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IRF4, MC1R and TYR genes are risk factors for actinic keratosis independent of skin color

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Abstract

Actinic keratosis (AK) is a pre-malignant skin disease, highly prevalent in elderly Europeans. This study investigates genetic susceptibility to AK with a genome-wide association study (GWAS). A full body skin examination was performed in 3194 elderly individuals from the Rotterdam Study (RS) of exclusive north-western European origin (aged 51–99 years, 45% male). Physicians graded the number of AK into four severity levels: none (76%), 1–3 (14%), 4–9 (6%) and ≥10 (5%), and skin color was quantified using a spectrophotometer on sun-unexposed skin. A GWAS for AK severity was conducted, where promising signals at IRF4 and MC1R (P<4.2×10⁻⁷) were successfully replicated in an additional cohort of 623 RS individuals (IRF4, rs12203592, Pcombined=6.5×10⁻¹³ and MC1R, rs139810560, Pcombined=4.1×10⁻⁹). Further, in an analysis of ten additional well-known human pigmentation genes, TYR also showed significant association with AK (rs1393350, P=5.3×10⁻⁴) after correction for multiple testing. Interestingly, the strength and significance of above-mentioned associations retained largely the same level after skin color adjustment. Overall, our data strongly suggest that IRF4, MC1R and TYR genes likely have pleiotropic effects, a combination of pigmentation and oncogenic functions, resulting in an increased risk of AK.

Introduction

Actinic keratosis (AK) is a pre-malignant skin disease, presenting as an irregularly shaped rough red scaly patch on sun-exposed skin sides. AK is a precursor of the invasive squamous cell carcinoma (SCC) of the skin. Many SCC arise from a pre-existing AK (20–27%) (1), and the transformation rate of AK to SCC is estimated at 0.075% per patch per year (2). In contrast to SCC, AK is diagnosed without histological confirmation. AK is highly prevalent (up to a quarter in northern Europeans of 50 years and older), individuals affected often develop multiple AK, and a considerable proportion of individuals with multiple AK have a history of skin cancer (3).

Cumulative UV exposure is known as the most important risk factor for AK. Other factors known to influence AK risk include increased age, male gender, baldness, light skin color, immunosuppression and human papilloma virus infections (3–7). Common genetic variants have also demonstrated to increase the susceptibility to AK. A recent study by Denny et al. re-examined a targeted set of 3144 single-nucleotide polymorphisms (SNPs), which showed significant association with any human phenotype in previous genome-wide association studies (GWAS), for association with 1358 different human phenotypes, including AK, in 13835 individuals (8). This study found significant association for SNPs at the IRF4/EXOC2 locus and suggestive association for SNPs in or close to the genes SLC45A2, HERC2, MC1R and TYR. All these loci are known to be involved in human pigmentation. Therefore, one obvious question is whether skin color serves as an intermediate variable explaining the observed genetic
association with AK. Furthermore, the study by Denny et al. investigated only a small fraction of genome-wide SNPs. Here we conduct the first GWAS of AK in 3817 elderly north-western Europeans from the Rotterdam Study (RS), taking into account skin color as a potential intermediate variable.

**Results**

The discovery cohort consisted of 3194 Dutch participants (45% male, mean age: 66.9 ± 8.0). Of these, 858 (72%) did not have AK, 16% had 1–3 AK lesions, 6% had 4–9 AK and 5% had 10 or more AK. Skin color was assessed digitally as color saturation, ranging from 15.5% (very white) to 45.7% (white to olive) (Table 1). Low skin color saturation was associated with an increased risk of AK (beta = −0.83, P = 5.8 × 10⁻³).

The GWAS in the discovery cohort (N = 3194) identified two SNPs in and close to the IRF4 gene showing genome-wide significant association with AK severity (Fig. 1 and Supplementary Material, Fig. S1A), deviating significantly from the expected null-distribution (Supplementary Material, Fig. S2). The most significant signal was found for SNP rs12203592(T) (beta = 0.22, P = 4.6 × 10⁻¹¹, Table 2). Other SNPs close to rs12203592 became non-significant (P > 0.05) when the analysis was conditioned on the effect of rs12203592. Interestingly, the same IRF4 SNP is well known to have a strong effect on human pigmentation traits (9).

The GWAS also highlighted SNPs in and nearby the MC1R gene on chr16q24 showing borderline significant association with AK severity (P < 4.2 × 10⁻⁷, Fig. 1 and Supplementary Material, Fig. S1B). The SNP rs139810560(A) located 24 kb downstream from MC1R showed the locus-wise most significant association (beta = 0.18, P = 8.6 × 10⁻⁸, Table 2). One of the other borderline associated SNPs was rs1805008(T) located in the single exonic gene MC1R (beta = 0.17, P = 1.9 × 10⁻⁷, Table 2), which is well-known as a loss of function variant causing red hair and pale skin (10,11). When conditioning the analysis on rs1805008(T), all borderline significantly associated SNPs at 16q24.3 became non-significant (P > 0.05).

Another genetic locus showing borderline genome-wide significance was found at chromosome 4q21.23 (Fig. 1), where the SNP rs11932985(C) was associated with an increased risk of AK (beta = 0.14, P = 2.9 × 10⁻⁷, Table 2). This SNP is located between the genes AGPAT9 and NKX6-1 (Supplementary Material, Fig. S1C), both of which are not known to be involved in cancer nor pigmentation.

To replicate our findings, we additionally collected phenotypes for 623 participants of the RS cohort. The replication cohort was slightly younger (42% male, mean age: 64.0 ± 6.6) and showed a smaller number of AK (88.3% had no AK) than the discovery cohort, but the skin color saturation was comparable to the discovery cohort (Table 1). We selected 13 SNPs which showed at least borderline significance in the discovery GWAS (P < 4.2 × 10⁻⁷) for replication. The three associated SNPs on chr6p25.3 (IRF4) all showed significant replication, and increased significance in the combined sample analysis (rs12203592(T), Preplication = 2.0 × 10⁻³, Pcombined = 5.8 × 10⁻³).

![Figure 1. Manhattan plot of the GWAS in 3194 north-western European individuals. The observed −log10 P-values (Y-axis) of the association between the SNPs and AK severity is shown. All SNPs are represented by dots and displayed per chromosome (X-axis). The horizontal line indicates the genome-wide significance threshold of P = 5 × 10⁻⁸.](image_url)
Table 2. SNPs associated with AK in the discovery GWAS (N = 3194) and replication results (N = 623)

<table>
<thead>
<tr>
<th>SNP</th>
<th>EA</th>
<th>fEA</th>
<th>Discovery (N = 3194)</th>
<th>P adj</th>
<th>Replication (N = 623)</th>
<th>P adj</th>
<th>Combined (N = 3817)</th>
<th>P adj</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chr: 4q21.23 (AGPAT9/NX6-1)</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>rs11932985</td>
<td>C</td>
<td>0.11</td>
<td>2.9 × 10^-7</td>
<td>3.2 × 10^-7</td>
<td>-0.02</td>
<td>0.685</td>
<td>0.788</td>
<td>0.12</td>
</tr>
<tr>
<td>rs62389423</td>
<td>A</td>
<td>0.08</td>
<td>3.7 × 10^-7</td>
<td>6.5 × 10^-7</td>
<td>0.16</td>
<td>0.006</td>
<td>0.10</td>
<td>0.17</td>
</tr>
</tbody>
</table>

**Table 3. Effect of pigmentation SNPs on AK and skin color saturation in 3817 Dutch individuals**

<table>
<thead>
<tr>
<th>SNP</th>
<th>Gene</th>
<th>EA</th>
<th>AK Beta</th>
<th>P Saturation</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs16891982</td>
<td>SLC45A2</td>
<td>C</td>
<td>-0.08</td>
<td>0.11</td>
<td>0.95</td>
</tr>
<tr>
<td>rs12203592</td>
<td>IRF4</td>
<td>T</td>
<td>0.21</td>
<td>6.5 × 10^-13</td>
<td>-0.76</td>
</tr>
<tr>
<td>rs1408799</td>
<td>TYR</td>
<td>T</td>
<td>-0.02</td>
<td>0.24</td>
<td>0.08</td>
</tr>
<tr>
<td>rs10756819</td>
<td>BNC2</td>
<td>G</td>
<td>-0.03</td>
<td>0.06</td>
<td>0.09</td>
</tr>
<tr>
<td>rs35264875</td>
<td>TPNC2</td>
<td>T</td>
<td>-0.02</td>
<td>0.25</td>
<td>0.04</td>
</tr>
<tr>
<td>rs1393350</td>
<td>TYR</td>
<td>A</td>
<td>0.07</td>
<td>5.3 × 10^-4</td>
<td>-0.45</td>
</tr>
<tr>
<td>rs12821256</td>
<td>KITLG</td>
<td>C</td>
<td>0.02</td>
<td>0.41</td>
<td>-0.16</td>
</tr>
<tr>
<td>rs17128291</td>
<td>SLC24A4</td>
<td>G</td>
<td>0.01</td>
<td>0.79</td>
<td>0.09</td>
</tr>
<tr>
<td>rs1800407</td>
<td>OCA2</td>
<td>T</td>
<td>-0.01</td>
<td>0.75</td>
<td>-0.07</td>
</tr>
<tr>
<td>rs12913832</td>
<td>HERC2</td>
<td>A</td>
<td>-0.04</td>
<td>0.10</td>
<td>0.24</td>
</tr>
<tr>
<td>rs1805008</td>
<td>MCM1</td>
<td>T</td>
<td>0.17</td>
<td>1.2 × 10^-8</td>
<td>-0.60</td>
</tr>
<tr>
<td>rs4911414</td>
<td>ASIP</td>
<td>T</td>
<td>0.04</td>
<td>0.02</td>
<td>-0.07</td>
</tr>
</tbody>
</table>

All SNPs with suggestive association (P < 4.2 × 10^-5) in the discovery GWAS are shown.

EA, effect allele; fEA, frequency of effect allele; P adj, p-value additionally adjusted for skin color saturation.

P_{combined} = 2.8 × 10^{-13} (Table 2). At the chr16q24.3 (MC1R) locus, eight of nine SNPs showed significant replication, and showed genome-wide significance in the combined sample analysis (rs139810560(A), P_{replication} = 4.8 × 10^{-10}, Table 2). However, the SNP on chr4q21.23 did not show significant replication (rs11932985(C), P_{replication} = 0.79, P_{combined} = 4.0 × 10^{-10}, Table 2) and therefore could represent a false positive finding of the discovery cohort. We further conducted a GWAS conditionional on the top 20 SNPs (rs12203592 and rs1805008) in the discovery sample, which did not show any residual genetic association that are significant at the genome-wide level (all P-values > 1 × 10^{-5}; a minimal P = 1.4 × 10^{-5} was found for SNP rs11932985 on chromosome 4, Supplementary Material, Table S1).

Both significant loci contain SNPs (rs12203592 (IRF4) and rs1805008 (MC1R)) that are well-known to be associated with pigmentation traits including skin color. Because light skin color is a known risk factor for AK and because the SNPs are significantly associated with skin color in our sample (P < 0.05, Table 3), we investigated whether the associated SNPs have an effect on AK independent of skin color, using the combined sample (N = 3817).

First, adjustment for saturation showed that the association of both SNPs with AK slightly reduced in significance and effect size, but retained genome-wide significance (rs12203592: P_{adjusted} = 2.8 × 10^{-14} and rs1805008: P_{adjusted} = 1.9 × 10^{-8}, Table 2), while color saturation became less significant (from P = 5.8 × 10^{-5}, to P_{adjusted} for rs12203295 = 0.03, and P_{adjusted} for rs1805008 = 0.02). Furthermore, in a skin color stratified analysis, the effects were comparable in all three skin color strata for both SNPs: rs12203592(T) (low S: beta = 0.22, P = 6 × 10^{-4}; medium S: beta = 0.22, P = 4 × 10^{-4}, high S: beta = 0.18, P = 8 × 10^{-4}, Supplementary Material, Table S2) and rs1805008(T) (low S: beta = 0.12, P = 2 × 10^{-4}, medium S: beta = 0.08, P = 8 × 10^{-4}, high S: beta = 0.09, P = 2 × 10^{-3}, Supplementary Material, Table S2). Interaction between the SNPs and saturation was absent (rs12203592: P_{interaction} = 0.08; rs1805008: P_{interaction} = 0.81). These results suggest that IRF4 and MC1R genes have a much stronger effect on AK risk than skin color.

Since both loci are known to be involved in pigmentation, we conducted a candidate SNP analysis by selecting 12 SNPs in 12 genes that have been shown to be significantly associated with skin color in previous studies (9,11–13), also including IRF4 and MC1R (Table 3). This analysis highlighted another SNP (rs1393350) in the TYR gene being significantly associated with AK after multiple testing correction (beta (A allele) = 0.07, P = 5.3 × 10^{-4}, Table 3). Interestingly, this SNP also showed significant association with skin color in our sample (P = 2.8 × 10^{-5}, Table 3) and its association with AK also remained significant after skin color adjustment (P = 1.1 × 10^{-5}, Table 4).

In a multivariable analysis we investigated the independent effects of age, sex, skin color saturation and the above described 12 pigmentation-associated SNPs on AK (Table 4). This analysis showed that sex, age, IRF4, MC1R and TYR were all significant risk factors for AK with an independent effect (P < 0.005). Saturation was not associated with AK (P = 0.17, Table 4), due to the inclusion of the pigmentation SNPs (without SNPs, P = 5.8 × 10^{-5} for saturation). Note that in our data HERC2 (rs12913832) and SLC45A2 (rs16891982) did not show any significant association.

Analysis of known pigmentation SNPs in association with AK and saturation. Saturation, continuous skin color measure, EA, effect allele.
Table 4. Multivariable analysis for AK in 3817 Dutch individuals

<table>
<thead>
<tr>
<th>Factors</th>
<th>Chr</th>
<th>Gene</th>
<th>EA</th>
<th>fEA</th>
<th>Beta</th>
<th>p</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (per year)</td>
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<td>3.5 x 10^-17</td>
<td>11.94</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Female sex</td>
<td>0.29</td>
<td>1.9 x 10^-32</td>
<td>3.15</td>
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<tr>
<td>Saturation</td>
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<td>0.15</td>
<td>0.01</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs16891982</td>
<td>5</td>
<td>SLC45A2</td>
<td>C</td>
<td>0.05</td>
<td>-0.09</td>
<td>0.09</td>
<td>0.08</td>
</tr>
<tr>
<td>rs12203592</td>
<td>6</td>
<td>IRF4</td>
<td>T</td>
<td>0.12</td>
<td>0.29</td>
<td>1.15</td>
<td></td>
</tr>
<tr>
<td>rs1408799</td>
<td>9</td>
<td>TYRP1</td>
<td>T</td>
<td>0.31</td>
<td>-0.02</td>
<td>0.32</td>
<td>0.02</td>
</tr>
<tr>
<td>rs10756819</td>
<td>9</td>
<td>BNC2</td>
<td>G</td>
<td>0.32</td>
<td>-0.04</td>
<td>0.05</td>
<td>0.08</td>
</tr>
<tr>
<td>rs35264875</td>
<td>11</td>
<td>TPNC2</td>
<td>T</td>
<td>0.17</td>
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<td>0.50</td>
<td>0.01</td>
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<td>rs1393350</td>
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<td>TYR</td>
<td>A</td>
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<td>1.1 x 10^-3</td>
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<td>rs17128291</td>
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<td>0.003</td>
</tr>
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<td>rs1800407</td>
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<td>OCA2</td>
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<td>0.05</td>
<td>-0.01</td>
<td>0.85</td>
<td>0.0007</td>
</tr>
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<td>HERC2</td>
<td>A</td>
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<td>-0.04</td>
<td>0.10</td>
<td>0.07</td>
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<td>rs1805008</td>
<td>16</td>
<td>MC1R</td>
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<td>2.6 x 10^-8</td>
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<td>0.02</td>
<td>0.09</td>
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<tr>
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<td></td>
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<td></td>
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<td>17.60</td>
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</tbody>
</table>

Multiple linear regression analysis of possible risk factors for AK. EA, effect allele; fEA, frequency of effect allele; R², percentage of explained AK variance, per predictor.

with AK. All analysed factors together explained 18 percent of the AK phenotypic variance (adjusted R² = 17.6%, Table 4). Although the majority of the AK variance was explained by age and sex (15%), SNPs in the three significant SNPs in IRF4, MC1R and TYR together explained 2.6% of the variance of AK, which is comparable to typical findings from GWAS of many human complex traits and diseases (14,15). A narrow sense heritability analysis based on all genotyped, autosomal, common SNPs suggests that about 17.2% AK variance may be explained by all SNPs (V(G)/Vp = 0.172, SE = 0.08, P = 0.02). In comparison, about 23.0% of variance in skin color saturation (V(G)/Vp = 0.230, SE = 0.08, P = 0.003) could be explained by the same set of SNPs. These estimates of narrow sense heritability are likely underestimated compared with family-based heritability estimates, because it is only based on SNPs, not on other heritable variations such as epigenetic factors. Nevertheless, the variance explained by the three top SNPs identified at IRF4, MC1R and TYR (2.6%), contributes a non-trivial portion of the overall variance explainable by our available SNPs (17.2%).

Finally, the IRF4 variant rs12203592(T) showed a clear dominant effect (P_dominant = 6 x 10^-11) but the known recessive effect of the MC1R variant rs1805008(T) was not so obvious (P_recessive = 4 x 10^-4, Supplementary Material, Table S3), which is likely explained by compound heterozygosity with other causal variants in the gene (16). The effect allele frequencies of both SNPs in the RS were comparable with those of the HapMap CEU population (approximately 10%, Supplementary Material, Table S4). These two SNPs appeared to be European specific, since the non-risk alleles are fixed or nearly fixed in Asian and African HapMap samples (Supplementary Material, Table S4).

Discussion

This first GWAS of AK reveals that IRF4, MC1R and TYR genes are involved in AK susceptibility in our European sample. These findings are largely consistent with those from a recent phenome-wide association study (8), except that our data did not support a role of HERC2 and SLC45A2. Additionally, we show that after careful adjustments for skin color, the strength and significance of the genetic association was hardly affected, indicating that the genetic effect is at least partially independent of skin color and having a much stronger effect than skin color on AK risk. IRF4, MC1R and TYR are all known to be involved in various kinds of human pigmentation traits including eye, hair and skin color, tanning ability and freckling (9,11,12,17-19) as well as with skin cancer, including basal cell carcinoma, SCC and melanoma of the skin (20-23).

IRF4 (chr6p25.3) encodes the interferon regulatory factor 4. The SNP rs12203592(T) was recently found to impair binding of the TFAP2A transcription factor which activates transcription of IRF4. Decreased expression of IRF4 will decrease expression of TYR, a key enzyme in the rate of melanin synthesis (24). Therefore, rs12203592(T) is an important risk allele for light pigmentation traits. Additionally, IRF4 is known to negatively regulate the toll-like-receptor signaling which is central in the activation of adaptive immunity (25). It has been suggested that a reduced IRF4 may have a role in a reduced host immune response against atypical keratinocytes and melanocytes (20), although there is no hard evidence for this. Interestingly, AK risk is known to be highly influenced by the host immune response. This is evident from the organ transplantation field where patients are under heavy immunosuppressive therapy and have a 250-fold higher risk to develop AK (27,28). Therefore, rs12203592(T) might have an additional immune suppressive effect next to its role in melanin synthesis, which may potentially explain the skin color independent effect on AK.

MC1R (chr16q24.3) encodes the melanocortin 1 receptor and is a key pigmentation gene. The MC1R is a melanocyte-specific transmembrane receptor. Binding to its ligand, melanocyte stimulating hormone (α-mSH), induces synthesis of the dark eumelanin. The SNP rs1805008(T) (among others) is known to induce loss of function of MC1R (10), which reduces MC1Rs ability to bind to α-mSH and subsequently switches the melanin synthesis to the yellow-red pheomelanin synthesis. Next to these melanin synthesis switches, additional effects of MC1R have been demonstrated in a few previous studies. For example, melanocytes with loss of function MC1R showed reduced DNA repair after UV exposure, independent of the total melanin content of the cell (29,30). Because failing of a DNA repair mechanism is an important skin cancer promoter (31), this likely plays a role in the pigmentation independent effect of MC1R on AK.

TYR (chr11q14.3) encodes the rate-limiting enzyme tyrosinase, which catalyzes at least three steps in the conversion of the amino acid tyrosine, to melanin. Active TYR results in dark eumelanin synthesis and inactive TYR results in yellow-red pheomelanin synthesis (32,33). Multiple polymorphisms in TYR are known to decrease activity of the enzyme and induce a lightly pigmented phenotype, where rs1393350(A) was shown to be the strongest associated SNP (11). Next to pigmentation and skin cancer risk, the same set of TYR polymorphisms has been associated with the auto-immune skin depigmentation disease vitiligo, showing an opposite risk effect, i.e. a protective effect (34). As TYR (wild type) is known as the major auto-antigen in vitiligo, showing an opposite risk effect, i.e. a protective effect (34). 

IRF4, MC1R and TYR all seem to exert effects on AK risk independent of skin color in our observational data. Because light skin color is an important risk factor for AK, skin color could well have been an intermediate phenotype in the association between the pigmentation genes and AK. However, adjustment for skin color did not affect the association of IRF4 and MC1R with AK, and still showed a highly significant genetic association.
Therefore, these genes likely have more than one biological effect, i.e. exert pleiotropic effects. As discussed above, these effects include a melanin synthesis function and an oncogenic function. In this study we did not find evidence for influence of HERC2 and SLC45A2 on AK. HERC2 is known to have a large influence on skin type (UV sensitivity) (12,19,37). However, most previous large-scale GWAS for skin cancer (basal cell carcinoma and melanoma) could not show any significant effect of HERC2 (22,23,38-40) independent of skin color. In our data SLC45A2 was significantly associated with skin color but not with AK risk. Likely because its effect size is relatively small and the allele frequency in Europeans is low (5%), our data has limited power to demonstrate its effect.

This population based study included a valuable set of AK count graded by physicians trained in dermatology. Although accuracy of grading by experienced physicians still seems to be of reasonable quality (41,42), it also appears unethical to aspire histological confirmation since AK is normally only diagnosed clinically. In this study, the number of AK was grouped in ordinal categories, which might increase power in the association analysis compared with a dichotomous outcome (43), as was also demonstrated by the slightly more significant P-value obtained from linear versus logistic regression when testing skin color association. Although the RS includes well documented data, we probably underestimated the AK prevalence in this cross-sectional design due to the waxing and waning nature of AK and a lack of knowledge on previous treatments for AK. Next to the AK data, a digitally quantified skin color measurement was available to investigate skin color independent effects. A digital skin color measurement is more objective than human perceived skin darkness, and it may capture subtle trait variation. Previously, skin color saturation has been shown to be an accurate skin color measure (12), and it was successfully used for the discovery of new skin color genes. In this study we did not have information about cumulative UV exposure, an important risk factor for AK. However, it is unlikely that identified association between some pigmentation genes and AK is confounded by UV exposure, which is more likely an essential prerequisite for the pigmentation genes to exert oncogenic effects.

In conclusion, this study showed that IRF4, MC1R and TYR genes are involved in AK development in Europeans, independent of skin color. This observation strongly suggests that a subset of pigmentation genes might exert effects on skin malignancies via additional pathways than the pigmentation pathway alone, which should guide future studies.

Materials and Methods

Study population

The RS is a population-based cohort study of 14 926 participants aged 45 years and older, living in the same suburb of Rotterdam, the Netherlands (44). The present study includes 3817 participants of Dutch European ancestry, who had undergone a full body skin examination and have quality controlled genome-wide microarray data available. The Medical Ethics Committee of the Erasmus MC University Medical Center approved the study protocol and all participants gave written informed consent.

Phenotypes

Skin phenotypes were collected during a full body skin examination at routine visits of the participants of the RS. Data collection is still ongoing and up to date the sample size (N = 3817) has almost doubled since a previous investigation of epidemiologic factors for AK (N = 2061) (3). For the GWAS, the sample was split in a discovery cohort (N = 3194), collected up to November 2013, and a replication cohort (N = 624), subsequently collected up to July 2014. In brief, trained physicians (including LCJ) examined the complete skin, excluding the underwear and socks areas, for multiple visible skin phenotypes. AK was defined as rough red scaling lesions, not fitting another diagnosis. AK presence was categorized into four levels according to the number of AK: 0, 1–3, 4–9 and ≥10. Skin color was assessed with a spectrophotometer (Konica Minolta Sensing, CM-600d, Singapore), measured at the inner upper arm, which is assumed to be sun-unexposed skin. The device is calibrated daily, and the mean color of three consecutive measurements is outputted. Color is displayed as a 3-dimensional continuous measure in the HSB color system, with H (hue, variations in color type), S (saturation, intensity of color) and B (brightness, luminance of the color). We here used the color saturation as it has been shown to be an accurate skin color measure (12).

Genotypes

Genotyping was carried out using the Infinium II HumanHap 550 K Genotyping BeadChip version 3 (Illumina, San Diego, CA, USA). Collection and purification of DNA have been described previously (45). All SNPs were imputed using MACH software (www.sph.umich.edu/csg/abecasis/MaCH/) based on the 1000-Genomes reference population information (46). Genotype and individual quality controls have been described in detail previously (15). After all quality controls, the current study included a total of 6 846 125 autosomal SNPs (MAF >0.03, imputation R² > 0.3, SNP call rate >0.97, HW 1 × 10⁻⁸) and 3817 individuals (individual call rate >0.95, pair-wise IBD coefficient <0.25, excluding x-matches and outliers from MDS analysis).

Statistical analysis

Linear regression (considering 4 AK severity levels as a continuous variable) and logistic regression (considering binary AK status) showed largely comparable but slightly more significant association results for saturation (P linear = 5.8 × 10⁻⁴, P logistic = 1.1 × 10⁻²). We chose linear regression in all association analyses and adjusted all analyses for age, sex and four genetic principal components. Genetic association was tested in the discovery cohort (N = 3194), assuming an additive allele effect. The P-value thresholds for genome-wide significance (5% type-I error rate) and suggestive significance (50% type-I error rate) were derived empirically by permuting (k = 1000) individual phenotype data. For each permutation, a GWAS was conducted and the minimal P-value was derived. The 5% (P = 5.0 × 10⁻⁵) and 50% (P = 4.2 × 10⁻³) quantiles of the k minimal P-values were considered as genome-wide and suggestive significance, respectively. The inflation factor lambda was close to 1.0 (λ = 1.005) and not further considered. All SNPs with suggestive association were further selected for replication in an additional cohort (N = 623), where P-values <0.05 were considered as significant replication. These SNPs were also analysed in the combined sample (N = 3817) and finally we conducted a GWAS conditioned on the top SNPs in the discovery sample.

In additional analyses, we tested the assumed functional SNP per significantly associated locus to a larger extent using the combined sample (N = 3817). First, skin color independent effects of these SNPs and AK were investigated. For this, we additionally adjusted the analysis of AK and the SNPs for saturation. Then, the combined sample was stratified for tertiles of saturation and per stratum the association of AK and the SNPs were tested.
Con of explained variance of AK (adjusted and the 12 pigmentation SNPs, we investigated the inde-
try of Health, Welfare and Sports; and the European Commission

BNC2 analyses were conducted in R (www.r-project.org).

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Additionally, interaction of the SNPs and saturation were tested. Furthermore, we tested whether a set of well-known human pigmentation SNPs were associated with AK. These SNPs were selected based on significant association with hair, eye or skin color in previous human pigmentation GWAS and included the most significant SNP of the following genes: MC1R, HERC2, OCA2, ASIP, TYR, TYRP1, IRF4, SLC45A2, SLC24A5, TPCN1, KITLG and BNC2 (9,11–13). P-values <0.005 were considered statistically significant, after bonferroni correction for 12 SNPs. Also saturation was tested for association with these same 12 pigmentation SNPs, to additionally validate saturation as skin color variable, and compare the associations of pigmentation genes with AK and skin color. In a multivariable model with age, sex, saturation and the 12 pigmentation SNPs, we investigated the independent effects of the different predictors, and the percentage of explained variance of AK (adjusted R²).

Genetic relatedness, PCA analysis and restricted maximum likelihood analysis were conducted using functions (–grm, –pca and –reml) implemented in the Genome-wide Complex Trait Analysis (GCTA) tool (http://gump.qimr.edu.au/gcta/) aiming to estimate the proportion of the phenotypic variance that can be explained by all autosomal SNPs assuming an additive model, i.e. narrow sense heritability (47). The top 20 eigenvectors from PCA, together with sex and age, were used as covariates in the REML analysis. This analysis included 477 201 genotyped autosomal SNPs with MAF > 0.01 and 3822 individuals.

Finally, we tested the assumed functional SNP per significantly associated locus for dominant or recessive allele effects and compared the effect allele frequencies of the study population with the frequencies of other populations in the HapMap data. All GWAS analyses were conducted using PLINK (48) and all other statistical analyses were conducted in R (www.r-project.org).

Supplementary material
Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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