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Pathway dysregulation analysis of the nucleotide excision repair mechanisms reveals it is not a common feature of melanomas

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Dear Editor,

Ultraviolet Radiation (UVR) is the major environmental mutagen driving the development of melanoma. The major UVR-induced DNA lesions, 6–4 photoproducts and cyclobutane pyrimidine dimers are primarily repaired by Nucleotide Excision Repair (NER). The very high levels of UV signature mutations (USM; Signature 7 mutations) that are observed in melanomas are the direct outcome of unrepaired UV-induced lesions (Alexandrov et al., 2013; Hodis et al., 2012), suggesting that there must be defects in the repair of these lesions, and thus potentially in NER. For further description of signature 7 mutations please refer to (Alexandrov et al., 2013). To date there have been contradictory reports on the efficiency of NER in melanomas. Most of these studies have used small numbers of melanoma cell lines (Belanger et al., 2014; Budden et al., 2016; Gaddameedhi et al., 2010). To address this question in a larger dataset we have examined the relationship between the expression of a comprehensive panel of NER component genes (KEGG NER pathway, 47 genes; Supp. Table 1) using The Cancer Genome Analysis (TCGA) melanoma dataset (The Cancer Genome Atlas, 2015) and USMs for each sample from (Alexandrov et al., 2013). Our analysis used 'Pathifier' (Drier et al., 2013), a validated tool developed to quantify pathway deregulation in gene expression data. Pathifier transforms gene-level information into pathway-level information to model a Pathway Deregulation Score (PDS) for each sample (Drier et al., 2013; Huang et al., 2014; Liu et al., 2016). We quantify the amount of dysregulation of a pathway in a sample by measuring the deviation of the sample from normal behavior. More specifically, the expression data is used to construct a principal curve using the Hastie and Stuetzle's algorithm, onto which each sample is projected. The PDS represents the projected distance of the sample along the curve, with a larger deviation from normal expression levels indicating a higher PDS, and therefore more dysregulation in the pathway. In our study, the PDS for each sample was related to the number of S7 mutations, to determine strength of association between dysregulation of the pathway and USM load. The TCGA data was filtered to only include Malignant Melanoma NOS and Nodular Melanoma. A total of 353 samples with complete RNAseq and USM data, including 67 primary melanomas was further analyzed (Supp. Table S2).

We assessed potential confounding variables prior to the analysis of the data. Analysis of the two subtypes included in the study: Malignant Melanoma NOS (338 samples) and Nodular Melanoma (15 samples) (Supp. Table S2), showed no significant difference in UV mutational load based on subtype, and no clustering of the expression data was evident with Principal Component Analysis (PCA). Likewise, age showed no association with USM load. Melanomas of the Head and Neck showed the expected elevated numbers of USMs (Supp. Figure S1A), but tumour location did not show a significant influence over NER expression (Supp Figure S1B). The expected elevated USM load in males was also found, but this did not significantly alter NER gene expression patterns (Supp. Figure S2). Metastases showed a greater USM loads than the primaries (median = 14.13 Mutations per Megabase (MpMb) vs 12.43 MpMB; Wilcox p-value = 0. 0.013; Supp. Figure S3A), but PCA did not highlight major differences in sample clustering (Supp. Figure S3B).

The samples were subgrouped based on USM load, determined by natural inflection points in the data; threshold values of USMs, >54.6 MpMB (High); 3.61- 54.6 MpMb (Mid); >0-3.6 MpMb (Low) (Supp. Figure S4). There were 12 samples with no USMs (zero mutation) (Supp. Table S3). PCA showed that the zero mutation melanomas had a broad variance in NER gene expression which was similar to the difference in NER pathway gene expression in the USM containing melanomas (Supp. Figure S5). Sparse version of PCA was used to determine whether the variation in the zero mutations samples was caused by a subset of genes with high variance, as opposed to the entire pathway collectively, however no small group of genes appeared to be disproportionally responsible for the effect**.**

Initial analysis showed that upregulated expression of the NER pathways genes was a common feature in melanomas (59% of the 470 analysed; z score >3; Supp. Figure S6). Interestingly, the general transcription factor complex TFIIH (GTF2H isoforms, ERCC3) and replication protein A (RPA) complexes are common targets of over-expression in >15% of the melanomas analysed, and the polymerase D (POLD1-4) complex and it associated replication factor C (RFC), are over-expressed in 10% of melanomas. The DNA damage binding proteins DDB1, DDB2, and core NER components XPA and XPC are rarely dysregulated at the mRNA level (Supp. Figure S6). There was little association between the expression of each gene in the NER pathway and the USM load for each tumour (Supp. Figure S7). To assess whether small changes in expression in multiple pathway genes might be the basis of failure of UV lesion

repair, Pathifier was used to calculate Pathways Deregulation Score (PDS). There was a very weak correlation between the PDS and USM in the primary samples (Spearman cor = 0.11 n = 67 ; Figure 1A), which extended when including all samples (Spearman cor = 0. 11 n = 353). The median PDS was similar for all categories of mutation level, although there was a non-significant trend from zero to high mutation of increasing PDS (Figure 1B; Supp. Table S4). The median scores for each group indicate a high level of altered expression between all samples including the zero mutation group. In a similar study using this approach to assess homologous recombination repair (HR) pathway expression in breast cancers, a PDS (HR score in the study) <0.4 was demonstrated to be HR proficient and >0.5 HR deficient (Liu et al., 2016). There was no clear association between PDS and mutation load in the outliers (PDS>0.6). To ensure a smaller cohort size in the primary melanoma data was not responsible for the lack of association, Pathifier analysis was performed 100 times each with a different permutation of 63 randomly selected metastatic samples. No correlation between PDS and USM load was observed (Spearman cor = -0.039; permutation p-value = 8.5 e-05). We identified 19 melanomas with USM information and homozygous deletion or putative loss of function mutation of key NER genes. Only 8/19 had increased USM loads when compared to the average load in melanomas from a similar anatomical location, suggesting that even this degree of NER dysfunction is not contributing to USM load (Supp. Table S5). An alternative approach is examining only USM in key melanoma oncogenes and tumour suppressors such as *PTEN*, *TP53*, *CDKN2A*. However, there would be no statistical power analysing this very small number of mutations (normally a single USM in a single gene for each tumour).

The lack of association between dysregulated NER gene expression and USM load suggests that the upregulated expression of the TFIIH and POLD complexes is not associated with NER. It might however indicate that these melanomas required more replication-associated proteins to facilitate replication, possibly as consequence of the high level of endogenous replication stress in melanomas (Brooks et al., 2013).

The Pathifier pathway analysis provided functional information based on collective gene expression. The method has been validated in several studies as being a suitable method of assessing pathway function and deregulation (Drier et al., 2013; Huang et al., 2014; Liu et al., 2016). Ideally however, Pathifier would use normal tissue as reference samples (Drier et al., 2013). This is not an explicit requirement, but it is an important consideration when interpreting

the results. As the TCGA data did not contain information on normal melanocytes, melanomas with no recorded S7 mutations were used as surrogates.

In summary, the absence of association between the NER pathway and USM load indicates that defects in other mechanisms involved in repairing UV lesions must contribute to the USM loads in melanoma. Cell cycle checkpoint and other DNA damage repair genes important to repairing UV-induced DNA lesions have been found to be commonly defective in melanoma cell lines (Belanger et al., 2014; Wigan et al., 2012), and these defects, rather than dysfunction of the central NER pathway, are likely responsible for the increased UV signature mutation load found in the majority of melanomas.

Figure Legend

Figure 1. Pathifier analysis on the NER pathway with respect to the number of Signature 7 (S7) mutations. **A**. PDS of all of samples are shown, along with the number of S7 mutations. The primary and metastatic tumours are indicated. The solid circles indicate the zero S7 mutation tumours. **B.** A boxplot of the samples PDS sub-divided based on UV mutational load. The primary and metastatic tumours are indicated. The median PDS for the different mutational load groups were as follows: Reference = 0.50, Low = 0.53, Mid = 0.51 and High = 0.58, the difference between these groups did not reach statistical significance (p-value = 0.089).

Conflict of Interest: The authors have no conflicts of interest to declare.

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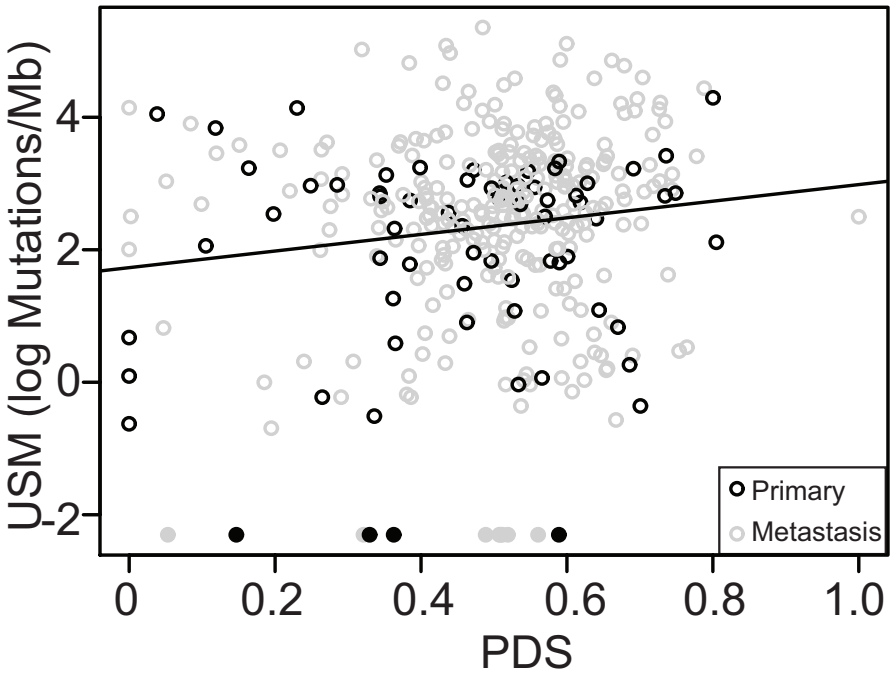
D'Arcy et al., Figure 1

A



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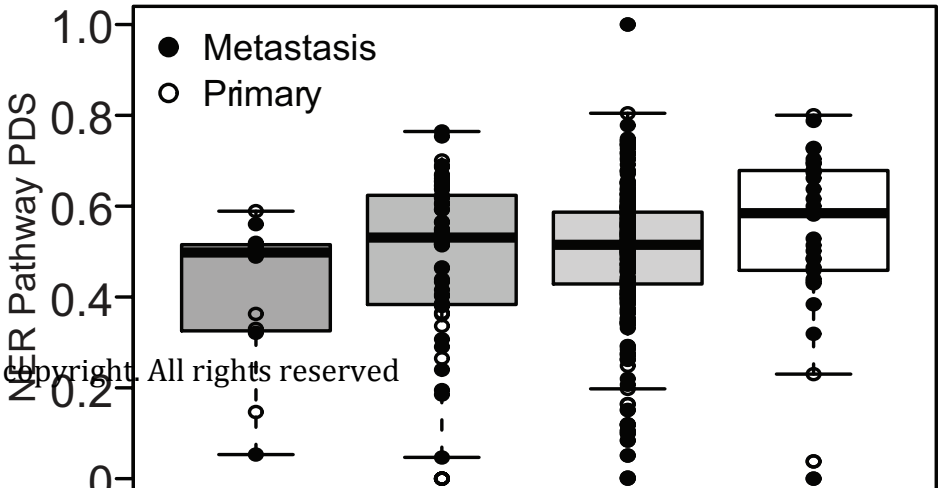

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