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Expression of cyclooxygenase-2 and peroxisome proliferator-activated receptor gamma during malignant melanoma progression

Background: Cancer chemoprevention using nonsteroidal anti-inflammatory drugs is frequently attributed to cyclooxygenase-2 (COX-2) inhibition, although recent studies suggest that peroxisome proliferator-activated receptor gamma (PPARγ) may also be involved. While surgical excision remains the treatment mainstay for localized malignant melanoma, certain high-risk patients may benefit from adjunctive chemotherapy. In this study, we compared COX-2 and PPARγ immunohistological staining in benign nevi, primary melanomas and metastatic melanomas to help predict the effectiveness of compounds targeting these markers.
Methods: COX-2 and PPARγ immunohistological staining was performed and reviewed in 99 melanocytic lesions, including 38 benign nevi, 32 primary melanomas and 29 metastatic melanomas.
Results: There was a significant increase in both COX-2 and PPARγ immunostaining in melanomas compared with benign nevi. Metastatic melanomas were more likely to have a higher number of PPARγ-immunopositive cells. They were also more likely to express COX-2 than primary melanomas. Neither COX-2 nor PPARγ expression was associated with a specific pathologic subtype.
Conclusions: COX-2 and PPARγ may help modulate the progression of melanocytic precursor lesions to disseminated malignant melanoma. As such, they may serve as candidate substrates for targeted cancer therapies and may be particularly useful as adjuncts to surgery.

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Carolyn Lee[1], James A. Ramirez[2], Joan Guitart[3] and Leslie K. Diaz[3]

[1]Department of Dermatology, Stanford University School of Medicine, Stanford, CA, USA,
[2]Department of Dermatopathology, St. Joseph Mercy Hospital, Ann Arbor, MI, USA, and
[3]Department of Pathology, Northwestern University School of Medicine, Chicago, IL, USA

Dr Carolyn Lee, Department of Dermatology, Stanford University School of Medicine, 900 Blake Wilbur Drive, Room W0069, Stanford, CA 94305, USA
Tel: +1 6507236316
Fax: 6507237711
e-mail: carilee@stanford.edu

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Nonsteroidal anti-inflammatory drugs (NSAIDs) have been shown to be beneficial in the prevention of colorectal and other types of cancer in humans and murine models.[1,2] This chemopreventive effect is most frequently attributed to cyclooxygenase (COX) inhibition. Expression of the COX-2 isoform has been particularly well studied and is known to be rapidly induced by growth factors and tumor promoters. Overexpression of COX-2 has been reported in premalignant and malignant lesions derived from a broad spectrum of tissues, including lung, breast, prostate, bladder and the gastrointestinal tract.[3] Deletion of COX-2 in cancer-prone mice suppresses tumor formation,[4] and selective COX-2 inhibitors have been shown to provide effective chemoprevention in humans and animal models of

carcinogenesis.[5,6] These observations suggest that overexpression of COX-2 is an early event in tumorigenesis and may play a role in the progression of precursor lesions to malignant neoplasms. As such, COX-2 may be a suitable candidate molecule for targeted cancer therapies.

Peroxisome proliferator-activated receptor gamma (PPARγ) is a transcription factor belonging to the nuclear receptor superfamily.[7,8] It appears to play a role in tumorigenesis, although the exact nature of that role has yet to be defined. A number of studies have shown that PPARγ is expressed in several types of human cancers but not in the normal tissues from which these cancers are derived.[9] PPARγ agonists have been shown to promote tumorigenesis in cancer-prone mice;[10–12] however, the same agonists have also induced differentiation and slowed the growth of human tumor xenografts in other murine models.[13–17]

Studies of PPARγ and COX-2 expression in human skin have yielded mixed results. Immunohistochemical analyses of COX-2 expression in benign nevi and malignant melanomas generally showed an increase in COX-2 expression with cancer progression.[18–20] Similar studies of PPARγ expression did not detect a correlation with tumor progression but did suggest that an association with COX-2 might exist.[21,22] Interestingly, PPARγ agonists did not reduce skin tumor formation in two well-characterized models of murine skin carcinogenesis[23] but did inhibit the growth of human melanoma cell lines *in vitro*.[24,25]

In an effort to clarify whether PPARγ and COX-2 are suitable molecular markers of melanoma progression, we studied their expression in benign nevi and primary and metastatic melanomas using immunohistochemistry. The data show a progressive increase in COX-2 expression during the malignant progression of melanocytic tumors. Similarly, melanomas, but not benign melanocytic lesions, show increased PPARγ expression. To our knowledge, the data contained herein represent the first immunohistochemistry study to correlate PPARγ expression with progression of malignant melanoma.

Methods

Histopathological specimens were selected from the files of the Department of Pathology at Northwestern University, Chicago, IL, USA. Each case was independently reviewed by two pathologists (L. K. D and J. A. R), and a consensus was obtained for any discrepant cases.

Immunohistochemical analysis for COX-2 and PPARγ was performed on 38 benign nevi (20 compound, 16 intradermal and 2 junctional), 32 primary melanomas (15 superficial spreading melanomas, 13 nodular melanomas, 2 lentigo maligna melanomas and 2 *in situ* melanomas) and 29 metastatic melanomas. In six cases, primary and metastatic melanomas were obtained from the same patient. Routine hematoxylin and eosin-stained sections were performed for histopathological evaluation.

Formalin-fixed, paraffin-embedded slides were stained using monoclonal antibodies against PPARγ (dilution 1 : 100; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or COX-2 (catalog number 160112; dilution 1 : 100; Cayman Chemical, Ann Arbor, MI, USA) and a standard avidin-biotin-peroxidase complex (ABC) immunoperoxidase procedure. Nuclear staining for PPARγ was classified into four grades on a scale of 0–3 based on the percentage of positive cells as follows: a score of 0 was given to specimens with 0% nuclear staining, a score of 1+ was given to specimens with 1–9% nuclear staining, a score of 2+ was given to specimens with 10–50% nuclear staining and a score of 3+ was given to specimens with greater than 50% nuclear staining. COX-2 staining was scored as either weak or strong based on the intensity of cytoplasmic staining.

Fisher's exact test was used to determine statistical significance. The chi-square test was used to determine independence. A p-value of < 0.05 was considered statistically significant.

Results

COX-2 expression increases with malignant progression of melanocytic lesions

Virtually all the melanocytic lesions studied showed some level of COX-2 expression. A cytoplasmic pattern of staining was observed (Fig. 1). As a significant number of cases showed a faint background blush, we interpreted COX-2 staining as either strong or weak instead of as a percentage of immunopositive cells. Specimens designated as strong showed intense and crisp cytoplasmic staining. Strong expression of COX-2 was seen in 33% (33/99) of all lesions studied. The percentage of specimens with strong expression of COX-2 was 3% (1/38) in benign nevi, 38% (12/32) in primary melanomas and 69% (20/29) in metastatic melanomas (Fig. 2 and Table 1). Fisher's exact test showed that these differences were statistically significant when comparing benign nevi and primary melanomas ($p = 0.0003$) as well as primary and metastatic melanomas ($p < 0.0001$, Table 2). These data show a progressive increase in COX-2 expression during the malignant progression of melanocytic tumors.

We then compared COX-2 expression in various melanoma subtypes. Thirty-eight percent (5/13) of nodular melanomas, 40% (6/15) of superficial spreading melanomas and 50% (1/2) of lentigo maligna melanomas exhibited strong COX-2 expression. There was no statistically significant association

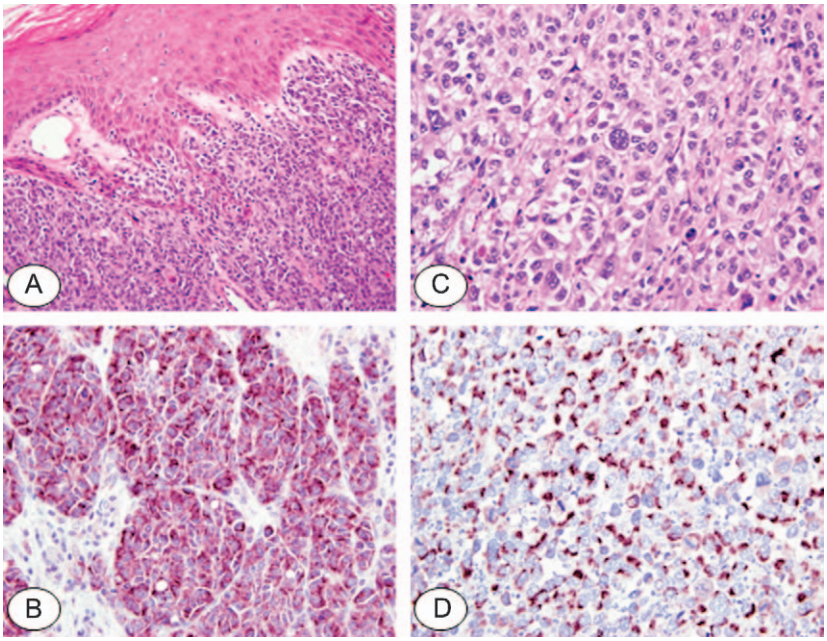

Fig. 1. Cyclooxygenase-2 expression in representative primary (B) and metastatic (D) melanomas by immunohistochemistry. Hematoxylin and eosin-stained primary and metastatic melanomas are shown in A and C, respectively.

between increased levels of COX-2 and melanoma subtype using Fisher's exact test. Of note, we observed enhanced COX-2 expression in the periphery of two nodular melanomas (40%, 2/5), consistent with a previous study.[19]

In our study, of the six individuals who had both primary and metastatic melanomas, only two showed strong COX-2 staining of their primary melanomas. These results suggest that strong COX-2 expression in a primary melanoma is not a prognosticator of future metastatic disease, although it is probable that our study was not adequately powered to detect a true difference in this subgroup.

PPARγ is expressed at high levels in primary and metastatic melanomas

Of the 99 samples studied, 22 (22%) showed immunoreactivity against PPARγ (> 10% immunopositive keratinocytes). Specifically, 3% (1/38) benign nevi, 28% (9/32) primary melanomas and 41% (12/29) metastatic melanomas expressed PPARγ (Table 1). A nuclear staining pattern was predominantly seen (Fig. 3), with cytoplasmic staining of PPARγ noted in only one metastatic melanoma specimen. Fisher's exact test (Table 2) showed that these differences were statistically significant when comparing benign nevi and primary melanomas ($p = 0.004$) and even more significant when comparing benign nevi and metastatic melanomas ($p < 0.0001$). While there appeared to be a trend toward increased PPARγ immunoreactivity in metastatic melanomas compared with primary melanomas, this relationship did not achieve statistical significance ($p = 0.30$). However, it is important to note that > 50% nuclear staining was only seen in metastatic melanomas (14%, 4/29, Table 3). Overall, the data show that increased PPARγ expression occurs in melanomas but not in benign melanocytic lesions.

When we compared PPARγ expression in various melanoma subtypes, 31% (4/13) of nodular melanomas, 27% (4/15) of superficial spreading melanomas and 50% (1/2) of lentigo maligna melanomas exhibited PPARγ immunoreactivity. These results

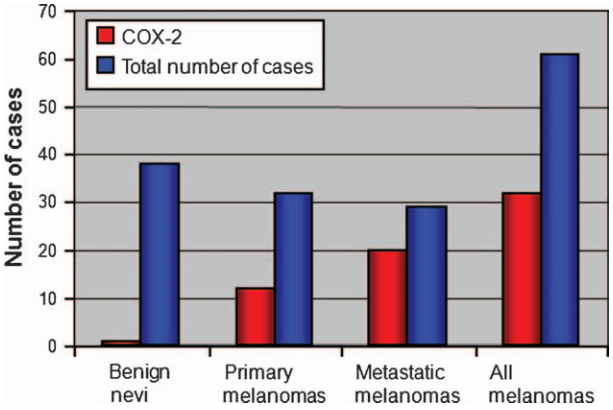

Fig. 2. Frequency of strong cyclooxygenase-2 expression in benign nevi and malignant melanomas.

Table 1. COX-2 and PPARγ expression in benign nevi and malignant melanoma

	Benign nevi (%)	Primary melanoma (%)	Metastatic melanoma (%)
COX-2	1/38 (3)	12/32 (38)	20/29 (69)
PPARγ	1/38 (3)	9/32 (28)	12/29 (41)

COX-2, cyclooxygenase-2; PPARγ, peroxisome proliferator-activated receptor gamma.

Table 2. p-Values of comparisons between melanocytic skin lesions for PPARγ immunoreactivity and strong COX-2 expression

Comparisons	COX-2*	PPARγ†
Benign nevi vs. primary melanomas	0.0003	0.004
Benign nevi vs. metastatic melanomas	< 0.0001	< 0.0001
Benign nevi vs. all melanomas	< 0.0001	< 0.0001
Primary melanoma vs. metastatic melanomas	< 0.0001	0.30

COX-2, cyclooxygenase-2; PPARγ, peroxisome proliferator-activated receptor gamma.
*Intense cytoplasmic immunostaining.
†> 10% immunopositive keratinocytes.

showed no statistically significant association between increased expression of PPARγ and melanoma subtype using Fisher's exact test.

In our study, of the six individuals who had both primary and metastatic melanomas, only two had primary melanomas that expressed PPARγ. These results suggest that PPARγ expression in a primary melanoma is not a prognosticator of future metastatic disease, although we cannot rule it out completely because of our limited sample size.

Discussion

Identifying molecular markers of cancer progression is critical for sustaining the current focus on targeted cancer therapies and rational drug design. An early intervention capable of interrupting or reversing disease progression would be particularly useful in malignant melanoma, where the current standards of care for metastatic disease have not resulted in a significant survival benefit. Targeted therapies could also be used as an adjunct to surgical excision in patients with localized disease who are at high risk of developing future metastases. To our knowledge, this is the first study to report increased expression of PPARγ in human malignant melanoma. This finding may explain why melanoma cell lines appear susceptible to the antiproliferative effects of selective PPARγ ligands, such as thiazolidinediones.[21,25]

In this study, we show that PPARγ is expressed in a significant proportion of primary and metastatic melanomas but rarely expressed in benign nevi. Furthermore, > 50% nuclear staining was only seen in metastatic melanomas, suggesting that PPARγ may have a role in the modulation of tumor invasion. We considered the possibility that strong expression of PPARγ in a primary melanoma might predict future metastases. However, our study was not adequately powered to perform this analysis. It is nonetheless interesting to note that thiazolidinediones were recently shown to inhibit cell migration and invasion in human breast and pancreatic cell lines.[26,27] Our data suggest that similar results may be seen in melanoma as well.

There are differing reports on the cellular localization of PPARγ in the skin. Some studies have observed a granular cytoplasmic pattern, while others have noted nuclear staining.[21,22] Our results are consistent with those obtained by others who used the same PPARγ antibody, suggesting that reagent selection may influence apparent localization. It is also possible that the discrepancy between the data described in Nijsten et al.[22] and those described herein is because of tumor-specific differences in PPARγ localization.

Increased expression of PPARγ has been reported in a wide range of human cancers. In melanoma cell lines, PPARγ agonists appear to suppress cellular

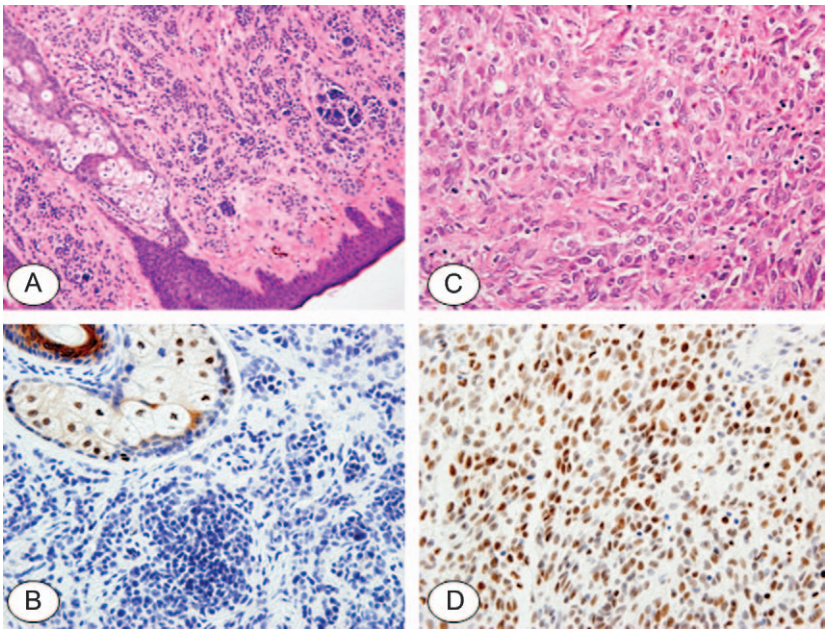

Fig. 3. Peroxisome proliferator-activated receptor gamma expression in a representative benign nevus (B) and metastatic melanoma (D) by immunohistochemistry. Of note, the sebaceous gland in B serves as an internal positive control. A hematoxylin and eosin-stained benign nevus and a metastatic melanoma are shown in A and C, respectively.

Table 3. Peroxisome proliferator-activated receptor gamma immunoreactivity in melanocytic skin lesions

	0–10% nuclear staining (%)	10–50% nuclear staining (%)	> 50% nuclear staining (%)
Benign nevi	37/38 (97)	1/38 (3)	0/38 (0)
Primary melanomas	23/32 (72)	9/32 (28)	0/32 (0)
Metastatic melanomas	17/29 (59)	8/29 (27)	4/29 (14)

proliferation and induce differentiation.[21,24,25,28] However, inhibition of PPARγ has produced similar results in other cancer types, including oral squamous cell carcinomas and hepatocellular carcinomas.[29,30] We are not certain why this discrepancy occurs, but it may be the result of off-target effects of PPARγ ligands or inherent differences between cell lines.

Strong COX-2 expression was observed in both primary and metastatic melanomas but rarely in benign nevi. Of note, we found that metastatic melanomas were significantly more likely to have strong COX-2 expression than primary melanomas. Our results are consistent with previous studies,[19,20] although we further extend the findings of Kuzbicki et al. to include distant cutaneous metastases as well as lymph node metastases. The data also raise the possibility that COX-2 promotes melanoma cell invasiveness. Our preliminary analysis did not detect a correlation between strong COX-2 expression in a primary melanoma and the presence of metastatic disease, but we recognize that the small sample size makes it difficult to rule out a type II error. Pharmacologic inhibition of COX-2 in melanoma cell lines has not consistently reduced prostaglandin E2 production or tumor cell invasiveness.[19,31] It is interesting to speculate whether this is related to off-target effects of NSAIDs, as a COX-independent mechanism of tumor suppression by these compounds has been shown.[32–34] Additional studies to clarify the role of COX-2 in melanoma progression and invasion seem to be warranted.

Studies on colorectal cancer have suggested that COX-2 and PPARγ may have opposing effects on tumorigenesis.[35] More recently, an immunohistochemical study showed that COX-2-positive squamous cell carcinomas and actinic keratoses were more likely to express PPARγ or its isoform, PPARβ.[22] As such, we expected to see a correlation between COX-2 and PPARγ expression in our study. A chi-square test for independence surprisingly did not show a relationship between COX-2 and PPARγ. While we cannot say for certain why this discrepancy occurred, it is possible that cancer-specific differences may be involved.

Our study examines the expression patterns of PPARγ and COX-2 in melanocytic lesions of the skin. The data suggest that both proteins play a role in the development and biological behavior of melanomas. More importantly, increased expression of these markers in melanoma may enable us to tailor treatment to selectively target malignant cells. The prothrombotic and gastrointestinal complications of COX-2 inhibitors have raised concerns about their long-term safety, although these risks may be outweighed by a potential benefit in survival in high-risk, disseminated or unresectable disease. Our data showing increased PPARγ expression in primary and metastatic melanomas also provide an explanation for the observed efficacy of thiazolidinediones in melanoma cell lines. As such, drugs that target COX-2 or PPARγ, particularly those with favorable safety profiles, may be effective adjuncts in the treatment of melanoma.

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