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The role of 8q24 copy number gains and c-MYC expression in amelanotic cutaneous melanoma

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Cutaneous melanomas may be quite heterogeneous in their clinical, histological and molecular findings. Correlating these features may help identify distinctive subgroups of melanomas and improve our overall understanding and prognostication of melanoma. We recently identified a subgroup of melanomas with increased chromosomal copy number gains in 8q24 at *MYC* having several distinctive clinical and histopathological characteristics, including an aggressive clinical course and an amelanotic clinical and histological appearance. It has been postulated that oncogenes such as *MYC* may have regulatory effects on genes critical to melanin pigment synthesis, specifically microphthalmia-associated transcription factor (MITF), which is known to have a key role in regulating the expression of tyrosinase (TYR), an important enzyme in the production of melanin pigment. We investigated the possible mechanism underlying the amelanotic appearance of melanomas with gains in 8q24 by evaluating the relationship between melanomas with and without 8q24 copy number gains and c-MYC, MITF and TYR protein expression. Immunohistochemical analysis of c-MYC, MITF and TYR was performed on 36 melanomas with gains in 8q24 and 40 melanomas without gains in 8q24. The melanomas with gains of 8q24 correlated with elevated c-MYC protein expression and melanomas without gains in 8q24 showed significantly decreased c-MYC protein expression. A direct relationship between the presence of gains in 8q24 and decreased MITF expression, as well as between c-MYC and TYR protein expression was also observed. Our results suggest that *MYC* can have a role in the pigmentary pathway of melanoma. In amelanotic melanomas with gains in 8q24, downregulation of TYR and other melanocyte-specific genes may be mediated by *MYC* leading to transcriptional suppression of *MITF*. As MITF is a frequently used marker to establish melanocytic lineage in melanoma, our study also raises the important clinical consideration that amelanotic melanomas, especially those with gains in 8q24 may lack expression of MITF.
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Correlating clinical, histopathological and molecular features may be helpful in improving our understanding of melanoma and could be useful in future classification systems for melanoma. Recently, we identified a subgroup of melanomas with chromosomal copy number gains involving v-myc myelocytomatosis viral oncogene homolog (*MYC*; alias *c-MYC*) at 8q24 having reproducible clinical and histological features including aggressive clinical course, occurrence in non-chronically sun damaged skin, amelanotic clinical appearance, histology showing a nodular or primary dermal growth (Figure 1a), epidermal consumption, cells with irregular nuclear contours, coarse chromatin and infrequent association with a nevus.[1,2] Specifically, 90% of the melanomas in our database having 8q24 copy number gains

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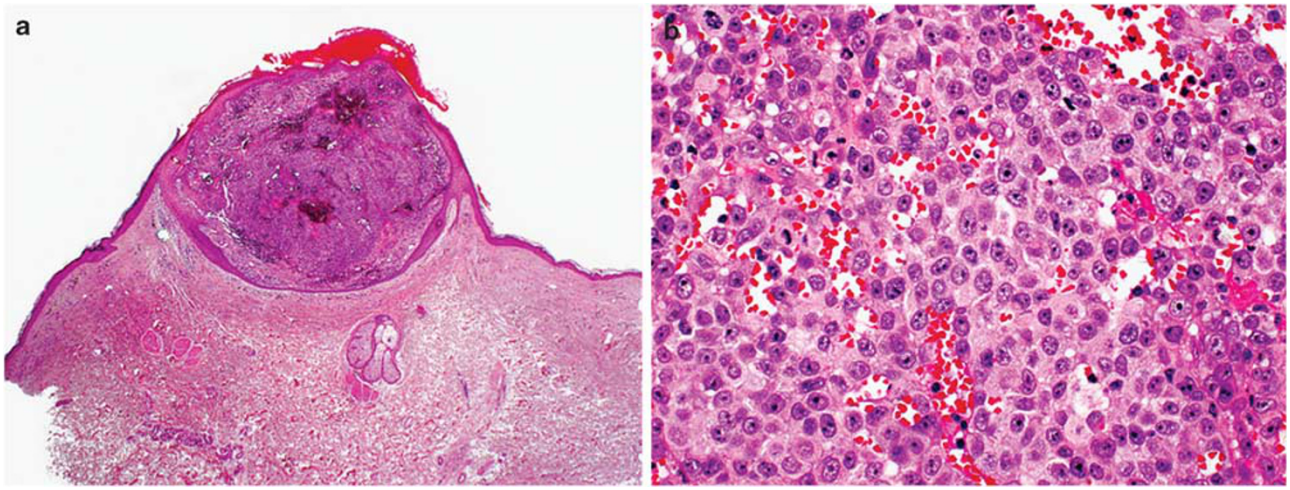

Figure 1 (**a**) Melanoma with gains in 8q24 with a nodular and primary dermal growth pattern and (**b**) an amelanotic appearance.

were clinically and histologically amelanotic (Figure 1b). Halaban *et al*[3] have proposed that a number of dominantly acting oncogenes, including *MYC*, may have upstream regulatory effects on genes critical to melanin pigment synthesis. Specifically they have proposed that upregulation of *MYC* in melanoma may downregulate one or more transcription factors critical in the pigmentary pathway in melanoma. Hence, it is our interest to further investigate the relationship between copy number gains in 8q24 in melanoma and the amelanotic clinical and histological phenotype.

It is now well understood that microphthalmia-associated transcription factor (MITF) is an essential regulator for melanocyte development, differentiation, proliferation and survival, as well as having a key role in regulating the expression of melanocyte-specific enzymes, including tyrosinase (TYR) and structural proteins important in the production of melanin.[4] The TYR protein is part of the final enzymatic pathway in melanin production and is the rate-limiting enzyme in melanin production.[5] It has been postulated that oncogenes such as *MYC* may have upstream regulatory effects in the control of melanin synthesis by suppressing *MITF* expression, probably by downregulating one or more of the transcription factors responsible for promoting *MITF* transcription.[3] Hence, in this study we wished to further investigate the relationship between 8q24 copy number gains, c-MYC protein expression, MITF expression and TYR expression in order to further elucidate the relationship between the 8q24 gains in the melanoma and the amelanotic appearance. Immunohistochemical analysis of c-MYC, MITF and TYR was performed on 40 melanomas with copy number gains in 8q24 (90% of these cases had an amelanotic phenotype on histopathology) and 40 randomly selected melanomas without copy number gains in 8q24 (28% of these cases had an amelanotic phenotype on histopathology). We performed a two-tailed Fisher's exact test to identify statistically significant relationships between 8q24 chromosomal gains and immunohistochemical expression of c-MYC, MITF and TYR.

Materials and methods

Case Selection and Immunohistochemistry

Approval for this study was obtained from the Northwestern University Cancer Center and Internal Review Board. From our previous study[1] we retrieved the hematoxylin and eosin slides, as well as the paraffin embedded tissue block from 40 cases of cutaneous melanomas characterized by increased copy numbers of 8q24 as identified by a probe targeting *MYC,* as well as 40 randomly selected cases of cutaneous melanomas lacking gains in 8q24. For all 80 melanoma cases immunohistochemistry was performed for c-MYC, MITF and TYR. Briefly, 3 micron slides were collected on Histobond slides from the paraffin embedded tissue blocks and dried overnight at 58 °C. Slides were rehydrated through graded alcohols to distilled water. Endogenous peroxidase was quenched using a solution of 0.5% hydrogen peroxide in methanol for 10 min. Retrieval was performed at 96.5 °C with EDTA pH 9.0 (Dako) S2367 for 60 min. Slides were rinsed in Tris buffer (Dako) S3006 and loaded on racks for Dako Autostainer Plus. The stainer was programmed for 60 min of primary antibody, 30 min secondary antibody, 10 min chromogen and 5 min counterstain. The antibodies used are detailed as follows: (1) c-MYC antibody (9E10)—ChIP Grade (ab32) Abcam diluted 1 μg/ml. (2) MITF (microphtalmia transcription factor) Clone D5, Dako M3621 diluted 1:50 with Dako diluents S0809. (3) TYR (T311) Cell Marque 344-98 pre-diluted mouse anti-human monoclonal antibody. Slide sections were then rinsed with Tris buffer followed by secondary antibody staining with Dako Envisions Dual link HRP polymer K0461 and then stained with DAB chromogen K3468; after

counterstaining with hematoxylin, slides were dehydrated through graded alcohols to clearing solution and covered.

All study cases underwent confirmatory review by two dermatopathologists participating in the study (PG and JG). In all 40 melanoma cases without gains in 8q24 there was ample tissue to allow for an adequate and interpretable antibody reaction with the tumor. In 36 of 40 cases of melanomas with gains in 8q24 there was enough tissue remaining in the paraffin block to allow for an adequate and interpretable antibody reaction with the tumor.

Immunohistochemistry Scoring

For the evaluation of c-MYC immunoreactivity, cytoplasmic and membranous staining of malignant cells was regarded as positive. Nuclear staining of c-MYC was partial and weak among all the cases in both categories and an observable difference was not identified between the two groups to allow for analysis of statistical significance with respect to nuclear immunoreactivity. For the evaluation of MITF immunoreactivity, nuclear staining of the malignant cells was regarded as positive. For the evaluation of TYR immunoreactivity, cytoplasmic staining of the malignant cells was regarded as positive. Expression analysis of c-MYC protein, MITF and TYR in melanoma cells was performed by evaluating the staining intensity of the immunoreactive melanoma cells, as well as the percentage of positive lesional cells. A case was scored as 0 if the percentage of positive staining melanoma cells was $<10\%$ and the staining intensity was graded as weak to no staining. A case was scored as 1 if the percentage of positive staining melanoma cells was 10–100% and the staining intensity was graded as strong. Blinded analysis of the slides was performed by two dermatopathologists participating in the study (PG and PP). Positive and negative tissue controls were performed for each antibody.

Statistical Analysis

A two-tailed Fischer's exact test was used to evaluate for significance in the relationship of immunohistochemical protein expression intensity of the tumor cells between the melanoma cases with gains in 8q24 and those without gains in 8q24 relative to c-MYC (both cytoplasmic and membranous), MITF and TYR expression. The following comparisons were performed: (1) *MYC* status (whether a case did or did not have gains in 8q24) *vs* c-MYC (both cytoplasmic and membranous), MITF and TYR immunoreactivity, (2) cytoplasmic *vs* membranous c-MYC immunoreactivity, (3) cytoplasmic c-MYC immunoreactivity compared with that of MITF and TYR, (4) membranous c-MYC immunoreactivity compared with that of MITF and TYR, and (5) immunoreactivity of MITF compared with that of TYR. A P-value <0.05 was considered statistically significant.

Results

Tables 1 and 2 summarize the results of the immunohistochemical staining for c-MYC, MITF and TYR among the melanoma cases with and without chromosomal gains in 8q24. Among the cases with gains in 8q24, 21 of 36 cases (58%) demonstrated strong cytoplasmic staining for c-MYC and 12/36 (33%) had strong membranous staining for c-MYC (Figure 2a). The cases with 8q24 gains also demonstrated weak to negative staining with MITF in 33 of 36 (91%) cases (Figure 2b) and had weak to negative staining with TYR in 17 of 36 (47%) cases (Figure 2c).

Alternatively, among the cases without chromosomal gains in 8q24, 27 of 40 cases (67%) demonstrated weak to negative cytoplasmic staining for c-MYC and 40/40 (100%) had weak to negative membranous staining for c-MYC (Figure 3a). In this group strong MITF expression was seen in 31/40 (77%) cases (Figure 3b) and strong staining with TYR was seen in 23 of 40 (57%) cases (Figure 3c).

Table 3 summarizes the statistical analyses comparing cytoplasmic and membranous c-MYC, MITF and TYR expression among the melanomas with and without gains in 8q24 using two-tailed Fisher's exact testing. The presence or absence of 8q24 gains was statistically significant in relation to both cytoplasmic and membranous c-MYC expression with P-values of 0.0371 and 4.035×10^{-5}, respectively. Hence, there was a statistically significant correlation between chromosomal gains in 8q24 and elevated cytoplasmic and membranous c-MYC expression. Cases with cytoplasmic c-MYC expression were also more likely to have membranous c-MYC expression and this relationship was statistically significant

Table 1 Total number of cases and the immunohistochemical reactivity of the melanomas with gains in 8q24

Staining score	*c-MYC (cytoplasmic)*	*c-MYC (membranous)*	*MITF*	*Tyrosinase*
0	15	24	33	17
1	21	12	3	19

Table 2 Total number of cases and the immunohistochemical reactivity of the melanomas without gains in 8q24

Staining score	*c-MYC (cytoplasmic)*	*c-MYC (membranous)*	*MITF*	*Tyrosinase*
0	27	40	9	17
1	13	0	31	23

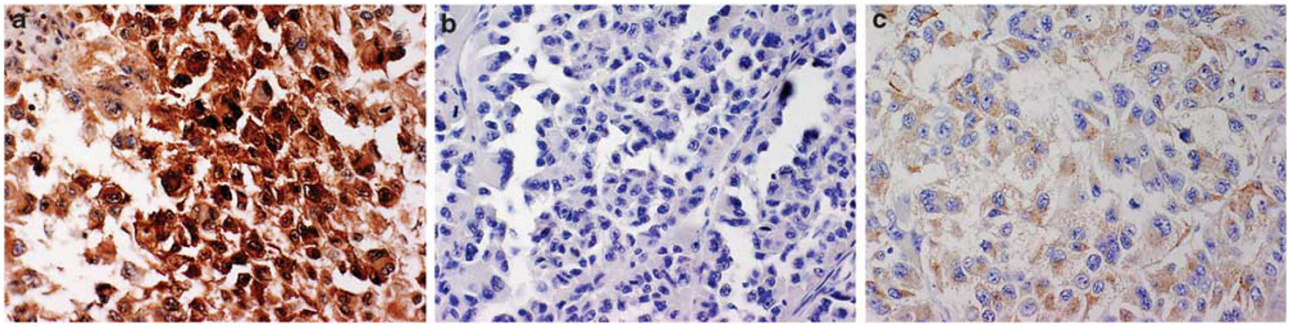

Figure 2 Melanoma with gains in 8q24 with (**a**) strong cytoplasmic and membranous c-MYC expression, (**b**) without MITF expression and (**c**) with weak to no TYR expression.

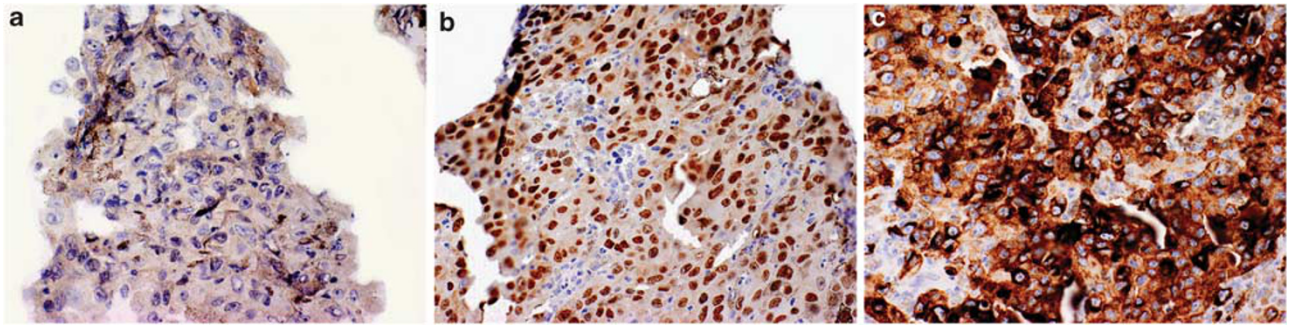

Figure 3 Melanoma without gains in 8q24 with (**a**) weak to no c-MYC expression, (**b**) strong nuclear MITF expression and (**c**) strong cytoplasmic TYR expression.

Table 3 Statistical analysis comparing c-MYC, MITF and tyrosinase expression of melanomas with and without gains in 8q24 using two-tailed Fischer's exact testing

	c-MYC (cytoplasmic)	*c-MYC (membranous)*	*MITF*	*Tyrosinase*
MYC Status (+/− gains in 8q24)	0.0371	4.035×10^{-5}	4.80×10^{-10}	0.8177
c-MYC (cytoplasmic)	/	1.768×10^{-5}	0.1674	0.0208
c-MYC (membranous)	/	/	0.0551	0.0091
MITF	/	/	/	1.00

with a *P*-value of 1.768×10^{-5}. The relationship between 8q24 chromosomal gains and MITF expression was also statistically significant, with cases having 8q24 chromosomal gains having significantly less MITF expression. A statistically significant relationship was also seen between both cytoplasmic and membranous c-MYC expression relative to TYR expression with *P*-values of 0.0208 and 0.0091, respectively. Hence, cases with cytoplasmic or membranous c-MYC expression had significantly less TYR expression.

There was a trend toward lower MITF expression with higher membranous c-MYC expression; however, this did not reach statistical significance with a *P*-value of 0.0551. Similarly, a statistically significant relationship was not identified between cytoplasmic c-MYC expression and MITF expression. Neither the 8q24 gain status nor MITF expression showed a statistically significant relationship to TYR expression.

Discussion

Cutaneous melanomas may be quite heterogeneous in their clinical, histological and molecular findings. Correlating these features may help identify distinctive subgroups of melanomas and improve our overall understanding and prognostication of melanoma. Recently we have identified a subgroup of melanomas with increased copy number gains in 8q24 having distinctive clinical and histopathological characteristics, which include aggressive clinical course, occurrence in non-chronically sun damaged skin, amelanotic clinical appearance, frequent histological appearance of a nodular and/or primary dermal growth pattern, frequent epidermal consumption, cells with irregular nuclear contours, coarse chromatin and infrequent association with a nevus.[1,2] Copy number gains in 8q24 at *MYC* not only

appear to have an important role in the aggressive clinical behavior of melanomas but may also be involved with the amelanotic clinical and histological appearance of melanomas harboring such chromosomal aberrations. In this study, we aimed to investigate the possible mechanism underlying the amelanotic appearance of melanomas with copy number gains in 8q24 by evaluating the relationship between melanomas with and without 8q24 copy number gains and c-MYC, MITF and TYR protein expression by immunohistochemical analysis.

We have shown that when increased chromosomal copy number gains of 8q24 are present in melanoma there is also a correlation with finding elevated cytoplasmic and membranous c-MYC expression. Cytoplasmic c-MYC expression was also strongly correlated to membranous expression of c-MYC. Conversely, melanomas without gains in 8q24 showed significantly decreased cytoplasmic c-MYC protein expression and often had absent membranous c-MYC protein expression. These results support a direct relationship with 8q24 chromosomal gains and c-MYC protein expression. We also found a statistically significant relationship between 8q24 copy number status and MITF protein expression. This also further supports our hypothesis that copy number gains in 8q24 resulting in elevated c-MYC protein expression may also downregulate MITF protein expression. Hence, melanomas harboring gains in 8q24 showed decreased or absent MITF expression while those without gains in 8q24 had elevated MITF levels. Also of importance, strong cytoplasmic and membranous c-MYC expression showed a statistically significant relationship to decreased TYR expression suggesting that presence of c-MYC protein may ultimately contribute to the repression of TYR expression, further supporting the view that *MYC* may have a regulatory influence on the pigmentary pathway in melanoma.

There was a trend toward decreased MITF expression among those melanomas with increased cytoplasmic and membranous expression of c-MYC although this did not reach statistical significance. Hence, although we believe it is likely that c-MYC has a role in downregulating MITF expression, the relationship may be complex involving many intermediaries possibly explaining our inability to show a directly statistically significant relationship.

There was also a trend toward decreased TYR expression in cases with 8q24 gains and increased TYR expression in cases with elevated MITF expression although neither of these relationships achieved statistical significance. It is possible that there may be a significant relationship between these markers that was not captured in our study because of our small sample size. Although MITF is thought of as having a central role in melanocyte development, differentiation, proliferation and survival of melanocytes and melanoma cells, there are likely many complex intermediary pathways and regulatory mechanisms involving coordination of gene expression and interactions among different transcription factors and various proteins involved in the pigmentary pathways in melanoma that were not captured by our study. For example, Hou *et al*[6] have shown that mouse embryonic melanocytes require the coordinated actions of both MITF and the transcription factor Sox10 for *TYR* induction to occur.

Our study showed a very strong relationship between elevated copy numbers of *MYC* and MITF expression consistent with the idea that *MYC* has significant regulatory control over MITF expression. A direct relationship between MITF and TYR, however, was not apparent. It is likely that there are many intermediaries between MITF and TYR, as well as other molecules from other pathways involved in the regulation of TYR expression that were not captured by our study. This likely explains why some of the melanoma cases with 8q24 gains expressed TYR as its regulation is likely complex and controlled by a variety of factors.

The potential complexity of the pigmentary pathway is reflected by the proposal from Halaban *et al*[3] that oncogenes such as *MYC* may have regulatory effects in the control of melanin synthesis in melanoma. In amelanotic melanomas with gains in 8q24, transcriptional downregulation of *TYR* and other melanocyte-specific genes may be mediated by dominantly acting oncogenes, such as *MYC* leading to transcriptional suppression of *MITF*. In this study, we found that there is a direct relationship between the presence of chromosomal copy number gains in 8q24 and decreased MITF expression. Therefore it may be plausible that *MYC* either directly or through a series of intermediaries represses *MITF* expression. One proposed mechanism for this has suggested that c-MYC may work through activating n-MYC downstream-regulated gene 2, a candidate tumor suppressor gene that is expressed in melanoma cells, which subsequently inhibits *MITF* expression.[7] Similarly, Brn-2, a POU class 3 homeobox transcription factor 2 has been shown to directly repress *MITF* expression.[8] Although an interaction between MYC and Brn-2 has yet to be established, if such a connection is found that would be another strong link tying the expression of *MYC* to the downregulation of *MITF* via an intermediary step. Additionally, it is known that at least four different transcription factors participate in the transactivation of the *MITF* gene in melanocytes: the paired box-containing transcription factor PAX3, a sex determining region Y family member SOX10, the Wnt/β-catenin pathway effector LEF-1 and the cAMP pathway effector cAMP response element binding.[4] Although confirmatory studies are necessary to demonstrate such interactions, it is plausible that *MYC* may act to downregulate one or more of these transcription factors, leading to the suppression of *MITF* activation.

This is the first study that investigates the relationship between the presence and absence of copy number gains in 8q24 in melanoma to protein

expression of c-MYC, MITF and TYR in an attempt to shed light on the relationship between melanomas with gains in 8q24 and the amelanotic clinical and histological. Our results suggest that *MYC* has a role in the pigmentary pathway in melanoma. This has important clinical implications as it allows for an improved understanding of the role *MYC* has not only in melanin pigment formation in melanomas but also in the pathogenesis of melanoma allowing for better correlation of clinical and molecular features, which could be integrated into an improved classification system for melanoma. Our study also raises the issue of the role of MITF as an immunohistochemical marker in the diagnosis of amelanotic melanomas. MITF is a frequently used marker to establish melanocytic lineage in melanoma. An important clinical consideration raised by our study is that MITF may not be a reliable melanocytic marker in the context of amelanotic melanomas, especially those with gains in 8q24. Further studies with larger sample sizes would be necessary to more completely establish the potential lack of utility of MITF in this clinical scenario.

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Disclosure/conflict of interest

Dr Gerami has served as a consultant to Abbott Molecular Labs and Neogenomics and has received honoraria for this. All other authors declare no conflict of interest.

Disclaimer
This work is original and has no prior presentation.

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