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ORIGINAL PAPER

FISH analysis of chromosomes 3 and 6 on fine needle aspiration biopsy samples identifies distinct subgroups of uveal melanomas

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Abstract
Purpose Circumstantial evidence suggests that development of uveal melanoma may be associated to two different pathogenetic pathways, either loss of chromosome 3 or extra copies of 6p (+6p). Chromosome 3 monosomy (−3) is detected in approximately half of uveal melanomas, and is strongly linked to metastatic disease, whereas +6p accounts for one-fourth of uveal melanomas with no clear clinical correlations. The aim of our study was to verify if the analysis of chromosomes 3 and 6 was able to distinguish two groups of patients for translating this approach in the clinical practise as prognostic tool.
Methods Fluorescence in situ hybridisation (FISH) with probes for chromosome 3, 6p and 6q was used to analyze cytological material obtained by fine needle aspiration biopsy (FNAB) from 28 primary uveal melanomas, just before brachytherapy.
Results Abnormalities affecting 6p and 6q were found in 14 tumors (50%), and −3 in 16 cases (57%). Interestingly, −3 and +6p were mutually exclusive in 23 cases (82%), whereas in two cases only (7%) they coexisted. In particular, +6p alone was present in 9 lesions (32%), −3 was the sole aberration in 11 cases (39%), and concomitant −3 and −6q in 3 other cases (11%).
Conclusions Although the patient cohort is limited, our findings confirm the hypothesis of a bifurcated pathogenetic model of uveal melanoma. Moreover, our results suggest that investigation of both markers on FNAB samples obtained in vivo could provide a clearer clinical picture of genetic lesions when no histological material is available for prognostic evaluation.

Keywords Uveal melanoma · FISH · Chromosomes 3 and 6 · FNAB

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Introduction

Uveal melanoma is the most common primary intraocular malignancy in adults with an annual incidence of 5–7 cases per million (Hu et al. 2005). Despite diagnostic advances and successful local therapy, about 50% of these patients develop metastatic disease causing death (Kivelä et al. 2006). Several clinical and histological features were correlated with poor prognosis, including large basal tumor diameter, ciliary body involvement, epithelioid cell presence, and vascular pattern (Mudhar et al. 2004). On the other hand, with the introduction of conservative radiation therapy these parameters lost their prognostic importance.

Recent cytogenetic analyses and imbalances studies have revealed recurrent chromosomal aberrations involving

chromosomes 3, 6, and 8 in uveal melanoma (Ehlers and Harbour 2006). Loss of chromosome 3 is detected in about 50% of tumors, and is considered as a poor prognostic marker since about 70% of patients with this abnormality die of metastases within 4 years after diagnosis (Prescher et al. 1996). Chromosome 6 alterations, instead, are found in approximately 25% of uveal melanomas, and consist of extra copies of 6p (+6p), mainly as a consequence of an isochromosome 6p, and loss of the long arm of the chromosome (6q−). +6p is rarely coupled with monosomy 3, whereas 6q− is identified more often in the metastases and their respective primary tumor, compared with non-metastasizing uveal melanomas (Mudhar et al. 2004). The prognostic significance of these abnormalities is not yet well understood, although they have been associated with favorable prognosis in a small group of patients (White et al. 1998a). Chromosome 8 abnormalities, mostly extra copies of 8q, are also common in uveal melanomas, and are strongly associated to monosomy 3 as well as with metastatic death (Sisley et al. 1997).

Recently, a pathogenetic model of uveal melanoma has been proposed to shed light on the different clinical behavior of this tumor (Höglund et al. 2004). Based on the cytogenetic data, this model envisages two distinct pathways of clonal evolution, the one that begins with loss of chromosome 3 and the other with +6p. It is interesting to note that, in this pathogenetic model, −3 and +8q belong to the same arm of the pathogenetic tree, distinct to +6p, while abnormalities of 6q such as 6q− represent a secondary event common to both −3 and +6p pathways (Höglund et al. 2004).

Since conservative radiation therapy obviously precludes obtaining histological material where to investigate the relative importance of chromosomes 3 and 6 abnormalities, we investigated the co-existence of chromosome 6 abnormalities (as +6p and 6q−) with monosomy 3 by fluorescence in situ hybridisation (FISH) analysis in cytological specimens obtained by transcleral fine needle aspiration biopsy (FNAB).

Materials and methods

Patients

Twenty-eight patients (19 males and 9 females aged 44–82 years) referred to the Ophthalmic Oncology Unit for plaque brachitherapy of uveal melanoma were included in the study. Before entering this trial, all the patients signed an informed consent form, approved by the Institutional Review Board of Ophthalmic Department of University Hospital of Padova. Thirteen tumors showed ciliary body involvement, and 15 were pure choroidal tumors; details on clinical data, such as tumor diameter and thickness are summarized in Table 1.

FISH analysis

Before the radioactive plaque application, cytological material was obtained by transcleral FNAB and was collected in RPMI 1640 medium (Euroclone, Milan, Italy) for cytospin preparation as previously described (Midena et al. 2006). FISH analysis for chromosome 6 was carried out with BAC clone RP11-513I15 located at 6p21.31 and RP11-697G4 for 6q located at 6q21 obtained from M. Rocchi (Resources for Molecular Cytogenetics, Bari, http://www.biologia.uniba.it/rmc/). FISH for chromosome 3 was performed with a centromeric probe labeled with SpectrumOrange fluorocrome (Vysis-Abbott, Downers Grove, IL, USA) according to manufacturer's instructions (Midena et al. 2006). BAC probes were biotin- and digoxigenin-labeled by nick translation using the biotin-nick and DIG-nick translation mix (Roche, Mannheim, Germany). For each experiment, 100 ng of labeled probe were used for hybridization following standard procedures. Signal detection was made using Avidin-Cy3 (Amersham Biosciences, Little Chalfont, UK) diluted 1:100 in phosphate-buffered saline (PBS; Gibco-BRL, Paisley, UK) and anti-digoxigenin–fluorescein (Roche) diluted 1:200 in PBS. Microscopic analyses were carried out using a fluorescent Zeiss Axioplan microscope (Carl Zeiss, Göttingen, Germany) with appropriate single band and triple band filters. At least 100 cells per sample were evaluated; cut-off limit for loss of chromosome 3 was 15%, whereas a cut-off of 10% for chromosome 6 probes was considered.

Results of hybridization with 6p (red) and 6q (green) probes were classified as follows: a signal pattern of 3R1G and 4R1G was considered as +6p, while a pattern with 2R1G was considered as −6q.

Results

Fluorescence in situ hybridization analysis for chromosome 6 gave an abnormal pattern in 14 out of 28 patients (50%), while 16/28 patients (57%) showed loss of chromosome 3 (Table 1). Three samples (11%) did not show any abnormality for either chromosome and were classified as normal for chromosomes 3 and 6 (Table 1).

Chromosome 6 abnormalities (Fig. 1) were classified as +6p in 9 cases (32%), 6q− in 3 cases (11%), and trisomy 6 in 2 patients (7%); in these latter, data were confirmed with a centromeric probe. Three patients with +6p showed coexistence of two cell populations, a +6p clone and a 6q− clone.

Table 1 Clinical details and FISH analysis results of the patients studied

Cases and gender (M/F)[a]	Age at diagnosis	Tumor diameter (mm)	Tumor thickness (mm)	Site[b]	Chromosome 3	Chromosome 6
1 M	82	11	9	CB	Normal	Normal
2 M	63	15	10	CB	Normal	+6p
3 M	75	15	9	CB	−3	Normal
4 M	75	15	10	CB	−3	Normal
5 F	66	12	10	C	Normal	+6
6 M	75	15	9	C	Normal	+6p
7 M	71	12	9	C	Normal	+6
8 M	58	13	10	C	−3	Normal
9 F	82	7	7	CB	−3	Normal
10 F	63	12	6	C	−3	Normal
11 F	44	11	9	CB	Normal	Normal
12 F	73	12	6.5	CB	−3	Normal
13 F	82	12	5	CB	−3	Normal
14 M	67	10	7	C	−3	Normal
15 M	81	10	10	C	−3	6q-
16 M	80	15	10	C	Normal	+6p/6q−
17 M	57	15	10	C	−3	6q−
18 M	74	10	5	C	−3	6q−
19 M	60	10	8	CB	−3	Normal
20 M	57	13	9	C	Normal	Normal
21 F	82	11	9.5	C	−3	+6p/6q-
22 M	70	15	9.5	C	Normal	+6p
23 M	60	9	5.5	C	Normal	+6p/6q−
24 M	78	10	6	CB	−3	+6p
25 M	58	14	7	CB	Normal	+6p
26 M	62	9	7	CB	−3	Normal
27 F	65	15	6.5	CB	Normal	+6p
28 F	60	15	8	C	−3	Normal

[a] *M* Male, *F* female

[b] *C* Choroidal, *CB* ciliary body

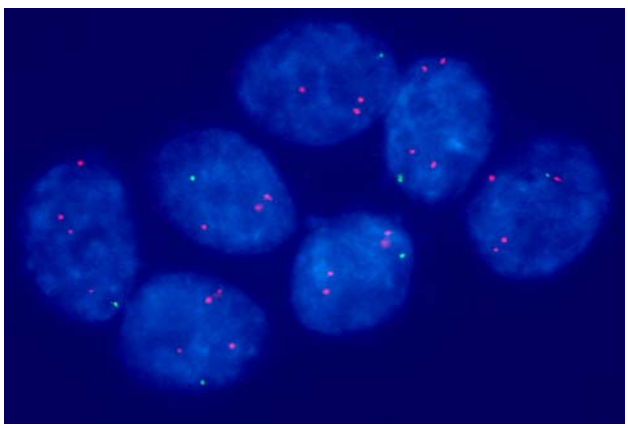

Fig. 1 FISH analysis with chromosome 6 probes in a case showing +6p: 6p probe SpectrumOrange; 6q probe SpectrumGreen

Of the 16 samples with loss of chromosome 3, 11 cases (69%) did not show abnormality for chromosome 6, while 3 patients (19%) presented loss of 6q (6q−), and 2 cases also showed a +6p pattern (12.5%). On the other hand, 7 out of 9 patients (78%) who presented a +6p profile did not show numerical abnormalities of chromosome 3, and the same results were found in the two samples with trisomy 6.

In reference to the tumor site (see Table 1), 13 tumors (46%) showed ciliary body involvement while 15 (54%) were pure choroidal tumors. Seven out of the 13 cases involving the ciliary body (54%) showed loss of chromosome 3 and were normal for 6p, while 3/13 samples (23%) had +6p, and only 1 patient (8%) showed the presence of both −3 and +6p. Among the 15 choroidal tumors, chromosome 3 loss and +6p showed an equal distribution (7 out of 15 cases for either abnormality), whereas only one sample (7%) showed coexistence of both alterations.

Three patients developed metastatic disease during the follow-up; all of them were characterized by monosomy 3 without +6p.

No complications were observed during the surgical procedure or during the follow-up.

Discussion

Conservative treatment of uveal melanoma, rather than enucleation, has deemed useless classic, histological parameters for defining patient prognosis, thus motivating scientists to the development of in situ techniques that are more suitable for investigating molecular markers in tumor samples of a few hundred cells, such as those collected ex vivo by fine needle aspiration. Previous studies have shown good correspondence between major clonal alterations found in FNAB material and those observed by cytogenetic techniques. Furthermore, it has been demonstrated that FISH could successfully be applied to FNAB specimens collected ex vivo and that this technique represents a reliable method for assaying genetic prognostic parameters (Sisley et al. 1998; Naus et al. (2002).

As we recently reported (Midena et al. 2006), we are able to apply FISH to cytological material collected in vivo, just prior to any local therapy, thus obtaining a sufficient number of cells to evaluate chromosome 3 loss. Nevertheless, in that paper, FISH was performed without previous morphological evaluation of the collected material, so the absence of the monosomy 3 might raise the question as to whether the sample was really representative of the neoplasia or if we were also analyzing normal cells. For this reason, we thought to increase the informativeness of FISH with the analysis of two distinct markers, possibly with different prognostic outlooks. Revision of the literature led us to look for chromosome 6 abnormalities since some evidence already exists suggesting a pathogenetic role distinct to the loss of chromosome 3 (White et al. 1998a; Höglund et al. 2004; Parella et al. 1999).

Previous studies investigating the clinical meaning of loss of chromosome 3 by different approaches all indicate an association of this abnormality to poor prognosis (Prescher et al. 1996; Sisley et al. 1997; Aalto et al. 2001; Kilic et al. 2005), moreover array-CGH confirmed that almost always is the entire chromosome to be lost rather than small interstitial regions (Hughes et al. 2005). On the other hand, only a few studies have simultaneously addressed chromosome 6 abnormalities, which are frequently found in uveal melanomas (Sisley et al. 2006). White et al. (1998a) first described an association between chromosome 6 aberrations and good prognosis in a small group of patients, and found a better outcome in patients with abnormalities 6 alone in comparison to those with associated abnormalities on chromosomes 3 and 8. Their analysis, however, did not discriminate between 6p and 6q aberrations, and thus it is impossible to understand what specific abnormalities were they looking to (White et al. 1998a). Subsequently, Parella et al. (1999) proposed a bifurcated tumor progression model in which, the allelic loss of 6p and chromosome 3 monosomy were mutually exclusive. This idea has been recently buttressed by the description of a pathogenetic model of karyotype evolution, based on cytogenetic data, in which +6p is presented as an alternative starting point to monosomy 3 at early stages of tumorigenesis (Höglund et al. 2004). In this model, loss of 6q is presented as a secondary imbalance in both −3 and +6p pathways, with some preference for the latter.

To our knowledge, our study is the first to focus the analysis on different chromosome 6 abnormalities in respect to chromosome 3 loss in uveal melanoma by FISH. By this approach, we were also able to discriminate 6p from 6q copy number changes, which was extremely useful since they act as separate alterations in the pathogenetic model; indeed, as demonstrated by cytogenetic studies, +6p is predominantly the result of an isochromosome of the short arm of chromosome 6 (i6p), and loss of 6q, considered a secondary change, could be the consequence of i(6p) or result from a different rearrangement.

Normal cases for both chromosomes 3 and 6 represent 11% of our case series; although, we cannot exclude sampling mistake or other rearrangements involving chromosomes 3 and 6 (White et al. 2006), this result may hind as well the isodisomy of chromosome 3 (White et al. 1998b). Indeed, FISH analysis is not able to distinguish the duplication of chromosome 3, which characterises 6% of uveal melanomas, from a normal disomy (Onken et al. 2007); for this purpose microsatellite analysis or single nucleotide polymorphisms (SNP) are techniques of choice but more time consuming and expensive than FISH, and since the low occurrence of isodisomy, they would be more appropriate as in-depth analysis in selected samples.

All the abnormal samples were homogeneous regarding the loss of chromosome 3. However two recent papers have showed that uveal melanoma might be heterogeneous in the distribution of monosomy 3 and they have questioned the utility of FISH to FNAB samples in predicting prognosis. By using FNAB, one may miss the cells with monosomy 3 when they occur only in low number or are restricted to a specific area of the tumor (Maat et al. 2007; Sandinha et al. 2006). The debate around the tumor heterogeneity of uveal melanoma is still open, perhaps molecular techniques on microdissected samples will clarify the real picture of this tumor.

Interestingly, almost 70% of the 16 samples with chromosome 3 monosomy showed a normal chromosome 6 profile, while 3 other cases demonstrated a −6q imbalance, that might be interpreted as a secondary event of chromosome 3 loss, as already suggested (Höglund et al. 2004). On the other hand, the vast majority of the cases with disomy 3 (7 out of 9) showed a pattern of +6p imbalance, while 2 samples showed trisomy 6, that might also be interpreted as +6p.

In reference to the tumor presentation the site, in our case series we noted an association among ciliary body

involvement, monosomy 3 and absence of chromosome 6 imbalances, even though no statistical significance could be achieved due to the limited number of samples studied. On the other hand, ciliary body involvement is considered as a poor prognostic factor (Mudhar et al. 2004), and a significant correlation between monosomy 3 and ciliary body component has already been described (Sisley et al. 1997).

Our results fit well with the statistical model of karyotype evolution proposed by Höglund et al. (2004), mostly because we were able to discriminate between 6p and 6q abnormalities. Previous findings concerning the clinical correlations of −3, 6q−, +8q and more recently −1p are clearly in favor of this model (White et al. 1998a; Sisley et al. 1997; Kilic et al. 2005; Hughes et al. 2005). All these abnormalities have been associated to the metastasizing potential of the primary tumor, and, in fact, belong to the same line of karyotypic evolution. Even though this issue needs to be confirmed in a larger population; our data are consistent with the hypothesis that loss of 3 and +6p are mutually exclusive, and that +6p potentially could be a favorable prognostic marker. In any case in the brachitherapy era, FISH analysis of a combination of different genetic markers proved to be a suitable and a low cost approach for prognostication when only small tumor samples are available.

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