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Misregulation of Rad50 expression in melanoma cells

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Abstract

DNA double-strand breaks are increased in human melanoma tissue as detected by histone H2AX phosphorylation^{1,2,3}. We investigated two of the downstream effectors of DNA double-strand breaks, Rad50 and 53BP1 (tumor suppressor p53 binding protein 1), to determine if they are altered in human primary melanoma cells. Melanoma cases demonstrated high Rad50 staining (81.8%; 9/11) significantly more frequently than conventional or atypical melanocytic nevi (0%; 0/18). In contrast, the staining pattern for 53BP1 appears similar between melanoma and nevi. This is the first study that demonstrates activation and misregulation of the DNA repair pathway in human melanoma cells. The staining features of Rad50, a component of an essential DNA double-strand break repair complex, are clearly increased in melanoma cells with regards to both staining intensity and the number of positive melanoma cells. Interestingly, among the melanoma cases with increased Rad50 staining, most demonstrated cytoplasmic rather than nuclear staining (88.9%, 8/9). Further studies are needed to determine the cause of this mislocalization and its affects, if any, on DNA double-strand break repair in melanoma.

Keywords

melanoma; Rad50; UV radiation; histone; phosphorylation

Introduction

Previously, it has been demonstrated that DNA double-strand breaks are increased in human melanoma cells as detected by histone H2AX phosphorylation^{1,2,3,4}. DNA double-strand breaks can alter gene transcription and destabilize chromosomes, which may contribute to oncogenesis⁵. One of the mechanisms by which mammalian cells repair double-strand breaks is through non-homologous end joining (NHEJ), a process by which DNA break ends are ligated together to form a continuous double helical strand^{6,7}. The MRN complex is a mammalian NHEJ double-strand break repair complex whose components include meiotic recombination 11 (Mre11), Rad50 and Nijmegen breakage syndrome 1 (Nbs1) proteins⁸.

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This complex facilitates double-strand break repair by forming a link between DNA molecules and by catalyzing resection of broken ends⁹.

Changes in localization of double-strand break repair proteins can cause oncogenic transformation of affected cells¹⁰. For example, repair protein Nbs1 when localized to the cytoplasm can activate the PI3-kinase/AKT pathway to promote tumorigenesis¹⁰. This is evidence that NHEJ repair proteins can have alternate functions based on subcellular localization. In the present study we investigate the expression and localization of DNA double-strand break repair protein Rad50 and the tumor suppressor 53BP1 in human melanoma tissues. Rad50 is an ATPase involved in tethering DNA double-strand break ends to an unbroken DNA strand for repair¹¹. It is transported to the nucleus along with Mre11 and Nbs1 by importin KPNA2. Interestingly, RNAi mediated depletion of KPNA2 resulted in a significant reduction in double-strand break repair¹⁰. 53BP1 co-localizes to MRN foci and mediates downstream activation of the DNA damage checkpoint and DNA damage response pathways^{12,13}. Inhibition of the DNA damage response pathway has been shown to increase tumor size and invasiveness¹⁴. Taken together, the mislocalization of repair proteins and the inhibition of DNA damage response pathways are both likely contributing to malignancy. Studying the expression and localization of repair proteins in benign and malignant tissues could improve our understanding of the role the MRN complex plays in melanoma oncogenesis.

Materials and Methods

Tissue procurement

This study was approved by the University of Arkansas for Medical Science institutional review board. All FFPE (formalin fixed paraffin embedded) tissue samples were obtained from the Pathology Department and the following cases were retrieved from our dermatopathology archives: 11 melanoma, 7 severely atypical melanocytic nevus, and 11 conventional or mildly atypical melanocytic nevi. There were no significance differences in age, sex, or location of the lesions between the melanocytic lesions ($p>0.5$; see Table 1). For the purpose of this study, conventional melanocytic nevi includes intradermal and compound melanocytic nevi. An atypical melanocytic nevi includes lesions that contain features of dysplastic nevi (shouldering, fibroplasias and/or bridging) with either mild cytologic features (mild) or severe cytologic features (severely).

Immunohistochemistry

Briefly, the tissue blocks were sectioned (4m) and baked onto Superfrost® Plus slides (Fisher). Slides were de-paraffinized by heating to 65C and washed in xylene and in decreasing percentage of EtOH. Antigen retrieval was performed in a Decloaking Chamber™ (Biocare Medical) using EnVision FLEX™ target retrieval solution, pH 9 (Dako). Samples were heated to 120C for 10 min at ~22psi. Immunohistochemistry was performed using standard technique and reagents from DAKO. Briefly, primary antibody to either Rad50 (13B3/2C6 clone; Abcam) or 53BP1 (#4937; Cell Signaling) was diluted to a 1:100 in phosphate buffered saline (PBS), pH 7.3 and was applied to each slide and slides were incubated at 4C overnight. After overnight incubation and washing with tris-buffered

saline with Tween (TBST), the secondary antibody was applied to each slide and slides were incubated at room temperature for 30 min. Slides were washed again and the tertiary (DAKO-Enhancer) was applied to each slide and slides were incubated for 30 min at room temperature. After three washes, Bio-Red substrate (DAKO) was applied and allowed to incubate for 1-5 min at room temperature. Slides were counterstained with Mayer's Hematoxylin (Thermo Scientific) for 1 min at room temperature.

Scoring of staining

The intensity of staining was scored as nil (0), low (1), medium (2), or high (3) by three independent dermatopathologists. The percentage of melanocyte or melanoma cell staining was scored as 0 (no staining), 1 (<25% staining), 2 (25-50%) and 3 (>50% staining). Two pathologists independently scored these results in a blinded fashion. The intensity was multiplied by the percentage of melanocyte or melanoma cells stained and the following product is then categorized as such: 0-1 is 0; 2-3 is 1+; 4-5 is 2+; 6-9 is 3+. High positive staining is considered 2+ or 3+. Chi square analysis was used to determine statistical significance with a $p < 0.05$ considered significant.

Results

Melanoma cases showed a statistically significant increase in Rad50 staining compared to cases of conventional and atypical melanocytic nevi (see Figure 1). Interestingly, most of the epidermal melanocytes from conventional or atypical melanocytic nevi show little or no staining for Rad50 (Figure 1A and B). There was positive (i.e. 1+) Rad50 staining in nine of eleven (82%) melanoma cases, two of seven (29%) severely atypical melanocytic nevus cases, and three of eleven (27%) conventional or mildly atypical melanocytic nevus cases and these are statistically significant differences ($p < 0.0027$, see Table 2). Whereas for high staining (2+ or 3+), melanoma cases showed an even more statistically significant increase in cases with high Rad50 staining (81.8%; 9/11) compared to cases of atypical melanocytic nevi (0%; 0/18) ($p < 0.0001$). Among the positive cases of melanoma, the majority of the cases had 3+ staining results. Importantly, among the positive Rad50 staining, the protein appeared to be present in the cytoplasm for eight of the nine high staining melanoma cases (88.9%). In contrast, for the positive cases of nevi, all of the Rad50 staining is in the nuclei and none in the cytoplasm. To determine if this was a specific finding, we also examined another nuclear protein that is involved in the DNA double-strand break repair pathway and found that there was no difference in staining pattern, intensity or localization for 53BP1 ($p = 0.98$, see Figure 2 and Table 3) between melanoma and nevus.

Discussion

Rad50 is an ATPase that binds to Mre11 and Nbs1 to form a repair complex that is transported to the nucleus in response to DNA double-strand breaks⁵. This complex localizes to double-strand break sites on chromosomes forming foci that can be visualized with electron and fluorescent microscopy. Foci remain co-localized with double-strand breaks until the break has been repaired⁸. 53BP1, also known as tumor suppressor p53 binding protein 1, has been shown to colocalize with MRN foci on DNA double-strand breaks⁹. It plays an important role in signaling DNA damage and is necessary for both intra-

S-phase and G₂-M checkpoints. Misregulation of expression or localization of components of this double-strand break repair complex can predisposes affected cells to oncogenesis.

Previous studies have shown that DNA double-strand breaks are increased in melanoma cells as detected by histone H2AX phosphorylation 1,2. In the current study, we compared cells from conventional nevi and mildly or severely atypical nevi to melanoma cells to determine if Rad50 or 53BP1 are misregulated in melanoma. Our staining results show significant differences in expression and localization of Rad50 between melanoma cells and nevi cells. Particularly, expression of Rad50 was greatly increased in most melanoma cases (9/11) and was detected only in the cytoplasm in eight of these nine cases. Conversely, Rad50 positive staining occurred in less than 30% of nevi cases and in these cases Rad50 was detected in the nucleus and not the cytoplasm. This indicates that Rad50 expression is increased in melanoma and is almost uniformly found in the cytoplasm, not in the nucleus as seen in positive staining nevi cases. In contrast, there were no significant differences seen in the expression and localization of 53BP1 in these tissues.

To our knowledge, this is the first study to demonstrate activation of a double-strand break repair pathway in melanoma, as seen by an increase in expression of Rad50. We have also shown that Rad50 is mislocalized to the cytoplasm of melanoma cells from patient samples. This altered localization may be inhibiting double-strand break repair and could be responsible for the increase in double-strand breaks seen in melanoma 1,2. Interestingly, Rad50 has previously been shown to be mislocalized into the cytoplasm in melanoma cell lines after UV radiation, indicating a possible link between UV irradiation and altered trafficking of Rad50 in melanoma 15. In addition, another component of the MRN complex NBS1, or nibillin, has a nuclear export signal. Nibillin has recently been shown to also transport into the nucleus after gamma-radiation in 3T3 fibroblast cell lines 16. Therefore, trafficking of MRN complex components can occur after UV or gamma radiation. Thus, the abnormal localization of Rad50 to the cytoplasm of melanoma cells may indicate an association between UV irradiation and inhibition of DNA double-strand break repair in melanoma.

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Figure 1.

Immunostaining results for Rad50 in conventional melanocytic nevus (A, shown at 100X) and in severely atypical melanocytic nevus (B, shown at 100X). In contrast, Rad50 staining was increased and largely cytoplasmic in invasive melanoma (C, shown at 200X and D, shown at 400X). Another invasive melanoma case with increased staining of Rad50 that is largely cytoplasmic is also shown (E, shown at 200X and F, shown at 400X).

Figure 2. Immunostaining results for 53BP1 in mildly atypical nevus (A, shown at 100X) and in melanoma (B, shown at 100X) show little change in staining pattern and intensity.

TABLE 1

Demographic information for cases used in this study.

	Conventional or mildly atypical nevi	Severely atypical nevi	Melanoma
# of cases	11	7	11
Age	47	47	52
Men	3	3	5
Women	8	4	6
Location			
Face	4	1	4
Torso	5	3	4
Extremities	2	3	3

Demographic composition and location of melanocytic lesions. Chi squared anaylasis did not reveal a statistically different between the melanocytic lesions for age, sex, or the lesions locations (all are p>0.5).

TABLE 2

Summary of the Rad50 staining results showing an increase in Rad50 staining in melanoma compared to melanocytic nevi.

Staining results (intensity and % of cells positive)				
	0	1+	2+	3+
Conventional or mildly Atypical Nevi	7	4	0	0
Severely Atypical Nevi	7	0	0	0
Melanoma	1	1	3	6

Summary of the 53BP1 staining results showing little change in 53BP1 staining pattern and intensity between melanocytic nevi and melanoma.

TABLE 3

Staining results (intensity and % of cells positive)				
	0	1+	2+	3+
Conventional or mildly Atypical Nevi	0	1	4	6
Severely Atypical Nevi	0	0	5	2
Melanoma	0	0	5	6