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Discrimination of Dysplastic Nevi from Common Melanocytic Nevi by Cellular and Molecular Criteria

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Dysplastic nevi (DNs), also known as Clark's nevi or atypical moles, are distinguished from common melanocytic nevi by variegation in pigmentation and clinical appearance, as well as differences in tissue patterning. However, cellular and molecular differences between DNs and common melanocytic nevi are not completely understood. Using cDNA microarray, quantitative RT-PCR, and immunohistochemistry, we molecularly characterized DNs and analyzed the difference between DNs and common melanocytic nevi. A total of 111 probesets (91 annotated genes, fold change > 2.0 and false discovery rate < 0.25) were differentially expressed between the two lesions. An unexpected finding in DNs was altered differentiation and activation of epidermal keratinocytes with increased expression of hair follicle-related molecules (keratin 25, trichohyalin, ribonuclease, RNase A family, 7) and inflammation-related molecules (S100A7, S100A8) at both genomic and protein levels. The immune microenvironment of DNs was characterized by an increase of T helper type 1 (IFNγ) and T helper type 2 (IL13) cytokines as well as an upregulation of oncostatin M and CXCL1. DUSP3, which regulates cellular senescence, was identified as one of the disease discriminative genes between DNs and common melanocytic nevi by three independent statistical approaches and its altered expression was confirmed by immunohistochemistry. The molecular and cellular changes in which the epidermal-melanin unit undergoes follicular differentiation as well as upregulation of defined cytokines could drive complex immune, epidermal, and pigmentary alterations.

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INTRODUCTION

Dysplastic nevi (DNs), also known as Clark's nevi or atypical moles, are benign melanocytic tumors that are important to discriminate from common, benign nevi, and melanoma (Duffy and Grossman, 2012a; Goldstein and Tucker, 2013). There is no established clinical definition of DNs, but some combination of the following criteria is generally adopted: (i) greater than 5 mm in size, (ii) variable pigmentation, (iii) asymmetry, (iv) indistinct or irregular borders, (v) existence of both raised and flat components, and (vi) red hue (Bergman et al., 1997; Tucker et al., 1997). Histologically, DNs are characterized by various degrees of architectural disorder and cytological atypia, as well as infiltration of inflammatory cells. These clinical and pathological features of DNs to some extent overlap with those of malignant melanomas.

There is active debate about whether DN is a distinct entity or just a subtype of common melanocytic nevus (CMN) (Ackerman, 1988; Elder, 2010). The usage of "dysplastic" has also been a subject of argument, because the majority of DNs do not transform to malignant lesions and are thus clearly distinct from melanomas in situ that are truly dysplastic (Ackerman, 1988).

DNs have clinical significance because their presence is an important risk factor for melanoma. Gandini et al. (2004) performed meta-analysis of 47 datasets to find risk factors for cutaneous melanoma. Having even one atypical nevus is a considerable risk factor (relative risk =1.60; 95% confidence interval: 1.38, 1.85). However, the risk of the lifetime transformation of a DN lesion to melanoma was estimated as 1/10,000 (Tsao et al., 2003). In addition, histological evaluation of melanoma coexisting with nevus revealed that roughly half of the nevi were CMN and half were DN (Duffy and Grossman, 2012a). It is thus unclear whether DNs are dysplastic melanocytic neoplasms with potential to be precursor lesions of melanoma, as seen in the cancer progression model of Fearon and Vogelstein (1990) for colon cancers.

The etiology of DN is not yet very well understood. Genetic mutations that are associated with melanoma have been studied in CMN and DN. For example, both CMN and DN harbor *BRAF* mutations (*V600E*) at approximately 60–70% of the frequency seen in melanoma (Uribe et al.,

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Abbreviations: CMN, common melanocytic nevus; DN, dysplastic nevus; NRML, normal skin; OSM, oncostatin M

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2006; Wu et al., 2007). Mutations in other genes, such as *CDKN2A*, *p53*, and *PTEN*, were also found at a similar level between CMN and DN (Duffy and Grossman, 2012b). However, several studies identified higher incidence of heterozygous or homozygous deletions in 9p21 that contains *CDKN2A* in DN and malignant melanoma compared with CMN (Birindelli et al., 2000; Park et al., 1998; Tran et al., 2002). Besides genetic factors, the tumor microenvironment may also have an important role in the pathogenesis of DN, because histology has shown the involvement of many cell types in this lesion. Scatolini et al. (2010) studied global gene expression profiles of 18 CMNs, 11 DNs, and 23 melanomas by a cDNA microarray. There were 24 differentially expressed genes when genomic profiles of CMNs and DNs were directly compared. They concluded that DNs are not very different from CMNs, but they noted the heterogeneity in global transcriptional changes of DNs that may reflect the various degrees of pathological atypia.

In this study, we used a molecular approach to identify differential expression of genes/proteins between DNs and CMNs and to map resulting alterations to cell types within DNs. Our data establish that DNs and CMNs can be discriminated at a molecular level, and mapping of differentially expressed genes establishes dysplasia in the epidermal-melanocyte unit with aberrant follicular differentiation of keratinocytes.

RESULTS

DNs showed distinct gene expression profiles from those of CMNs

DNs have various degrees of architectural disorder and cellular atypia among cases. A previous report that studied the range of mild-to-severe atypia was not able to find major differences in gene expression between DNs and CMNs (Scatolini et al., 2010). Hence, DNs included in this study were specifically chosen to be compound nevi with moderate to severe architectural disorder and cytological atypia (Figure 1a, b and d, e, patient demographics in Supplementary Table S1 online). These lesions were often characterized by epidermal elongation and bridging (Figure 1b and c). In addition, marked increases in CD3+ T cells and CD11c+ dendritic cells were detected in DNs compared with CMNs (Figure 1f–k). Prior work from our group has demonstrated that dendritic cells in both CMNs and DNs are characterized as largely myeloid, immature, and inflammatory (Gulati et al., 2015). By double immunofluorescent staining, DNs contained both CD3+CD8+ cytotoxic T cells and CD3+CD8- helper T cells (Supplementary Figure S1 online). Gene expression profiles of DNs (n = 7) and CMNs (n = 5) were then compared with those of normal skin (NRML, n = 6). There were 745 up- and 885 downregulated probesets in CMNs compared with NRMLs (fold change > 2.0 and false discovery rate < 0.10). A total of 376 up- and 166 downregulated probesets were found in DNs compared with NRMLs. There were 364 commonly regulated probesets between CMNs and DNs. A total of 178 probesets were uniquely regulated in DNs, whereas 1,266 were specific for CMNs. Table 1A shows the top 20 upregulated probesets in DNs compared with NRMLs (complete list can be found in Supplementary Tables S2 and S3 online). Melanocyte-related genes such as TRPM, TYR, and MLANA were consistently upregulated in both DNs and CMNs compared with NRMLs. There were several groups of genes whose expression levels were significantly higher in DNs versus NRMLs compared with CMNs versus. NRMLs. These included follicular keratinocyte-related genes (KRT25, TCHH, KRT27, and KRT71) and inflammatory molecules such as S100A8 and S100A7. These results suggest that DNs contain distinct gene expression profiles from those of CMNs, and the larger number of uniquely regulated genes in CMNs as compared with DNs could be due to the different types of CMNs included in the analysis.

DNs were characterized by increased expression of hair follicle-related genes

The purpose of this study was to provide a molecular characterization of DNs and to compare it with that of CMNs. We thus directly compared gene expression profiles of these two lesions (Table 1B and C). KRT25, TCHH, and KRT71 were significantly upregulated in DNs compared with CMNs (false discovery rate < 0.25). Overall, 111 probesets (representing 91 annotated genes) were differentially expressed between DNs and CMNs (Supplementary Table S4 online). The expression of selected genes found through microarray analysis was confirmed by RT-PCR. To generalize our observations, an additional eight DNs and six CMNs were included for RT-PCR. MLANA, MITF, and TYR, three melanocyte markers, were equally expressed in the two lesions (Supplementary Figure S2a–c online). KRT25 and TCHH were upregulated in DNs (TCHH approached significance; $P = 0.06$, Supplementary Figure S2d and e). S100A8 and S100A7 were both significantly upregulated in DNs (Supplementary Figure S2f and g). RNASE7, a follicular keratinocyte-derived microbial peptide (Reithmayer et al., 2009), was also significantly elevated in DNs compared with CMNs (Supplementary Figure S2h). Further RT-PCR studies of malignant melanoma samples (n = 19) demonstrated the presence of melanoma-specific genes such as PRAME (Clarke et al., 2015), CCL8, MAGEA3, and MAGEA6, which were not present in DN (Supplementary Figure S2i–l). For example, PRAME and MAGEA3 were expressed >32-fold higher in melanoma samples compared with DN or CMN samples.

Epidermal activation and focal expression of hair follicle-related genes were found in DNs

To evaluate the corresponding protein expression to the genes identified through microarray and RT-PCR analysis, immunohistochemistry was carried out. Anagen phase hair follicles from normal scalp tissues and psoriasis tissues were also stained as positive controls for several molecules. MLANA staining showed clear dermal nests in both CMNs and DNs (Figure 2b and c). Psoriatic epidermis contained increasing MLANA positive cells compared with normal skin as we have reported previously (Figure 2d and a) (Wang et al., 2013). MLANA-positive melanocytes were also clustered in the anagen hair bulb (Figure 2e). KRT25 weakly stained the basal layer of the epidermis of normal skin and CMN (Figure 2f and g), but strong positivity was observed in the hair bulb region (Figure 2j). The epidermis of DNs was strongly stained for KRT25 (Figure 2h), whereas psoriatic epidermis was only weakly positive for KRT25 (Figure 2i). KRTAP2 weakly stained the