melanocytes. UM may arise in the iris (5%), ciliary body (23%) or choroid (72%). Choroidal melanomas are the most common and usually display a discoid, dome-shaped or mushroom shaped growth pattern. Approximately 80% of the primary intraocular tumours are diagnosed as UM in patients above the age of 20 years, with a mean age of 60 years (Singh & Topham, 2003). Despite a shift towards more conservative eye treatments, survival has not improved during 1973 to 2008 (Singh et al, 2011). Growth of the primary tumour is related with histopathological features, as well as the genetic changes within these tumours. In this chapter we will not discuss iris melanoma, as this shows a different clinical and genetic behaviour, compared to ciliary body and choroidal melanoma. The clinical features, histopathological profile and genetic alterations of UM, as well as therapeutic options for primary tumours and metastases will be discussed.

2. Epidemiology

The incidence of UM ranges from 4.3 to 10.9 per million (Singh et al, 2009). For the past fifty years, the incidence has remained stable, unlike trends indicating a higher incidence of cutaneous melanoma. The incidence in Europe and United States is comparable to that in Australia and New Zealand. In Europe, a lower incidence is reported in Spain and the south of

Italy, about 2 per million, whereas registries in France, the Netherlands, Switzerland and Germany has intermediate values around 4 to 5 per million. The United Kingdom registered over 6 per million, and the highest incidence is up to > 8 per million in Norway and Denmark (Virgili et al, 2007).

3. Predisposing factors

Men and women with UM are more or less affected equally (Damato & Coupland, 2012; Singh et al, 2011). Iris melanoma is more common in women than in men (Damato & Coupland, 2012). Several phenotypes, like blue or grey eyes and fair skin have been suggested to predispose for UM (Schmidt-Pokrzywniak et al, 2009). This might explain why Caucasians are approximately 150 times more frequently affected than Africans (Margo et al, 1998; Singh et al, 2005a). In Asians UM is less common (Biswas et al, 2002).

From all the parts of the uvea the iris is most exposed to ultraviolet light, because of filtering effects of the lens and retinal pigment epithelium (RPE), the choroid receives less light (Singh et al, 2004). Although several epidemiologic and case control studies have been performed to investigate the influence of sunlight exposure on UM, the results are not conclusive (Guenel et al, 2001; Holly et al, 1990; Pane & Hirst, 2000; Shah et al, 2005; Vajdic et al, 2002). UM may occur as a part of familial syndromes, like xeroderma pigmentosa, Li-Fraumeni syndrome and familial breast and ovarian cancer. Of all UM 0.6% is considered to be familial (Singh et al, 1996). In a retrospective study 0.0017% of the primary UM patients were in the setting of familial atypical mole and melanoma syndrome (FAMM). These patients were relatively young with a mean age of 40 years (Singh et al, 1995). Furthermore, an association of neurofibromatosis type 1 and UM has been suggested, since both are of neural crest origin, however this association remains unclear (Honavar et al, 2000). Ocular and oculodermal melanocytosis (Nevus of Ota), dysplastic nevi and cutaneous melanoma are correlated with an increased risk of UM development (Carreno et al, 2012; Gonder et al, 1982; Hammer et al, 1995; Richtig et al, 2004; Singh et al, 1998; Toth-Molnar et al, 2000; van Hees et al, 1994). Additionally, in UM patients ocular and oculodermal melanocytosis are about 35 to 70 times more common (Carreno et al, 2012; Singh et al, 1998).

4. Clinical presentation

Depending on de location and size of the tumour, patients can present with visual complaints. Most UMs are detected during a routine ophthalmic examination. Approximately 30% of the patients have no symptoms at time of diagnosis, and if there are any complaints these consist mostly of blurred vision, floaters, photopsias and visual field loss (Damato, 2010) (figure 1). Usually patients do not present with severe ocular pain, however, this can occur secondary to inflammation or neovascular glaucoma.

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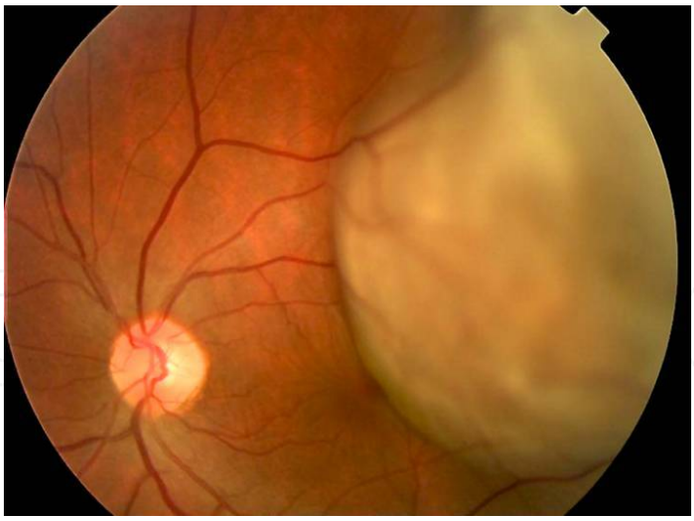

Figure 1. A large amelanotic uveal melanoma leads to a visual field defect.

5. Diagnosis

Diagnosis of UM is based on a combination of clinical examination with slit lamp biomicroscopy, indirect ophthalmoscopy (figure 1, 2a, 3a) and ultrasonography (US) (figure 2b, 3b). Iris melanomas are readily detectable by slit lamp biomicroscopy, whereas ciliary body tumours are hidden behind the iris and can be visualized by US. Choroidal tumours, depending on their location, are diagnosed by dilated indirect ophthalmoscopy and US. In suspect cases of intravenous fluorescein angiography can be helpful in differentiating melanomas from other diagnoses. Also optical coherence tomography (OCT) and autofluorescence can provide additional information (Lavinsky et al, 2007; Shields et al, 2008). In selected cases, when in doubt, an intraocular biopsy is taken of the tumour.

Indirect ophthalmoscopy through a dilated pupil provides a correct diagnosis in more than 95% of the cases (Char et al, 1980). Accuracy of the right diagnosis is established to be over 99% by experienced clinicians with US, ophthalmoscopy, and fluorescein angiography and confirmed by histopathology (Collaborative Ocular Melanoma Study Group, 1990). The ability to differentiate melanoma from other lesions has improved over the last decades. When comparing studies of 1964 and 1973, in 19% of the enucleated patients with the clinical diagnosis melanoma no histopathological evidence of a melanoma was found (Ferry, 1964; Shields, 1973). The accuracy in diagnosing medium to small sized tumours is quite challenging. Nine percent of presumed melanomas are found to have another diagnosis by fine needle aspiration biopsy (Char & Miller, 1995). Most important is to minimise the delay in referring patients with melanoma to a specialised centre. It is reported that in 29% of the patients a melanoma is missed during the first visit by an ophthalmologist, and that 31.5% of the patients referred to an oncology centre with the diagnosis of melanoma actually had a mimicking lesion (Eskelin & Kivelä, 2002; Khan & Damato, 2007).

5.1. Characteristics

Melanoma are generally pigmented, but one fourth are relatively non-pigmented or amelanotic (figure 1). Melanoma can develop into two different directions: towards the vitreous and outwards, through the underlying sclera. Having broken through Bruch's membrane, into the vitreous, UMs achieve a characteristic shape, even pathognomonic, like a 'collar button' or 'mushroom'. Small melanomas can appear flat or dome shaped.

5.2. Clinical prognostic factor

Well-known clinical prognostic factors are age and location of the tumour. Older patients tend to have a worse prognosis (Shields et al, 2012). One study found that UMs were located predominantly posterior and temporal or had a preference for macular zone, while others found a more equal distribution of melanoma (Krohn et al, 2008; Li et al, 2000; Shields et al, 2009b). Patients with larger tumours, tumours that ruptured through Bruch membrane and in patients who have developed metastasis, the tumours were significantly more often located anterior to the equator (Krohn et al, 2008).

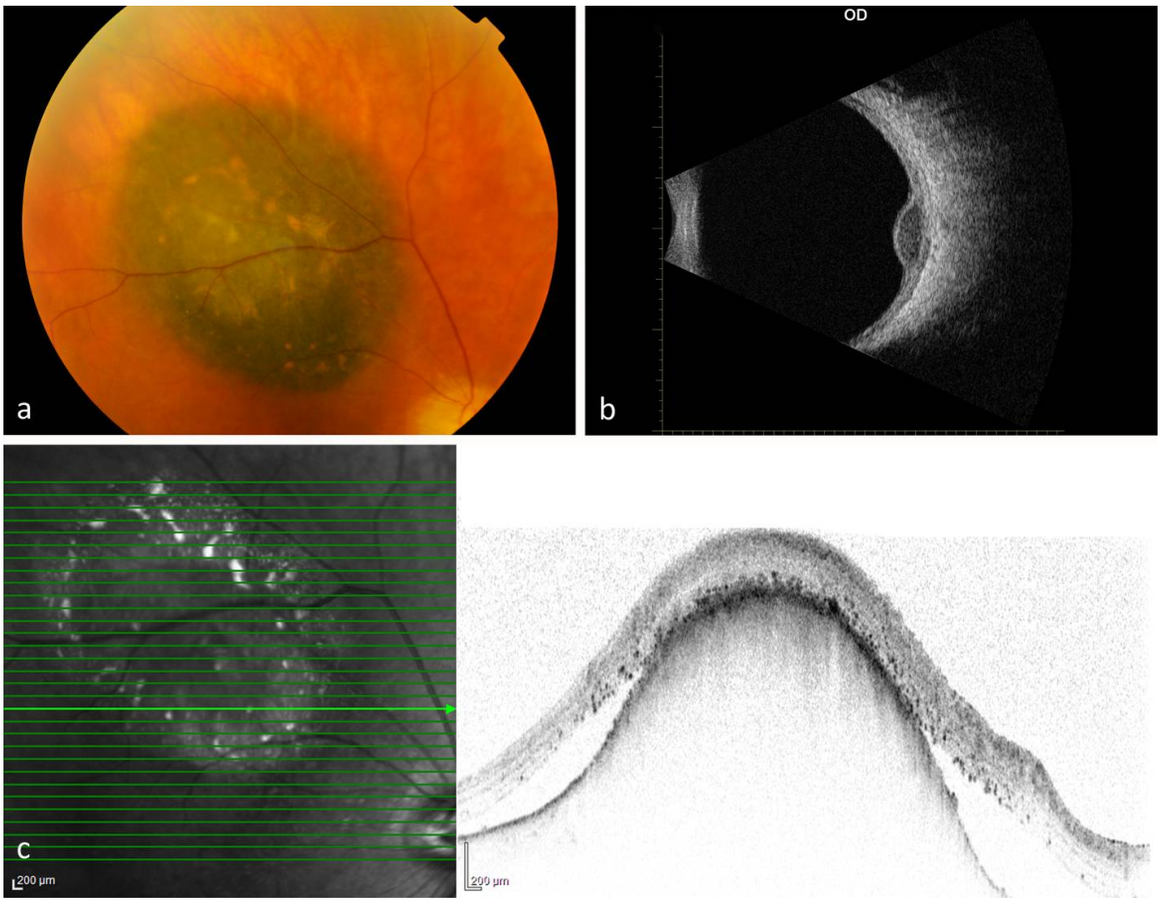

Figure 2. a: A dark pigmented uveal melanoma with orange pigment; 2b: On B-scan ultrasonography acoustic hollowing and choroidal excavation is present, 2c: Subretinal fluid and retinal pigment epithelial alterations are visible on optical coherence tomography scan at the top of the tumour.

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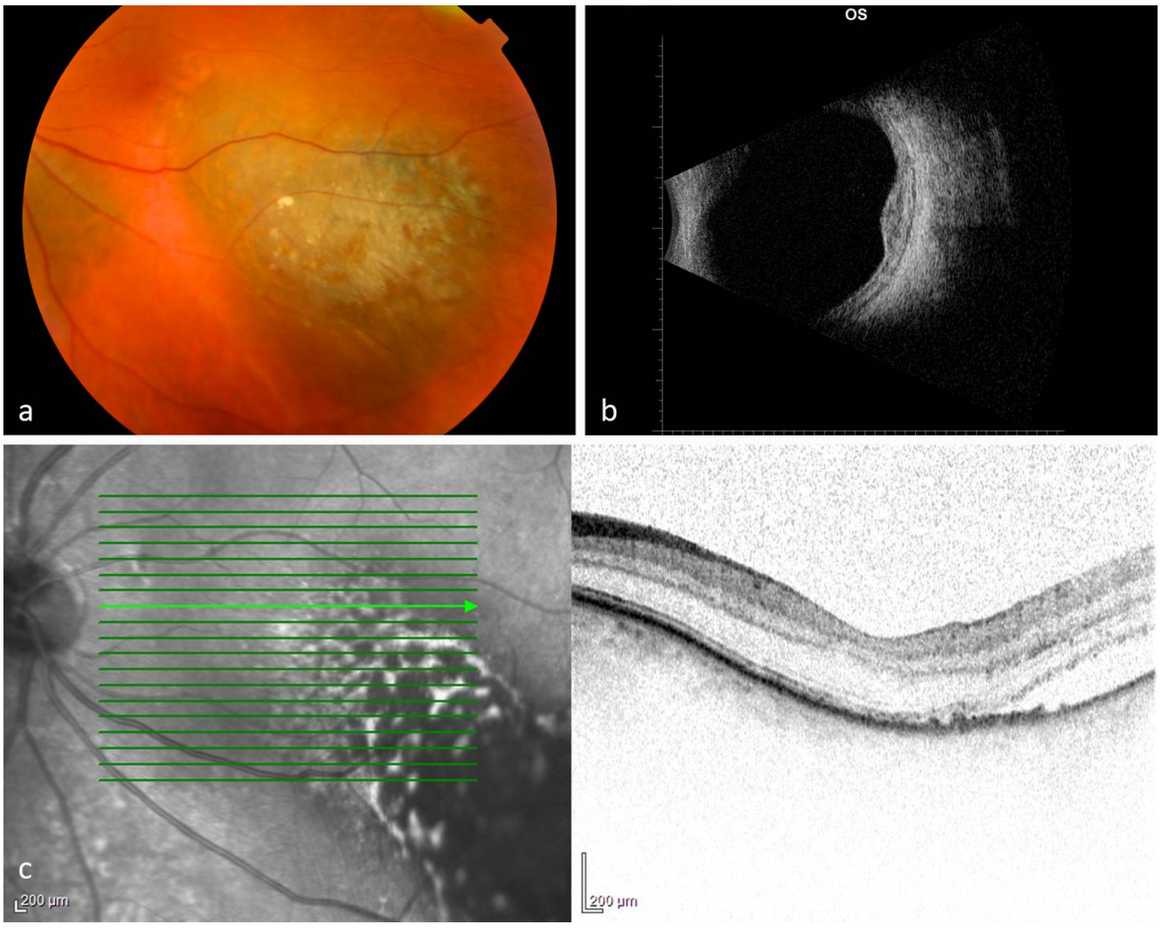

Figure 3. a: Pigmented uveal melanoma with orange pigment (lipofuscin); 3b: A homogeneous grey scale in the tumour and choroidal excavation on B-scan ultrasonography; 3c: Optical coherence tomography of the same tumour with subretinal fluid.

The most important clinical prognostic factor is tumour size, and is often used for selection of the treatment. There are several treatment options, which will be discussed later in this chapter. UM are subdivided into different categories depending on the apical size and diameter, however, many centres use their own definition. Most widely used definition is suggested by the COMS study. Small melanomas are 1.0 - 2.5 mm in apical height and > 5.0 mm in largest basal dimension (Collaborative Ocular Melanoma Study Group, 1997). Medium tumours are defined as tumours 2.5 to 10 mm in apical height and ≤ 16 mm in largest basal diameter. Large tumours are ≥ 2 mm in apical height and > 16 mm in maximal basal diameter, or a melanoma > 10 mm in apical height, regardless of the basal diameter (Collaborative Ocular Melanoma Study Group, 2003). One large study described that each increase in millimeter of tumour thickness increased the risk for metastasis by 5% (Shields et al, 2009b). The mortality rate for small (< 2 - 3 mm height), medium (3 - 8 mm height) and large (> 8 mm height) melanoma was 16%, 32% and 53% in 5 years, respectively, and has not changed in recent years (Diener-West et al, 1992). This supports the model of tumour doubling time of melanoma and its' related metastasis. The model suggests that micrometastasis already exist

several years before diagnosis of the primary tumour (Eskelin et al, 2000). This emphasizes the importance of identifying small melanoma and reducing the risk of metastases.

5.3. Clinical predictive factors of small melanoma

In general, choroidal nevi have a less than 5 mm basal diameter and are minimal in height (< 2 mm), although several definitions of nevi have been proposed. Due to different examination methods and definitions, the prevalence of nevi is between 0.2% and 30% (Gass, 1977; Wilder, 1946). Overall in a Caucasian population the incidence is 6.5% (Sumich et al, 1998). Whenever, growth of a nevus is measured on US in a short time a transformation into a small melanoma is suspected. On the other hand benign nevi can also grow slowly. Mashayekhi *et al* observed in 31% of nevi a slight growth, without evidence of development into a melanoma over a mean follow up of 15 years (Mashayekhi et al, 2011). As described by Singh and co-workers, assuming that all melanoma result from nevi, 1 out of 8845 choroidal nevi can undergo malignant transformation into melanoma in the Caucasian population in the USA (Singh et al, 2005b). In Australia this is estimated 1 out of 4300 nevi (Sumich et al, 1998).

It is important to differentiate melanoma form other choroidal pathologies, such as choroidal nevi, by identifying indicators of potential malignancy which may differentiate nevi from small UM. Shields *et al* constructed a mnemonic "TFSOM", i.e. "to find small ocular melanoma" to assist in identifying small choroidal melanoma at risk for growth (Shields et al, 1995). The letters of the mnemonic indicate: Thickness > 2 mm, subretinal Fluid, Symptoms, Orange pigment and Margin to the optic disc. Tumours with no, one or more than two factors have 4%, 36% or > 45% chance of growth, respectively (Shields et al, 2000). A tumour with a thickness of more than 2 mm is considered suspect of being a melanoma rather than a nevus. Subretinal fluid is the strongest indicator of malignancy. Exudative retinal detachment, overlying or adjacent to the tumour, is associated with tumour growth (Augsburger et al, 1989). Presence of symptoms, as mentioned earlier or a change in symptoms is a risk factor for malignancy. Orange pigment is formed on melanomas of the posterior pole, although this can also be seen on the surface of presumed benign nevi and haemangioma. Orange pigment is an accumulation of lipofuscin within the RPE. In amelanotic tumours it appears brown-black of colour. Besides orange pigment as a risk factor, a tumour margin within 3 mm of the optic disc is also suspect for malignant potential (figure 4a).

Later "Using Helpful Hints Daily" was added to "TFSOM" mnemonic (Shields et al, 2009a). These features indicate a low acoustic profile or Ultrasound Hollowness, absence of a Halo around the tumour and absence of Drusen over the tumour. US hollowness is shown in 25% of nevi that transformed into melanoma, compared to the 4% with growth without US hollowness (Shields et al, 2009a). A halo around a tumour is a pigmented lesion with a surrounding depigmentation, as can also be noticed in dysplastic nevi. Drusen suggest a chronic lesion and usually indicate that the tumour is benign, however this is not conclusive.

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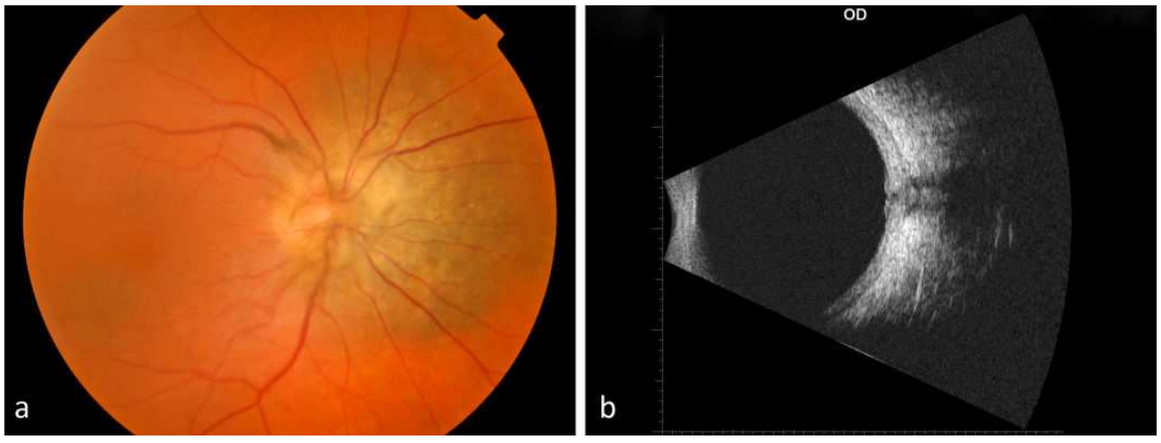

Figure 4. a: Peripapillary nevus, barely elevated, with margin located < 3 mm to the optic disc in the right eye of a 72 year-old man; 4b: High reflectivity on B-scan ultrasonography.

5.4. Ancillary testing

5.4.1. Ultrasonography

US is a non-invasive tool and helps to establish the diagnosis of UM, despite media opacities or whether the tumour is located far peripherally. UM shows characteristic low to medium internal reflectivity on A-scan. B-scan US is primarily used to plan therapy based on the first measurement, and to periodically measure tumour prominence (height) and basal diameter for follow-up. The B-scan can identify possible extraocular extension as an empty area behind the sclera. On B-scan US the internal structure of the tumour is typically seen as a relative homogeneous grey scale, although this pattern is not specifically diagnostic (figure 3b). At the base of the tumour an acoustically silent zone (called acoustic hollowing) is seen, as well as choroidal excavation and shadowing in the orbit (figure 2b). Eighty-eight percent of the UM show US hollowness or low acoustic reflectivity (Boldt et al, 2008). Choroidal excavation is not observed in all melanomas and varies from 42% to 70% (Coleman et al, 1974; Sobottka et al, 1998; Verbeek, 1985). US provides accurate measurements with an interobserver variability of 0.5 mm (Char et al, 1990).

5.4.2. Fluorescein angiography

The diagnostic value of fluorescein angiography in UM is limited. Fluorescein angiography does not show pathognomonic patterns and is especially helpful in differentiating lesions, which simulate melanoma. The pigmentation, size and effect on the RPE of the tumour influence the fluorescein angiogram. It is of little help in some medium to large melanomas that have an intrinsic tumour circulation. This 'double circulation' (simultaneous visualization of retinal and choroidal circulation) consists of late staining of the lesion and multiple pin-point leaks at the level of the RPE, which is evident in the early phase of the angiogram. Blockage of background fluorescence and late staining, when fluorescein leaks from the vessels can be seen on an angiogram as well (Atmaca et al, 1999). Characteristic signs are hypo-

fluorescence in the early phase followed by diffuse hyperfluorescence and hyperfluorescent spots (due to changes in RPE). In the late phase the dye accumulates in the tumour tissue and hyperfluorescents (figure 5b). Hypofluorescent spots correspond with deposits of orange pigment on the surface of the tumour.

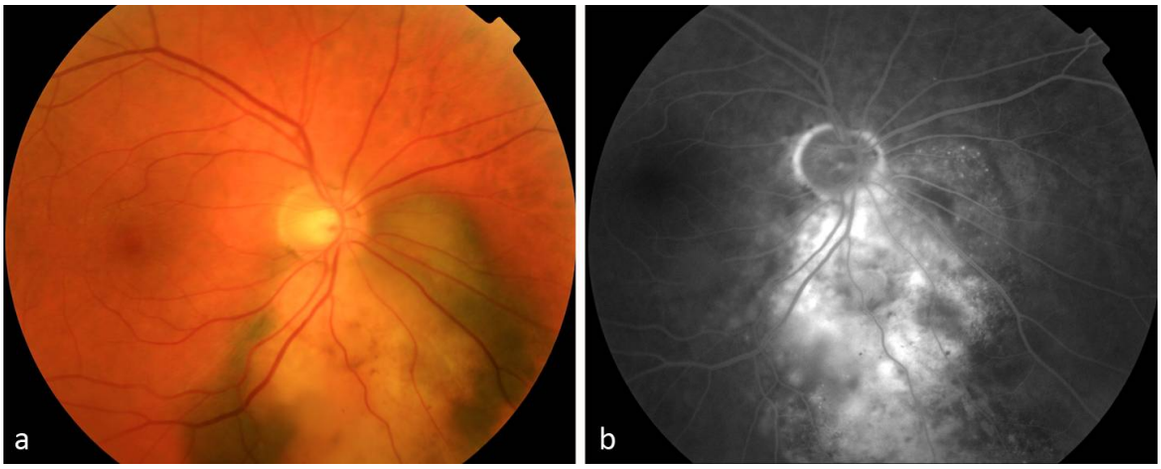

Figure 5. a: A partly pigmented and non-pigmented uveal melanoma; 5b: Fluorescein angiogram with blockage of the background and fluorescein leaking from the vessels.

5.4.3. Indocyanine green angiography

Indocyanine green angiography is designed to visualize the choroidal vessels and provides more information than fluorescein angiography. Whether an evident pattern can be seen on an angiogram depends on the pigmentation, thickness, disruption through Bruch's membrane and vascularisation of the tumour (Atmaca et al, 1999). More fluorescence is seen in less pigmented and larger tumours. The choroidal vasculature can be better visualised with indocyanine green than fluorescein. On indocyanine green late staining is observed, because of the leaking of indocyanine green in the extracellular space of the tumour (Frenkel et al, 2008; Guyer et al, 1993; Stanga et al, 2003).

5.4.4. Optical coherence tomography and fundus autofluorescence

OCT and fundus autofluorescence imaging have limited use in detecting changes in the choroid, however, both techniques are non-invasive and of help in identifying subtle changes in the RPE, retina and vitreoretinal interface. By means of an OCT subretinal fluid can be visualized and quantified, small tumours can be measured, whereas with fundus autofluorescence orange pigment can be shown. Spectral domain OCT can be useful in the detection of subretinal deposits, vitreous seeding and transretinal tumour extension (Heindl et al, 2009).

Although OCT itself is not useful in diagnosing uveal melanoma, it aids in differentiating other pigmented lesions from melanomas (Schaudig et al, 1998). For example, melanocytoma tend to have a high reflective signal anteriorly, corresponding with the nerve fibre layer, and an optical shadowing posteriorly (Muscat et al, 2001). In most choroidal nevi no charac-

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teristic or subtle patterns of autofluorescence were observed (Lavinsky et al, 2007; Shields et al, 2008). Choroidal melanoma and related retinal and RPE changes, show different autofluorescence patterns, and secondary changes, such as subclinical retinal detachments associated with presence of small amounts of subretinal fluid can discriminate small choroidal melanoma and nevi at risk for growth (Muscat et al, 2004). Like some nevi UM show brighter hyperautofluorescence in overlying orange pigment, RPE detachment and subsequently decreased brightness in subretinal fluid and drusen (Shields et al, 2008) (figures 2c and 3c).

5.4.5. Magnetic resonance imaging and computed tomography

Magnetic resonance imaging (MRI) and computed tomography (CT) can be of additional value in the differential diagnosis of UM. On CT an UM appears as a hyperdense lesion with moderate contrast enhancement. Tumours thinner than 2 mm are not detectable on CT. Besides that, CT is less accurate than US in differentiating melanoma and is more expensive (Mafee et al, 1986; Peyster et al, 1985). For extrascleral extension CT is inferior to US (Scott et al, 1998). On the other hand, MRI seems more sensitive and more specific than US for detection of extraocular extension of UM (Hosten et al, 1997). A choroidal melanoma appears hyperintense on a T1 and hypointense on a T2 weighted scan. As this can also be the appearance of a melanocytoma, MRI is not specific for uveal melanoma. Due to the higher expenses of CT and MRI and the superiority of US, both techniques are not routinely used for diagnostic evaluation.

5.5. Differential diagnosis

About 54 different conditions are able to simulate UM. The most frequent diagnosis is choroidal nevus, accounting for 49% of the approximately 1739 presumed melanoma patients referred to a large tertiary Oncology Department in the USA (Shields et al, 2005b). The differentiation between small melanomas and choroidal nevi remains a clinical challenge. Clinical features that are more prevalent in *choroidal nevi* than in melanomas are drusen and RPE changes, whereas retinal detachment, choroidal neovascularisation or haemorrhagic retinal detachment can occur in both. On B-scan US, nevi have a high internal reflectivity (figure 4b). Also orange pigment and subretinal fluid, which are features of potential malignancy as mentioned previously, can be present in nevi. Ten percent of the nevi have orange pigment and 18% have subretinal fluid.

Congenital hypertrophy of the retinal pigment epithelium (CHRPE) has sharper edges than melanoma and usually sharply bordered nonpigmented areas (lacunae), or a depigmentated or pigmented halo within. The lesions might be slightly elevated and are black or grey of colour. CHRPE is a benign lesion and is typically located in the peripheral fundus. On the other hand, adenocarcinomas arising from a CHRPE have been reported (Shields et al, 2009e).

Optic disc melanocytoma is a heavily pigmented benign lesion with a fibrillated or feathery margin. Although it can occur anywhere in the uveal tract, the tumour is most often located unilateral and on or nearby the optic disc. Optic disc melanocytoma is a variant of melanocytic nevus. Most patients (75%) have no visual complaints, whereas patients with visual

loss were related to neuroretinitis from tumour necrosis and secondary subretinal fluid of the fovea (Shields et al, 2006; Shields et al, 2004). In addition, visual field defects have been described (Meyer et al, 1999; Shields et al, 2006). Ocular melanocytosis is associated with melanocytoma in 8% of cases, and melanocytoma enlargement is noticed in 57% within 8 years (Lee et al, 2010) and 32% within 10 years (Shields et al, 2004). Although malignant transformation is extremely rare, it has been reported (Meyer et al, 1999; Shields et al, 2004).

Hyperplasia of the RPE is a common ocular finding, which is idiopathic or develops in response to trauma, inflammation, haemorrhage and retinal detachment. It is characterised as a black irregular usually small retinal lesion consisting of proliferated RPE cells. Intraretinal pigmented spicules can be seen, and when it manifests as a subretinal localized mass, a melanoma can be suspected.

Choroidal haemangioma is a benign tumour consisting of blood vessels with a typical red to orange colour. Some areas of increased pigmentation can be observed, which makes it difficult to differentiate from melanoma. On angiography typical early hyperfluorescence is shown and on US a characteristic high internal reflectivity is present.

Choroidal metastases are the most common intraocular malignancies. The prevalence of uveal metastases from all forms of carcinoma is between 2% and 9%, with a mean of 7% for breast cancer and 5% for lung cancer (Kanthan et al, 2007). The origin of choroidal metastases is predominantly breast cancer in woman and lung cancer in man. Less frequently patients are diagnosed with other primary tumours, such as gastrointestinal tract, kidney, skin and prostate carcinoma (Shields et al, 1997). Choroidal metastases typically develop after the diagnosis of breast cancer and in some cases systemic metastases have already been detected. In 66% to 97% of lung cancer patients, choroidal metastases are detected after the primary tumour has been diagnosed (Kanthan et al, 2007). In conclusion, uveal metastases can also be observed before the diagnosis of breast or lung cancer (Demirci et al, 2003; Singh et al, 2012). The median interval between diagnosis of the primary tumour and uveal metastasis is 1 - 4.5 years (Amer et al, 2004; Ratanatharathorn et al, 1991; Rosset et al, 1998; Rottinger et al, 1976; Tsina et al, 2005). Choroidal metastases are creamy yellow, flat or elevated and often multilobulated lesions that can occur bilateral. More than half of the patients may develop subretinal fluid (Demirci et al, 2003). The lesion can show clumps of brown pigmentation, known as leopard spots and RPE alterations. Metastases grow in a different fashion than primary UMs, they infiltrate and replace the normal choroidal architecture more diffusely. On US metastases from breast carcinoma show a higher internal reflectivity than UM (Sobottka et al, 1998).

Choroidal osteoma is a rare ossifying benign lesion of the choroid that appears as a yellowish to orange well-defined, juxtapapillary or macular choroidal tumour. These lesions mostly occur in young women with a mean age of 26 years; usually it occurs unilateral, although in 20-30% of cases it appears to be bilateral. Over time an osteoma may enlarge and decalcify partially or totally (Ross & Kemp, 2009; Shields et al, 2005a). There is a 31% chance of developing choroidal neovascularisation after 10 years (Shields et al, 2005a). On B-scan US a highly reflective lesion that shadows the orbit can be seen.

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Peripheral exudative hemorrhagic chorioretinopathy (PEHC) lesions, unilateral and often bilateral, have peripheral (> 3 mm outside the fovea) subretinal or sub-RPE haemorrhage that arises from choroidal neovascularisation. In the periphery signs of macular degeneration, such as lipid exudation, subretinal fluid and fibrosis can be observed (Mantel et al, 2009; Shields et al, 2009c). Also in the macula drusen, RPE alterations or choroidal neovascularisation can be present, which is then consistent with macular degeneration (Shields et al, 2009c). On B-scan internal lesion characteristics show a solid or hollow acoustic quality and no choroidal excavation (Mantel et al, 2009; Shields et al, 2009d). The majority of the peripheral lesions resolve spontaneously over time, leaving a scar.

Hemorrhagic detachment of the retina and RPE may also simulate melanoma.

Choroidal haemorrhage may be distinguished from UM by partially or totally resorption of the haemorrhage over a few weeks, and on US an after-movement can be noticed by kinetic evaluation. Key features are elevated eye pressure, forward movement of diaphragm combined with severe pain (Yang et al, 2003).

Posterior nodular scleritis is rare, but often underdiagnosed. It is twice as common in women as in men, and in 35% of the patients it occurs in both eyes. The most common symptoms are periocular pain, pain with eye movement and decreased vision. The differentiation between scleritis and melanoma can be made by US. On B-scan echogenic scleral nodules, fluid in Tenon's capsule, swelling of the optic disc and serous retinal detachment are found (McCluskey et al, 1999).

Intraocular leiomyoma is a rare benign amelanotic tumour of the uvea and mimics an UM. It presents as a dome-shaped lesion, showing light translucency and often contains dilated episcleral vessels, with a predilection in young females (Shields et al, 1994). Sometimes the diagnosis cannot be made by non-invasive examination and intraocular biopsy is necessary (Richter et al, 2003).

Adenoma of the RPE is infrequently diagnosed before enucleation. RPE adenoma is dome-shaped and has in contrast to melanoma a higher internal reflectivity on A-scan US (Nakamura et al, 2012). Compared to UM, RPE adenoma has more frequently retinal feeder vessels, retinal or subretinal exudates and exudative retinal detachment (Wei et al, 2010).

6. Classification and histopathologic features

UMs develop from melanocytes of the uvea that are derived from neural crest cells. Initially Callender and colleagues described several melanoma cell types, (Callender, 1931) currently three histopathological uveal melanoma categories are being recognised: spindle, epithelioid and mixed cell type (Campbell et al, 1998). Haematoxylin and eosin (H&E) staining is used to differentiate between cell types. Spindle cells exhibit elongated nuclei that may contain eosinophilic nucleoli. In general, Ums containing spindle cells grow slowly and might be associated with better prognosis. On the other hand, UMs consisting of faster growing epithelioid cells, have a more aggressive behaviour, and are therefore associated with poor clinical

outcome. Epithelioid cells have more polygonal cytoplasm and contain eccentric placed large pleomorphic nuclei and prominent eosinophilic nucleoli (figure 6). The mixed-cell type melanoma has variable proportion of spindle and epithelioid cells with a minimum of 10% of any one type (Edge & American Joint Committee on Cancer, 2010). Other inter-tumour factors, like the presence of certain extracellular matrix patterns (three closed loops located back to back identified by Periodic-acid Schiff (PAS) staining) and increased mitotic figures (number of mitoses per 50 high-power fields equal to 8mm2) can both provide additional adverse prognostic information (Folberg et al, 1993; Mooy et al, 1995). Other histological features associated with mortality and metastases are mean diameter of ten largest nucleoli, degree of pigmentation, presence of inflammation and tumour necrosis (Gill & Char, 2012). Extrascleral extension by perineural, perivascular, intravascular or direct scleral invasion is correlated with a worse prognosis, especially when the orbital fat resection margin is positive (Collaborative Ocular Melanoma Study Group, 1998).

Immunohistochemistry may be of diagnostic value. S-100 is expressed by cells of neuroectodermal origin. HMB-45 binds to gp100, an antigen expressed by melanocytes that can be useful in differentiating UM from nonmelanocytic tumours (Burnier et al, 1991).

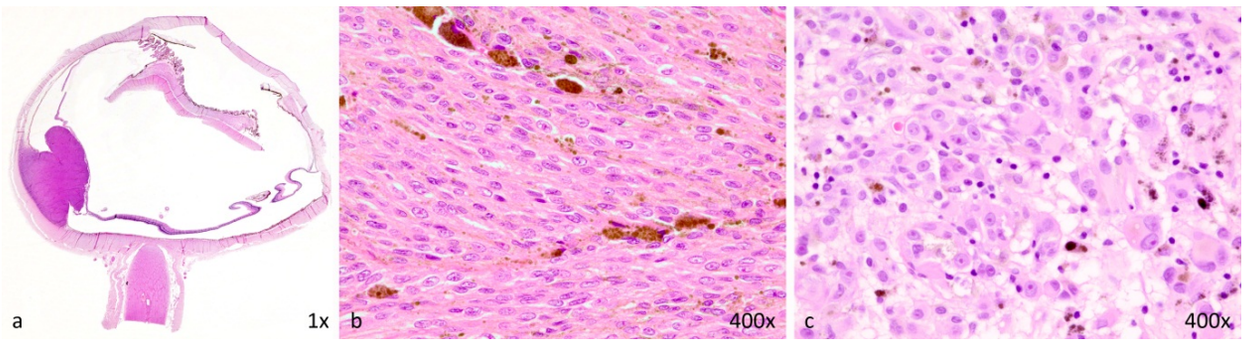

Figure 6. a: Haematoxylin and eosin staining of formalin fixed and paraffin embedded eye sample with a typical mushroom shaped melanoma.; 6b: Uveal melanoma tissue with spindle cell type characterised by elongated nuclei; 6c: Uveal melanoma tissue with epithelioid cells containing large pleomorphic nuclei and prominent eosinophilic nucleoli.

7. Genetic classification

Cytogenetic studies in solid tumours have been a greater challenge than in haematological malignancies since metaphase chromosome spreads of good quality are more difficult to obtain. Solid tumours frequently have highly complex chromosome alterations and are more heterogeneous. Despite this, UM has been well studied since the late eighties with different techniques, such as cytogenetic and fluorescent in situ hybridization (FISH) analysis. Over the years, we have learned that the majority of UMs contain non-random chromosomal

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anomalies on either the short arm (p) and or long arm (q) of chromosomes 1, 3, 6 and 8, which can serve as prognostic markers.

7.1. Cytogenetic and molecular techniques in UM research

To examine chromosomal changes in UM tissue several cytogenetic and molecular techniques are available. UMs are quite suitable for cytogenetic analysis because of their relatively simply karyotype. Large chromosomal gains, deletions and translocations can be visualized with conventional karyotyping and spectral karyotyping (SKY) (figure 7a). However, for the detection of smaller abnormalities other techniques are necessary, such as FISH (figure 7b), comparative genomic hybridization (CGH) or quantitative polymerase chain reaction (qPCR) based techniques. An approach is the multiplex ligation probe amplification (MLPA) which allows the relative quantification of multiple loci in one single reaction. MLPA can detect patients at risk for metastatic disease using the results for chromosome 3 and 8 with similar accuracy as FISH (Damato et al, 2009; Vaarwater et al, 2012). MLPA and other qPCR-based techniques as Multiplex Amplicon Quantification (MAQ) fill the gap between more expensive genome-wide screening assays and cheaper methods that only provide information on a single locus (Kumps et al, 2010). A different technique is microsatellite analysis (MSA). Microsatellites are tandem repeats of polymorphic sequences located in the non-coding regions of DNA. An extreme form of microsatellite instability was first described in hereditary nonpolyposis colorectal cancer syndrome (Thibodeau et al, 1993). This technique is used to study loss of heterozygosity (LOH) as an indicator of chromosomal loss. A drawback of MSA is that only a limited number of markers can be analyzed in one experiment.

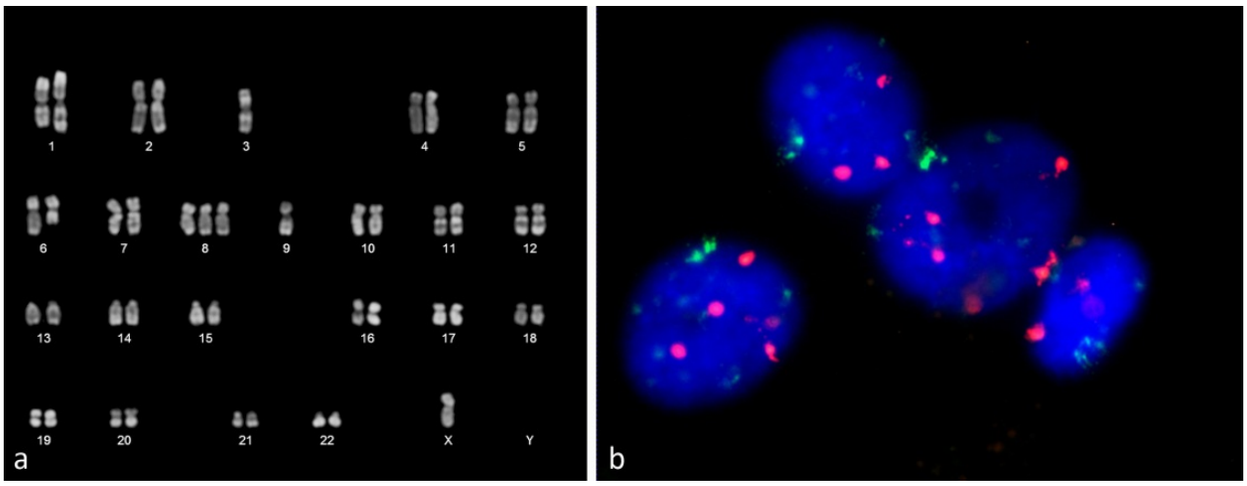

Figure 7. a: Example of a karyogram showing monosomy 3 and trisomy of chromosome 8; 7b: FISH analysis of a tumour demonstrates 1 signal for the probe on centromere 3 (green signals) and 3 to 4 signals of the probe on centromere 8 (red signals).

After completion of the human genome project, genome-wide DNA assays became available. Micro-assay based CGH, single nucleotide polymorphism (SNP) analysis and gene expression profiling (GEP) analysis are the frequently applied techniques. With the

development of Next Generation Sequencing (NGS) technologies, the genome can be analyzed at base pair level. Genome-wide mutation analysis of tumour samples led to the discovery of a subset of genes in UM such as *GNAQ* and *BAP1*.

7.2. Chromosomal anomalies

7.2.1. Monosomy 3

Monosomy of chromosome 3 is observed in approximately 50% of the cases of UM and is strongly associated with clinical and histopathological prognostic factors and with metastatic death (Horsman et al, 1990; Prescher et al, 1990; Sisley et al, 1990). Prescher and associates were the first to find a strong correlation between loss of chromosome 3 and a poor prognosis of the patient (Prescher et al, 1996). Since then several groups have confirmed the prognostic value of monosomy 3 (Kilic et al, 2006; Sisley et al, 2000; Sisley et al, 1997; White et al, 1998). It is assumed that loss of chromosome 3 is a primary event, as it often occurs with other chromosomal aberrations in UM such as 1p loss, and gain of 6p and 8q (Prescher et al, 1995). Kiliç and colleagues established that tumours with concurrent loss of chromosome 1p and 3 are at higher risk of metastasizing than the tumours with other aberrations (Kilic et al, 2005). Mostly one entire copy of chromosome 3 is lost, although in some cases, isodisomy of chromosome 3 is acquired (Aalto et al, 2001; Scholes et al, 2001; White et al, 1998). Partial deletions or translocations have rarely been described on this chromosome making it difficult to map putative tumour suppressor genes. However, recently a mutation in the *BAP1* gene, located on chromosome 3, has been identified in UMs and this gene seems to play an important role in the tumour progression (Harbour et al, 2010). This gene will be discussed in more detail later in this chapter.

7.2.2. Chromosome 8

Abnormalities in chromosome 8, and in particular gain of 8q or an isochromosome 8q, are thought to be a secondary event in UM as variable copy numbers can be present in one melanoma (Horsman & White, 1993; Prescher et al, 1994). Gain of chromosome 8q is frequently found in tumours that also have loss of chromosome 3, and this is associated with a poor patient outcome (Aalto et al, 2001; Prescher et al, 1995; White et al, 1998). A SNP array analysis with this chromosome status is depicted in figure 8. The relationship between the percentages of aberrant copy numbers within UM cells and patient outcome has been investigated. A higher percentage of monosomy 3 and chromosome 8q gain in primary UM cells shows a strong relation with poor disease-free survival compared to low percentage aberrations (van den Bosch et al, 2012).

7.2.3. Chromosome 6

Rearrangements on chromosome 6 affect both arms of the chromosome, resulting in deletions of 6q and gains of 6p. The relative gain of chromosome 6p can occur either through an isochromosome of 6p or a deletion of 6q. Tumours with gain of 6p are thought to be a separate group within UM with an alternative genetic pathway in carcinogenesis, since gain of

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6p is frequently found in tumours with disomy 3 (Ehlers et al, 2008; Hoglund et al, 2004; Sisley et al, 1997). However, this combination of gain of 6p with disomy 3 could not be confirmed by others (Mensink et al, 2009). Aberrations resulting in a relative increase of 6p have been found to be related with both a longer survival (White et al, 1998) or a decreased survival (Aalto et al, 2001). The effect of chromosome 6 aberrations on patient outcome is not conclusive.

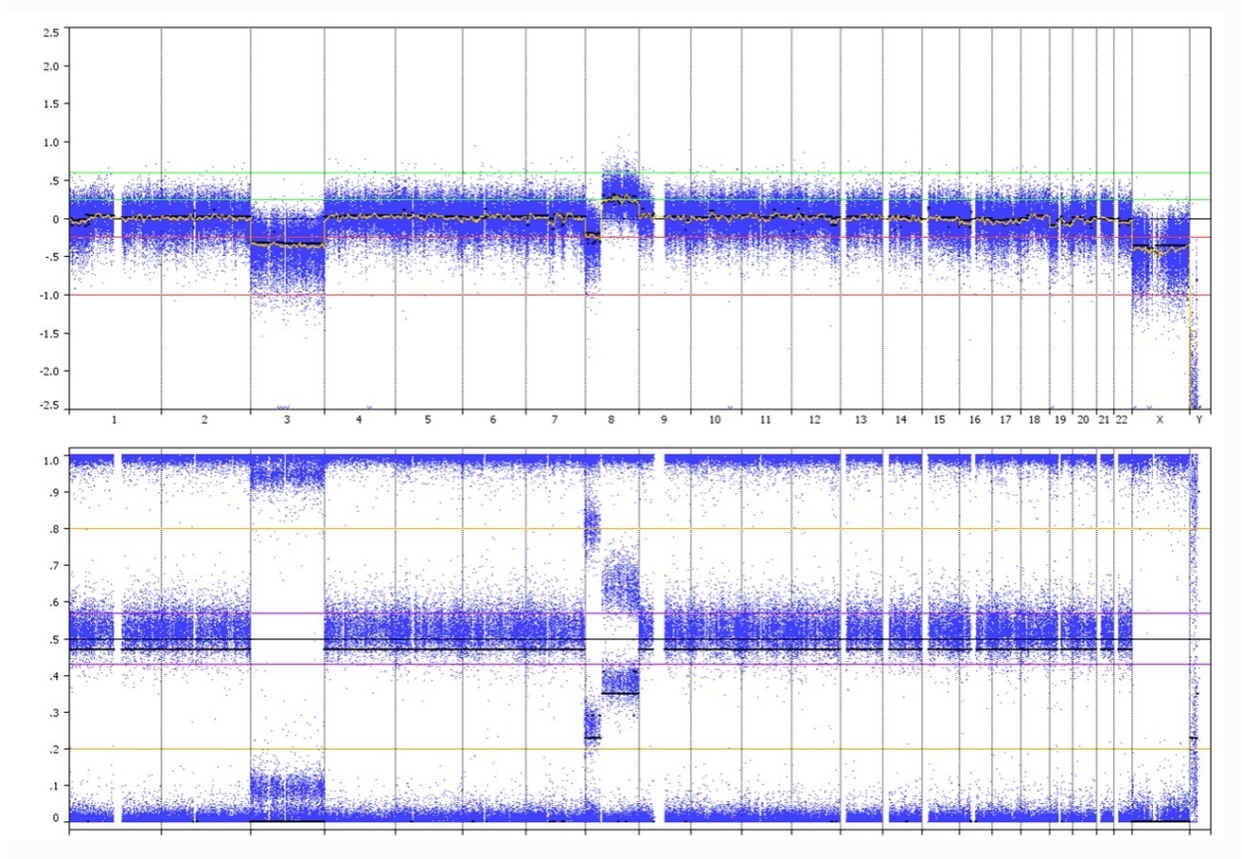

Figure 8. Single nucleotide polymorphism (SNP) array of an uveal melanoma. The upper panel (LogR ratio) shows loss of chromosome 3, partial loss of chromosome 8p and gain of chromosome 8q. The lower panel depicts the B-allele frequency representing allelic imbalance at these chromosomes.

7.2.4. Chromosome 1

In cutaneous melanoma rearrangements on the short arm of chromosome 1 are a common abnormality, occurring in about 80% of all cases (Fountain et al, 1990; Zhang et al, 1999). In UM this region on 1p is also frequently affected, giving rise to a deletion of 1p. However, these anomalies on chromosome 1 are less common than those in skin melanomas with a frequency of approximately 30% (Horsman & White, 1993; Parrella et al, 1999; Prescher et al, 1990; Prescher et al, 1995; Sisley et al, 2000).

Aberrations on other chromosomes have been explored, such as chromosome 9p21 (Scholes et al, 2001), chromosome 11q23 (Sisley et al, 2000), chromosome 18q22 (Mensink et al, 2008;

White et al, 2006), and chromosome 16q (Kilic et al, 2006; Vajdic et al, 2003). The impact on the prognosis, however, remains unclear due to contradictory findings.

7.2.5. Gene expression profiling

Using GEP UMs can be classified into two classes of tumours that correspond remarkably well with the ability of the tumour to metastasize. In a study of 25 UMs, class 1 tumours had a low risk of metastasizing and class 2 tumours had a high risk of developing metastasis (Onken et al, 2004). This molecular classification strongly predicts metastatic death and outperforms other clinical, histopathological and cytogenetic prognostic indicators (Petrausch et al, 2008; van Gils et al, 2008; Worley et al, 2007). Class 1 tumours predominantly show disomy of chromosome 3, whereas class 2 tumours consist mostly of monosomy 3 (Worley et al, 2007).

7.3. Candidate genes

After identifying the non-random chromosomal alterations in UM, the search for potential oncogenes and tumour suppressor genes followed. By narrowing down altered regions on chromosomes, researchers have tried to identify genes involved in tumourigenesis or progression towards metastasis. This way, studies have been conducted on chromosome 8q revealing potential oncogenes such as *MYC*, which is amplified in about 30% of the UMs (Parrella et al, 2001). Other oncogenes on chromosome 8q have been described, such as *DDEF1* and *NBS1* (now referred to as *ASAP1* and *NBN*, respectively) (Ehlers & Harbour, 2005; Ehlers et al, 2005). Yet, no specific oncogenic mutations on this region have been reported thus far. Other candidate genes were proposed, such as *HDM2*, *BCL-2* and *CCND1*. However, the pathogenic significance for any of these genes has not been established.

Mutations in certain genes have been well described for cutaneous melanoma. Examples of such genes are the oncogenes *NRAS*, *BRAF* and *AKT3*, and the tumour suppressors *CDKN2A*, *PTEN* and *TP53*. In contrast to skin melanomas, *PTEN* mutations were not observed in a study of nine cell lines (Naus et al, 2000). Nevertheless, in 15% of the UM cases mutations in *PTEN* were found resulting in activation of *AKT* and overexpression of the PI3K-PTEN-AKT pathway preventing apoptosis (Abdel-Rahman et al, 2006; Ehlers et al, 2008; Ibrahim & Haluska, 2009).

7.3.1. The RAS-RAF-MEK-ERK pathway

In a large proportion of the UMs the RAS-RAF-MEK-ERK pathway or mitogen-activated protein kinase (MAPK) pathway is constitutionally activated, leading to excessive cell proliferation and suggesting the presence of activating mutations upstream in the pathway (Weber et al, 2003; Zuidervaart et al, 2005). Mutation analysis on potential mutation sites in the *BRAF* gene were performed, since a single substitution (p.V600E) in *BRAF* occurs frequently in benign and premalignant cutaneous nevi (Davies et al, 2002; Pollock et al, 2003). However, *NRAS* and *BRAF* mutations have been reported in a few UMs but

overall these mutations are rare (Cohen et al, 2003; Kilic et al, 2004; Mooy et al, 1991; Saldanha et al, 2004).

7.3.2. GNAQ and GNA11 gene

With the recent discovery of activating *GNAQ* and *GNA11* mutations new light has been shed on the MAPK pathway. Van Raamsdonk and co-workers demonstrated an alternative route to MAPK activation through G-protein signalling in melanocytic neoplasia including UMs. They reported a *GNAQ* mutation in 83% of blue naevi and in 46% of UM cases (Van Raamsdonk et al, 2009). Other studies confirmed these results, *GNAQ* mutations were found in half of the UM cases (Bauer et al, 2009; Onken et al, 2008). *GNAQ* and its paralog *GNA11* encode the heterotrimeric guanine nucleotide-binding protein G subunit alpha q and 11, respectively. Through mutations these subunits become activated and abrogate their intrinsic GTPase activity, which is required to return them to an inactive state. This oncogenic conversion is suggested to be the cause of constitutive MAPK pathway activation. A subsequent study reported that 83% of UM samples harboured Gα-protein mutations (*GNAQ* or *GNA11* mutations) affecting specific regions on either exon 4 or 5 (codon R183 or Q209, respectively) in a mutually exclusive pattern (Van Raamsdonk et al, 2010). There is no relation between *GNAQ* mutations and prognosis (Bauer et al, 2009). Hence, the presence of Gα-protein mutations in tumours at all stages of malignant progression and in melanocytic lesions of the choroid, suggests that they are early events in UM (Onken et al, 2008; Van Raamsdonk et al, 2009).

7.3.3. BAP1 gene

Exome genome sequencing led to the discovery of the BRCA1 associated protein 1 (*BAP1*) gene in UM (Harbour et al, 2010). *BAP1,* a nuclearly localized enzyme, was originally identified as an ubiquitin hydrolase that binds to the RING finger domain of BRCA1 (Farmer et al, 2005; Jensen et al, 1998). It has de-ubiquitinating activity and is involved in several biological processes, including regulation of cell cycle and cell growth, chromatin dynamics and DNA damage response (Farmer et al, 2005). *BAP1* is located on chromosome 3p21.1 and is thought to be a tumour suppressor gene (Ventii et al, 2008). Mutations in this gene first have been reported in a small number of breast and lung cancer cell lines (Jensen et al, 1998). Recently, inactivating somatic mutations were found in 84% of the metastasizing UMs. These mutations were only found in 1 out of 26 investigated class 1 tumours against 26 out of 31 class 2 tumours, implicating that *BAP1* mutations occur late in the UM progression (Harbour et al, 2010). In addition, co-segregating germline *BAP1* mutations have been described in several families with different range of diseases, such as cutaneous melanomas (Wiesner et al, 2011), malignant pleural mesotheliomas (Testa et al, 2011), and other cancers such as meningioma (Abdel-Rahman et al, 2011). Given the functional complexity of *BAP1*, different germline mutations in *BAP1* may predispose to divergent tumour types. To understand more about the impact of *BAP1* mutations on UM and other types of cancers, more extensive clinical, molecular genetic, and functional studies are ongoing.

8. Metastases

Irrespective of primary treatment of the UM nearly half of the patients develop metastases (Gilissen et al, 2011). UM spreads haematogenous, with a high tendency to metastasize to the liver in 90-95% of the patients. One explanation for the development of new distant metastasis years after the control of primary tumour is the presence of circulating tumour cells at time of the initial diagnosis (Manschot et al, 1995). In other words, the disease is often already disseminated at time of tumour diagnosis. Several pathways have been implicated in the preferential homing of tumour cells to the liver, such as hepatocyte growth factor (HGF) and it's corresponding receptor c-Met, insulin-like growth factor 1 (IGF-1), and chemokine CXCL12 (Bakalian et al, 2008). In case of liver metastasis prognosis is poor with a median survival of approximately 8 months (Eskelin et al, 2003).

Despite the fact that there a no therapeutic options for metastatic UM that improve survival or quality of life, the following methods can be used for screening of liver metastasis: liver function tests (gamma-glutamyl transpeptidase (γGT) and lactate dehydrogenase (LDH) from blood), liver imaging with US, CT and MRI. Although screening annually or semi-annually for liver metastasis by liver function tests are being widely used, there are reports of disseminated liver metastases and normal liver function tests (Donoso et al, 1985; Eskelin et al, 1999).

Patients have 97.5% chance or more of having no metastasis in the case of normal liver function tests, because of the high negative predictive value. However, isolated or combined liver function tests for aspartate aminotransferase (AST), alanine transaminase (ALT), yGT, LDH and phosphatidic acid (PA) are not indicated for detection of early liver metastasis (Mouriaux et al, 2012). Other upcoming screening options make use of serum markers, Among which S-100β (neural crest marker), melanoma inhibitory activity (MIA), tissue polypeptide specific antigen (TPS) and osteopontin (OPN). MIA and S-100β showed significant increase in levels before clinical diagnosis of metastasis (Barak et al, 2011). In a lead time of more than 6 months before clinical metastasis a significant increase in OPN and steeper trendlines in MIA and S-100β levels were demonstrated (Hendler et al, 2011).

9. Treatment of primary UM

Conservation of the eye in UM with useful vision has improved with advances in local irradiation (brachytherapy), heavy particle radiation techniques (proton and helium ion beam), stereotactic radiotherapy (SRT), endoresection, exoresection, transpupillary thermotherapy and photodynamic therapy (Spagnolo et al, 2012). If the tumours are larger, advanced and, in particular, if there is evidence of extraocular extension enucleation is advised (Spagnolo et al, 2012). In addition, enucleation is also performed after serious treatment induced complications (Hungerford, 1993; Shields et al, 1991). Choice of treatment depends on the location and size of the tumour and goals of therapy. Even though enucleation is sometimes required, eye-preserving approaches have shown to be equally successful regarding overall

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survival and metastasis-free survival (Seddon et al, 1985; Seddon et al, 1990). Brachytherapy is the most common method for treating UM, and currently the ruthenium-106 (Ru-106) and iodine-125 (I-125) applicators are the most frequently used. Brachytherapy can be used in combination with other methods of treatment of UM, such as local resection or transpupillary thermotherapy (Pe'er, 2012). Local control with plaque radiotherapy has provided overall survival comparable to enucleation. Radiation-induced side effects have necessitated secondary enucleation in 10-22% of the patients (Bell & Wilson, 2004; Char et al, 1993; Finger, 1997; Garretson et al, 1987; Gunduz et al, 1999; Lommatzsch et al, 2000; Packer et al, 1992; Shields et al, 1991; Tjho-Heslinga et al, 1999; Vrabec et al, 1991). Local recurrences after brachytherapy are reported between 4 - 28%, depending on the size of the tumour and the follow up time (Damato & Foulds, 1996; Gragoudas, 1997; Karlsson et al, 1989; Seregard et al, 1997; Tjho-Heslinga et al, 1999; Wilson & Hungerford, 1999; Zografos et al, 1992). Radiation-induced complications include radiation retinopathy, radiation maculopathy, radiation opticopathy as well as recurrences (Gragoudas et al, 1999; Kinyoun et al, 1996; Summanen et al, 1996). Heavy particle radiation with positive charged particles (protons or helium-ions) enables treatment of small, medium- and large-choroidal melanomas. The local recurrence rate for proton beam irradiation is similar to brachytherapy and at 10 years is usually around 5% (Gragoudas, 1997; Zografos et al, 1992). Secondary enucleation is performed in 10 - 15% of patients either due to complications or local recurrence. Other complications, such as maculopathy, opticopathy, cataract, glaucoma, vitreous haemorrhage, retinal detachment and dryness have also been described (Desjardins et al, 2012). In concordance with proton beam irradiation radiogenic side effects are also reported after SRT. Side effects, such as radiation retinopathy, opticopathy and neovascular glaucoma are responsible for the majority of secondary visual loss and secondary enucleations after SRT (Mueller et al, 2000; Zehetmayer et al, 2000). The efficacy of SRT for UM has been proven in different studies with local tumour control rates reported over 90%, 5 and 10 years after treatment (Zehetmayer, 2012). Local resection (endoresection and exoresection) of UM aims to conserve the eye and remain a useful vision. The tumour can be removed in several manners, through the vitrous and retinal with a vitreous cutter, endoresection, or through a scleral opening exoresection. Variations of exoresection include iridectomy, iridocyclectomy, cyclochoroidectomy, and choroidectomy. Endoresection as well as exoresection can be used as a primary procedure, after another conservative therapy as a treatment option for recurrences or toxic tumour syndrome. An advantage of local resection is that eyes that would otherwise be inoperable can be preserved, while relative large tumour samples are available for prognostication and research (Damato & Foulds, 1996; Damato, 2012; Robertson, 2001).

10. Treatment of liver metastases

Although treatment options for small to medium sized melanoma improves visual outcome, there has not been any standardized therapy that improves survival in metastatic disease. Systemic treatment options, such as intravenous chemotherapy and immunotherapy do not seem to give promising results or survival benefit (Augsburger et al, 2009).

Several locoregional techniques are available, for example immunoembolization, chemoembolization, isolated liver perfusion and hepatic intra-arterial chemotherapy. In highly selected patients, surgical resection of liver metastases can be beneficial. Operating on patients with a time from diagnosis of the primary tumour to liver metastases of > 24 months, ≤ 4 liver metastatic lesions and absence of 'miliary' disease (multiple, diffuse, millimetre-sized, dark punctuate lesions on CT) is associated with prolonged survival. A median survival of 27 months has been described in patients with microscopically complete liver resection versus 14 months in patients with microscopically or macroscopically incomplete liver resection (Mariani et al, 2009).

11. Future prospects

With the discovery of *GNAQ* and *BAP1* mutations, new therapeutic strategies based on the specific mutated gene content seem promising. For tumours with Gα-protein mutations, the therapeutic goal is to inhibit downstream signalling molecules in the MAPK pathway that are activated. Preclinical studies show that inhibition of MAPK pathway in UM cell lines results in decreased cell proliferation (Van Raamsdonk et al, 2009). There are several key molecules in the MAPK pathway, which have been explored as potential therapeutic targets. One of such is MEK, and Gα-protein mutant UM cells showed to be mildly sensitive to the MEK inhibitor AZD6244 (Gill & Char, 2012). Another recent preclinical study proposed to target both the MAPK and PI3K/AKT pathway since both pathways are activated in UM. A combination of MEK and PI3K inhibition treatment resulted in induction of apoptosis in a Gα-mutant UM cells (Khalili et al, 2012). Other potential targets in the MAPK pathway are currently being investigated, including protein kinase C, which is a component of signalling from *GNAQ* to Erk1/2 (Wu et al, 2012).

Therapeutically targeting UMs with a *BAP1* mutation works in a different manner than the Gα-protein mutations, since *BAP1* acts as a tumour suppressor gene. Regaining lost functions of suppressor genes are in general more challenging than inhibiting an overactive oncogene. Nevertheless, ongoing studies show that histone deacetylase (HDAC) inhibitors may have therapeutic potential in UM. Landreville and colleagues established that HDAC inhibitors can reverse the histone H2A hyperubiquitination that occurs in cultured UM cells depleted of *BAP1*, and it induces morphologic differentiation, cell-cycle exit, and shifts to a differentiated, melanocytic GEP (Landreville et al, 2012). Examples of HDAC inhibitors are valproic acid, trichostatin A, LBH-589, and suberoylanilide hydroxamic acid. Clinical trials are needed to evaluate the effect of these compounds in UM patients, and hopefully UM specific treatment based on mutational content will lead to improved patient survival.

Abbreviations

UM: uveal melanoma

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RPE: retina pigment epithelia

FAMM: familial atypical mole and melanoma syndrome

US: ultrasonography

OCT: optical coherence tomography

MRI: magnetic resonance imaging

CT: computed tomography

CHRPE: congenital hypertrophy of the retinal pigment epithelium

PEHC: peripheral exudative hemorrhagic chorioretinopathy

H&E: haematoxylin and eosin

PAS: Periodic-acid Schiff

FISH: fluorescent in situ hybridization

SKY: spectral karyotyping

CGH: comparative genomic hybridization

qPCR: quantitative polymerase chain reaction

MLPA: multiplex ligation probe amplification

MAQ: multiplex amplicon quantification

MSA: microsatellite analysis

LOH: loss of heterozygosity

SNP: single nucleotide polymorphism

GEP: gene expression profiling

NGS: next generation sequencing

MAPK: mitogen-activated protein kinase

HGF: hepatocyte growth factor

IGF-1: insulin-like growth factor 1

γGT: gamma-glutamyl transpeptidase

LDH: lactate dehydrogenase

AST: aspartate aminotransferase

ALT : alanine transaminase

PA: phosphatidic acid

MIA: melanoma inhibitory activity

TPS: tissue polypeptide specific antigen

OPN: osteopontin

SRT: stereotactic radiotherapy

Ru-106: ruthenium-106

I-125: iodine-125

HDAC: histone deacetylase

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