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Implications of DNA Damage and Induction of DNA Repair Gene Expression in Cutaneous vs Mucosal Melanoma

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Abstract

Background: This study addressed implications of DNA damage and repair in radiation induced cutaneous melanoma (CM) compared to de novo arising mucosal melanoma (MM). The role of DNA repair was assessed through two DNA repair genes: the human DNA repair genes *XRCC3* and *RAD5.* These genes were selected based on significant homology to the radio-resistant *Deinococcus radiodurans RecA* (46.8% and 42.9% homology, respectively).

Methods: DNA damage in melanoma was assessed and quantified by immunoassay for a marker of DNA damage, 8-hydroxy-2′-deoxyguanosine (8-OHdG). Gene expression analysis was measured by RT-qPCR.

Results: In cutaneous melanoma, DNA damage was significantly higher in tumor than adjacent non-malignant tissue ($p = 0.001 < 0.05$). In contrast, for MM, DNA damage was similar in the non-malignant tissue and tumor ($p = 0.965 > 0.05$). Alcohol use was correlated with higher DNA damage in the MM ($p = 0.036 < 0.05$) than in the cutaneous melanoma patients ($p = 0.104 > 0.05$). The high DNA damage in mucosal tissue was not accompanied by induction of *XRCC3* and *RAD51* expression, compared to non-malignant tissue adjacent to CM.

Conclusions: These observations are consistent with a pre-cancerous condition in MM, one in which repair functions are not induced and DNA damage is allowed to accumulate. Defects in repair functions may increase susceptibility to therapy with DNA damaging agents.

Introduction

Cutaneous Melanoma (CM) is one of the most common and aggressive types of cancer; the prognosis of the patient is directly related to early detection of the disease. Melanoma is most commonly diagnosed by physical examination of the skin, upon observation of an abnormal mole or lesion [1]. Mucosal melanomas are not well characterized. They arise in mucosal tissues apparently in the absence of radiation. The cause of these tumors, therefore, is unclear. Mucosal melanoma is rare compared to CM and is often diagnosed at a much later and more advanced stage [2]. By studying the human repair genes homologous to those associated with DNA repair in *Deinococcus*, there is potential to discover new pathways of mutagenesis, pathological mechanisms, and mutations that could provide early detection markers or targets for therapies [3]. Evaluating the amount of DNA damage associated with the two types of melanoma could also lead to greater clarity on how the two melanomas are different and how each may arise.

Deinococcus radiodurans is an extremophilic bacterium found in many diverse geographical areas. It was first

characterized in 1956 when a can of meat had spoiled after exposure to X-rays. A culture from the spoiled meat produced red pigmented, gram positive cocci. Further examination of the bacterium yielded evidence that suggested it was highly resistant to damage caused by radiation as well as damage caused by hydrogen peroxide, UV light, and desiccation [4]. The ability to withstand the damaging effects of such harsh agents has led to many industrial uses for the organism, such as environmental reclamation from toxic spills of heavy metals and other hazardous wastes.

Conservation of structure and function from bacteria to vertebrates occurs in key genes. A well-known example is the mismatch repair system in *E. coli* (*MutL*, *MutS*) and *MLH1, MSH2* loss causing microsatellite instability in Lynch syndrome. Projects have addressed the ability of bacterial gene homologs to convey resistance to other organisms. In a study conducted by Wen et al. [5], *Deinococcus* gene (*pprI*) was integrated into a human lung epithelial cell line (BEAS-2B). The transformed human lung cells demonstrated increased radiation resistance.

The human DNA repair protein *XRCC3* has 46.8% homology to *Deinococcus Rec.* The *XRCC3* gene has been studied and correlated with melanoma in the past but with conflicting results. In a meta-analysis conducted by Zeng et al. [6], the *XRCC3* rs861539 (T241M) polymorphism was found to have no association with CM. Conversely, in a meta-analysis conducted by Fan et al. [7], the same polymorphism was found to be correlated with melanoma. This meta-analysis initially found no correlation between the *XRCC3* T241M polymorphism and melanoma when analyzing 6 case-control studies that included 2,133 patients and 3,141 controls. However, in a subsequent meta-analysis of 3 studies that consisted of 500 cases, there was a significant association made between the *XRCC3* T241M polymorphism and melanoma [2]. These studies have analyzed the mutational status in *XRCC3*in regard to melanoma but do not address gene expression. The loss of or decrease in expression of the wild type gene may have mechanistic significance in radiation sensitivity/resistance.

Human *RAD51* is another gene that has homology (42.9%) to *Deinococcus RecA*. *RAD51* is physically coupled with *XRCC3* when homologous DNA repair is initiated. In an early study by Bishop et al. [8], *RAD51* was shown to be necessary for initiation of homologous DNA repairs and stabilized by *XRCC3*, increasing the effectiveness of DNA repair. A separate study demonstrated the importance of the coupling between *RAD51* and *XRCC3*, reporting a 25-fold decrease in error-free double strand DNA breaks when XRCC3 was absent [9].

XRCC3 and *RAD51* have implications in different types of cancer, including melanoma. Thus, data regarding the role of *XRCC3*/*RAD51* in developing melanoma and much of the literature on somatic variants is conflicting. This study will address the role of *XRCC3*/*RAD51*gene expression in melanoma.

Methods

The patient cohort for this study included 20 CM and 20 MM samples collected in the form of formalin-fixed paraffin embedded tissue. Data regarding gender, alcohol use, and smoking status were also obtained from patient medical records, followed by de-identification of the patients. A 5 micron section (at least 2 mm^2) of each tissue underwent a hematoxylin and eosin stain (H&E) and was reviewed by a pathologist to confirm the presence and location of tumor and non-malignant tissue on the slide. After the pathologist reviewed each H&E slide, DNA was extracted from dewaxed tissue by microdissection.

DNA damage was assessed using an ELISA method detecting 8-hydroxy-2′-deoxyguanosine (8-OHdG; Epigentek, Farmingdale, NY, IN) according to manufacturer's protocol. 8-OHdG is a biomarker that has strong associations to DNA damage caused by many carcinogenic agents, including radiation from UV light. Normal (non-malignant) tissue and malignant tissue from both cutaneous and mucosal specimens were analyzed. The ELISA color readings were used to quantify the relative and absolute amounts of 8-OHdG. The equation used to calculate the relative amount of 8-OHdG was

$$8-\text{OHdG}\% = \frac{(Sample\ OD - NC\ OD) \div S}{(PC\ OD - NC\ OD) \div P} X100\%$$

where OD is the optical density obtained from the microplate reader from the sample, negative control (NC) and positive control (PC), S is the amount of sample DNA in ng, and P is the amount of positive control in ng.

Gene expression was measured in RNA isolated from tumor and adjacent non-malignant tissue for each melanoma sample with the Recover All isolation system (Life Technologies, Carlsbad, CA) according to the manufacturer's protocol. RNA was converted to cDNA with MMLV Reverse Transcriptase (Invitrogen) in a pre-programmed thermocycler. The cDNA was analyzed

by TaqMan quantitative PCR on the QuantStudio 6 flex real time thermal cycler (Life Technologies). Microsoft excel was used to calculate relative expression from the C_T values from *XRCC3*and *RAD51* and C_T values from corresponding β2-microglobulin.

Statistical analysis

A review of the current literature on *Deinococcus* was performed to find specific genes that confer radiation resistance. The *Deinococcus* gene of interest is *RecA*, which has been found to be essential for radiation resistance in *Deinococcus*. Using the Basic Local Alignment Search Tool (BLAST) hosted by uniprot.org, *RecA* was compared to the human proteome. Once the search was completed, the results were changed from score (a numerical grade that compares homology) to % identity. The human homologs that displayed the highest % identity to the *Deinococcus* homologs were chosen to be evaluated in this study. A second pBLAST-search was conducted with identical parameters in the NCBI protein database to confirm the initial BLAST-search.

Non-parametric statistics were used to analyze the data obtained in this study. Tissue type (cutaneous normal tissue, cutaneous tumor tissue, mucosal normal tissue, and mucosal tumor tissue) was compared for median DNA damage by using the Kruskal-Wallis test. The Mann-Whitney U test was used to compare the use of alcohol to median DNA damage. The Pearson Chi-Square test was used to see if there was a significant association between the expression of *XRCC3*/*RAD51* and tumor type. The data was dichotomized in a way where neither or one of the genes were expressed or both of the genes were expressed.

Results

DNA damage analysis

A Kruskal-Wallis statistic was used to determine if there is a significant difference in DNA damage between the tumor and normal tissue of each type of melanoma. A significant difference ($p = 0.001 < 0.05$; Figure 1) in DNA damage was observed between the normal (non-malignant) tissue adjacent to tumor tissue in CM.

The graph in figure 1 shows the significant difference between the DNA damage observed in the cutaneous normal and cutaneous tumor tissue, with no significant difference in DNA damage between the mucosal normal and mucosal tumor tissue (confirmed by Mann-Whitney U test; $p = 0.965 > 0.05$). It is also evident that DNA damage between the cutaneous tumor and both the mucosal normal and tumor tissue are relatively equal in damage ($p = 0.837 > 0.05$). Results of the same analysis done on a subset of the MMs, sinonasal locations only, were the same as those observed for the MM group.

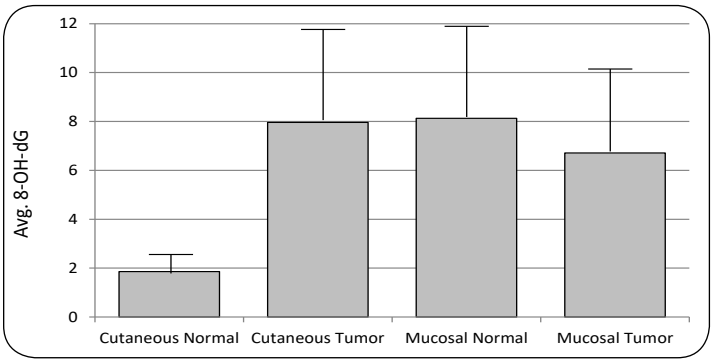

Figure 1: Measurement of 8-OHdG by tumor type based on quantification according to the formula in the methods section. Normal mucosal tissue had more DNA damage than normal cutaneous tissue, similar to levels in tumor tissues.

The effects of smoking and alcohol use on each type of melanoma was also assessed (Figure 2). Smoking was found to have no significant correlation in either type of melanoma. Alcohol use, however, was found to have a significant correlation ($p = 0.036 < 0.05$) in the mucosal tumors but no significant correlation in the cutaneous tumors ($p = .104 > 0.05$).

Homology search

XRCC3 and *RAD51* are human DNA repair genes that encode for proteins of the same name. *XRCC3*/*RAD51*are components of a complex responsible for repairing DNA double stranded breaks via homologous recombination repair. *XRCC3* was found to be 46.8% identical to the *Deinococcus* DNA repair protein *RecA*. *RecA* was also found to share 42.9% identity to the human DNA repair protein *RAD51*.

Repair gene expression

XRCC3 and *RAD51* gene expression data was measured and normalized to β2-microglobulin. The data was dichotomized because *XRCC3* and *RAD51* are thought to be physically joined in a complex when accurately repairing DNA. *RAD51* can repair DNA on its own but at a 25-fold decrease in error free homologous DNA repair [9]. Expression of both *XRCC3* and *RAD51* was found more frequently in the tumor specimens than in the normal tissues (Table 1; $p = 0.027 < 0.05$). Since the DNA damage

Table 1: Gene expression of XRCC3 and RAD51.

Gene expression	Tumor Type				
	Normal Cutaneous	Tumor Cutaneous	Normal Mucosal	Tumor Mucosal	Total
Neither or one expressed	11	11	11	9	42
Both expressed	0	6	0	4	10
Total	11	17	11	13	52

in the mucosal normal tissue was found to have almost an equal amount of DNA damage as the cutaneous tumor tissue, the expression of *XRCC3* and *RAD51* were expected to be similar between the two but in fact the opposite was found to occur.

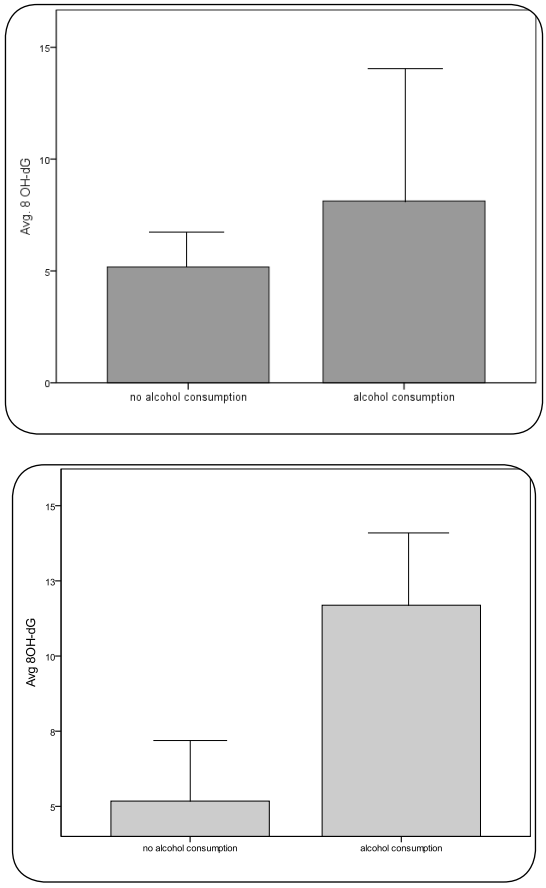

Figure 2: Alcohol use resulted in increased DNA damage in mucosal tumors, but not in cutaneous tumors. Alcohol use in cutaneous tumor (left; p =.104 > 0.05) and mucosal tumor (right; p = 0.036 < 0.05).

Discussion

The DNA damage assessment of the CM patients supports the expected relatively low levels of DNA damage in the non-malignant cutaneous tissue and higher levels of DNA damage in the cutaneous tumor tissue. The DNA damage analysis of the mucosal patients did not fit this pattern. High amounts of DNA damage were seen in both the non-malignant and tumor tissue in the mucosal samples. There are several explanations for the high level of DNA damage observed in the non-malignant mucosal tissue. All are based on the physical proximity of the non-malignant tissue tested to the mucosal tumor. A localized field effect may be in place, including pre-cancerous DNA damage and lack of its repair. Predisposition is another possibility, however this would likely show effects on other organs and tissues not seen in the MM cases. The results do provide evidence that CM and MM are two distinct diseases that afflict melanocytes with differing mechanisms of pathogenesis.

DNA homologous repair systems are induced upon DNA damage. As predicted, repair genes *XRCC3* and *RAD51* are not coordinately expressed in non-malignant cutaneous tissue with low levels of DNA damage and are expressed in both cutaneous and mucosal tumor with DNA damage. None of the non-malignant mucosal tissues tested, however, showed induction of both genes. This suggests that DNA damage goes unrecognized in the non-malignant tissues adjacent to the MM. These results also support the idea that MM may progress from a pre-cancerous state, one in which DNA damage is allowed to occur until a tumor phenotype is evident. This pre-cancerous state affecting melanocytes could play a role in MM and provide a somatic predisposition in these cells, where DNA damage is not recognized and/or improper cell cycle regulation may not be implemented correctly. A pre-cancerous state in melanocytes could be a confounding factor in the characterization of this type of cancer and lead to more specific treatment options.

Although smoking history showed no significant association, there was an association between alcohol consumption and high DNA damage in both tumor types, more so in the MM cases. The mucosal tissues in the

cases studied may have sensitivity to alcohol leading to DNA damage. Patients with MM may lack the ability to effectively repair damage caused by chemical exposure as in the case of alcohol consumption and could potentially develop a tumor in the affected/damaged areas.

With regard to the gene expression data, both *XRCC3* and *RAD51* were expressed more frequently among the tumor specimens compared to normal (non-malignant) tissues; this observation was not surprising, as a malignant cell would require DNA repair mechanisms in order to compensate for the apparent damage. The DNA damage analysis would suggest that the adjacent normal mucosal tissue would exhibit similar expression of *XRCC3* and *RAD51* as the cutaneous tumor tissue and the mucosal tumor tissue but this was not observed. The expression pattern more closely resembled the expression found in cutaneous normal tissue, tissue that was observed to have significantly less damage. These rare mucosal tumors, therefore, might arise from a delay of repair in cells with a particular genetic background, until a malignant state is achieved. Furthermore, with an uncoupling of induction of *XRCC3* and *RAD51* in mucosal tissue, incidental DNA damage would accumulate, increasing the likelihood of tumor development.

XRCC3 and RAD51 proteins accomplish DNA repair by homologous recombination where a damaged chromosome is repaired using the undamaged copy as a template. If one homologue is damaged and the damage goes unrecognized, the cell will continue to proliferate but with one defective chromosome. Should damage occur to the second copy of the chromosome, the homologous recombination repair pathway would be ineffective when tasked with repairing the DNA as the first chromosome that would be used as a template for repair has preexisting damage. In this case, late induction of homologous repair may actually facilitate the proliferation of malignant cells. A study conducted by Yao et al. [10], has also suggested there may be a pre-cancerous condition associated with MM. The Yao study followed a 54-year-old patient who presented with a nasal melanocytic hyperplasia without the presence of melanoma. The patient was serially biopsied in order to track the status of the melanocytic region. Over a period of four years, the patient developed a malignant melanoma in *situ* and eventually succumbed to his condition.

Previous studies in our laboratory investigated epigenetic contributions to the mucosal phenotype through promoter methylation of the pro-apoptosis genes *PTEN* and *BAX*. Significant methylation was observed in *PTEN* in sinonasal tumors compared to non-malignant tissue. This observation predicts survival of cells despite the presence of DNA damage. More studies regarding the implication of apoptosis in sinonasal (mucosal) melanoma could lead to further characterization and more options for treatment. Another gene that could have major implications in developing MM is *BAP1. BAP1* is a de-ubiquinating protein that has many functions including apoptosis, cell cycle regulation, and DNA damage and repair. A loss of function in *BAP1* due to lack of expression or point mutations has been observed to be significantly correlated with developing melanoma in the oral mucosa [11]. If the MM patients are more sensitive to the damaging effects caused by alcohol or are unable to accurately repair the damage potentially caused by alcohol, damaged cells that normally would proceed to apoptosis may survive as a result. Carcinogens in smokeless ("chewing") tobacco are also linked to increased epithelial cancer incidence. No data was available on smokeless tobacco use in this patient group.

The most common treatment option for both cutaneous and MM is tumor excision. Once the cancer becomes metastatic radiation therapy and immunotherapy are also implemented. Combining chemotherapy drugs with immunotherapy drugs such as interferon-alpha and/or interleukin-2 may be more effective than the chemotherapy alone. Previously observed aberrant promoter hypermethylation of the *PTEN* gene in MM patients suggests that an epigenetic approach such as methyltransferase inhibitors used in myeloid cell malignancies could be effective against metastatic tumor in MM patients. The presumptive lack of DNA repair presented here may sensitize tumor cells to alkylating agents such as dacarbazine or temozolomide. Further analysis of these relatively rare tumors may reveal additional distinctive characteristics targetable by therapy.

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