Detection of c-myc Amplification in Uveal Melanoma by Fluorescent In Situ Hybridization

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PURPOSE. Genetic abnormalities of chromosomal arm 8q have been reported by many studies in uveal melanoma. To better understand the role of 8q abnormalities in uveal melanoma development, copy number anomalies of the c-myc oncogene (located on 8q24.1) have been investigated.

METHODS. Forty-three uveal melanomas were analyzed by fluorescent in situ hybridization (FISH) with probes for c-myc and the chromosome 8 centromere. Results of the FISH analysis were compared with genetic changes previously detected by microsatellite analysis on chromosomes 3 and 6p.

RESULTS. Thirty uveal melanomas (70%) had extra copies of c-myc, 2 tumors (5%) had loss of c-myc, and 11 tumors (25%) had no abnormalities in c-myc copy number. Of those with extra copies of c-myc, 13 tumors (43%) had amplification of the c-myc gene, 14 tumors (47%) had an intermediate relative increase in the c-myc copy number, and 3 tumors (10%) had a simple gain of chromosome 8. An association between larger tumor size and c-myc amplification was found (P < 0.01). Although extra copies of c-myc were seen in tumors with retention of chromosome 3, remarkably only tumors with monosomy 3 showed amplification of c-myc (P = 0.03).

CONCLUSIONS. The specific amplification of the c-myc oncogene detected in at least 30% of primary uveal melanomas cannot be explained by the simple 8q abnormalities observed in cytogenetic studies. The striking association between c-myc amplification and monosomy 3 suggests a unique pathway of genetic progression in a subset of uveal melanomas. (Invest Ophthalmol Vis Sci. 2001;42:1679–1684)

Chromosome 8 abnormalities in uveal melanoma, the most common intraocular cancer in adults and the second most common site of origin of metastatic melanoma, 1 have been reported by many cytogenetic studies 2 and have been correlated with relatively poor patient prognosis. 3-5 Trisomy 8, partial duplication of 8q, or isochromosome 8q are present in approximately 50% of tumors and are often associated with monosomy 3 (M3). 2 Comparative genomic hybridization (CGH) studies have revealed gain of chromosomal arm 8q in 65% of uveal melanomas, with the smallest duplicated region represented by 8q24–qter, including the c-myc oncogene locus. 3-7

The oncogene c-myc participates in the control of cellular proliferation, programmed cell death, and differentiation. 8-10 c-myc belongs to a family of transcription factors and has been implicated in both activation and repression of transcription. 11 Since its initial identification as a proto-oncogene in the early 1980s, alterations in c-myc at the genomic and/or expression level have been demonstrated in many types of cancer. 12

Although abnormalities of chromosomal arm 8q have been widely reported in uveal melanoma, only a few studies have investigated the role of the c-myc oncogene in the development of this neoplasm. Multiplication of 8q alleles at the c-myc locus was found by Southern blot analysis in 6 of 11 informative cases. 13 Overexpression of the c-myc protein was demonstrated by immunohistochemistry 14-16 and flow cytometry. 17,18 In those studies, c-myc expression was correlated with either poor prognosis 16 or better prognosis. 14,17,18 However, no published studies have analyzed anomalies of c-myc gene copy number and their numeric relationship with the chromosome 8 centromere in uveal melanoma.

To better understand the role of chromosome 8q abnormalities in uveal melanoma development, we have investigated anomalies in the c-myc gene copy number and their correlation with the genetic abnormalities found on chromosomes 3 and 6 by our published allelotype. 19 Forty-three tumors from the original allelotype cohort of 50 uveal melanomas were analyzed by fluorescent in situ hybridization (FISH) with a region specific probe for c-myc and a chromosome enumeration probe for chromosome 8. Extra copies of c-myc were demonstrated in 70% of cases, and of these, 43% showed substantial amplification of the c-myc gene. When these results were compared with the genetic alterations on chromosomes 3 and 6p as detected by the allelotype, all tumors with c-myc amplification were also M3, suggesting a unique pathway of genetic progression in a subset of uveal melanomas.

MATERIALS AND METHODS

Pathologic Specimens

Forty-three uveal melanomas from our original allelotype cohort of 50 tumors 19 were analyzed in this study. The distribution of tumor location, size, and cell type in this subset did not differ significantly from the original cohort.

Preparation of Nuclear Suspension

Tumor cells were isolated from 12-μm paraffin sections and deparaffinized as described previously. 20 Cellular disaggregation was per-

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nucleus and an overall mean c-myc copy number for either c-myc or CEP8. In six cases, we were unable to obtain an optimal preparation for FISH. In one case, the preparation lacked a sufficient number of nuclei to count. Dual probe hybridization was performed with a chromosome enumeration probe for chromosome 8 (centromere 8 probe [CEP8]; Vysis Inc., Downer Grove, IL) and a region specific probe for B2q.4.1 (LSL-c-myc; Vysis Inc.). Probes and target DNA were codenaturated at 85°C for 5 minutes and incubated at 37°C overnight. After hybridization, samples were washed in 2× SSC (pH 7.2) + 0.1% NP40 (Sigma) at 75°C for 2 minutes and 2× SSC (pH 7.2) at RT for 1 minute. Nuclei were then counterstained with 4,6-diamino-2-phenylindole (DAPI; Sigma) and the antifade compound, P-phenylenediamine (Vector Laboratory Inc., Burlingame, CA). FISH signals were counted using a fluorescent microscope equipped with a triple-pass filter. Two hundred nonoverlapping interphase nuclei were counted for c-myc and CEP8 signals. Occasionally, split signals were observed and were counted as one, when the signals appeared to be derived from sister chromatids in cells in S or G2 phase of the cell cycle (barely a perceivable distance between them; Fig. 1E). All 50 tumors from our original alleleotype cohort were tested by FISH analysis, but in seven cases we were unable to obtain an optimal preparation for FISH. In one case, the preparation lacked a sufficient number of nuclei to study (at least 200 nuclei). In six cases, >15% of nuclei had no signal either for c-myc or CEP8. On those samples, FISH analysis was repeated at least once on a different nuclear preparation with similar results. The number of c-myc and CEP8 signals were counted for each nucleus and an overall mean c-myc:CEP8 ratio was calculated for each tumor. Figure 2A summarizes typical data from an example case, UM11. Figure 2A is divided into three sectors based on the individual tumor. Figure 2A summarizes typical data from an example case, UMs8. The 30 tumors with extra copies of c-myc could be divided into three groups: amplification of c-myc was present in 13 tumors (43%), IRI in 14 tumors (47%), and +8 in 10 tumors (10%). Representative results are shown in Figures 1A through 1D. Of the 13 uveal melanomas with c-myc amplification, 2 tumors showed >40% of nuclei in the high c-myc:CEP8 sector, 4 had 20% to 31%, and 7 had fewer than 20% of nuclei in such sector. The mean ± SD of the c-myc:CEP8 ratio for the high ratio sector was 3.3 ± 0.52 (range 3.4–14).

Tumor UM58 has two histologically distinct regions, which were processed separately as tumor samples A (unpigmented) and B (pigmented). FISH analysis was performed using the SigmaStat 1.02. The association between the discrete variables was assessed using Fisher’s exact test. Mean values were compared using the two-tailed t-test. Differences were considered statistically significant for P < 0.05.

**Results**

**FISH Analysis of Uveal Melanomas**

Of the 43 uveal melanomas analyzed, 30 (70%) showed extra copies of c-myc, 2 tumors (5%) had a relative loss of c-myc copy number compared with the chromosome 8 centromere, and 11 tumors (25%) showed no increase in the c-myc copy number. The 30 tumors with extra copies of c-myc could be divided into three groups: amplification of c-myc in 13 tumors (43%), IRI in 14 tumors (47%), and +8 in 3 tumors (10%). Representative results are shown in Figures 1A through 1D. Of the 13 uveal melanomas with c-myc amplification, 2 tumors showed >40% of nuclei in the high c-myc:CEP8 sector, 4 had 20% to 31%, and 7 had fewer than 20% of nuclei in such sector. The mean ± SD of the c-myc:CEP8 ratio for the high ratio sector was 3.3 ± 0.52 (range 3.4–14).

Tumor UM58 has two histologically distinct regions, which were processed separately for FISH. Although the unpigmented region (UM58A) and the pigmented region (UM58B) both showed c-myc amplification, the distributions of the cell populations of the two regions were different. The percentage of cells in the high c-myc:CEP8 ratio sector was 20% for UM58A and 12% for UM58B (Figs. 1E, 1F). In this sector, the high c-myc:CEP8 ratio sector, the percentage of nuclei with 7 or more c-myc signals was 50% for UM58A (17 of
nuclei) compared with 4% (1 of 23 nuclei) for UM58B ($P < 0.0002$).

The FISH results were correlated with tumor size (basal diameter [BD] and apical height [AH]) and tumor localization (choroidal or ciliochoroidal), parameters previously shown to be prognostic. A statistically significant association with BD was found for both $c$-myc amplification and IRI. BD was $13.2 \pm 2.9$ mm in tumors with $c$-myc amplification, $13.1 \pm 3.2$ mm in tumors with IRI, and $9.7 \pm 3.4$ mm in tumors with no increase in $c$-myc copy number ($P = 0.012$ and $P = 0.01$, respectively). An association was also found between AH and $c$-myc amplification, but not with IRI. AH was $9.0 \pm 4.7$ mm in tumors with $c$-myc amplification and $5.4 \pm 2.5$ mm in tumors without increase in $c$-myc copy number ($P = 0.03$). Extra copies of $c$-myc were found more often in tumors with ciliary body involvement (86%) than in tumors located solely in the choroid (64%), but the difference was not statistically significant.

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**Figure 1.** Dual probe FISH using a region specific probe for $c$-myc (orange) and a probe for the chromosome 8 centromere (green). Representative isolated nuclei from uveal melanomas are shown. Nuclei are counterstained with 4',6-diamidino-2-phenylindole (DAPI, blue). (A) Tumor UM13 with a normal $c$-myc copy number. Overall $c$-myc:CEP8 ratio, 1.01; intermediate sector, 11%; high sector, 0%. *Arrows*, nuclei with two signals for $c$-myc and CEP8. (B) Tumor UM22 with relative loss of $c$-myc. Overall $c$-myc:CEP8 ratio, 0.85; intermediate sector, 8%; high sector, 1%. *Arrows*, nuclei with one signal for $c$-myc and two signals for CEP8. (C) Tumor UM9 with IRI of $c$-myc. Overall $c$-myc:CEP8 ratio, 1.4; intermediate sector, 50%; high sector, 7%. *Arrow*, a nucleus with four $c$-myc signals and two CEP8 signals. (D) Tumor UM44 with amplification of $c$-myc. Overall $c$-myc:CEP8 ratio, 2.0; intermediate sector, 50%; high sector, 31%. *White arrow*, a nucleus with seven $c$-myc signals and one CEP8 signal. *Red arrow*, a nucleus with four $c$-myc signals and two CEP8 signals. (E) Tumor UM58A (unpigmented) with amplification of $c$-myc. Overall $c$-myc:CEP8 ratio, 1.6; intermediate sector, 56%; high sector, 20%. *White arrow*, a nucleus with eleven $c$-myc signals and four CEP8 signals; two split signals for $c$-myc and one split signal for CEP8 were each counted as one. *Red arrow*, a nucleus with seven $c$-myc signals and two CEP8 signals. (F) Tumor UM58B (pigmented) with amplification of $c$-myc. Overall $c$-myc:CEP8 ratio, 1.6; intermediate sector, 60%; high sector, 12%. *Arrows*, nuclei with four $c$-myc signals and two CEP8 signals.
Comparison between FISH and Microsatellite Analysis Results

Although extra copies of \(c\)-\(myc\) were found in tumors with retention of both chromosome 3 and chromosomal arm 6p (R3-R6p), increase in \(c\)-\(myc\) copy number (amplification, IRI and +8) was more frequent in tumors with M3 (Table 1). \(c\)-\(myc\) amplification was demonstrated in 13 of the 26 tumors with M3 by microsatellite analysis compared with 0 of 10 tumors with 6p AI (\(P = 0.006\), Fisher’s test) and in 0 of 7 tumors with R3-R6p (\(P = 0.03\), Fisher’s test). Thus, all tumors with specific \(c\)-\(myc\) amplification also displayed monosomy of chromosome 3.

Of the 28 uveal melanomas with 8q AI by microsatellite analysis, 25 showed an increase in \(c\)-\(myc\) copy number (either amplification, IRI or +8), 2 showed loss of \(c\)-\(myc\), and only 1 case showed no variation in \(c\)-\(myc\) copy number. Of the 15 tumors with retention of 8q by microsatellite analysis, 5 showed IRI (\(n = 3\) ) or +8 (\(n = 2\) ), but none showed amplification of \(c\)-\(myc\).

DISCUSSION

Karyotypic abnormalities of chromosomal arm 8q have been repeatedly described in uveal melanoma, suggesting that a gene(s) on this chromosomal arm may be involved in the development of this tumor type. We have demonstrated that extra copies of the \(c\)-\(myc\) gene, located on 8q, are present in 70% of uveal melanomas. By cytogenetic studies, trisomy 8,

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<th>% of nuclei with the indicate number of (c)-(myc) signals</th>
<th>Total for CEP8</th>
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<td>Total for (c)-(myc)</td>
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Total \(c\)-\(myc\)/CEP8 ratio = 2.1

LOW SECTOR: individual nuclei \(c\)-\(myc\):CEP8 ratio ≤ 1 (26%).

INTERMEDIATE SECTOR: individual nuclei \(c\)-\(myc\):CEP8 ratio >1 to ≤ 2 (29%).

HIGH SECTOR: individual nuclei \(c\)-\(myc\):CEP8 ratio > 2 (44%).

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<th>(0) or (1) signal</th>
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<td>(c)-(myc)</td>
<td>31.5 ± 7.5</td>
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<td>CEP8</td>
<td>36.2 ± 6.4</td>
<td>62.1 ± 6.8</td>
<td>1.3 ± 1.9</td>
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isochromosome 8q, and duplication of large region(s) of 8q, were found in approximately 50% of the tumors analyzed.\(^1,13,26-32\) Thus, the percentage of tumors with extra copies of \(c-myc\), as detected by FISH in our study, exceeded that reported from simple karyotypic abnormalities of 8q.

Almost half (47%) of the tumors with an increase in 8q copy number have IRI in the \(c-myc\) copy number. Jenkins et al.\(^{23}\) have suggested that the principal mechanism for IRI is isochromosome 8q formation. This hypothesis is consistent with our observation and data from karyotypic studies in which isochromosome 8q is the most commonly detected chromosome 8 abnormality.\(^{1,13,26-32}\)

Amplification of the \(c-myc\) oncogene, not explained by isochromosome 8q or trisomy 8 formation, was detected in 43% of the tumors with extra copies of \(c-myc\). In those tumors, the percentage of nuclei containing \(>5\) \(c-myc\) signals per chromosome 8 centromere ranged from 11% to 45%. In the presence of isochromosome 8q or trisomy 8, the average \(c-myc\):CEP8 ratio cannot exceed 2. The specific \(c-myc\) amplification we have detected is most likely the result of intrachromosomal rearrangement or translocation of a small region of 8q containing the \(c-myc\) oncogene rather than an extrachromosomal amplification (double minutes), which has not been observed cytogenetically in this tumor type.

On the basis of our published allelotype, we proposed a bifurcated model for the progression of genetic changes that lead to uveal melanoma.\(^19\) The most common pathway involved loss of one copy of chromosome 3 (M3), and the secondary pathway was characterized by alteration of chromosomal arm 6p.\(^19\) Further analysis of five tumors (initially non-informative for markers from chromosomal arm 3p or 6p in the original allelotype) with additional microsatellite markers confirmed the mutual exclusivity of M3 and 6p alterations.

In our original allelotype, because all tumors with 8q alterations showed M3 or 6p abnormalities, we proposed that 8q AI imbalance follows chromosome 3 or chromosome 6p alterations. In fact, the presence of 8q abnormalities without M3 or chromosome 6 AI seems to be a very rare event in uveal melanomas. In the present study, only three tumors, which were R3-R6p by microsatellite analysis, had extra copies of \(c-myc\) by FISH (IRI in one case, \(+8\) in two cases). CGH studies have reported 8q gain without a concomitant loss of chromosome 3 or a gain of chromosome 6p in just 1 of 21 uveal melanomas (4.7%).\(^{5,6}\) Likewise, in those cytogenetic studies in which both chromosome 3 and chromosome 6 status was reported, only a few tumors were found to harbor solely 8q abnormalities.\(^{1,27-29,31-33}\)

\(c-myc\) amplification also appears to follow M3. Remarkably, all 13 tumors with \(c-myc\) amplification were M3, but only 13 of the 26 tumors with M3 showed \(c-myc\) amplification. \(c-myc\) amplification was not present in any tumor without M3. These striking findings suggest that \(c-myc\) amplification not explained by isochromosome 8q or trisomy 8 typically follows the loss of one copy of chromosome 3.

Several of our observations are consistent with \(c-myc\) amplification occurring later in the genetic progression of uveal melanoma. First, larger BD and greater tumor thickness were associated with amplification of \(c-myc\). Second, for both regions of UM58, microsatellite analysis showed loss of heterozygosity of the same allele for all informative markers on chromosome 3 but amplification of different 8q alleles.\(^{19}\) Third, tumor heterogeneity with respect to the absolute number of copies of \(c-myc\) was observed in our tumors with additional copies of \(c-myc\) (a representative example is seen for UM11 in Fig. 2A). As well, histopathologically distinct regions of tumor UM58 showed amplification of \(c-myc\), but the overall percentage of cells with \(c-myc\) amplification (cells in the high \(c-myc\)/CEP8 ratio sector) was approximately 30% higher in the unpigmented region (UM58A) of the tumor. We believe that UM58A is less differentiated and farther along the genetic progression pathway because it had a total of 10 additional chromosomal arms with LOH when compared with UM58B.

Either extra copies of \(c-myc\) (25 cases) or loss of one copy of the \(c-myc\) gene locus (2 cases) were demonstrated in all but one of the tumors with AI at 8q loci by microsatellite analysis, demonstrating that allelic amplification can be readily detected by microsatellite analysis in addition to allelic loss. A minimal increase in \(c-myc\) copy number was found in 5 tumors (8 in 2 cases and IRI in 3 cases) with retention of 8q loci by microsatellite analysis. One explanation for the discrepancy is that in those 5 tumors, only a small proportion of cells harbored 8q abnormalities falling below the threshold of detection by microsatellite analysis. It is also possible that the increase in \(c-myc\) copy number in these 5 tumors did not lead to a measurable imbalance between the microsatellite alleles tested.

Others have already proposed a potential role for \(c-myc\) expression as a prognostic indicator in uveal melanomas. In studies based on protein expression levels, \(c-myc\) overexpression correlated with either a poor\(^{10}\) or better prognosis.\(^{14,17,18}\) To date, we have been unable to correlate the molecular data with patient survival, as our overall mean patient follow-up period is less than 3 years.

Although our results cannot exclude the direct involvement of another gene(s) on chromosomal arm 8q in uveal melanoma tumorigenesis, specific amplification of the \(c-myc\) oncogene was demonstrated in at least 30% of primary uveal melanomas. Moreover, this level of amplification cannot be explained by the simple 8q abnormalities that have been observed in cytogenetic studies (trisomy 8, isochromosome 8q). The striking association between \(c-myc\) amplification and M3 in uveal melanoma further suggests that \(c-myc\) amplification (and probably overexpression) generally follows loss of a critical gene(s) on chromosome 3.
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