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Higher Nevus Count Exhibits a Distinct DNA Methylation Signature in Healthy Human Skin: Implications for Melanoma

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High nevus count is the strongest risk factor for melanoma, and although gene variants have been discovered for both traits, epigenetic variation is unexplored. We investigated 322 healthy human skin DNA methylomes associated with total body nevi count, incorporating genetic and transcriptomic variation. DNA methylation changes were identified at genes involved in melanocyte biology, such as *RAF1* ($P = 1.2 \times 10^{-6}$) and *CTC1* (region: $P = 6.3 \times 10^{-4}$), and other genes including *ARRDC1* ($P = 3.1 \times 10^{-7}$). A subset exhibited coordinated methylation and transcription changes within the same biopsy. The total analysis was also enriched for melanoma-associated DNA methylation variation ($P = 6.33 \times 10^{-6}$). In addition, we show that skin DNA methylation is associated in *cis* with known genome-wide association study single nucleotide polymorphisms for nevus count, at *PLA2G6* ($P = 1.7 \times 10^{-49}$) and *NID1* ($P = 6.4 \times 10^{-14}$), as well as melanoma risk, including in or near *MC1R*, *MX2*, and *TERT/CLPTM1L* ($P < 1 \times 10^{-10}$). Our analysis using a uniquely large dataset comprising healthy skin DNA methylomes identified known and additional regulatory loci and pathways in nevi and melanoma biology. This integrative study improves our understanding of predisposition to nevi and their potential contribution to melanoma pathogenesis.

INTRODUCTION

The total body number of melanocytic nevi is the strongest risk and predictive factor for melanoma in Caucasian populations (Gandini et al., 2005; Olsen et al., 2009). Melanoma is the most aggressive of skin tumors with an increasing incidence (Siegel et al., 2014). These malignancies arise from an existing benign nevus in 20% to 50% of cases (Haenssle et al., 2016; Purdue et al., 2005; Shitara et al., 2014; Weatherhead et al., 2007). The vast majority of nevi never progress to melanoma; however, nevi count is still a predisposition marker for

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Abbreviations: BMI, body mass index; CGI, CpG island; DMP, differentially methylated position; DMR, differentially methylated region; eQTL, expression quantitative loci; EWAS, epigenome-wide association study; FDR, false discovery rate; GWAS, genome-wide association studies; MAF, minor allele frequency; SNP, single nucleotide polymorphism

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melanoma arising de novo (Chang et al., 2009). Therefore, further understanding of the biology of nevi will give insights into the development and pathology of melanoma.

Typically, the number of nevi decrease after the age of 40; however, in individuals at high risk of melanoma, this loss of nevi is delayed, reflecting an altered senescence (Newton et al., 1993). Furthermore, a higher total body nevus count has also been associated with longer telomere length in blood in individuals from the TwinsUK cohort (Bataille et al., 2007). This link with senescence may indicate that the total numbers of nevi reflect differences in senescence pathways between individuals that can be detected in skin tissue where nevi are found. The usefulness of nevus counts as an intermediate phenotype to melanoma has already been shown in genome-wide association studies (GWAS), as common single nucleotide polymorphisms (SNPs) in the loci *PLA2G6* and *MTAP* were first associated with total body nevus count (Falchi et al., 2009; Nan et al., 2011) and then subsequently with melanoma risk (Barrett et al., 2011; Bishop et al., 2009). For nevus count, variants in *PLA2G6* were replicated across two studies that also identified additional associations in *NID1*, *c11orf74*, and *MTAP*. Although GWAS have identified genetic variation for nevus count and melanoma, the variance in nevus counts explained by these genes is low and no study has previously examined epigenetic variation in this context.

Here, we explore epigenome-wide DNA methylation variation in healthy human skin tissue in relation to total body nevus counts in 322 female individuals from the TwinsUK cohort. The focus of this study is on the potential to identify a predisposing DNA methylation signature in normal skin to the number of nevi and not the malignant changes occurring in melanocytes themselves. It has become increasingly
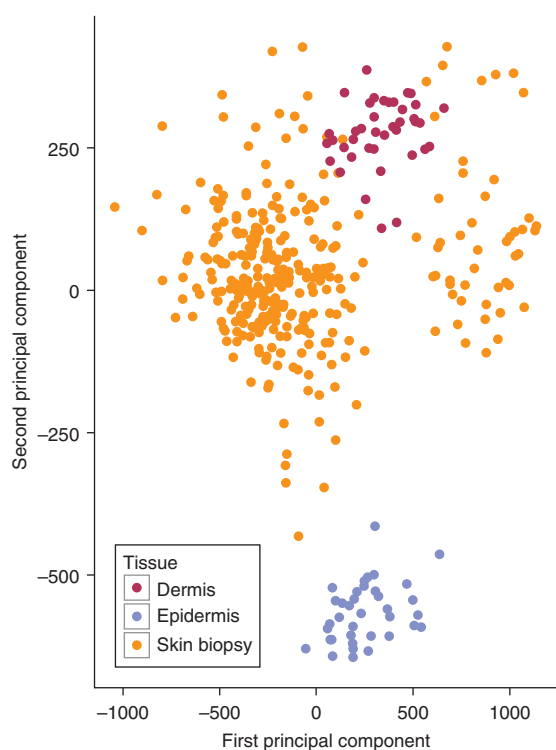
Figure 1. Global DNA methylation profiles and skin tissue specificity. First two principal components colored by layer specificity; red for dermal tissue, blue for epidermal tissue, and yellow for our data (see the legend).

acknowledged that crosstalk between all cells within the skin and melanocytes is important in the progression to melanoma (Kaur et al., 2016; Kim et al., 2013; Li et al., 2003; Shih et al., 1994). We investigated individual CpG differentially methylated positions (DMPs), as well as differentially methylated regions (DMRs) in healthy skin tissue, and corresponding gene expression changes within the same tissue. To study the potential interaction between genetic variants and DNA methylation, we examined the association between the skin DNA methylome and genetic variants previously associated with nevus count or melanoma risk by GWAS.

RESULTS

The skin DNA methylome and its tissue layer specificity

As expected, because of the differing cell types in skin within the dermis and epidermis, these tissue layers harbor distinct DNA methylation profiles (Vandiver et al., 2015). In our study, skin tissue DNA was derived from a periumbilical punch biopsy (adipose tissue was removed from the biopsy before freezing) from 322 healthy female twins and profiled using the Infinium HumanMethylation450 BeadChip. To confirm which skin layer was represented in our biopsy sample, we compared our DNA methylation dataset with recently published DNA methylation profiles of mechanically separated epidermal (36 individuals) and dermal tissue (36 individuals) from Vandiver et al. (2015). Principal component analysis was performed on unadjusted DNA methylation profiles of the three groups of samples (dermis [n = 40], epidermis [n = 38], and our whole skin sample [n = 322]). The first two principal components explain 55.6% of the variance, capturing the skin layer specificity of the dermis and epidermis samples as

previously shown (Vandiver et al., 2015). Our whole skin DNA methylation profiles cluster closely with the dermal layer DNA methylation profiles (Figure 1).

Single CpG site differential skin DNA methylation in relation to total body nevus count

We first explored evidence for differential skin DNA methylation associated with total body nevus count at the single CpG-site level across the genome in 322 healthy female twins. We fitted a linear mixed effects model regressing DNA methylation levels on fixed effects (age, body mass index [BMI], smoking status, chip, order on the chip, and bisulfite conversion efficiency) and random effects (family structure and zygosity). Three DMPs were identified to be significantly associated with total body nevus count (n-DMPs) at a false discovery rate (FDR) of 5% and a further 45 associations were observed at an FDR of 10% (Figure 2a, Table 1, Supplementary Table S1 online). The 48 n-DMPs are enriched for strong enhancers (ChromHMM state 4) in the normal human epidermal keratinocyte cell line derived from epidermal keratinocytes ($P = 0.03$) and for CpG island (CGI) shores (2 kb either side of the CGI, $P = 0.04$), while depleted for signals located in open sea genomic regions that are more than 4 kb beyond CGIs ($P = 2.2 \times 10^{-3}$).

The strongest signals are shown Table 1 and Figure 2b. The most associated signal (cg06244240, $P = 5.5 \times 10^{-8}$) lies in a CGI shore approximately 6.5 kb downstream of *METRNL*, which is expressed in skin (Ushach et al., 2015) and is also involved in neural cell formation. The second ranked signal (cg06123942, $P = 2.2 \times 10^{-7}$) is in the 5′ CGI promoter of *C15orf48*, which displays reduced expression in squamous cell carcinoma (Freiberger et al., 2015). The third ranked signal (cg25384157, $P = 3.1 \times 10^{-7}$) is positively associated with the number of nevi and is in a CGI shore approximately 1.5 kb upstream of the transcription start site of *ARRDC1*, a negative regulator of the Notch signaling pathway (Puca et al., 2013). The CpG (cg11297934, $P = 1.2 \times 10^{-6}$) lies approximately 200 bp upstream of the transcription start site of proto-oncogene *RAF1* (also known as *CRAF*), which is a member of the RAF family in the extracellular signal-regulated kinase/mitogen-activated protein kinase pathway that includes the key player *BRAF*.

Six of our top 10 n-DMPs did not show direct association with age ($P = 0.05$) including two of the top four results (*ARRDC1* and *RAF1*), with no n-DMP associations with age surpassing FDR 10% correction (Figure 2c). The remaining four signals did not show stronger evidence for association with age alone compared with nevus count. These results suggest that the majority of top-ranked n-DMPs are not directly associated with age.

Regional differential skin DNA methylation associated with total body nevus count

We next aimed to identify DMRs, that is, small genomic regions that contain multiple CpG sites and show consistent directional association with total body nevus count (n-DMRs). DMRs have been found in general to be enriched for functionally relevant regions as well as for GWAS SNPs from the GWAS catalog (Ziller et al., 2013). We applied the BumpHunter (Jaffe et al., 2012) algorithm and identified