

Patterns of Expression of DNA Repair Genes and Relapse From Melanoma

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Abstract

Purpose: To use gene expression profiling of formalin-fixed primary melanoma samples to detect expression patterns that are predictive of relapse and response to chemotherapy.

Experimental Design: Gene expression profiles were identified in samples from two studies (472 tumors). Gene expression data for 502 cancer-related genes from these studies were combined for analysis.

Results: Increased expression of DNA repair genes most strongly predicted relapse and was associated with thicker tumors. Increased expression of *RAD51* was the most predictive of relapse-free survival in unadjusted analysis (hazard ratio, 2.98; $P = 8.80 \times 10^{-6}$). *RAD52* (hazard ratio, 4.73; $P = 0.0004$) and *TOP2A* (hazard ratio, 3.06; $P = 0.009$) were independent predictors of relapse-free survival in multivariable analysis. These associations persisted when the analysis was further adjusted for demographic and histologic features of prognostic importance (*RAD52* $P = 0.01$; *TOP2A* $P = 0.02$). Using principal component analysis, expression of DNA repair genes was summarized into one variable. Genes whose expression correlated with this variable were predominantly associated with the cell cycle and DNA repair. In 42 patients treated with chemotherapy, DNA repair gene expression was greater in tumors from patients who progressed on treatment. Further data supportive of a role for increased expression of DNA repair genes as predictive biomarkers are reported, which were generated using multiplex PCR.

Conclusions: Overexpression of DNA repair genes (predominantly those involved in double-strand break repair) was associated with relapse. These data support the hypothesis that melanoma progression requires maintenance of genetic stability and give insight into mechanisms of melanoma drug resistance and potential therapies. *Clin Cancer Res*; 16(21); 5211–21. ©2010 AACR.

The established predictors of outcome for melanoma patients relate to the histologic characteristics of the primary tumor (Breslow thickness, presence of ulceration (1), mitotic rate (2)), tumor site, sex (2), and age (3). These histologic characteristics are used to estimate prognosis as part of the American Joint Committee on Cancer staging system (1) and in various algorithms (2, 3), but much of the variance in survival remains unexplained. To identify prognostic and predictive biomarkers and to better establish the biological pathways of relevance, molecular studies on primary melanomas are necessary.

Genomic and transcriptomic studies in melanoma tumors have hitherto been few, using limited numbers of samples because of the small size of melanoma primaries. A number of studies using cryopreserved tissue have identified gene signatures or altered expression in groups of genes within a biological pathway that are predictive of survival or progression of a tumor (4–7). One such study by Kauffmann et al. (7) showed in 60 cryopreserved melanomas that overexpression of DNA repair genes was associated with metastasis, a finding validated in a further seventeen tumors.

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Translational Relevance

This article presents gene expression results from melanoma primary tumors in a study using formalin-fixed tissue. This is the largest gene expression study to date in melanoma and has identified prognostic and potential predictive markers. Overexpression of DNA repair genes was shown to be associated with reduced relapse-free survival, thicker tumors, and tumors with higher mitotic rate. Preliminary data are also reported suggesting that DNA repair genes are overexpressed in tumors from patients who do not respond to chemotherapy. This study highlights the importance of DNA repair gene expression in the progression of melanoma and gives insight into the biological basis of chemoresistance. It also provides preliminary evidence that this may be of predictive value in terms of resistance to chemotherapy. This article furthermore confirms that gene expression studies are possible on a large-scale using formalin-fixed tissue and may be used to identify further prognostic and predictive markers.

We have recently reported the use of Illumina cDNA mediated annealing, selection, extension, and ligation (DASL) assay to discover prognostic biomarkers in formalin-fixed, paraffin-embedded primary melanomas (8). We obtained gene expression detection from 76% of unselected primary tumor blocks and identified increased expression of osteopontin as being the single change most predictive of relapse in samples from a cohort of melanoma patients from the United Kingdom (8). Here, we report that, among the 502 cancer-related genes studied, the group of genes whose expression was most strongly predictive of relapse overall in a larger pooled data set were a number of DNA repair genes or genes involved in this process (predominantly in double-strand break repair). We went on to explore the possibility that increased expression of these genes might also be useful predictive biomarkers of response to chemotherapy.

Materials and Methods

Patient samples

Formalin-fixed, paraffin-embedded cutaneous primary tumors were identified from two study sets as previously described (ref. 8; Supplementary Table S1). In study 1, the Leeds Melanoma Cohort Study (9), population ascertained melanoma patients were recruited to a cohort in the period from 2000 to 2006. Two hundred fifty-four blocks identified from participants within the cohort with tumors thicker than 0.75 mm with the longest follow-up were used in this study. In study 2, 218 blocks identified from participants with the longest follow-up in a study designed to identify predictors of sentinel node positivity and relapse were sampled. Both studies were approved

by United Kingdom national ethics committees (Multicentre Research Ethics Committee and Patient Information Advisory Group). Here, we report the combined gene expression data analysis from these studies.

Sample preparation

Primary tumor blocks were identified, and a hematoxylin and eosin-stained slide was examined to identify the deepest part of the tumor containing the lowest admixture of inflammatory or stromal cells. This area was marked using a fine-tipped permanent marker, and a 0.8-mm tissue microarray needle was used to sample the tumor block horizontally. This technique produces sufficient RNA yields from the deepest parts of tumors in a study using hundreds of samples while preserving the block for future pathology (8).

RNA extraction and quality control

Tissue cores were dewaxed using xylene, which was removed using two changes of absolute ethanol. RNA was extracted from tissue cores using the High Pure paraffin RNA kit (Roche Diagnostics Ltd.) according to the manufacturer's protocol and eluted in 26 μ L nuclease-free water. Details of RNA quality control procedures have been described previously (8).

Gene expression profiling with the DASL assay

The DASL assay was done by ServiceXS using the Human Cancer Panel (Illumina, Inc.). The Cancer Panel includes 1,536 unique sequence specific probes targeting 502 genes (listed in Supplementary Information). Each gene is targeted in three locations by three separate probe pairs designed by a proprietary algorithm (10).

Data preprocessing

The data were normalized using Beadstudio software (Illumina) before exporting to STATA version 10 for statistical analyses. The normalization methods used were background correction, cubic spline smoothing (11), and plate scaling. Normalization was conducted relative to a synthetic reference array, which was created in each study by averaging all melanoma samples as described previously (8). Each study was normalized separately before the data sets being combined for analysis.

Statistical methodology

In the DASL assay, the number of genes detected in each sample (probe signal significantly greater than average signal from negative controls with $P < 0.05$) was used as a measure of the quality of the results. Samples with <250 detected genes were classified as "failed" and excluded from further analysis.

Mean gene expression was used for replicate samples. Fold change calculations were done using normalized data. Differential gene expression, survival analyses, and chemotherapy analyses were done using log-transformed normalized data (\log_2). Within the sample sets, expression of each gene was compared with histologic features of interest or response status to chemotherapy using

linear regression. Chemotherapy response was defined as stable disease, mixed response, partial response, or regression of disease determined radiologically (by computed tomography scan) following two or three cycles of chemotherapy.

Survival analysis was done using a Cox proportional hazards model to estimate hazard ratios and 95% confidence intervals (95% CI) for each gene. Significance values were ranked to identify the genes most differentially expressed between groups of interest. Relapse-free survival was defined as the period between diagnosis and date of first relapse at any site. To identify DNA repair genes that were independently predictive of survival, a multivariable Cox proportional hazards model was generated using all DNA repair genes significantly associated with Breslow thickness, mitotic rate, or survival in the previous analyses.

Ingenuity Pathway Analysis (Ingenuity Systems) was used to identify the pathways and networks of genes co-regulated with the most significant DNA repair genes. To

achieve this goal, a three-stage approach was adopted. (a) To summarize into one variable the expressions of the most significant DNA repair genes, principal components analysis was applied to the pooled data set adjusting for study. (b) r was calculated between the first principal component (summary variable) and each of the genes on the cancer panel. (c) The Ingenuity Pathway Analysis was interrogated to find pathways and networks involving genes significantly correlated with the principal component at a level of $P < 10^{-10}$.

For analyses identifying genes predictive of survival and genes associated with histologic features, the Bonferroni method was used to correct for multiple testing (12); therefore, the significance level was set at 0.0001 for these analyses. For chemotherapy and survival analysis assessing the expression of single genes in each test, the significance level for highlighting results of interest was set at 0.05. All analyses were adjusted for the study from which the patients were recruited and survival analysis was further adjusted for whether the patient

Table 1. Top 25 genes with expression levels in tumors most strongly related to relapse-free survival (adjusted for study and SNB status)

Gene	Mean fold change between relapsers and nonrelapsers	Hazard ratio	95% CI	Significance value
<i>RAD51</i>	1.22	2.98	1.84-4.83	8.80×10^{-6}
<i>TK1</i>	1.12	4.68	2.29-9.58	0.00002
<i>ING1</i>	1.11	5.87	2.59-13.32	0.00002
<i>RAD52</i>	1.16	4.49	2.24-9.02	0.00002
<i>TFAP2C</i>	0.83	0.49	0.35-0.69	0.00004
<i>CCNA2</i>	1.16	2.66	1.67-4.23	0.00004
<i>BIRC5</i>	1.19	2.74	1.67-4.50	0.00007
<i>TOP2A</i>	1.12	3.84	1.96-7.52	0.00009
<i>CDH13</i>	0.80	0.48	0.33-0.71	0.0002
<i>HLF</i>	0.78	0.45	0.30-0.69	0.0002
<i>SPP1</i>	1.41	1.67	1.26-2.21	0.0003
<i>ITGB4</i>	0.79	0.58	0.43-0.79	0.0004
<i>FLI1</i>	0.83	0.51	0.35-0.75	0.0005
<i>MLF1</i>	1.15	2.74	1.55-4.83	0.0005
<i>EPHB4</i>	0.89	0.41	0.25-0.69	0.0008
<i>VEGFB</i>	1.04	9.84	2.57-37.71	0.0009
<i>ELK3</i>	0.95	0.20	0.07-0.53	0.001
<i>RAD54B</i>	1.18	2.08	1.33-3.25	0.001
<i>E2F1</i>	1.10	2.78	1.47-5.25	0.002
<i>WNT2</i>	1.34	2.49	1.41-4.41	0.002
<i>TERT</i>	1.29	1.79	1.24-2.58	0.002
<i>CDC2</i>	1.22	1.84	1.26-2.70	0.002
<i>CCNH</i>	1.08	3.39	1.58-7.27	0.002
<i>IFNGR1</i>	0.89	0.47	0.29-0.76	0.002
<i>PML</i>	0.96	0.21	0.07-0.58	0.003

NOTE: DNA repair genes or those associated with this process are highlighted in dark grey and genes involved in the cell cycle or cell proliferation are highlighted in light grey. Hazard ratios for reduced relapse-free survival are calculated for doubling of gene expression value. P values are from the proportional hazards model.

Table 2. The association of genes involved in DNA repair with relapse-free survival in univariable and multivariable models

Gene	(i) Association between single genes and RFS						(ii) Multivariable model with all DNA repair genes predictive of RFS, Breslow thickness, or mitotic rate						
	1. Analysis adjusted for study and SNB status only			2. Analysis further adjusted for age and sex of patient and site of tumor			1. Analysis adjusted for study and SNB status only		2. Analysis further adjusted for age and sex of patient and site of tumor		3. Analysis further adjusted for Breslow thickness, ulceration, and mitotic rate of tumor		
	Fold	HR (95% CI)	P	HR (95% CI)	P	HR (95% CI)	P	HR (95% CI)	P	HR (95% CI)	P	HR (95% CI)	P
<i>RAD51</i>	1.22	2.98 (1.84-4.83)	8.80 × 10 ⁻⁶	3.22 (1.95-5.32)	4.67 × 10 ⁻⁶	2.55 (1.46-4.47)	0.001	1.91 (0.98-3.72)	0.06	2.13 (1.09-4.18)	0.03	2.01 (0.98-4.15)	0.06
<i>RAD52</i>	1.16	4.49 (2.24-9.02)	0.00002	4.32 (2.12-8.82)	0.00006	2.79 (1.27-6.15)	0.01	4.73 (1.99-11.26)	0.0004	4.20 (1.77-9.95)	0.001	3.15 (1.27-7.76)	0.01
<i>TOP2A</i>	1.12	3.84 (1.96-7.52)	0.00009	3.87 (1.97-7.61)	0.00009	3.39 (1.62-7.11)	0.001	3.06 (1.33-7.06)	0.009	2.78 (1.20-6.45)	0.02	3.14 (1.23-8.00)	0.02

NOTE: Part (i) of the table shows the association between single genes and survival. Data adjusted for study and SNB status only are presented in column 1. The association was adjusted for sex, patient age, and tumor site (as known predictors of outcome) in column 2. In column 3, further adjustment is made for known histologic predictors of outcome: Breslow thickness, mitotic rate, and ulceration. Part (ii) of the table presents results of a multivariable Cox proportional hazards model with DNA repair genes identified as being predictive of relapse-free survival, Breslow thickness, or mitotic rate (listed in Table 3). Significance values for genes that are independent predictors of survival are highlighted in bold. It can be seen that upregulation of all three genes was associated with reduced relapse-free survival when adjusted for other known prognostic factors. *RAD52* and *TOP2A* remained significant in multivariable analysis. Abbreviations: RFS, relapse-free survival; HR, hazard ratio.

had undergone a sentinel node biopsy and the effect of the biopsy result (SNB status). Analyses were further adjusted for demographic and histologic factors of prognostic importance in melanoma.

Target validation by quantitative real-time reverse transcription PCR

DNA repair genes identified as significantly associated with relapse were further investigated by quantitative real-time reverse transcription PCR on samples from study 1 using Taqman Gene Expression Assays (Applied Biosystems). The comparative Ct method (13) was used to calculate relative fold changes in gene expression (Supplementary Information). To identify genes associated with chemotherapy response, a selection of samples from patients treated with chemotherapy was analyzed using a customized Taqman Array microfluidic quantitative real-time reverse transcription PCR card (Chemosensitivity Gene Expression Assay, CanTech Ltd.) containing 92 genes known or hypothesized to be involved in cytotoxic resistance or sensitivity as reported previously (14). The comparative Ct method (13) was used to calculate relative fold changes in gene expression between nonresponders and responders to chemotherapy (Supplementary Information).

Results

Patient and tumor characteristics of the two samples sets were similar and are representative of the larger studies

that the test sets were derived from as previously described (ref. 8; Supplementary Table S1).

RNA yields from tumor samples and performance using the DASL assay

Adequate RNA yields were obtained from 361 (76%) of 472 of blocks as described previously (8). Four hundred twenty-three RNA samples, including replicate samples (359 unique samples and 64 replicate samples), were supplied to ServiceXS. Fewer than 250 genes were detected in six (1.4%) samples (failed samples). The failure rate was 2.1% in study 1 and 0.9% in study 2 (8).

Genes predictive of relapse

Genes whose expression levels in tumors were most strongly related to relapse-free survival are presented in Table 1 when analysis is adjusted for study type and SNB status only. The predominant group of genes differentially expressed was DNA repair genes or genes associated with these processes. Within this group, overexpression of *RAD51*, *RAD52*, and *TOP2A* were most predictive of poor relapse-free survival (Table 2) with hazard ratios of 2.98 ($P = 8.80 \times 10^{-6}$), 4.49 ($P = 0.00002$), and 3.84 ($P = 0.00009$), respectively, for a doubling of expression levels. Overexpression of these genes continued to be predictive of relapse-free survival when the analysis was adjusted for host variables known to predict relapse (age, sex, and tumor site) and, when adjusted additionally for Breslow thickness, mitotic rate (number per square millimeter) and the presence of tumor ulceration (Table 2). Expression of *RAD51*

Table 3. DNA repair genes differentially expressed in relation to Breslow thickness and mitotic rate

Gene	Breslow thickness				Mitotic rate			
	Fold changes in gene expression between indicated group and tumors <2 mm thick		Significance level adjusted for study only	Significance level adjusted for age and sex of patient and site of tumor	Fold changes in gene expression between indicated group and tumors with mitotic rate <1/mm ²		Significance level adjusted for study only	Significance level adjusted for age and sex of patient and site of tumor
	2-4 mm	>4 mm			1-6/mm ²	>6/mm ²		
<i>RAD54B</i>	1.20	1.39	1.24×10^{-8}	1.71×10^{-7}	1.29	1.62	5.03×10^{-8}	6.28×10^{-7}
<i>MSH2</i>	1.20	1.25	1.27×10^{-8}	1.52×10^{-7}	1.22	1.37	3.03×10^{-6}	0.00001
<i>RAD51</i>	1.18	1.33	9.18×10^{-8}	3.20×10^{-7}	1.22	1.53	1.36×10^{-8}	7.65×10^{-8}
<i>RAD52</i>	1.07	1.21	9.18×10^{-8}	4.98×10^{-7}	1.07	1.16	0.003	0.009
<i>CHEK1</i>	1.21	1.36	4.63×10^{-6}	0.00005	1.13	1.42	0.00005	0.0004
<i>TOP2A</i>	1.07	1.19	0.00003	0.00007	1.21	1.38	4.12×10^{-9}	8.87×10^{-9}
<i>RAD54L</i>	1.20	1.29	0.00004	0.0004	1.09	1.31	0.001	0.004
<i>BRCA1</i>	1.20	1.25	0.00005	0.0002	1.18	1.48	2.92×10^{-6}	0.00001
<i>BRCA2</i>	1.18	1.20	0.00009	0.0006	1.22	1.32	0.001	0.004
<i>MSH6</i>	1.04	1.08	0.0008	0.001	1.07	1.15	1.30×10^{-6}	1.28×10^{-6}

NOTE: Genes listed were identified as significantly differentially expressed ($P < 0.0001$) in tumors with greater Breslow thickness or mitotic rate using linear regression. Fold changes in gene expression are presented for each tumor thickness and mitotic rate group compared with baseline groups. Analyses are adjusted for study only and further adjusted for age of patient at diagnosis, site of tumor, and sex of patient.

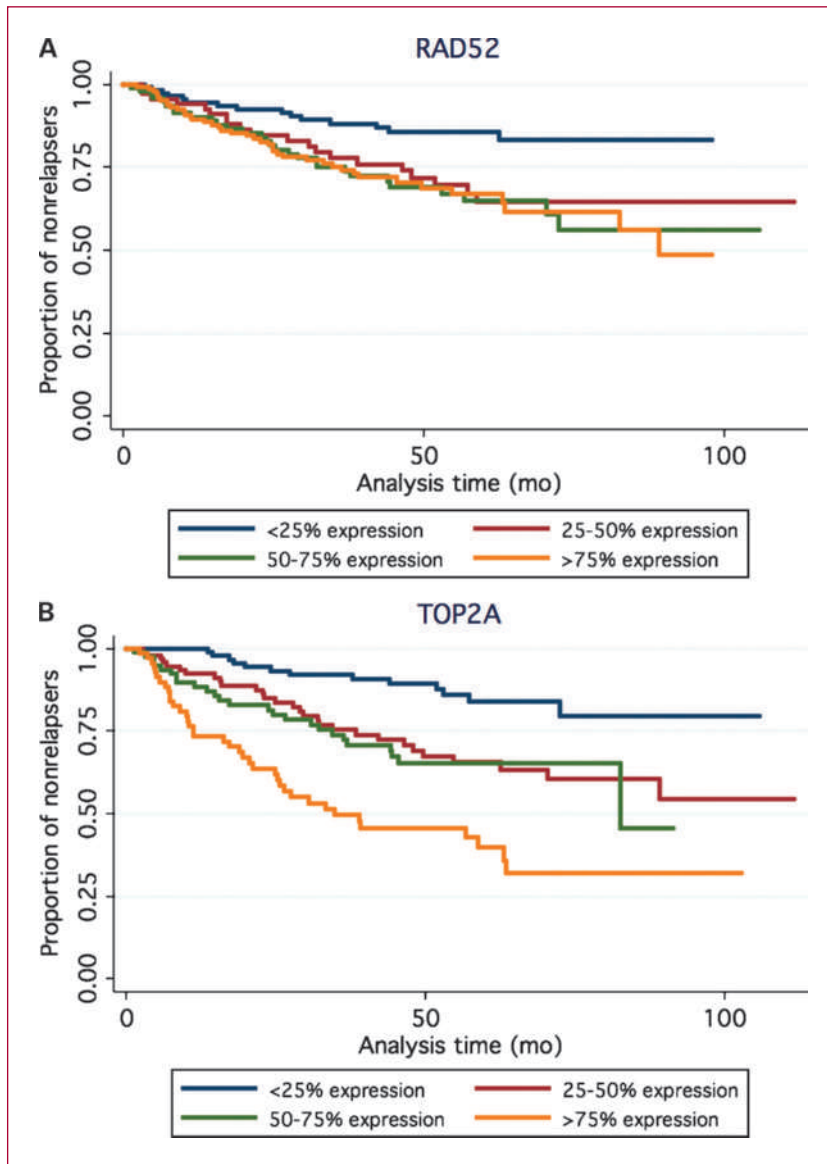


Fig. 1. Kaplan-Meier curves of relapse-free survival for *RAD52* (A) and *TOP2A* (B) gene expression. Survivor functions have been estimated for each quartile of gene expression. Hazard ratios compared with the lowest quartile increase for each successive quartile. Analysis is adjusted for study and SNB status.

was 1.22 times greater in tumors from patients who relapsed versus those that did not; the fold changes between tumors from relapsers and nonrelapsers for *RAD52* and *TOP2A* were 1.16 and 1.12, respectively (Table 1). *RAD54B*, *RAD52*, *TOP2A*, and *RAD51* were also overexpressed in tumors from patients who died versus surviving patients (fold changes of 1.15, 1.11, 1.09, and 1.10, respectively).

Independent predictors of relapse-free survival

Overexpression of *RAD52* and *TOP2A* were independent predictors of poor relapse-free survival in a model with the other DNA repair genes identified (listed in Table 3; multivariable model Table 2 and Fig. 1). In analysis adjusted for study and SNB status only, hazard ratios for *RAD52* and *TOP2A* were 4.73 ($P = 0.0004$) and 3.06 ($P = 0.009$), respectively, for a doubling of

levels. When analyzed by quartiles of *RAD52* and *TOP2A* gene expression, hazard ratios increased for each quartile of gene expression: using the lowest quartile as baseline, for *RAD52*, 25% to 50% expression, hazard ratio 1.57 (95% CI, 0.80-3.09; $P = 0.19$); 50% to 75% expression, hazard ratio 1.79 (95% CI, 0.95-3.37; $P = 0.07$); and >75% expression hazard ratio 2.90 (95% CI, 1.57-5.36; $P = 0.001$); and for *TOP2A*, 25% to 50% expression, hazard ratio 2.82 (95% CI, 1.46-5.47; $P = 0.002$); 50% to 75% expression, hazard ratio 2.67 (95% CI, 1.34-5.29; $P = 0.005$); and >75% expression, hazard ratio 3.59 (95% CI, 1.78-7.24; $P < 0.0001$; Fig. 1). Both *RAD52* and *TOP2A* continued to have a significant independent predictive influence on relapse-free survival when analyses were adjusted for host variables (age, sex, and tumor site) and further adjusted for

Breslow thickness, mitotic rate, and ulceration of the tumor (Table 2).

Gene expression in tumors with poor prognostic histopathologic features

Genes involved in DNA repair were also relatively over-expressed in tumors with greater Breslow thickness and mitotic rate (Table 3).

Target validation by quantitative real-time PCR

To seek corroborative data for the DASL assay results, quantitative real-time reverse transcription PCR was done on study 1 RNA samples for four DNA repair genes identified as associated with relapse. Increased expression of *RAD51* (fold change, 1.11), *RAD54B* (fold change, 1.08), and *TOP2A* (fold change, 1.37) was detected with quantitative real-time reverse transcription PCR in association with reduced relapse-free survival in samples from study 1 compared with fold changes of 1.22, 1.18, and 1.12, respectively, in the merged DASL assay analysis. We were unable to validate the association between *RAD52* expression and survival using quantitative real-time reverse transcription PCR in samples from study 1. There was considerable variation in DASL gene expression results for *RAD52* across the three probes, with results from one probe correlating better with quantitative real-time reverse transcription PCR results than the other two. Because DASL gene expression data is calculated from the mean of three probes, this may explain the discrepancy

between DASL and quantitative real-time reverse transcription PCR results.

Coexpression of genes with DNA repair genes

The expression of most DNA repair genes identified as being associated with reduced survival or poor prognostic histologic features correlated well with each other (Table 2s). An exception was *RAD52*, with expression levels that did not correlate with the levels of *RAD54L*, *BRCA2*, and *TOP2A*.

Principal components analysis of the top 10 significant DNA repair genes (listed in Table 3) identified one component that explained 40% of total variance in DNA repair gene expression while each of the other components explained $\leq 10\%$ (study adjusted). Principal component 1 was also the only one that correlated with each of the 10 genes from which it was generated (r , 0.40-0.74). Association analysis between this component and relapse-free survival, Breslow thickness, and mitotic rate showed that the component was predictive of these phenotypes when adjusted for study, sex, age, and tumor site ($P = 1.1 \times 10^{-7}$ for Breslow thickness, $P = 1.1 \times 10^{-10}$ for mitotic rate, and $P = 3.1 \times 10^{-5}$ for relapse-free survival). None of the other components was predictive of these phenotypes. In Table 4, we report the list of genes correlated with the first principal component at a level of $P < 10^{-10}$. A large number of these genes are associated with cell cycle control (e.g., *CDC2*, *CCNA2*) or DNA repair (e.g., *PCNA*, *BLM*, *XRCC2*).

Table 4. Genes most correlated with principal component 1

Gene	Correlation	P	Gene	Correlation	P
<i>CDC2</i>	0.54	1.3×10^{-27}	<i>XRCC2</i>	0.38	2.4×10^{-13}
<i>TYMS</i>	0.51	8.4×10^{-25}	<i>MYBL2</i>	0.37	3.8×10^{-13}
<i>BIRC5</i>	0.50	1.2×10^{-23}	<i>FYN</i>	-0.37	5.7×10^{-13}
<i>HMMR</i>	0.49	1.8×10^{-22}	<i>XRCC4</i>	0.37	1.2×10^{-12}
<i>CCNA2</i>	0.48	9.0×10^{-22}	<i>PDGFRB</i>	-0.37	1.3×10^{-12}
<i>CDC25A</i>	0.48	1.1×10^{-21}	<i>BCL6</i>	-0.36	2.8×10^{-12}
<i>TK1</i>	0.48	1.7×10^{-21}	<i>CDH11</i>	-0.36	4.0×10^{-12}
<i>PCNA</i>	0.45	2.8×10^{-19}	<i>FOSL2</i>	-0.36	4.4×10^{-12}
<i>CDC25C</i>	0.45	5.6×10^{-19}	<i>MAF</i>	-0.36	5.5×10^{-12}
<i>DEK</i>	0.45	8.6×10^{-19}	<i>MLF1</i>	0.36	5.9×10^{-12}
<i>DCN</i>	-0.44	5.0×10^{-18}	<i>COL18A1</i>	-0.36	6.3×10^{-12}
<i>CDK4</i>	0.42	7.1×10^{-17}	<i>PBX1</i>	-0.35	6.8×10^{-12}
<i>WEE1</i>	0.42	9.9×10^{-17}	<i>JUNB</i>	-0.35	1.1×10^{-11}
<i>ETS2</i>	-0.41	4.9×10^{-16}	<i>IFNGR1</i>	-0.35	2.2×10^{-11}
<i>PDGFRA</i>	-0.41	8.9×10^{-16}	<i>AXL</i>	-0.34	3.5×10^{-11}
<i>BLM</i>	0.40	7.2×10^{-15}	<i>E2F3</i>	0.34	4.2×10^{-11}
<i>ITGB4</i>	-0.39	2.4×10^{-14}	<i>MCF2</i>	0.34	7.7×10^{-11}
<i>CDKN2C</i>	0.39	2.4×10^{-14}	<i>RECQL</i>	0.34	9.0×10^{-11}
<i>E2F1</i>	0.38	1.5×10^{-13}			

NOTE: r 's were adjusted for study. These 37 genes with an r significant at $P < 10^{-10}$ were used in Ingenuity Pathway Analysis to infer cellular and molecular functions and to build gene networks.

The gene network in Supplementary Fig. S1 built using Ingenuity Pathway Analysis contains 35 genes, among which 22 are correlated with the principal component of DNA repair genes at a significance level of $P < 10^{-10}$. The major cellular or molecular functions making up this network are cell cycle and cell death, confirming the strong association between these processes and DNA repair.

Genes predictive of chemotherapy response

Forty-two patients had received chemotherapy (11.9%), so we looked at response to chemotherapy in these indi-

viduals as a pilot study. Of these patients, tumor response following chemotherapy had been recorded for 36 patients. Most patients received dacarbazine (DTIC), with three patients receiving treosulfan as first-line chemotherapy. Six patients (17%) responded to the chemotherapy (DTIC). *RAD51* and *TOP2A* had significantly higher expression in tumors from nonresponders compared with responders (*RAD51* fold change, 1.66; $P = 0.01$; *TOP2A* fold change, 1.43; $P = 0.03$). *BRCA1* was also overexpressed in tumors from nonresponders (fold change, 1.36), but this difference barely reached statistical significance ($P = 0.05$).

Table 5. Genes differentially expressed in tumors unresponsive to chemotherapy using the Chemosensitivity Gene Expression Assay 1 array

	Gene	No. of nonresponders/ no. of responders	Mean fold change in gene expression between nonresponders and responders
DNA repair genes	<i>MSH6</i>	17/4	4.61
	<i>TOPO1</i>	21/4	3.15
	<i>MSH2</i>	16/4	3.06
	<i>XRCC1</i>	13/4	2.95
	<i>XRCC5</i>	22/5	2.87
	<i>TOP2A</i>	17/3	2.53
	<i>XRCC6</i>	22/5	2.47
	<i>MGMT</i>	9/2	2.21
	<i>RAD51</i>	17/3	2.13
	<i>ERCC1</i>	22/5	2.07
	<i>TOP2B</i>	22/4	1.96
	<i>XPA</i>	20/5	1.87
	<i>ATM</i>	18/3	1.81
	<i>BRCA1</i>	17/3	1.77
	<i>ERCC2</i>	18/4	1.73
<i>GTF2H2</i>	21/4	1.51	
<i>MLH1</i>	16/2	1.21	
Genes most overexpressed in nonresponding tumors	<i>TAP4</i>	15/2	24.63
	<i>TS</i>	21/3	24.53
	<i>KI67</i>	9/2	12.06
	<i>MTII</i>	22/5	8.06
	<i>mTOR</i>	21/4	7.33
	<i>cN II</i>	19/5	6.62
	<i>PIK3CA</i>	21/4	5.02
	<i>MSH6</i>	17/4	4.61
	<i>HSP90</i>	22/5	4.51
	<i>SOD 1</i>	22/5	4.40
Genes most underexpressed in nonresponding tumors	<i>MCL1</i>	21/4	0.38
	<i>FAS</i>	16/4	0.45
	<i>FPGS</i>	12/2	0.71
	<i>BAX</i>	21/5	0.86
	<i>P53</i>	19/5	0.92

NOTE: DNA repair genes on the array are listed followed by genes most overexpressed and underexpressed in unresponsive tumors. The number of samples from nonresponders and responders is presented with fold change difference in gene expression between nonresponders and responders.

Thirty-one of these samples (five from responders) were additionally sent for quantitative real-time reverse transcription PCR analysis using the Chemo-sensitivity Gene Expression Assay 1 array in Portsmouth. Genes for which there were <10 results or less than two results for responders were excluded from further analyses. All of the 17 genes involved in DNA repair on the array were overexpressed in tumors from patients who did not respond to chemotherapy (Table 5). These genes included *RAD51* (fold change, 2.13), *TOP2A* (fold change, 2.53), and *BRCA1* (fold change, 1.77), as well as genes involved in nucleotide excision repair (e.g., *ERCC1*, *XPA*), base excision repair (e.g., *XRCC1*), removal of damaged DNA bases (*MGMT*), mismatch repair (e.g., *MLH1*, *MSH2*), and DNA nonhomologous end joining (e.g., *XRCC5*, *XRCC6*; refs. 14, 15). Genes encoding proteins associated with apoptosis, cellular proliferation and membrane transport molecules were differentially expressed in tumors that did not respond to chemotherapy (Table 5; refs. 14, 16). As a result of the small numbers of samples included in these analyses, the power was too low to achieve statistical significance.

Discussion

Cutaneous melanoma carries a good prognosis for most patients, but ~20% overall die of the disease. Response rates to chemotherapeutic regimens are poor at around 12% to 15% (17), and there are no means of identifying patients who might benefit from treatment. Poor progress in identification of prognostic and predictive biomarkers has been in part a result of the fact that primary melanomas are physically small, and pathologists are therefore reluctant to cryopreserve tumor. Using the Illumina DASL assay Cancer Panel, we have produced gene expression profiles, using formalin-fixed material, that have prognostic significance (8), giving hope for larger scale biomarker studies in the future.

In this large study, overexpression of DNA repair genes was associated with reduced relapse-free survival, greater Breslow thickness, and higher tumor mitotic rate. Our results are in agreement with the findings of Kauffmann et al. who showed that overexpression of genes involved in recovery of stalled replication forks was associated with metastasis in cryopreserved tumors (7). It has been hypothesized that, for a melanoma cell to continue to divide and give rise to metastases, the cell needs to maintain genomic integrity, and therefore, those tumors with upregulated genes associated in DNA repair processes (18) prove to be more aggressive. Overexpression of DNA repair genes in melanoma tumors may help to explain why melanoma tumors are so resistant to treatment with chemotherapy and radiotherapy (18), a hypothesis that gains some support from our observations in a relatively small number of chemotherapy treated patients.

Many of the genes we have identified as associated with relapse in these analyses are involved in double-strand break repair through homologous recombination. *RAD51*

is the central protein involved in the double-strand break process and is overexpressed in many tumors (19). *RAD52*, *RAD54*, *BRCA1*, and *BRCA2* are required for double-strand break repair (20), with *RAD52* also involved in single-strand annealing independently of *RAD51* (21). *CHEK1* is involved in cell-cycle control; it encodes a protein kinase that prevents progress of the cell from G₂ to M phase when activated by DNA damage, such as double-strand breaks and stalled DNA replication forks (7, 15). *MSH2* and *MSH6* are mismatch repair genes that code for proteins that repair errors made in base pairing by DNA polymerases during replication (7, 22). *TOP2A* induces transient DNA breaks to allow changes in DNA topology during DNA replication (23). Expression of *TOP2A* closely reflects the proliferative activity of cells (23).

Formation of double-strand breaks and other DNA damage occurs during replication of cells, so it is unsurprising that, in addition to identifying overexpression of DNA repair genes associated with survival, we have also identified overexpression of genes associated with the cell cycle and cell proliferation in tumors from patients who relapse and in patients with thicker tumors and with higher mitotic rates. Using principal component analysis of genes associated with relapse-free survival, Breslow thickness, and mitotic rate, we have shown that many of the genes with expression levels that correlate with the first principal component are involved in DNA repair processes and the cell cycle, indicating that overexpression of genes involved in these pathways is common in tumors with poorer prognosis, perhaps reflecting increased proliferation in these cells. *RAD52* is an exception because expression levels were not correlated with expression of other genes involved in double-strand break repair such as *RAD54L* and *BRCA2*. This may reflect the additional function of *RAD52* in repair by single-strand annealing (21).

Previous reports have identified the association between *TOP2A* expression and aggressive tumor features in prostate (24), hepatocellular (25), and colorectal cancers (26). Recent work has confirmed that high *TOP2A* gene expression is associated with shorter metastasis-free interval in breast cancer (27). *TOP2A* overexpression in breast tumors correlates with response to anthracycline-based chemotherapy regimens (27, 28) but has also been linked to resistance to chemotherapy regimens in hepatocellular carcinoma (doxorubicin resistance; ref. 25) and colorectal tumors (irinotecan and etoposide resistance; ref. 26).

DTIC remains the standard first-line chemotherapy for metastatic melanoma (29) internationally. DTIC is an alkylating agent that attaches alkyl groups to DNA at the O⁶-position of guanine (30), causing interstrand and intrastrand cross-links and mutation during cell replication. Finally, double-strand breaks develop in DNA causing cell death (30). The critical toxic lesion induced by DTIC is the addition of alkyl groups, and accordingly, the activity of DNA repair genes such as O⁶-methylguanine-DNA methyltransferase (*MGMT*), which removes alkyl groups from DNA, is associated with resistance to alkylating

agents in glioblastoma tumors (31) and in melanoma cell lines (32). A limitation of our study is that the 502 genes assessed on the DASL assay did not include *MGMT*, however, in the smaller number of samples assessed using the Chemo-sensitivity Gene Expression Assay 1 array there was increased expression of *MGMT* in tumors unresponsive to chemotherapy, which supports these previous findings. The finding that overexpression of *RAD51*, *TOP2A*, and *BRCA1* was associated with poor response to chemotherapy is intriguing, suggesting for the first time that genes involved in double-strand break repair may also be associated with resistance to chemotherapy of melanoma. Because double-strand breaks represent the final common pathway of DTIC action in cancer cells (30), this association is biologically plausible. Using the Chemo-sensitivity Gene Expression Assay 1 array, there was increased expression of genes involved in base-excision repair, nucleotide excision repair, and DNA nonhomologous end joining (15) in tumors unresponsive to chemotherapy. In contrast, genes encoding proteins involved in apoptosis, cellular proliferation, and drug pumps exhibited increased and decreased expression. This observation suggests that increased expression of a number of DNA repair pathways is associated with resistance to chemotherapy in melanoma, with some (limited) evidence of additional mechanisms of chemotherapy resistance involving more complex interactions between cellular pathways. Gene expression studies assessing multiple genes in melanoma tumors have previously been small and not designed to identify predictive markers; however, these current data were derived from a small sample, and the observed effect only reached statistical significance for *RAD51* and *TOP2A*, so these are preliminary observations only. The relationship between higher expression of DNA repair and failure to respond to DTIC may also simply reflect the fact that these tumors are more aggressive, with a higher proliferative rate, rather than a specific relationship with the drug used.

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In summary, in this large-scale assessment of gene expression profiles in formalin-fixed, paraffin-embedded primary melanomas we have identified overexpression of DNA repair genes as being associated with shorter relapse-free survival and poor prognostic histopathologic features. This validates the findings of Kauffmann et al. (7) in a much larger study, suggesting that DNA repair pathways are essential biological pathways involved in progression of melanoma and perhaps treatment responses. Although there is hope of new therapeutic agents for the treatment of melanoma, it is very likely that a role for DTIC will remain and the identification of predictive biomarkers will remain of crucial importance.

Disclosure of Potential Conflicts of Interest

I.A. Cree is a director of CanTech Ltd.

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